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Cation-selectivity of the L-glutamate transporters of *Escherichia coli*, *Bacillus stearothermophilus* and *Bacillus caldotenax*: dependence on the environment in which the proteins are expressed

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**Summary**

L-Glutamate transport by the H\(^{+}\)-glutamate and Na\(^{+}\)-glutamate symport proteins of *Escherichia coli* K-12 (GltP\(_{EC}\) and GltS\(_{EC}\), respectively) and the Na\(^{+}\)--H\(^{+}\)-glutamate symport proteins of *Bacillus stearothermophilus* (GltT\(_{BS}\)) and *Bacillus caldotenax* (GltT\(_{BC}\)) was studied in membrane vesicles derived from cells in which the proteins were either homologously or heterologously expressed. Substrate and inhibitor specificity studies indicate that GltP\(_{EC}\), GltT\(_{BS}\) and GltT\(_{BC}\) fall into the same group of transporters, whereas GltS\(_{EC}\) is distinctly different from the others. Also, the cation specificity of GltS\(_{EC}\) is different; GltS\(_{EC}\) transported L-glutamate with (at least) two Na\(^{+}\), whereas GltP\(_{EC}\), GltT\(_{BS}\) and GltT\(_{BC}\) catalysed an electrogenic symport of L-glutamate with \(\geq 2\) H\(^{+}\), i.e. when the proteins were expressed in *E. coli*. Surprisingly studies in membrane vesicles of *B. stearothermophilus* and *B. caldotenax* indicated a Na\(^{+}\)--H\(^{+}\)--L-glutamate symport for both GltT\(_{BS}\) and GltT\(_{BC}\). The Na\(^{+}\) dependency of the GltT transporters in the *Bacillus* strains increased with temperature. These observations suggest that the conformation of the transport proteins in the *E. coli* and the *Bacillus* membranes differs, which influences the coupling ion selectivity.

**Introduction**

In the mesophile *Escherichia coli* three L-glutamate transport systems have been identified: (i) a binding-protein-independent sodium-independent system; (ii) a binding-protein-independent sodium-independent system (GltP\(_{EC}\)); and (iii) a binding-protein-independent sodium-dependent system (GltS\(_{EC}\)) (Halpern et al., 1973; Miner and Frank, 1974; Schellenberg and Furlong, 1977). In the thermophilic bacteria *Bacillus stearothermophilus* and *Bacillus caldotenax*, only one L-glutamate transport system is present: GltT\(_{BS}\) and GltT\(_{BC}\), respectively (Tolner et al., 1992a). The transport by the GltT proteins is driven by the proton motive force (\(\Delta p\)) and also by inwardly directed Na\(^{+}\) gradients (\(\Delta p\)Na), and transport of L-glutamate probably occurs in symport with one H\(^{+}\) and one Na\(^{+}\) (De Vrij et al., 1989; Heyne et al., 1991). A similar type of Na\(^{+}\)--H\(^{+}\)--glutamate symport has been found in the thermophile *Bacillus IS1* (GltT\(_{BI}\*)) (B. Tolner and B. Poolman, unpublished). In order to discriminate between Na\(^{+}\)--glutamate, H\(^{+}\)--glutamate and Na\(^{+}\)--H\(^{+}\)--glutamate transport proteins, the gene designation gltS, gltP and gltT is used. Additionally, the subscripts Bs, Bc, Bi, Bsu and Ec are used to discriminate between the genes/proteins of *B. stearothermophilus*, *B. caldotenax*, *B. IS1*, *B. subtilis* and *E. coli*, respectively. The glutamate transporters GltS\(_{EC}\), GltP\(_{EC}\) and GltT\(_{BS,Bc,Bi}\) seem to differ from each other with respect to their cation selectivity. So far, the kinetic parameters of transport and the cation and substrate specificity of GltP\(_{EC}\) and GltS\(_{EC}\) have only been determined in membrane vesicles and intact cells in which multiple glutamate transport systems were expressed, and under conditions in which ion gradients and Na\(^{+}\) concentrations were not controlled. In order to enable a better comparison of the different transport proteins, the energetic and cation coupling properties and the substrate specificity of GltP\(_{EC}\) (marker K-12), GltS\(_{EC}\) (marker K-12), GltT\(_{BS}\) and GltT\(_{BC}\) were studied after expression in a genetically well-defined *E. coli* strain (ECOMUT1). Transport studies in membrane vesicles of this *E. coli* ECOMUT1 indicate that L-glutamate transport by GltS\(_{EC}\) occurs by electrogenic sodium symport, whereas GltP\(_{EC}\), GltT\(_{BS}\) and GltT\(_{BC}\) transport L-glutamate by electrogenic proton symport. GltT\(_{BS}\) and GltT\(_{BC}\)-mediated transport is dependent on Na\(^{+}\) and exhibits a 10-fold decreased affinity constant for L-glutamate uptake when the proteins are expressed in their original lipid environment.
Fig. 1. Construction of plasmid pECO118. (i) The 546 bp EcoRI-Hincll fragment (gltT promoter/RBS region) of pGBT102 was ligated into phage M13mp19 (pBST102) and a XbaI site (primer: 5'-AAAAGAAAGGGCTCTAGAATGAGAAAAATTGG-3') was introduced immediately upstream of the translation initiation codon of gltT (pBST100). Subsequently, the gltT promoter/RBS region of pBST100 was isolated as a PCR product (primers: T7 universal and M13 reverse) and ligated into EcoRV-digested pSKII to form pBST111. (ii) The 941 bp BamHI fragment (gltP promoter/RBS region) of pGBT521 was ligated into phage M13mp18 (pECO521) and a XbaI site (primer: 5'-CCATTGAGGAAGTCTCTAGAATGAAAAATATAAATTCAGCC-3') was introduced immediately upstream of the translation initiation codon of gltP (pECO100). The Smal- BamHI gltP promoter/RBS region of pECO100 was ligated into Smal/BamHI-digested pUC19 (pECO103) and, subsequently, the Aval–EcoRI gltP fragment of pGBT521 was ligated into Aval/EcoRI-digested pECO103, yielding pECO113. By substituting the 121 bp XbaI-Sacl fragment of pECO113 for the XbaI-Sacl fragment (gltP-coding region) of pECO113, the gltP gene was placed behind the gltT promoter/RBS region (pECO116). The Hindlll-Sacl fragment was ligated into Hindlll/Sacl-digested pTAQ1 to form pECO118, which has Ptac and Pbsr in tandem 5' of the gltP gene.

