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Characterization and functional expression in *Escherichia coli* of the sodium/proton/glutamate symport proteins of *Bacillus stearothermophilus* and *Bacillus caldotenax*

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Summary

The genes encoding the Na⁺/H⁺/L-glutamate symport proteins of the thermophilic organisms *Bacillus stearothermophilus* (gltTBs) and *Bacillus caldotenax* (gltTC) were cloned by complementation of *Escherichia coli* JC5412 for growth on glutamate as sole source of carbon, energy and nitrogen. The nucleotide sequences of the gltTBs and gltTC genes were determined. In both cases the translated sequences corresponded with proteins of 421 amino acid residues (96.7% amino acid identity between gltTBs and gltTC). Putative promoter, terminator and ribosome-binding-site sequences were found in the flanking regions. These expression signals were functional in *E. coli*. The hydropathy profiles indicate that the proteins are hydrophobic and could form 12 membrane-spanning regions. The Na⁺/H⁺ coupled L-glutamate symport proteins GltTBs and GltTC are homologous to the strictly H⁺ coupled L-glutamate transport protein of *E. coli* K-12 (overall 57.2% identity). Functional expression of glutamate transport activity was demonstrated by uptake of glutamate in whole cells and membrane vesicles. In accordance with previous observations (de Vrij et al., 1989; Heyne et al., 1991), glutamate uptake was driven by the electrochemical gradients of sodium ions and protons.

Introduction

To date, two types of L-glutamate transport mechanisms have been reported for thermophilic bacteria. In *Bacillus stearothermophilus* L-glutamate (or L-aspartate) transport proceeds via a sodium/proton-symport mechanism with a 1:1 stoichiometry (de Vrij et al., 1989; 1990; Heyne et al., 1991). In *Clostridium thermocellum* an electrogenic sodium symport mechanism with a stoichiometry of 2 has been identified (Speelmans et al., 1989). In the mesophilic organism *Escherichia coli* three L-glutamate transport systems have been identified: (i) a binding-protein-dependent, sodium-independent, glutamate-aspartate system (inhibited by cysteate); (ii) a binding-protein-independent, sodium-independent, glutamate–aspartate system (inhibited by β-hydroxyaspartate and cysteate); and (iii) a binding-protein-independent, sodium-dependent, glutamate-specific system (inhibited by α-methylglutamate) (Halpern et al., 1973; Miner and Frank, 1974; Schellenberg and Furlong, 1977). Genes encoding the sodium-motive and proton-motive transport systems, designated gltS and gltP, respectively, have been cloned (Deguchi et al., 1989; Kalman et al., 1991; Wallace et al., 1990) and their nucleotide sequences have been reported (Deguchi et al., 1990; Kalman et al., 1991; Wallace et al., 1990). Recently the reported sequence of the gltP gene of *E. coli* has been corrected (Tolner et al., 1992).

The mechanism of energy coupling to glutamate transport in *B. stearothermophilus* has been described (de Vrij et al., 1989; Heyne et al., 1991). To elucidate the molecular properties of the sodium/proton/L-glutamate-symport transport system of *B. stearothermophilus* in more detail, a strategy was devised to clone the gene encoding the glutamate transport protein. This strategy is based on the complementation of an *E. coli* K-12 strain for growth on glutamate as sole source of energy, nitrogen and carbon. *E. coli* K-12 strains do not grow in media containing glutamate as sole source of energy, nitrogen and carbon because of an insufficient capacity to accumulate glutamate (Halpern and Lupo, 1965). Another thermophilic bacillus is *Bacillus caldotenax*, which has a higher optimum temperature of growth (70 versus 63°C of *B. stearothermophilus*), and can grow much faster than *B. stearothermophilus* on glutamate as sole source of energy, nitrogen and carbon (t₅₀: 30 min versus 5 h for *B. stearothermophilus*). The L-glutamate transport gene of *B. caldotenax* has also been isolated and characterized.

