Chapter 4
Relative rates of amino acid import via the ABC transporter GlnPQ determine the growth performance of *Lactococcus lactis*

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Submitted for publication
Abstract

The GlnPQ amino acid transporter belongs to the ATP-Binding Cassette superfamily and is found widespread in both Gram-positive and Gram-negative bacteria. The system is essential in various Gram-positive bacteria, including Lactococcus lactis and several pathogens. The GlnPQ transporter from L. lactis has the remarkable feature of having two substrate-binding domains (SBD) fused to the N-terminus of the transmembrane domain (TMD). The system thus has four SBDs in the homodimeric complex. Here, we describe the role of the individual substrate-binding domains (SBDs) of GlnPQ in the import of amino acids in relation to the growth performance of L. lactis. Specifically, we show at varying media compositions how the amino acids compete with each other for binding to the SBDs and how the SBDs compete with each other for substrate delivery to the transporter.
1.1 INTRODUCTION

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. ABC transporters translocate solutes across the cell or organellar membrane at the expense of ATP hydrolysis. The basic unit of ABC transporters consists of two transmembrane domains (TMD) and two nucleotide-binding domains (NBD), together referred to as the translocator. While the sequence and structure of the TMDs vary, the nucleotide-binding domain (NBD) of the proteins is highly conserved and is the hallmark of the ABC superfamily. 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 (Gram-positive bacteria, Archaea). In a subset of ABC transporters the SBP is fused to the TMD, resulting in most cases in more than one SBDs per functional complex.

GlnPQ is an ABC importer that belongs to the PAO (polar amino acids and opines) family. Numerous studies have reported the important roles of this transporter in pathogenic and non-pathogenic bacteria. In Lactococcus lactis, a non-pathogenic Gram-positive bacterium, GlnPQ provides the main route for fast import of glutamine, glutamate and asparagine. 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.

Two distinct substrate-binding domains (SBDs) are present in GlnPQ from L. lactis, namely SBD1 and SBD2. These SBDs are arranged in tandem and fused to each of the TMD, 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) (chapters 2). Although the two SBDs have a similar fold and similar binding pockets, the ligand affinity and specificity of SBD1 and SBD2 differ enormously. SBD1 has relatively high affinity for asparagine (K_D = 0.2 μM) and low affinity for glutamine (K_D = 92 μM), while SBD2 binds glutamine with high affinity (0.9 μM). On the basis of uptake experiments, glutamate is transported by both
SBD1 and SBD2, and the pH dependence suggests that glutamic acid is the translocated species \(^{10,12}\). However, the \(K_D\) for glutamate (glutamic acid) binding could not be determined by isothermal titration calorimetry. Differential titration calorimetry (DSC) showed that 5 mM glutamate shifts the temperature of unfolding of SBD2 by 2.6°; such an effect was not observed for SBD1, yet glutamate uptake was detected (this study). The amino acid specificity of the SBDs was confirmed by \textit{in vivo} uptake assays using GlnPQ variants, in which either SBD1 or SBD2 were deleted (GlnPQ-SBD2 and GlnPQ-SBD1, respectively) \(^9\).

The conformational dynamics of the SBDs in GlnPQ has been probed by single-molecule spectroscopy \(^9\). It was found that \textit{apo} SBDs could visit open and closed conformations. In the presence of ligand, the SBDs visit the closed-state more frequently but the lifetime of the closed-ligated 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 life-time 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 determine the growth of \textit{L. lactis} as a function of glutamine, glutamate and asparagine availability in the medium and perform transport assays in parallel. We employ \textit{L. lactis} strain GKW9000 which has the chromosomal \textit{glnPQ} genes deleted \(^9\). The strain is complemented \textit{in trans} using plasmids containing \textit{glnPQ} or mutant derivatives. The cell growth and transport analyses reveal how amino acids compete with each other for uptake via GlnPQ and shows the advantage of having two SBDs with different specificity.