Symbols: Av, B, E, Ev, Hc, Hd, S, Sm and X represent Aval, BamHI, EcoRI, EcoRV, Hincll, Hindlll, Smal and XbaI restriction endonuclease, respectively. P Bs, P Ec and P tac correspond to the gltT, and gltP promoter/RBS region, and lac promoter, respectively. Black, open and shaded boxes correspond to gltT fragment, gltP fragment and vector sequence, respectively. Arrow below sequence: coding region of respective gene. SDM, site-directed mutagenesis.

**Results**

**Expression of L-glutamate transport proteins**

In membrane vesicles derived from ECOMUT1/pKK223-3, L-glutamate transport was completely absent, as expected since both gltSEc and gltPEc were inactivated in this strain. Membrane vesicles of ECOMUT1/pGBT521 (GltPEc), in which gltPEc was expressed from its own promoter/RBS (ribose-binding site), exhibited in the presence of potassium ascorbate (K-asc)/phenazine methosulphate (PMS) an initial L-glutamate uptake rate of 0.03 nmol min mg⁻¹ protein. A much higher initial rate of uptake (0.56 nmol min mg⁻¹ of protein) was observed when the coding region of gltPEc was fused to the promoter/RBS region of gltTbs (Fig. 1, for details on the construction of pECO118). GltTBS- and GltTBo-mediated transport in membrane vesicles of ECOMUT1(pGBT112) (GltTbs) and ECOMUT1(pGBT231) (GltTBo), respectively, occurred with initial uptake rates of 0.78 and 0.80 nmol min mg⁻¹ protein, respectively. Using membrane vesicles of ECOMUT1(pMK15) (GltSEc), the initial rate of GltSEc-mediated transport was 1.98 nmol min mg⁻¹ protein. The transport rates were measured in duplicate (less than 10% variation between the two measurements) and the assays were performed in buffer containing 50 mM NaCl. Without added NaCl, the buffer contained <10 µM Na⁺ but the transport rates for GltPEc, GltTbs and
The L-glutamate transporters GltPEc, GltSEc, GltTgs and GltTBc were expressed in E. coli ECOMUT1 harbouring pECO118 (GltPEc), pMK15 (GltSEc), pGBT112 (GltTBc) or pGBT231 (GltTBc). The solutes used to examine the substrate specificity of the proteins are expressed in E. coli ECOMUT1 harbouring pECO118 (GltPEc), pMK15 (GltSEc), pGBT112 (GltTBc) or pGBT231 (GltTBc). Values for L-glutamate uptake by GltTBc and GltTBc in membrane vesicles of E. coli ECOMUT1 were determined in the presence of Na+ ions. After 1 min of incubation, L-[14C]-glutamate (2-400 μM) was added and transport assays were further handled as described in the Experimental procedures.