In this paper we report the cloning of the genes encoding the Na⁺/H⁺/L-glutamate symport proteins of *B. stearothermophilus* (gltTBs) and *B. caldotenax* (gltTC), their nucleotide sequence, deduced amino acid sequence

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Fig. 1. Expression of the glt genes of B. stearothermophilus and B. caldotenax in minicells of E. coli P678-54. Proteins were labelled in the presence of [35S]-methionine (>1000 Ci mmol⁻¹) and 100 µM IPTG, and separated on a 15% SDS/PAA gel. Lanes 1–4: P678-54 containing pUC18 (vector control), pGBT231 (GltT<sub>Be</sub>), pGBT102 (GltT<sub>Bs</sub>), and pGBT112 (GltT<sub>Bs</sub> expressed in the opposite direction from the lac promoter), respectively. Molecular size markers (in kDa) are indicated. Solid arrow: glutamate transport proteins. Open arrow: product of the ampicillin-resistance gene.

and deduced hydropathy profile. We conclude that Glt<sub>Bs</sub> and Glt<sub>Bc</sub> are homologous. Furthermore, these proteins are homologous to the H⁺/glutamate symport protein of E. coli K-12.

Results

Cloning of the glutamate transport genes of B. stearothermophilus and B. caldotenax

The glt<sub>Bs</sub> and glt<sub>Bc</sub> genes were cloned using the strategy outlined in the Experimental procedures. In the case of glt<sub>Bs</sub>, 61 Glu<sup>+</sup> transformants able to grow on M9G plates (supplemented with carbenicillin and IPTG) were collected after 48 h. The cells were grown in liquid media and their plasmid content was analysed with respect to insert size. All transformants did harbour plasmid pKK223-3, with inserts ranging from 2.5 to 7 kb in length. A total of 30 of these plasmids conferred a Glu<sup>+</sup> phenotype on E. coli JC5412 upon retransformation. One transformant harbouring pGBT38 (insert 2.5 kb) was used to perform uptake experiments in whole cells. In these cells, sodium-stimulated glutamate transport activity was significantly higher than in cells harbouring plasmid pKK223-3 (data not shown). To obtain the smallest insert that allowed JC5412 to grow on M9G, subclones of pGBT38 were constructed in pUC18. The two smallest hybrid plasmids which resulted in a Glu<sup>+</sup> phenotype of JC5412, were pGBT102 and pGBT112. Both plasmids contained a 1537 bp EcoRI fragment of pGBT38 but in opposite orientation. Since the glt<sub>Bs</sub> gene in pGBT112 is expressed in the opposite direction of the lac promoter, the glt<sub>Bs</sub> promoter may have been cloned along with the glt<sub>Bs</sub> gene.

The glt<sub>Bc</sub> gene was cloned essentially as described above for the glt<sub>Bs</sub> gene, and was located on a 1535 bp EcoRI fragment (pGBT231). The glt<sub>Bc</sub> promoter could also be expressed independently of its orientation relative to the lac promoter of pUC18.

Expression of the glutamate transport genes of B. stearothermophilus and B. caldotenax

In the minicell-producing strain E. coli P678-54, in which pGBT102 and pGBT112 were used to express Glt<sub>Bs</sub>, one additional protein band with an apparent molecular mass of 33 kDa was found which was not present in a control strain containing pUC18 (Fig. 1).

Uptake of L-glutamate and L-aspartate by whole cells (strain E. coli JC5412) harbouring pGBT102 (Glt<sub>Bs</sub>) was several-fold higher than in cells harbouring pUC18 (Fig. 2). The initial rate of uptake and steady-state level of uptake were compared by expressing the glutamate transport protein of B. stearothermophilus. Uptake by cells harbouring plasmid pUC18 (vector control; □, ■) and pGBT102 (Glt<sub>Bs</sub>; ○, ●) were compared. Concentrated cells were diluted to a final concentration of 0.75 mg protein per ml into 50 mM potassium phosphate, pH 6.0, 5 mM MgSO<sub>4</sub>, and 10 mM glucose buffer, with (●, ○) or without (□, ■) 20 mM NaCl. After 1 min of incubation, L-[<sup>14</sup>C]-glutamate (1.75 µM) or L-[<sup>14</sup>C]-aspartate (2.23 µM) was added and transport assays were further handled as described in the Experimental procedures.
Glutamate transport genes of thermophilic bacilli

Fig. 3. L-glutamate counterflow activity by membrane vesicles of E. coli (JC5412(pUC18 or pGBT102)). Counterflow by membrane vesicles prepared from E. coli harbouring either pUC18 (vector control; D) or pGBT102 (GltT_Bc; O). Membrane vesicles loaded with 1 mM L-glutamate were diluted 100-fold into 50 mM potassium phosphate, pH 6.0, 5 mM MgSO_4 and 3.5 µM L-[14C]-glutamate. The transport reaction was stopped at different time intervals as indicated in the Experimental procedures.