\section*{1.2 \textbf{RESULTS AND DISCUSSIONS}}

\subsection*{1.2.1 Amino acid dependence of growth}

Based on genome sequencing, \textit{L. lactis} IL1403 and MG1363 have all the genes necessary to synthesize the 20 standard amino acid \(^{13,14}\), however some of the genes are not functional due to mutations or small deletions as reported for \textit{leuA} and \textit{leuB} that is involved in BCAA (branched-chain amino acid) biosynthesis \(^{15}\). Furthermore, regulation of amino acid metabolism involves control of the enzymes activity and their synthesis in response to the presence of certain metabolites \(^{16}\). In \textit{L. lactis}, regulation of amino acid biosynthesis is globally controlled by CodY \(^{16}\). Excess of isoleucine has been shown to block several CodY-dependent amino acid
biosynthesis pathways, thus inhibiting growth \textsuperscript{16-18}. Moreover, an excess of one branched-chain amino acid inhibits growth by competing out others that are taken up via the same transport system \textsuperscript{19}. The amino acids requirement for optimal \textit{L. lactis} growth varies from one strain to another. Generally, synthetic medium for \textit{L. lactis} should contain at least six amino acids, that are, isoleucine, leucine, valine, histidine, methionine, and glutamate (or glutamine) \textsuperscript{20-22}. Several other amino acids such as asparagine, alanine, arginine and threonine are important as growth stimulating factors \textsuperscript{19,21,22}. The composition and concentration of amino acids in the growth medium gives rise to variation in the growth rate of \textit{L. lactis}.

Glutamine (or glutamate) is known to be essential for \textit{L. lactis}, but the organism is able to synthesize asparagine itself \textsuperscript{14,15}. \textit{L. lactis} converts intracellular glutamine into glutamate and \textit{vice versa}, using glutaminase and glutamine synthetase, respectively \textsuperscript{23,24}. Figure 1 shows that glutamine (or glutamate) and asparagine are required for growth of \textit{L. lactis} NZ9000 (panel M and panel P, respectively; see also panel D, G, and J, where the medium lacks either of glutamine, glutamate or asparagine). \textit{L. lactis} NZ9000 is a derivative of \textit{L. lactis} MG1363 containing the \textit{nisRK} genes in the \textit{pepN} locus of the genome \textsuperscript{25}. Apparently, under our experimental conditions the synthesis of asparagine is insufficient to support growth (panel G and J), even though the amino acid is not considered to be essential for \textit{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 \textsuperscript{26}. Deletion of the \textit{glnPQ} gene results in the inability of \textit{L. lactis} (GKW9000) to grow on medium containing glutamine and asparagine (panel N), but the strain can grow in the presence of alanyl-glutamine (panel T); or if the strain is complemented \textit{in trans} with the \textit{glnPQ} gene (panel O). We also found that GlnPQ is not the only transporter for glutamate or asparagine as slow but significant growth of \textit{L. lactis} GKW9000 was detected when these amino acids were added to the medium (panel Q). Glutamate may be taken up via a secondary transporter AcaP \textsuperscript{27} or GltPQ \textsuperscript{13}, a protein that has been annotated as 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.
Figure 1. Growth dependence of *L. lactis* on asparagine, glutamine, and glutamate. Growth of *L. lactis* NZ9000, GKW9000 (pNZ8048; empty plasmid), and GKW9000 expressing wildtype *glnPQ* in *trans*. Cells were grown in 96 well plates in special chemically defined medium (CDM*) supplemented with either 3 mM of glutamine, 3 mM glutamate, 2.7 mM asparagine or a combination of these amino acids as indicated in the figure.
1.2.2 Glutamate-binding probed by differential-scanning calorimetry

We have previously shown by isothermal titration calorimetry (ITC) that SBD1 binds glutamine and asparagine, whereas SBD2 binds glutamine. 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. We now used differential-scanning calorimetry (DSC) to determine the unfolding temperature (T_M) 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 of SBD1 and SBD2 by 3.4 °C and 14.3 °C, respectively (Figure 2A and 2B, dashed line). Glutamate in contrast causes a shift in T_M of SBD2 by 2.6 °C, but gives no shift to with SBD1 (Figure 2B and 2A, respectively; dotted line); the shift is larger with decreasing pH (see Table 1), 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 T_M values for SBD1 and SBD2 in the tandem SBD12 were comparable to what is seen in the individual SBDs (Figure 2C and Table 1), indicating that the SBDs do not stabilize each other.

Figure 2. The apparent melting temperature (T_M) of SBD1 (A), SBD2 (B) and SBD12 (C) determined by DSC. Measurements were performed in 50 mM KPi 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 also Table 1 for T_M measurements at different pH. C_p is heat capacity.
Table 1. Apparent melting temperature ($T_M$) of SBDs determined by DSC.