Table 1. Kinetic parameters of glutamate transport in membrane vesicles of B. stearothermophilus (GltTBc), B. caldotenax (GltTBc) and E. coli ECOMUT1 harbouring pECO118 (GltPEc), pMK15 (GltSEc), pGBT112 (GltTBc) or pGBT231 (GltTBc).

<table>
<thead>
<tr>
<th>Vesicles derived from</th>
<th>V_{\text{max}}^\text{app} (nmol min^{-1} mg protein^{-1})</th>
<th>K_{\text{m}}^\text{app} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECOMUT1/pECO118 (GltPEc)</td>
<td>3.6 ± 1.2</td>
<td>6.2 ± 0.4</td>
</tr>
<tr>
<td>ECOMUT1/pMK15 (GltSEc)</td>
<td>36.9 ± 11.1</td>
<td>37.7 ± 2.1</td>
</tr>
<tr>
<td>ECOMUT1/pGBT112 (GltTBc)</td>
<td>56.9 ± 18.3</td>
<td>24 ± 2.3</td>
</tr>
<tr>
<td>ECOMUT1/pGBT231 (GltTBc)</td>
<td>38.0 ± 13.6</td>
<td>16.8 ± 1.3</td>
</tr>
<tr>
<td>B. stearothermophilus (GltTBc)</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>B. caldotenax (GltTBc)</td>
<td>3</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 2. Inhibition constants of glutamate transport in membrane vesicles of E. coli ECOMUT1/pMK15 (GltSEc). Glutamate uptake was only observed when Na+ was present in the assay buffer, irrespective of pH. Under these conditions and at pH 6.0, L-glutamate uptake was partially inhibited by GltTBc and GltTBc in E. coli is an electrogenic process in which only protons are transported in symport with the substrate.

Effect of ionophores on L-glutamate transport

To establish the nature of the cations transported with L-glutamate, the effect of ionophores on sodium and/or proton motive force-driven transport (at pH 6.0) was studied in membrane vesicles of B. stearothermophilus, B. caldotenax and E. coli ECOMUT1 harbouring pECO118 (GltPEc), pGBT112 (GltTBc), pGBT231 (GltTBc) or pMK15 (GltSEc).

Both nigericin (electroneutral exchange of K+/H+) as well as valinomycin (K+ ionophore) inhibit the uptake of L-glutamate via GltTBc partially, while complete inhibition was observed in the presence of both ionophores. Also, L-glutamate transport mediated by GltTBc and GltTBc were similarly affected by valinomycin and nigericin. Furthermore, the presence of an additional sodium motive force (50 mM NaCl in the assay buffer; the standard assay buffer contained <10 μM Na+) had no effect on L-glutamate transport by GltTBc. GltTBc and GltTBc. These observations strongly suggest that L-glutamate transport mediated by GltTBc, GltTBc and GltTBc in E. coli is an electrogenic process in which only protons are transported in symport with the substrate.

Table 2. Inhibition constants of glutamate transport in membrane vesicles of E. coli ECOMUT1/pMK15 (GltSEc). Glutamate uptake was only observed when Na+ was present in the assay buffer, irrespective of pH. Under these conditions and at pH 6.0, L-glutamate uptake was partially inhibited by GltTBc and GltTBc in E. coli is an electrogenic process in which only protons are transported in symport with the substrate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>GltP_Ec</th>
<th>GltS_Ec</th>
<th>GltTBc</th>
<th>GltTBc</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamate</td>
<td>8</td>
<td>37</td>
<td>21</td>
<td>83</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>17</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Glutamate</td>
<td>650</td>
<td>450</td>
<td>650</td>
<td>900</td>
</tr>
<tr>
<td>D-MG</td>
<td>&gt;1000</td>
<td>950</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>β-HA</td>
<td>20</td>
<td>&gt;1000</td>
<td>59</td>
<td>210</td>
</tr>
<tr>
<td>Cysteate</td>
<td>13</td>
<td>&gt;1000</td>
<td>108</td>
<td>187</td>
</tr>
</tbody>
</table>

By comparison, GltSEc showed a high affinity for L-glutamate and low affinity for α-glutamate and DGlu (Table 2).

Substrate specificity

The solutes used to examine the substrate specificity of the L-glutamate transporters GltPEc, GltSEc, GltTBc and GltTBc were found to be highly specific for L-glutamate, L-aspartate, β-hydroxyaspartate (β-HA) and cysteic acid. D-Glutamate was somewhat inhibitory whereas α-methylglutamate (α-MG), L-glutamine and L-asparagine had no significant effect on the transport rate, not even at a 500-fold excess of the substrates (K_{m}^{app}>1 mM; Table 2).
inhibited by monensin (electroneutral Na⁺/H⁺ exchange) and nigericin, whereas valinomycin had no effect on the rate of transport. Complete inhibition of L-glutamate uptake was observed in the presence of nigericin plus valinomycin. Under these conditions, not only the Δp but also the ΔpNa is abolished. At pH 8.0 when the proton motive force is largely composed of an electrical potential, monensin as well as nigericin did not affect L-glutamate transport, whereas valinomycin abolished transport completely (data not shown). These findings suggest that transport of L-glutamate mediated by GltT is an electrogenic process in which only Na⁺ is co-transported.