Fig. 4. Sequencing strategy for the EcoRI fragments containing the gltT_Bs gene of B. steaothermophilus (pGBT102) (A) and the gltT_Bc gene of B. caldotenax (pGBT231) (B). Part of the vector sequence (open box), the cloned fragment (shaded box) and the position and direction of transcription of the glt gene (large arrow above the sequence) are shown. The regions sequenced are indicated by black arrows. Symbols: E, HindIII; H, EcoRI; R, SalI; S, Sau3A; and H, HincII restriction endonuclease, respectively.

Table 1. Apparent kinetic parameters for glutamate transport by membrane vesicles of B. stearothermophilus, B. caldotenax, E. coli JC5412 (pGBT102) and JC5412 (pGBT231).

<table>
<thead>
<tr>
<th>Vesicles derived from:</th>
<th>K_m (µM)</th>
<th>V_max (nmol mg protein^-1 min^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. stearothermophilus</td>
<td>4.7^p</td>
<td>11.4^p</td>
</tr>
<tr>
<td>B. caldotenax</td>
<td>2.9</td>
<td>17.4</td>
</tr>
<tr>
<td>E. coli JC5412 (pGBT102)</td>
<td>31.8</td>
<td>4.8</td>
</tr>
<tr>
<td>E. coli JC5412 (pGBT231)</td>
<td>25.1</td>
<td>6.2</td>
</tr>
</tbody>
</table>

a. Uptake experiments were performed by diluting membrane vesicles 100-fold in 50 mM potassium phosphate, pH 6.0, 5 mM MgSO_4, 40 mM glucose and 500 mM NaCl. After 3 min of preincubation PQQ (920 µM) was added. After another min of incubation L-[14C]-glutamate (1.75 µM) was added and transport assays were further handled as described in the Experimental procedures.

Fig. 5. Nucleotide sequence of the EcoR I fragments containing the gltB gene of B. stearothermophilus (pGBT-102) (A) and the gltB gene of B. caldothermus (pGBT231) (B). The start and stop codons, putative promoter (-35 to -10), possible ribosome-binding site (RBS) and possible terminator sequences (....) are indicated. The amino acid sequence deduced from the DNA sequences of the gltB gene is shown below the DNA sequence. The 12 possible membrane-spanning regions are underlined and in bold face. These data are available in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession numbers M86500 (gltB) and M86509 (gltB).
Table 2. Amino acid compositions of the GltT proteins of B. stearothermophilus and B. caldotenax.