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(1)b and (2)c correspond to $T_M$ of SBD1 and SBD2, respectively.

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$^a$ $T_M$ (°C) of SBD1, SBD2 and SBD12 of GlnPQ was measured at different pH either in the absence or presence of 5mM glutamine (Gln) or glutamate (Glu) as indicated in the table.

$^b,c$ (1) and (2) correspond to $T_M$ of SBD1 and SBD2 in the tandem SBD12, respectively.
1.2.3 Glutamate transport by GlnPQ

In order to determine the contribution 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 (Figure 3A). The transport via SBD1 was low but significantly above background and saturated with an apparent \( K_M \) of about 500 \( \mu \text{M} \) (Figure 3B). If we take a \( pK_a=4.2 \) for glutamate = glutamic acid and a medium pH of 6, then the data correspond to a \( K_M \) of \( \sim 7 \mu \text{M} \) 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 SBD2 is by far the preferred path.

![Figure 3](image)

(A) Glutamate transport in *L. lactis* GKW9000 carrying pNZ8048 empty plasmid (□), GlnPQ (△), GlnPQ-SBD1 (○) or GlnPQ-SBD2 (▼). The uptake of 250 \( \mu \text{M} \) [\(^{14}\text{C}\)]-glutamate is shown as a function of time. (B) The glutamate uptake rate of cells carrying GlnPQ-SBD1 (○) and empty plasmid pNZ8048 (□) were determined as a function of [\(^{14}\text{C}\)]-glutamate concentration.

1.2.4 Glutamate dependence of growth and role of SBD1 and SBD2

To determine the role 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* (see Table 2) supplemented with glutamate. As stated earlier, GlnPQ is not the only transporter for glutamate but is needed for maximal cell growth (Figure 4A). With glutamate instead of glutamine as source of glutamine and glutamate, *L. lactis* expressing wildtype GlnPQ (Figure 4B) or GlnPQ-SBD2 (Figure 4D) grew well with concentrations as low as 1 mM (red line), but GlnPQ-SBD1 required a higher concentration of glutamate (Figure 4C). The difference in glutamate requirement can be understood when the amino acid affinity and specificity of the SBDs are taken into account. Asparagine binds with high affinity
to SBD1\textsuperscript{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 of asparagine present in the CDM outcompetes the glutamate when present at \(1\) mM concentration (red trace in Figure 4C), which is not the case in wildtype GlnPQ or GlnPQ-SBD2 (red trace in Figure 4B and 4D, respectively). In wildtype GlnPQ (Figure 4B), glutamate can be taken up via SBD1 and SBD2 and the competition by asparagine is less severe.

![Figure 4](image)

Figure 4. Growth of \textit{L. lactis} as a function of glutamate concentration in CDM\* containing high (upper graphs) or low (lower graphs) concentration of potassium phosphate buffer. \textit{L. lactis} GKW9000 carrying pNZ8048 empty plasmid (A and E), GlnPQ (B and F), GlnPQ-SBD1 (C and G), or GlnPQ-SBD2 (D and H) were grown in CDM\* containing \(2.7\) mM asparagine and glutamate at a concentration of: 0 (black), 1 mM (red), 3 mM (green), 10 mM (blue) or 30 mM (cyan).

In our experimental setup the high concentration of potassium phosphate (~100 mM) in CDM supports a higher cell yield relative to standard CDM, which contains ~ 20 mM. We also determined the growth of cells expressing GlnPQ, GlnPQ-SBD1, GlnPQ-SBD2 and empty plasmid in standard CDM (Figure 4F, 4G, 4H, and 4E, respectively), and found that the outcome is similar to that of high-phosphate CDM. In addition, we tested full-length GlnPQ with either SBD1 or SBD2 inactivated via E184F and D417F mutations, respectively; we noted that SBD2(D417F) no longer binds glutamine, whereas SBD1(E184F) has some residual glutamine and asparagine uptake activity (Figure 5). The results were (qualitatively) similar to those in the corresponding SBD deletion mutants. In summary, GlnPQ supports growth of \textit{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

