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* (gltTbc) 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 gltTbc 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 gltTbc). 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 GltTbc 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 1:1:1 stoichiometry (de Vrij et al., 1989; 1990; Heyne et al., 1991). In *Clostridium fervidus* 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-hydroxylaspartate 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 (Halpern and Lupo, 1965). 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* (gltTbc), their nucleotide sequence, deduced amino acid sequence...
Fig. 1. Expression of the gltT 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_Bc), pGBT102 (GltT Bs), and pGBT112 (GltT Bs 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 GltT Bs and GltT_Bc 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 gltTBs and gltTBc genes were cloned using the strategy outlined in the Experimental procedures. In the case of gltTBs, 61 Glu⁺ 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⁺ 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⁺ phenotype of JC5412, were pGBT102 and pGBT112. Both plasmids contained a 1537 bp EcoRI fragment of pGBT38 but in opposite orientation. Since the gltT Bs gene in pGBT112 is expressed in the opposite direction of the lac promoter, the gltT Bs promoter may have been cloned along with the gltT Bs gene.

The gltT_Bc gene was cloned essentially as described above for the gltT Bs gene, and was located on a 1535 bp EcoRI fragment (pGBT231). The gltT_Bc gene 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 GltT Bs, 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 (GltT Bs) was several-fold higher than in cells harbouring pUC18 (Fig. 2). The initial rate of uptake and steady-state level of uptake of L-glutamate and L-aspartate by whole cells (strain E. coli JC5412) harbouring pGBT102 (GltT Bs) was 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 MgSO₄, and 10 mM glucose buffer, with (●, ■) or without (○, □) 20 mM NaCl. After 1 min of incubation, L-[¹⁴C]-glutamate (1.75 µM) or L-[¹⁴C]-aspartate (2.23 µM) was added and transport assays were further handled as described in the Experimental procedures.
Glutamate transport genes of thermophilic bacilli

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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; ) or pGBT102 (GltT Bs). Membrane vesicles loaded with 1 mM L-glutamate were diluted 100-fold into 50 mM potassium phosphate, pH 6.0, 5 mM MgSO4 and 3.5 µM L-[14C]-glutamate. The transport reaction was stopped at different time intervals as indicated in the Experimental procedures.

accumulation of L-glutamate increased significantly upon the addition of 20 mM NaCl (Fig. 2). Similar observations were made for (sodium)-proton motive force driven L-glutamate uptake in membrane vesicles derived from strain JC5412 harbouring pGBT102 (data not shown). Membrane vesicles derived from strain JC5412 harbouring pGBT102 also showed significantly higher L-glutamate counterflow activity than membrane vesicles derived from strain JC5412 harbouring pUC18 (Fig. 3).

The kinetic parameters (apparent Km and Vmax) of L-glutamate uptake in membrane vesicles of B. stearothermophilus and membrane vesicles of E. coli JC5412 expressing GltT Bs and GltT Be were determined (Table 1). Uptake of glutamate mediated by GltT Bs and GltT Be yielded lower Vmax and Km values when the proteins were assayed in membrane vesicles of E. coli relative to membrane vesicles of B. stearothermophilus and B. caldotenax.

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>Kmapp (µM)</th>
<th>Vmax (nmol mg protein-1 min-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. stearothermophilus (GltT Bs)</td>
<td>4.7</td>
<td>11.4</td>
</tr>
<tr>
<td>B. caldotenax (GltT Be)</td>
<td>2.9</td>
<td>17.4</td>
</tr>
<tr>
<td>E. coli JC5412(pGBT102) (GltT Bs)</td>
<td>31.8</td>
<td>4.8</td>
</tr>
<tr>
<td>E. coli JC5412(pGBT231) (GltT Be)</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 MgSO4, 40 mM glucose and 500 µM 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.


Glutamate transport genes of thermophilic bacilli

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The kinetic parameters (apparent Km and Vmax) of L-glutamate uptake in membrane vesicles of B. stearothermophilus and membrane vesicles of E. coli JC5412 expressing GltT Bs and GltT Be were determined (Table 1). Uptake of glutamate mediated by GltT Bs and GltT Be yielded lower Vmax and Km values when the proteins were assayed in membrane vesicles of E. coli relative to membrane vesicles of B. stearothermophilus and B. caldotenax.

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>Kmapp (µM)</th>
<th>Vmax (nmol mg protein-1 min-1)</th>
</tr>
</thead>
<tbody>
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<td>11.4</td>
</tr>
<tr>
<td>B. caldotenax (GltT Be)</td>
<td>2.9</td>
<td>17.4</td>
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<tr>
<td>E. coli JC5412(pGBT102) (GltT Bs)</td>
<td>31.8</td>
<td>4.8</td>
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<tr>
<td>E. coli JC5412(pGBT231) (GltT Be)</td>
<td>25.1</td>
<td>6.2</td>
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</table>

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Fig. 5. Nucleotide sequence of the EcoRI fragments containing the \( \text{glT}_{\text{B}} \) gene of \( B. \) \textit{thermotolerans} (pGBT102) (A) and the \( \text{glT}_{\text{A}} \) gene of \( B. \) \textit{caldotenax} (pGBT231) (B). The start and stop codons, putative promoter (−35/−10), possible ribosome-binding site (RBS) and possible terminator sequences (→→) are indicated. The amino acid sequence deduced from the DNA sequences of the \( \text{glT}_{\text{A}} \) and \( \text{glT}_{\text{B}} \) genes are shown below the DNA sequence. The 12 possible membrane-spanning regions are underlined and in bold face. These data sequences appear in the EMBL/GenBank/DDJB Nucleotide Sequence Data Libraries under the accession numbers M86508 (pG673) and M86509 (pG672).
Table 2. Amino acid compositions of the GltT proteins of B. steathermophilus and B. caldotenax.