<table>
<thead>
<tr>
<th>Amino acid residues</th>
<th>No. of amino acids in transport system:</th>
<th>GltTBs</th>
<th>GltTBc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Non-polar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>32</td>
<td>7.6</td>
<td>35</td>
</tr>
<tr>
<td>Cys</td>
<td>1</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>Gly</td>
<td>40</td>
<td>9.5</td>
<td>40</td>
</tr>
<tr>
<td>Ile</td>
<td>56</td>
<td>13.3</td>
<td>53</td>
</tr>
<tr>
<td>Leu</td>
<td>42</td>
<td>9.9</td>
<td>44</td>
</tr>
<tr>
<td>Met</td>
<td>15</td>
<td>3.6</td>
<td>15</td>
</tr>
<tr>
<td>Phe</td>
<td>26</td>
<td>6.7</td>
<td>28</td>
</tr>
<tr>
<td>Pro</td>
<td>15</td>
<td>3.6</td>
<td>14</td>
</tr>
<tr>
<td>Trp</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Tyr</td>
<td>12</td>
<td>2.9</td>
<td>12</td>
</tr>
<tr>
<td>Val</td>
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<td>10.0</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>67.7</td>
<td></td>
</tr>
<tr>
<td>Polar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>10</td>
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<td>11</td>
</tr>
<tr>
<td>Glu</td>
<td>16</td>
<td>3.8</td>
<td>16</td>
</tr>
<tr>
<td>Arg</td>
<td>4</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>His</td>
<td>3</td>
<td>0.7</td>
<td>3</td>
</tr>
<tr>
<td>Lys</td>
<td>26</td>
<td>6.2</td>
<td>25</td>
</tr>
<tr>
<td>Asn</td>
<td>13</td>
<td>3.1</td>
<td>12</td>
</tr>
<tr>
<td>Gin</td>
<td>17</td>
<td>4.0</td>
<td>16</td>
</tr>
<tr>
<td>Ser</td>
<td>24</td>
<td>5.7</td>
<td>26</td>
</tr>
<tr>
<td>Thr</td>
<td>23</td>
<td>5.5</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>32.3</td>
<td></td>
</tr>
</tbody>
</table>

of plasmid pGBT231 containing the glutamate transport gene of B. caldotenax is shown in Fig. 5B. An ORF is found between positions 109 and 1372, encoding a polypeptide of 421 amino acid residues (molecular mass 45 345 Da).

Amino acid composition, sequence homology and hydropathy

The amino acid compositions of GltTBs and GltTBc are shown in Table 2. GltTBs contains 67.7% non-polar and 32.3% polar residues, indicating a composition typical of membrane proteins (Büchel et al., 1980). Of the 421 residues present in GltTBs, 30 (7.2%) are basic (His residues were not taken into account) and 26 (6.2%) were acidic. The GltTBs protein is therefore a basic protein with an excess of four positive charges at neutral pH. The theoretical isoelectric point is 9.3. Similar data were obtained for the GltTBc protein, although this protein has an excess of three positive charges at neutral pH and a theoretical isoelectric point of 9.1. Alignment of the polypeptide sequences of gltTBs and gltTBc revealed 72 mismatches (in 68 triplets). However, they resulted in only 14 mismatches at amino acid level, i.e. 96.7% identical amino acid residues (Fig. 6 and Table 3). The deduced amino acid sequences of the Na"/H"/glutamate symport proteins of B. stearothermophilus and B. caldotenax were compared with the revised sequence of the H"/glutamate symport protein of E. coli K-12 (Tolner et al., 1992) and the sequence of the Na"/glutamate symport proteins of E. coli B (Deguchi et al., 1990) and E. coli K-12 (Kalman et al., 1991). Sequence comparisons revealed extensive similarity between the thermophilic Na"/H"/glutamate symport proteins and the H"/glutamate symport system of E. coli, comprising 57.2% identity (Fig. 6 and Table 3). There was no significant similarity between the thermophilic Na"/H"/glutamate symport proteins and the Na"/glutamate symport proteins of E. coli B and K-12 (Table 3). Also, no similarity was found between the glutamate transport proteins of the thermophilic bacilli and any other protein in the SWISSPROT Protein Sequence Data Bank (Version 1.40), except for some local similarity with other Na"-dependent transport proteins.

The method of Eisenberg et al. (1984) predicts, for both thermostable proteins, 12 membrane-spanning regions (Fig. 7). The 12 membrane-spanning regions of GltTBs and GltTBc are located in similar positions as the 12 membrane-spanning segments predicted for the E. coli H"/glutamate transport protein (Fig. 7), although the putative membrane-spanning helices 4 and 12 in GltTBs and GltTBc do have a somewhat lower hydrophobicity than the corresponding regions in GltPEc.

Codon usage

The codon usage in the gltTBs and gltTBc genes is nearly identical (Table 4). The low-GC content of the gltTBs and gltTBc genes (40.3 and 40.7%, respectively), when compared with the gltPEcK12 gene (53.2%), is reflected in the codon usage. At all codon positions, but particularly at the third position, a strong preference for A or U over G or C can be seen.