Glutamine is only transported by GlnPQ, unlike glutamate, for which multiple transporters are present in \emph{L. lactis}. As shown in Figure 6A, cells expressing empty plasmid pNZ8048 show no growth when cells are made dependent on glutamine as source of glutamate and glutamine. The growth dependence of \emph{L. lactis} expressing GlnPQ-SBD2 (Figure 6E) or the E184F-mutant (Figure 6F) on glutamine was comparable to that of wildtype GlnPQ (Figure 6B), whereas cells expressing GlnPQ-SBD1 required an order of magnitude more glutamine than the wildtype GlnPQ (Figure 6C). 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\textsuperscript{11}. In wildtype GlnPQ, glutamine can be taken up via SBD1 and SBD2 of GlnPQ, and thus is the competition by
Chapter 4

asparagine less severe (Figure 6B). Cells expressing GlnPQ (D417F) (Figure 6D) grew less well than those expressing GlnPQ-SBD1 (Figure 6C), which can be explained by the lower level of expression of transporters with two rather than one SBD.

Figure 6. Growth of *L. lactis* as a function of glutamine concentration in CDM* containing high concentration of potassium phosphate buffer. *L. lactis* GKW9000 carrying pNZ8048 (A), GlnPQ (B), GlnPQ-SBD1 (C), GlnPQ (D417F) (D), GlnPQ-SBD2 (E), or GlnPQ (E184F) (F) were grown in CDM* containing 2.7 mM asparagine and glutamine at a concentration of: 0 (black), 3 mM (red), 10 mM (blue) or 30 mM (grey).

1.2.6 Growth inhibition by asparagine

Next, we determined the growth of *L. lactis* at various concentrations and ratios of glutamine and asparagine (Figure 7A). *L. lactis* GKW9000 expressing GlnPQ-SBD1 grew poorly in medium containing 1 mM glutamine plus 0.1 mM of asparagine. 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
Relative rates of amino acids import via GlnPQ

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.

Figure 7. Competition between asparagine and glutamine (A), and between asparagine and glutamate (B) for transport via GlnPQ affects the growth of *L. lactis* GKW9000. The GKW9000 cells were complemented in trans with GlnPQ (I) GlnPQ-SBD1 (II) or GlnPQ-SBD2 (III). Cells were grown in CDM* plus asparagine and glutamine or asparagine and glutamate with different concentration combinations as indicated in the figure.

Presumably a metabolic pathway is induced by asparagine. In fact asparagine is important for CodY-dependent amino acid biosynthesis, especially in media with excess of isoleucine. 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 wildtype GlnPQ, growth is less severely affected by asparagine (Figure 7A; panel I), albeit that asparagine-bound SBD1 will compete for translocation with glutamine-bound SBD2. Growth of cells expressing GlnPQ-SBD2 is simpler to explain than that of cells with GlnPQ-SBD1. For cell growth, a minimum of 2.5 mM of asparagine and 1 mM of glutamine are needed (Figure 7A; panel III). 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 of glutamate instead of glutamine, the effects of asparagine competition are less pronounced (Figure 7B, panel II), presumably because L. lactis then uses one or more alternative transporter(s) for glutamate.

1.2.7 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 8A shows the rate of uptake of [14C]-glutamine transport in cells expressing GlnPQ (panel i), GlnPQ (D417F) (panel ii) or GlnPQ-SBD1 (panel iii). 25 and 250 μM of radiolabelled glutamine was used to report the effect of a 10- or 100-fold excess of asparagine on the transport of glutamine. At 25 μM of glutamine, SBD1 (K_D = 92 μM) is not saturated with substrate whereas SBD2 is (K_D = 0.9 μM); SBD1 is saturated with substrate at 250 μM glutamine. For comparison, we also determined the effect of a 10- and 100-fold of excess of glutamine on the uptake of [14C]-glutamine. (Figure 8A, panel i) shows that the excess of glutamine has larger impact on the uptake rate of [14C]-glutamine than 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 wildtype 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.

1.2.8 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 (Figure 8B). What is striking is that the rate of uptake of [3H]-asparagine is increased 3-fold when SBD2 is inactivated by the D417F mutation (Figure 8B, panels ii compare to i); the expression levels of GlnPQ mutation and GlnPQ(D417F) are comparable. We have previously shown that the D417F mutation prevents closing of the SBD2 (in the absence and
Relative rates of amino acids import via GlnPQ

presence of glutamine) and that the closed-unliganded state of SBD2 inhibits (asparagine) import via SBD1. 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 (Figure 8B, 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 GlnPQ-SBD1 (Figure 8B, panel ii versus iii).