The cation-coupling mechanism of the GltT transporters, expressed in E. coli, differs from previous observations in B. stearothermophilus and B. caldotenax (De Vrij et al., 1989; Heyne et al., 1991). The isolation of glutamate transport mutants of B. caldotenax, using [3H]A as a toxic analogue, yielded transport phenotypes that were consistent with the inactivation of a single gene product, i.e. GltT (data not shown). To address the apparent discrepancy between the cation-coupling mechanisms of GltT in E. coli (this study) and previous studies, L-glutamate transport was analysed further in membrane vesicles of B. stearothermophilus and B. caldotenax. The effects of ionophores in the Bacillus membranes were indeed different from those observed in E. coli membrane vesicles. In the Bacillus vesicles and at pH 6.0, nigericin did not affect transport, whereas dissipation of the ΔΨ (membrane potential) by valinomycin inhibited L-glutamate transport partially. Furthermore, an approximately threefold increase in the initial rate of L-glutamate transport was observed in the presence of monensin, possibly as a result of the conversion of the ΔpH into a ΔpNa. These results suggest that L-glutamate transport in B. stearothermophilus and B. caldotenax is electrogenic and are consistent with the previously reported Na⁺—H⁺—L-glutamate co-transport (De Vrij et al., 1989; Heyne et al., 1991).

**Artificial ion gradients**

Since L-glutamate is an anionic species at physiological pH, the electrogenic nature of the transport processes in membrane vesicles of ECOMUT1 harbouring pECO118 (GltPEc), pGBT112 (GltTBs), pGBT231 (GltTBc) or pMK15 (GltSEc), as well as in membrane vesicles of B. stearothermophilus and B. caldotenax, suggests that at least two cations are transported in symport with the substrate. Although the use of ionophores allows the selective manipulation of the components of the proton and sodium motive force across the membrane, dissipation of one of the components usually results in a partially compensatory increase of another component. Furthermore, the ionophores may affect transport not only through their effects on the driving force but also by altering parameters such as the internal pH (Poolman et al., 1987). To specify the nature of the co-transported cation(s) more precisely, experiments were carried out in which L-glutamate uptake was driven by artificially imposed ion gradients.

The Δp as well as its components ΔΨ and ΔpH, were able to drive L-glutamate uptake in membrane vesicles of ECOMUT1 harbouring pECO118 (GltPEc), pGBT112 (GltTBs) or pGBT231 (GltTBc) (Fig. 2). An Na⁺ gradient, either alone or in addition to an artificially generated Δp, ΔΨ or ΔpH, had no effect on L-glutamate uptake. The results at 45 C were similar to those obtained at 37 C (data not shown). Again, these data strongly suggest that GltPEc, GltTBs and GltTBc only transport protons in symport with L-glutamate. L-Glutamate transport by ECOMUT1 harbouring pMK15 (GltSEc) was only observed when a ΔpNa was generated; transport was not observed when only Δp,
Fig. 3. Uptake of L-glutamate in membrane vesicles of ECOMUT1/pMK15 (GitS\textsubscript{EC}) driven by artificially imposed ion gradients. L-Glutamate uptake was measured in the presence of a Δp + ΔpNa (●) or ΔpH + ΔpNa (△) (A); and a ΔpNa (○), ΔΨ + ΔpNa (△), ΔpH + ΔpNa (△), ΔpH (△) or ΔΨ (△) (B), as described in the Experimental procedures. The assay temperature was 37°C. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (○).

Fig. 4. Uptake of L-glutamate in membrane vesicles of B. stearothermophilus (GitT\textsubscript{BS}) driven by artificially imposed ion gradients. L-Glutamate uptake was measured in the presence of a ΔpNa (●), Δp (○) or Δp + ΔpNa (△) (A); and a ΔpNa (○), ΔpH (△), ΔpH + ΔpNa (△), ΔΨ (△) or ΔΨ + ΔpNa (△) (B), as described in the Experimental procedures. The assay temperature was 45°C. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (○).