Discussion

Uptake of L-glutamate and L-aspartate by whole cells and membrane vesicles of E. coli JC5412 harbouring pGBT102 (GltTBs) increased significantly upon addition of 20 mM sodium (Fig. 2). These results are in accordance with those of glutamate transport in membrane vesicles of

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Amino Acid Identity (Similarity) With:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GltTBs</td>
</tr>
<tr>
<td>GltTBs</td>
<td>100</td>
</tr>
<tr>
<td>GltTBc</td>
<td>96.7</td>
</tr>
<tr>
<td>GltPEcK12</td>
<td>(2.1)</td>
</tr>
<tr>
<td>GltSEcK12/EcB</td>
<td>(8.5)</td>
</tr>
</tbody>
</table>

Table 3. Amino acid identity (similarity) between pairs of proteins.
Glutamate transport genes of thermophilic bacilli

### Fig. 6. Multiple alignment of the deduced amino acid sequences of the GltT Bs, GltTBc and GltPEc glutamate transport proteins. The best fit was achieved by introducing gaps in order to maximize the identity score. The overall identity was 57.2%. Identical residues and conserved substitutions are indicated by asterisks and full points, respectively. Symbols: S, residues involved in the putative Na⁺ recognition or binding motif; #, mismatches between the GltT Bs and GltT Be proteins. GltPEc-12 sequence was taken from Tolner et al. (1992).

B. steaothermophilus (de Vrij et al., 1989; Heyne et al., 1991) and therefore suggest that the gene encoding the previously described sodium/proton/glutamate symport protein of B. steaothermophilus (de Vrij et al., 1989; Heyne et al., 1991) has been cloned.

The GltT Bs and GltT Be proteins both consist of 421 amino acid residues, corresponding with molecular masses of 45 469 and 45 345 Da, respectively. These values are higher than the apparent molecular masses of 33 000 Da estimated from SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1). However, aberrant electrophoretic behaviour is often observed for integral membrane proteins (e.g. Büchel et al., 1980; Oeguchi et al., 1990; Ehring et al., 1980; Nakao et al., 1987; Poolman et al., 1989; Van der Rest et al., 1990; Wallace et al., 1990; Yazyu et al., 1984), and is probably explained by increased binding of sodium dodecyl sulphate due to the hydrophobic nature of the proteins.
existence of a sigma factor in \textit{B. stearothermophilus},
which is similar to $\sigma^{43}$ in \textit{B. subtilis} and $\sigma^{70}$ in \textit{E. coli},
that are involved in transcription of genes for housekeeping functions.
A putative ribosome-binding site (RBS) is located at proper distance
($4$ bp) from the translation initiation
A translation may be initiated from the promoter indicated in Fig. 5A.
The RBS shows extensive complementarity to the $3'$ end
of \textit{B. stearothermophilus} $16$S rRNA (Douthwaite et al., 1983).
The stop codon (TAA at position $1373$–$1375$) is followed by an inverted repeat
($\Delta G^o = -114.2$ kJ mol$^{-1}$, calculated according to Tinoco et al., 1973) with features
of a putative rho-independent transcription terminator sequence (Rosenberg and Court, 1979).
The $-35$ and $-10$ promoter regions of \textit{gltTBs} are
identical to those of \textit{gltTBs} of \textit{B. stearothermophilus}.
The putative ribosomal binding site (Fig. 5B), however, shows major differences
and is probably much weaker than the one upstream of \textit{gltTBs} of \textit{B. stearothermophilus}.
\textit{B. stearothermophilus} glutamate transport gene ($\Delta G^o = -75.6$ kJ mol$^{-1}$, calculated
according to Tinoco et al., 1973). The differences in the expression signals of \textit{gltTBs}.