Figure 8. Amino acids transport in L. lactis GKW9000 carrying GlnPQ (i), GlnPQ(D417F) (ii) or GlnPQ-SBD1 (iii). (A) The uptake rate at 25 and 250 μM of [14C]-glutamine in the presence of a 10- or 100-fold excess of glutamine or asparagine is shown. (B) The uptake rate at 25 and 250 μM of [3H]-asparagine in the presence of a 10- or 100-fold excess of asparagine or glutamine is shown. The blue and the red circles in the GlnPQ cartoons correspond to glutamine and asparagine, respectively.

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 (Figure 9).

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; (iii) transport via SBD2 is more efficient than via SBD1. The higher capacity to transport glutamine is consistent with the higher needs of protein synthesis for glutamine and glutamate than asparagine 19, and the role of these amino acids for cell volume regulation 28. Glutamate is the most abundant amino acid in bacteria, incl. *L. lactis* 12, and glutamate is readily converted from glutamine when this amino acid is imported. We also show that for optimal growth the composition and concentrations of media need to be designed carefully.

Figure 9. Asparagine transport in *L. lactis* GKW9000 carrying GlnPQ-SBD2 (○) or empty plasmid pNZ8048 (□). The uptake rate was determined as a function of asparagine concentration.

### MATERIALS AND METHODS

#### 1.3.1 Strains and growth conditions

*Lactococcus lactis* NZ9000 25 and GKW9000 9 were used as hosts to express GlnPQ, GlnPQ-SBD2 or GlnPQ-SBD1. 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 can be found in Gouridis et al. L. lactis cells were cultivated semi-anaerobically at 30°C in a special chemically defined medium (CDM*) containing free amino acids, 1% (w/v) glucose and 5 μg/ml chloramphenicol. The CDM composition is described in Table 2. All the CDM stock solutions were stored at -20°C, except basic medium that was kept in the dark at room temperature. CDM* is CDM without glutamine and asparagine, while CDM** is without glutamine.

1.3.2 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 OD600 ~0.05 in 5 ml fresh medium and allowed to grow to OD600 ~0.5. The cells were harvested and washed twice with 30 ml of 50 mM cold and sterile K2HPO4/KH2PO4 pH 6.5 and resuspended in this buffer to OD600 ~0.5. 30 μl of pre-culture were used to inoculate a total volume of 300 μl of CDM* supplemented with 0.01% of L. lactis NZ9700 culture medium (containing the inducer, nisin A29), 5 μg/ml chloramphenicol, and 1% (w/v) glucose. Combinations of asparagine, glutamine, glutamate, and dipeptide were added to the CDM* as indicated in the figure legend of each experiment. 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 hours with either 15 min or 30 min intervals in between OD measurements. The growth rate (μ) was determined by fitting the curves to a modified logistic model as described in 30. Model fitting was performed using Mathematica software.

1.3.3 Differential scanning calorimetry

The SBDs were isolated and purified as described previously 11. The purified SBDs without histag were dialyzed overnight against 50 mM potassium phosphate buffer plus 1 mM EDTA and 1 mM NaN₃ at pH 5.0, 6.0 or 7.0. 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 (Tₘ) 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 the protein precipitated during/after unfolding. As a result the melting temperatures can only be compared under the same experimental conditions and quantitative thermodynamic information cannot be extracted.
1.3.4 Amino acid transport assay

*L. lactis* strains GKW9000 harboring the expression constructs or the empty vector pNZ8048\textsuperscript{25} were grown in M17 medium supplemented with 1.0 % (w/v) glucose (GM17 medium), to OD\textsubscript{600} ~0.4 and induced for 1 hour with 0.01% of culture supernatant of the Nisin A-producing strain NZ9700 [containing ~10 μg/L of nisin A]\textsuperscript{29}. Cells were harvested, washed and resuspended to OD\textsubscript{600} ~50 in ice-cold 10 mM 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 – 250 μg/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 plus 5 mM MgCl\textsubscript{2} were added. After 3 min, the uptake reaction was started by addition of either [\textsuperscript{14}C]-glutamine or [\textsuperscript{3}H]-asparagine; the specific radioactivity was adjusted in the different experiments to have the disintegrations per minute (dpm) at least 10-fold above the background; the final amino acid concentrations are indicated in the figures or figure legends. For studies of (competitive) inhibition by amino acids, unlabelled glutamine or asparagine was mixed with the labelled 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 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 in time.
Relative rates of amino acids import via GlnPQ