**Discussion**

In this study, we report the construction of an E. coli strain (ECOMUT1) which is devoid of secondary glutamate transport activity. The GitP and GitS proteins of E. coli and GitT of B. stearothermophilus and B. caldotenax have been expressed in this strain and their functional properties with regard to substrate specificity, cation selectivity and nature of the driving force for transport have been investigated. An important conclusion that follows from these studies is that the cation specificity and the apparent affinity constant for substrate uptake is highly

ΔΨ or ΔpH was imposed (Fig. 3B). The generation of a Δp, ΔΨ or ΔpH on top of a ΔpNa stimulated the transport (Fig. 3). These results are in accordance with the notion that Na\textsuperscript{+} is obligatory for L-glutamate uptake mediated by GitT\textsubscript{EC}. The stimulatory effect of ΔpNa on ΔpNa driven L-glutamate transport may reflect an activation of GitT\textsubscript{EC} by an increased internal pH (Poolman et al., 1987).

The Δp as well as its components ΔΨ and ΔpH were able to drive L-glutamate uptake in membrane vesicles of B. stearothermophilus and B. caldotenax. Furthermore, uptake of L-glutamate was enhanced several-fold when a ΔpNa was applied in addition to Δp, ΔΨ or ΔpH (Fig. 4). Since B. stearothermophilus and B. caldotenax are thermophilic organisms, the effect of temperature on the ΔpNa stimulation of L-glutamate transport was studied. Interestingly, the results revealed that the stimulation of transport by a ΔpNa was highly dependent on temperature (Fig. 5).

The data demonstrate that, in addition to H\textsuperscript{+}, Na\textsuperscript{+} also plays a role in GitT-mediated L-glutamate uptake in membrane vesicles of B. stearothermophilus and B. caldotenax.

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dependent on the lipid environment in which the proteins are expressed.

Studies in membrane vesicles of *E. coli* ECOMUT1 harbouring pECO118 (GltPEc), pGBT112 (GltTBe) or pGBT231 (GltTBC) and of *B. stearothermophilus* and *B. caldotenax* revealed that the GltP- and GltT-type L-glutamate transporters are specific for the substrates L-glutamate and L-aspartate (Table 2). The $K_{\text{app}}$ values for L-glutamate and L-aspartate are in the micromolar range (Table 1). For GltTBe and GltTBC the $K_{\text{app}}$ values are approximately 10-fold higher when the proteins are expressed in *E. coli* as compared with the *Bacillus* strains. L-Glutamate transport via GltPEc, GltTBe and GltTBC is inhibited by β-HA and cysteic acid, as previously reported for GltPEc of *E. coli* (Schellenberg and Furlong, 1977). In contrast to Deguchi et al. (1989), who characterized GltPEc of *E. coli* B, no significant inhibition of L-glutamate uptake by L-glutamine and L-asparagine was found (at a 500-fold excess over L-$[^{14}$C]-glutamate). Since only the sequence of gltP of *E. coli* K-12 is known (Tolner et al., 1992b), it cannot be excluded that differences in the amino acid sequences of GltP from *E. coli* strain K-12 and B are responsible for the observed differences. The inhibitor of GltS α-MG (Schellenberg and Furlong, 1977) did not affect transport of the GltPEc, GltTBe and GltTBC. The substrate specificity of these L-glutamate transporters is similar to that of GltP in *B. subtilis* (Tolner et al., 1995).

Studies in membrane vesicles of ECOMUT1 harbouring pMK15 (GltSEc) revealed that the GltS of *E. coli* K-12 is highly specific for L-glutamate (Table 2). Our data indicate a low affinity of GltS for D-glutamate ($K_{\text{app}}$ 450 µM), whereas L-glutamine does not significantly inhibit L-glutamate uptake. These observations are at variance with those of Deguchi et al. (1989) in *E. coli* B, who reported a strong inhibition of GltS-mediated L-glutamate transport by D-glutamate and L-glutamine. The gltS-coding regions of *E. coli* K-12 and B differ in six bases, but these differences are translationally silent (Deguchi et al., 1990; Kalman et al., 1991) and therefore cannot be responsible for the discrepancies in substrate specificity. Surprisingly, GltS exhibited a low affinity for α-MG, which is often reported to be a specific inhibitor of GltS-mediated transport in *E. coli* (Deguchi et al., 1990; 1989; Kalman et al., 1991; Schellenberg and Furlong, 1977).