\begin{table}
\centering
\begin{tabular}{lcccc}
\hline
Codon & Amino Acid & \textbf{No. of times used} & \textbf{Bs} & \textbf{Be} \\
\hline
TTT & Phe & 19 (20) & TAT & Tyr & 8 (10) \\
TTC & Phe & 9 (8) & TAC & Tyr & 4 (2) \\
TTA & Leu & 17 (17) & TAA & --- & 1 (1) \\
TTG & Leu & 7 (8) & TAG & --- & 0 (0) \\
CTT & Leu & 11 (12) & CAT & His & 3 (3) \\
CTC & Leu & 3 (4) & CAC & His & 0 (0) \\
CTA & Leu & 1 (0) & CAA & Gin & 11 (10) \\
CTG & Leu & 3 (3) & CAG & Gin & 6 (6) \\
ATT & Ile & 40 (36) & AAT & Asn & 8 (6) \\
ATC & Ile & 13 (14) & AAC & Asn & 5 (6) \\
ATA & Ile & 3 (3) & AAG & Lys & 18 (18) \\

t & Tyr & 8 (10) \\

t & Asp & 3 (4) \\

t & Glu & 11 (13) \\

t & Glu & 5 (3) \\

t & Cys & 1 (0) \\
CYS & Cys & 0 (0) \\

t & Tga & 0 (0) \\

t & Trp & 2 (2) \\

t & Arg & 2 (1) \\

t & Arg & 1 (2) \\
CAG & Arg & 0 (0) \\

t & Asp & 0 (0) \\

t & Gly & 12 (11) \\
GCC & Gly & 8 (9) \\
\hline
\end{tabular}
\caption{Codon usage of the glutamate transport gene of \textit{B. stearothermophilus} (Bs) and \textit{B. caledonae} (Be).}
\end{table}

Transcription of \textit{gltTBs} is probably initiated from the promoter indicated in Fig. 5A.
The $-35$ and $-10$ promoter regions, and the spacing of $17$ bp between the two regions,
is similar to that of consensus promoter sequences ($-35$: TTGACA; $-10$: TATAAT; spacing $16$–$18$, $17$–$19$ bp) that are recognized by the $\sigma^{43}$ and $\sigma^{70}$
factors of the holoenzyme form of RNA polymerase of \textit{B. subtilis} (Heimann and Chamberlin, 1988; Moran et al., 1982) and \textit{E. coli} (Hawley and McClure, 1983; Heimann and Chamberlin, 1988), respectively. This suggests the
and GltTbc are not reflected in the expression levels of the proteins both in E. coli and the thermophilic bacilli (Table 1). Since the expression levels of GltT in B. stea{thermophilus and B. caldotenax are similar, the observed differences in growth rates on glutamate as sole carbon, energy and nitrogen source cannot be explained at the level of transport. The presence of putative promoter and transcription termination sequences flanking the glutamate transport genes of B. stea{thermophilus and B. caldotenax suggests that both genes are transcribed as single cistronic messages.

The apparent $K_m$ and $V_{max}$ values for $L$-glutamate transport in membrane vesicles derived from B. stea{thermophilus and B. caldotenax are very similar (Table 1). However, B. caldotenax can grow approximately 10-fold faster than B. stea{thermophilus in media with 50 mM glutamate as sole source of energy, carbon and nitrogen (data not shown). Therefore it is unlikely that the GltTbc transport protein is limiting the growth of B. stea{thermophilus on glutamate as sole source of energy, carbon and nitrogen.

The deduced amino acid sequence of the Na$^+/H^+/gluta{mate symport proteins of B. stea{thermophilus and B. caldotenax were initially compared with the H$^+/glutamate symport protein of E. coli K-12 (Wallace et al., 1990). This did reveal regions of homology while other regions differed completely. By translating the nucleotide sequence of gltPEcK-12 in different reading frames, and by comparing the translated sequences with those of GltTbc and GltTbc it became apparent that the sequence divergence between GltPEcK-12 and GltTbc and GltTbc was probably caused by sequencing errors, i.e. base substitutions, deletions and insertions, in the L-glutamate transport gene of E. coli. The gltPEcK-12 sequence, as published by Wallace et al. (1990), was therefore resequenced and revised (Tolner et al., 1992). Sequence comparisons revealed extensive similarity between the GltTbc and GltTbc and the revised GltPEcK-12 sequence (Fig. 6 and Table 3).