Table 2. Composition of chemically defined medium (CDM)

<table>
<thead>
<tr>
<th>Amino acid mix, 20x solution (adjusted to pH 7.0, filter sterilized)</th>
<th>g/L</th>
<th>Vitamin mix 100x solution (adjusted to pH 7.0, filter sterilized)</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>4.75</td>
<td>nicotinic acid (niacin amid)</td>
<td>0.2</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>7.8</td>
<td>thiamine hydrochloride</td>
<td>0.1</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>7</td>
<td>riboflavin</td>
<td>0.1</td>
</tr>
<tr>
<td>L-arginine</td>
<td>2.5</td>
<td>Ca-pantothenate</td>
<td>0.1</td>
</tr>
<tr>
<td>L-lysine</td>
<td>8.75</td>
<td>4-aminobenzoic acid</td>
<td>1</td>
</tr>
<tr>
<td>L-isoleusine</td>
<td>4.25</td>
<td>biotin</td>
<td>1</td>
</tr>
<tr>
<td>L-methionine</td>
<td>2.5</td>
<td>folic acid</td>
<td>0.1</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>5.5</td>
<td>vitamin B12 (cyanocobalamin)</td>
<td>0.1</td>
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<tr>
<td>L-serine</td>
<td>6.75</td>
<td>orotic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>L-threonine</td>
<td>4.5</td>
<td>2-deoxythymidine (thymidine)</td>
<td>0.5</td>
</tr>
<tr>
<td>L-tryptophane</td>
<td>1</td>
<td>inosine</td>
<td>0.25</td>
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<tr>
<td>L-valine</td>
<td>6.5</td>
<td>DL-6,8 thiocetic acid</td>
<td>0.5</td>
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<tr>
<td>Glycine</td>
<td>3.5</td>
<td>pyridoxamine dihydrochloride</td>
<td>0.2</td>
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<tr>
<td>L-histidine</td>
<td>3</td>
<td>pyridoxal-HCl</td>
<td>0.2</td>
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<tr>
<td>L-leucine</td>
<td>9.5</td>
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<td></td>
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<tr>
<td>L-proline</td>
<td>13.5</td>
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<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Metal mix, 400x solutions, filter sterilized (in H₂O)</th>
<th>g/L</th>
<th>Base mix, 100x solution (in 0.2 M NaOH, filter sterilized)</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. MgCl₂</td>
<td>160</td>
<td>adenine</td>
<td>1</td>
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<tr>
<td></td>
<td>CaCl₂</td>
<td>20</td>
<td>uracil</td>
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<tr>
<td></td>
<td>ZnSO₄·7H₂O</td>
<td>2</td>
<td>xanthine</td>
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<tr>
<td>B. CuSO₄·5H₂O</td>
<td>1.2</td>
<td>guanine</td>
<td>1</td>
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<tr>
<td></td>
<td>CoCl₂</td>
<td>0.08</td>
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<tr>
<td>C. FeCl₂·4H₂O</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. MnSO₄</td>
<td>14</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Basic medium 2.7x solution (set to pH 6.4 with HCl, autoclaved)</th>
<th>g/L</th>
<th>Per liter CDM, 2x solution</th>
<th>mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled H₂O (in ml)</td>
<td>750</td>
<td>Basic medium</td>
<td>750</td>
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<tr>
<td>tyrosine</td>
<td>0.58</td>
<td>Vitamin mix</td>
<td>20</td>
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<tr>
<td>KH₂PO₄</td>
<td>27.2</td>
<td>Amino acid mix</td>
<td>100</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>50.2</td>
<td>Metal mix 1</td>
<td>5</td>
</tr>
<tr>
<td>(NH₄)₃·citrate</td>
<td>1.2</td>
<td>Metal mix 2</td>
<td>5</td>
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<tr>
<td>Na-acetate-3H₂O</td>
<td>2</td>
<td>Metal mix 3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metal mix 4</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Base mix</td>
<td>5</td>
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</table>
REFERENCES