L-Glutamate transport in membrane vesicles of *B. stearothermophilus* (GltTBe), *B. caldotenax* (GltTBC) and *E. coli* ECOMUT1 harbouring pECO118 (GltPEc), pMK15 (GltSEc), pGBT112 (GltTBe) or pGBT231 (GltTBC) was found to be electrogenic and to occur in symport with at least two cations. This was shown by the effect of ionophores on
K-asc/PMS (or N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD))-energized transport as well as by transport driven by artificially imposed ion gradients (Figs 2–4). The studies have revealed distinct differences with respect to the nature of coupling ion(s) to transport. (i) GltSEc transports L-glutamate in symport with at least two Na⁺. The involvement of Na⁺ in GltS-mediated transport has been suggested before (Frank and Hopkins, 1969; Kahane et al., 1975; Marcus and Halpern, 1969; Miner and Frank, 1974; Schellenberg and Furlong, 1977), but in most of these studies a role of protons as alternative coupling ion (Hasan and Tsuchiya, 1977; MacDonald et al., 1977; Tsuchiya et al., 1977) or a symport of L-glutamate with Na⁺ plus H⁺ could not be excluded (Fujimura et al., 1983a,b). By complementation of the E. coli ECOMUT1 strain (GltS⁻, GltP⁻) and the use of membrane vesicles, we have shown that only GltSEc is responsible for the observed Na⁺ dependency of L-glutamate transport in E. coli. (ii) A surprising observation was that GltTBc and GltTBs, when expressed in E. coli, transport L-glutamate in symport with at least two H⁺. Previous studies on the energetics of these transporters in their native environment revealed a H⁺–Na⁺ coupled symport mechanism (De Vrij et al., 1989). The observations made for GltPEc mimic those of the GltP protein of B. subtilis, which has recently been identified as an electrogenic H⁺–glutamate symporter (Tolner et al., 1995). (iii) When GltTBc and GltTBs are expressed homologously in the corresponding Bacillus strains, a ΔpNa indeed stimulates L-glutamate transport, i.e. when a ΔΨ, ΔpH or Δp are generated simultaneously. Furthermore, the stimulation of the initial rate of L-glutamate uptake by a ΔpNa increased from threefold at 25°C to sevenfold at 55°C. Under the conditions in which a ΔpNa was generated, no increase in the ΔΨ and/or ΔpH was observed (data not shown) which could have occurred as a result of Na⁺/H⁺ antiport activity. If Na⁺ is taken up by a Na⁺/H⁺ antiporter, the ΔpH (inside alkaline) can be raised as a result of the coupled efflux of H⁺ in the antiport reaction. Since this was not observed, the effect of Na⁺ must be exerted on the GltT proteins directly.

It is concluded that, in the E. coli lipid environment, L-glutamate is probably transported with two H⁺ while in the Bacillus lipid environment L-glutamate transport is mediated with one H⁺ and one Na⁺. Apparently, the conformation of the GltT transporters in E. coli is such that H⁺ (H₂O⁺; Boyer, 1988), but not Na⁺, can be accommodated in the cation-binding pocket. These observations need further clarification at the molecular level. For such analysis, membrane vesicles are too complex and studies of the purified and reconstituted L-glutamate transporters are needed.

Finally, the present study calls for precautions when the cation selectivity of transporters is studied in membranes (heterologous expression or reconstituted into artificial membranes) that are different from the native environment.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table 3. B. stearothermophilus was grown at 63°C, with vigorous aeration in a medium containing 2% (w/v) tryptone, 1% (w/v) yeast extract and 170 mM NaCl, and adjusted to pH7.0. E. coli strains were grown at 37°C with vigorous aeration in Luria–Bertani (LB) or M9 media (Sambrook et al., 1989). The mineral media were supplemented with essential nutrients as indicated by the auxotrophic markers. When needed, carbenicillin, kanamycin, chloramphenicol, X-gal and IPTG were added to a final concentration of 100, 20 and 30 μg ml⁻¹, 20 mg ml⁻¹ and 100 μM, respectively.

Isolation of membrane vesicles

For transport studies in membrane vesicles, cells of B. stearothermophilus or B. caldotenax were grown to an A₆₀₀ of 1.0. Cells were harvested and membrane vesicles were isolated essentially as described by Konings et al. (1973), except that the incubation temperature of the cells in the presence of lysozyme was increased to 50°C. Cells of E. coli strain ECOMUT1 harbouring plasmid pKK223-3, pEC1118, pGBT112 or pMK15 were grown to an A₆₀₀ of 1.0 in M9 containing 1% (v/v) LB and 1 mM IPTG, and membrane vesicles were isolated as described previously by Kaback (1971). Cytoplasmic membranes of B. stearothermophilus, B. caldotenax and E. coli were resuspended to about 15 mg protein per ml in 50 mM potassium phosphate pH 6.0 and stored in liquid nitrogen.