A conserved amino acid sequence has been proposed to be involved in Na$^+$ recognition or binding (SOB-motif $\text{G}_{38}^-\text{A}_{40}^-\text{G}_{46}^-\text{G}_{71}^-\text{K}_{72}^-$; see also Fig. 6). However, apart from one mismatch, the SOB motif can also be found in the GltPEc protein $\text{G}_{35}^-\text{A}_{46}^-\text{A}_{62}^-\text{G}_{71}^-\text{K}_{72}^-$; see also Fig. 6). If indeed this SOB motif is essential for Na$^+$ binding this mismatch could explain the inability of GltPEc to use Na$^+$ as coupling ion despite the extensive similarity between GltTbc and GltPEc. On the other hand, the SOB motif of GltTbc and GltTbc is located in a short hydrophilic region and might be involved in retention of the three-dimensional shape of these proteins, as is proposed for this region in the 'consensus glucose transport protein' (Henderson, 1990).

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and phages used in this study are listed in Table 5. B. stea{thermophilus and B. caldotenax were grown at 63 and 70°C, respectively, with vigorous aeration in a medium containing 2% (w/v) tryptone, 1% (w/v) yeast extract and 170 mM NaCl, and adjusted to pH 7.0. For growth experiments, mineral medium of pH 7.0 was used, containing 1 ml of trace element solution (Vishniac and Santer, 1957) per litre of medium, 34 mM Na$_2$HPO$_4$, 22 mM KH$_2$PO$_4$, 10 mM NaCl, 1 mM MgSO$_4$, 0.1 mM CaCl$_2$, and 50 mM L-glutamate as sole source of energy, nitrogen and carbon. E. coli strains were grown at 37°C with vigorous aeration in LB, M9, M9G (M9 in which ammonium-chloride was replaced by L-glutamate at a final concentration of 10 mM) or M9CA medium (Sambrook et al., 1989). The mineral media were supplemented with essential nutrients as indicated by the auxotrophic markers. When needed, carbenicillin and IPTG were added to a final concentration of 100 µg ml$^{-1}$ and 100 µM, respectively.

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 the rubidium chloride (Sambrook et al., 1989) or by the electrotransformation (Dower et al., 1988) method. Other DNA techniques were performed as described previously (Sambrook et al., 1989).

Cloning of the glutamate transport gene

The strategy for cloning the gltTbc and gltTbc genes is based on the complementation of E. coli K-12 strain JC5412, which does not grow on glutamate as sole source of energy, nitrogen and carbon. Partially EcoRI-, HindIII-, PstI- or Sau3A-digested chromosomal DNA of B. stea{thermophilus or B. caldotenax was fractionated by polyacrylamide gel (6% w/v) electrophoresis. Fragments of 2 to 20 kb were electroeluted from the gel and ligated into linearized and dephosphorylated pKK223-3. The resulting hybrid plasmids were used to transform E. coli JC5412 by electrotransformation. Transformants able to grow on M9G plates (supplemented with carbenicillin and IPTG) were analysed with respect to their plasmid content. Purified plasmids were used to retransform E. coli JC5412 in order to distinguish between Glu$^+$ revertants and true transformants. Transformants again were selected on M9G plates.

Sequence determination of the glutamate transport genes

The nucleotide sequences of both strands of the EcoRI fragment of pGBT102 and pGBT231, or subclones derived thereof...
Table 5. Bacterial strains, plasmids and phages used.

<table>
<thead>
<tr>
<th>Bacterial strain, plasmid or phage</th>
<th>Relevant characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td></td>
<td>ATCC7954</td>
</tr>
<tr>
<td>B. caldotenax</td>
<td></td>
<td>Heinen and Heinen (1972)</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM101</td>
<td>∆(lac-proAB) (F'&lt;lac')∆M15 doesn't grow on L-glutamate as sole carbon, nitrogen and energy source</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>JC5412</td>
<td></td>
<td>Willettts and Clark (1969)</td>
</tr>
<tr>
<td>P678-54</td>
<td>Minicell-producing</td>
<td>Adler et al. (1967)</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; expression vector</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pKK223-3</td>
<td>pKK223-3, carrying gltT of B. stearothermophilus on a 2500 bp EcoRI-EcoRI fragment</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pGBT102</td>
<td>pUC18, carrying gltT of B. stearothermophilus on a 1537 bp EcoRI-EcoRI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT112</td>
<td>pUC18, carrying gltT of B. stearothermophilus on a 1537 bp EcoRI-EcoRI fragment (in reverse orientation relative to pGBT102)</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT231</td>
<td>pUC18, carrying gltT of B. caldotenax on a 1535 bp EcoRI-EcoRI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>Phage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13mp18/19</td>
<td></td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, ampicillin-resistant.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