Transport assays: Na⁺ and H⁺ motive force-driven uptake in E. coli membrane vesicles

Uptake of L-[¹⁴C]-Glutamate was assayed at 37°C, under continuous aeration. The electron donor system K-asc/ PMS was used to generate Δp. Membrane vesicles were diluted 100-fold into 50 mM potassium phosphate pH 6.0, 5 mM MgSO₄, 10 mM K-asc and 100 μM PMS (<30 μM Na⁺). The effect of the sodium gradient (ΔpNa) was assessed by adding 50 mM NaCl to this assay buffer. When appropriate, valinomycin (2 μM), nigericin (1 μM) or monensin (20 mM) was added to abolish the ion gradients across the membrane. After 1 min of incubation, the uptake was initiated by adding L-[¹⁴C]-glutamate to a final concentration of 1.9 μM. The uptake reactions were terminated by adding a 10-fold excess of ice-cold 0.1 M KCl, followed by immediate filtration over cellulose nitrate filters (0.45 μm pore size). The filters were washed once with 2 ml ice-cold KCl.

Transport assays: Na⁺ and H⁺ motive force-driven uptake in vesicles from Bacillus sp.

The assays were similar to those described above, except that K-asc (10 mM)/TMPD (100 μM) was used as electron donor system; the temperature was kept at 50°C.
### Table 3. Bacterial strains, plasmids and phages used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. steaothermophilus</strong></td>
<td></td>
<td>ATCC 7954</td>
</tr>
<tr>
<td>JM101</td>
<td></td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>EC101</td>
<td></td>
<td>Department of Genetics, Groningen University</td>
</tr>
<tr>
<td>MK416</td>
<td></td>
<td>Kalman et al. (1991)</td>
</tr>
<tr>
<td>ECOMUT1</td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>CJ236</td>
<td></td>
<td>Laboratory collection</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18/19</td>
<td>Ap^R, only replicates in Rep^A^ strains, Tc^R</td>
<td>Yanisch-Perron et al. (1995)</td>
</tr>
<tr>
<td>pSKII^−</td>
<td></td>
<td>Stratagene</td>
</tr>
<tr>
<td>pTAQI</td>
<td>Lacl^+, Ap^R (expression vector)</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pORI24</td>
<td>Rep^A^, only replicates in Rep^A^ strains, Tc^R</td>
<td>Department of Genetics, Groningen University</td>
</tr>
<tr>
<td>pHE450CMR</td>
<td>Carrying a Cm^R^ gene on a 3.7 kb fragment in the multiple cloning site, Ap^R^, Cm^R^</td>
<td>Fellay et al. (1987)</td>
</tr>
<tr>
<td>pGBT102</td>
<td>pUC18, carrying ghtT_po10−10, Ap^R^</td>
<td>Toiiler et al. (1992a)</td>
</tr>
<tr>
<td>pGBT112</td>
<td>pUC18, carrying ghtT_po10−10 (in reverse orientation relative to pGBT102), Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT231</td>
<td>pUC18, carrying ghtT_iso10−10, Ap^R^</td>
<td>Toiiler et al. (1992a)</td>
</tr>
<tr>
<td>pGBT521</td>
<td>pUC18, carrying ghtPsecR10−10, Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT521S</td>
<td>pSKII^− (EcoRV digested), carrying ghtT promoter/RBS region of pBSt100 (PCR product: T7 universal and M13 reverse primer), Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pBST111</td>
<td>pUC18, carrying ghtPsecR10−10, Smal at position 479, Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pECHO103</td>
<td>pUC19 (SmaI/BamH1 digested), carrying ghtP promoter/RBS region of pECHO100, Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pECHO113</td>
<td>pECHO103 (XbaI/EcoRI digested), carrying ghtP terminator region of pBGT521, Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pECHO116</td>
<td>pECHO113 (XbaI/SacI digested), carrying ght promoter/RBS region of pBST100, Ap^R^</td>
<td>This work</td>
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<tr>
<td>pECHO128</td>
<td>pTAQI (HindIII/SacI digested), carrying ghtT_glpq fragment of pECO116, Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT550</td>
<td>pUC19 (Aval/HincII digested), carrying a 926 bp Aval–HincII ghtP fragment of pBGT521, Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT557</td>
<td>pSKII^− (KpnI/HincII digested), carrying the KpnI–HincII ghtP fragment of pBGT550, Ap^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT557CM</td>
<td>pGBT557 (EcoRI digested), carrying the Cm^R^ gene (EcoRI fragment) of pBGT557CM, Ap^R^, Cm^R^</td>
<td>This work</td>
</tr>
<tr>
<td>pORI557CM</td>
<td>pORI24, carrying the KpnI–PstI fragment of pBGT557CM (ghtP fragment and Cm^R^ gene), Ap^R^, Cm^R^, Tc^R^</td>
<td>This work</td>
</tr>
</tbody>
</table>

### Transport assays: artificial ion gradients

Transport of L-glutamate driven by artificial gradients was assayed essentially as described previously (Tolner et al., 1995). Membrane vesicles were washed twice in 20 mM morpholine-ethanesulfonic acid (Mes), 100 mM acetic acid (HAc) plus 100 mM KOH (adjusted to pH 6.0 with H2SO4) and subsequently incubated for 2 h at 4°C in the same buffer. After centrifugation for 5 min at 20000 g, the membranes were concentrated to approximately 40 mg protein per ml. Uptake driven by specific ion gradients was initiated by diluting the membrane vesicles 100-fold into the appropriate buffer containing L-[^14]C-glutamate (1.9 μM) with or without valinomycin (2 nmol mg^-1^). In addition, the buffers contained for Ap, 120 mM Mes, 100 mM methylglucamine (Mglu) (+valinomycin); ΔY^+^, 20 mM Mes, 100 mM HAc, 100 mM Mglu (+valinomycin); ΔpH, 120 mM Mes, 100 mM KOH (+valinomycin); ΔpNa, 20 mM Mes, 100 mM HAc, 100 mM NaOH; ΔpNa + ΔY^+^, 20 mM Mes, 100 mM HAc, 100 mM NaOH (+valinomycin); ΔpNa + ΔpH, 120 mM Mes, 100 mM HAc, 100 mM NaOH (+valinomycin); and ΔpNa + Δp, 120 mM Mes, 100 mM HAc, 100 mM NaOH (+valinomycin). The buffers were adjusted to pH 6.0 with Mglu or H2SO4 and all contained 5 mM MgSO4. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (MES/HAc/KOH). The reaction was terminated...
The internal 926 bp AvaI-HindIII gltP fragment of pGBT521 was ligated into AvaI–HindIII-digested pUC19. Subsequently, the resulting plasmid (pGBT550) was KpnI–HindIII-digested and the gltP-containing fragment was ligated into the compatible sites of pSKII to form pGBT557. After ligation of the CmR gene containing the EcoRI fragment of pH450CMR into EcoRI-linearized pGBT557, the resulting plasmid, pGBT557CM, was digested with KpnI and PstI. The KpnI–PstI fragment of pGBT557CM was ligated into KpnI–PstI-linearized pORI24 to yield pORI557CM.

Symbols as in Fig. 1. In addition, K represents KpnI and P PstI. Black, open and shaded box: CmR gene, gltP (plus promoter/RBS) and vector sequence, respectively. Arrow below sequence: coding region of respective gene. OriUC, OriPSK and OriWV represent origin of replication of pUC19, pSKII- and pWV01, respectively.

as described above. Care was taken to avoid contamination of the buffers with Na++; disposable plastic materials and ultrapure chemicals were used in all experiments (concentration of Na+ contamination <10 μM). The uptake experiments were performed at 37°C or 50°C for E. coli and B. stearothermophilus (and B. caldotenax), respectively, unless stated otherwise.

The kinetic parameters of transport, apparent K_m and V_max, were estimated from the uptake of (labelled) amino acid (2 to 400 μM) determined after 10 s. Results were analysed by fitting the data to the Michaelis equation.

DNA manipulations

Mini- and large-scale preparations of plasmid DNA were obtained by the alkaline lysis method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981). Chromosomal DNA was isolated essentially as described previously (Leenhouts et al., 1990), except that mutanolysine was omitted. The strains were transformed by heat-shock after rubidium chloride treatment of the cells (Sambrook et al., 1989) or by electrotransformation (Dower et al., 1988). Site-directed mutagenesis was performed according to Kunkel (1985). Other DNA manipulations were performed as described (Sambrook et al., 1989). Polymerase chain reactions (PCR) (Mullis and Falona, 1987) were carried out with Vent DNA polymerase (New England BioLabs), using the recommended buffer, 20 ng primers, 200 ng phage DNA (M13 replicative form) and 400 μM deoxynucleoside triphosphates in a total volume of 100 μl and 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 70°C.

Construction of strain ECOMUT1

An E. coli gltS−, gltP− strain was constructed by Campbell-like integration of vector pORI557CM into the chromosome of E. coli MK416 (gltS−); pORI557CM carries an internal fragment of the E. coli gltP gene (Fig. 6, for details). E. coli MK416 was transformed with pORI557CM and transformants were selected on LB agar plates containing 20 μg ml−1 kanamycin and 30 μg ml−1 chloramphenicol. The disruption of the gltP gene was confirmed by Southern blot analysis (using an
Aval–HindII internal gtp fragment and PstI-linearized pORI24 as probes).

Sequence determination

The nucleotide sequence of both strands of PCR products and of fragments that had been subject to site-directed mutagenesis was determined by the dideoxy-chain termination method (Sanger et al., 1977). Double-stranded DNA was sequenced using a T7 sequencing kit (Pharmacia). PCGene (release 6.26, Genofit) was used for computer-assisted sequence analysis was determined by the dideoxy-chain termination method except for the specific mutations that were introduced (see Table 1).

Protein determination

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

References