in pUC18 or M13mp18/19 (Rsal, Sau3A, HpaI, HindIII or HincII fragments), were determined by using the dideoxy-chain termination method (Sanger et al., 1977). A T7 sequencing kit (Pharmacia) was used in sequencing either single- or double-stranded DNA. MICROGENIE (Release 5.0, Beckman, Palo Alto, Cal., USA) and PCGENE (release 6.26, Genofit) were used for computer-assisted sequence analysis.

Transport assays with membrane vesicles

Cells (15 ml) of strain JC5412 harbouring plasmid pUC18, pGBT102, or pGBT231, were grown to an A<sub>660</sub> of 0.7 in LB (supplemented with carbenicillin and IPTG). Cells were harvested and membrane vesicles were isolated as described previously (Kaback, 1971). Membranes were finally resuspended to 15 mg protein per ml in 50 mM potassium phosphate, pH 6.0, and stored in liquid nitrogen.

Modes of transport

Counterflow activity. Membrane vesicles were washed twice with 50 mM potassium phosphate, pH 6.0, 5 mM MgSO<sub>4</sub> and resuspended in the same buffer supplemented with 1 mM L-glutamate. After 2 h of incubation at room temperature, membrane vesicles were pelleted by centrifugation and resuspended to 20 mg protein per ml in the same buffer. Counterflow was initiated by diluting membrane vesicles 100-fold with buffer consisting of 50 mM potassium phosphate, pH 6.0, 5 mM MgSO<sub>4</sub> and 3.5 µM L-[<sup>14</sup>C]-glutamate. The reaction was terminated as described for whole cells.

Sodium/proton motive force driven uptake. The electron donor system 2,7,9 tricarboxy-1-H-pyrrolo-(2,3)-quinoline-4,5-dione (PQQ)/glucose was used to generate a Δp (van Schie et al., 1985). Membrane vesicles were diluted 100-fold with buffer consisting of 50 mM potassium phosphate, pH 6.0, 5 mM MgSO<sub>4</sub> and 3.5 µM L-[<sup>14</sup>C]-glutamate. The reaction was terminated as described for whole cells.
MgSO₄, and 40 mM glucose. After 3 min of preincubation the electron mediator POQ was added to a final concentration of 20 µM. To initiate the uptake experiment, L-[¹⁴C]-glutamate or L-[¹³C]-aspartate was added after another minute of incubation to a final concentration of 1.75 and 2.23 µM, respectively. Further handling was the same as described for whole cells. All transport assays were carried out at 37°C.

Determination of kinetic parameters
The kinetic parameters for transport, apparent $K_m$ and $V_{max}$, were estimated from the uptake of labelled amino acid in the first 10 s. Results were analysed by Eadie–Hofstee Plots.

Minicells
Minicells of strain P678–54 were purified in three subsequent sucrose gradient centrifugations (Maeger et al., 1977). The in vivo labelled ([³⁵S]-methionine) proteins were resolved by 15% (w/v) (SDS–PAGE) and visualized by autoradiography.

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

Nomenclature
In order to discriminate between the proton/glutamate and the sodium/glutamate transport proteins of E. coli the gene designations gltP and gltS are used. The L-glutamate transport systems of B. stearothermophilus and B. caldotenax translocate glutamate in symport with sodium ions and protons. For the gene encoding these proteins the designation gltT was used. To discriminate between the genes and proteins the substracts Bs, Bc and Ec (B or K-12) were added, for B. stearothermophilus, B. caldotenax and E. coli (B or K-12), respectively.

References


Leenhouts, K.J., Kok, J., and Venema, G. (1990) Stability of integrated plasmids in the chromosome of Lactococcus lac-