<table>
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<tr>
<th>Amino acid residues</th>
<th>No. or % of amino acids</th>
<th>GltT&lt;sub&gt;BS&lt;/sub&gt;</th>
<th>%</th>
<th>GltT&lt;sub&gt;BC&lt;/sub&gt;</th>
<th>%</th>
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<td>Non-polar</td>
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<tr>
<td>Ala</td>
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<td>44</td>
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<td>3.6</td>
<td>15</td>
<td>3.6</td>
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<tr>
<td>Phe</td>
<td>28</td>
<td>6.7</td>
<td>28</td>
<td>6.7</td>
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<tr>
<td>Pro</td>
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<td>3.6</td>
<td>14</td>
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<tr>
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<td>2</td>
<td>0.5</td>
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<tr>
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<td>12</td>
<td>2.9</td>
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<tr>
<td>Val</td>
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<td>10.0</td>
<td>42</td>
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<tr>
<td>Total</td>
<td></td>
<td>67.7</td>
<td></td>
<td>67.9</td>
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<tr>
<td>Polar</td>
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<td>Asp</td>
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<td>2.6</td>
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<tr>
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<tr>
<td>Lys</td>
<td>26</td>
<td>6.2</td>
<td>25</td>
<td>5.9</td>
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<tr>
<td>Asn</td>
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<td>3.1</td>
<td>12</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Gln</td>
<td>17</td>
<td>4.0</td>
<td>16</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
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<td>5.7</td>
<td>26</td>
<td>6.2</td>
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<tr>
<td>Thr</td>
<td>23</td>
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<td>21</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>32.3</td>
<td></td>
<td>32.1</td>
<td></td>
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</tbody>
</table>

The amino acid compositions of GltT<sub>BS</sub> and GltT<sub>BC</sub> are shown in Table 2. GltT<sub>BS</sub> 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 GltT<sub>BS</sub>, 30 (7.2%) are basic (His residues were not taken into account) and 26 (6.2%) were acidic. The GltT<sub>BS</sub> 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 GltT<sub>BC</sub> 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 nucleotide sequences of gltT<sub>BS</sub> and gltT<sub>BC</sub> revealed 72 mismatches (in 68 triplets). However, they result 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<sup>+</sup>/H<sup>+</sup>/glutamate symport proteins of B. steathermophilus and B. caldotenax were compared with the revised sequence of the H<sup>+</sup>/glutamate symport protein of E. coli K-12 (Tolner et al., 1992) and the sequence of the Na<sup>+</sup>/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<sup>+</sup>/H<sup>+</sup>/glutamate symport proteins and the H<sup>+</sup>/glutamate symport system of E. coli, comprising 57.2% identity (Fig. 6 and Table 3). There was no significant similarity between the thermophilic Na<sup>+</sup>/H<sup>+</sup>/glutamate symport proteins and the Na<sup>+</sup>/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<sup>+</sup>-dependent transport proteins.

The method of Eisenberg et al. (1984) predicts, for both thermophilic proteins, 12 membrane-spanning regions (Fig. 7). The 12 membrane-spanning regions of GltT<sub>BS</sub> and GltT<sub>BC</sub> are located in similar positions as the 12 membrane-spanning segments predicted for the E. coli H<sup>+</sup>/glutamate transport protein (Fig. 7), although the putative membrane-spanning helices 4 and 12 in GltT<sub>BS</sub> and GltT<sub>BC</sub> do have a somewhat lower hydrophobicity than the corresponding regions in GltP<sub>Ec</sub>.

Codon usage

The codon usage in the gltT<sub>BS</sub> and gltT<sub>BC</sub> genes is nearly identical (Table 4). The low-GC content of the gltT<sub>BS</sub> and gltT<sub>BC</sub> genes (40.3 and 40.7%, respectively), when compared with the gltP<sub>EcK-12</sub> 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 JCS412 harbouring pGBT102 (GltT<sub>BS</sub>) increased significantly upon addition of 20 mM sodium (Fig. 2). These results are in accordance with those of glutamate transport in membrane vesicles of E. coli K-12 (Tolner et al., 1992) and the sequence of the Na<sup>+</sup>/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<sup>+</sup>/H<sup>+</sup>/glutamate symport proteins and the H<sup>+</sup>/glutamate symport system of E. coli, comprising 57.2% identity (Fig. 6 and Table 3). There was no significant similarity between the thermophilic Na<sup>+</sup>/H<sup>+</sup>/glutamate symport proteins and the Na<sup>+</sup>/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<sup>+</sup>-dependent transport proteins.

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Table 3. Amino acid identity (similarity) between pairs of proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>GltT&lt;sub&gt;BS&lt;/sub&gt;</th>
<th>GltT&lt;sub&gt;BC&lt;/sub&gt;</th>
<th>GltP&lt;sub&gt;EcK-12&lt;/sub&gt;</th>
<th>GltS&lt;sub&gt;EcK-12/EcB&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>GltT&lt;sub&gt;BS&lt;/sub&gt;</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GltT&lt;sub&gt;BC&lt;/sub&gt;</td>
<td>95.7</td>
<td>100</td>
<td></td>
<td></td>
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<tr>
<td>GltP&lt;sub&gt;EcK-12&lt;/sub&gt;</td>
<td>60.1</td>
<td>60.3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GltS&lt;sub&gt;EcK-12/EcB&lt;/sub&gt;</td>
<td>8.5</td>
<td>9.5</td>
<td>12.5</td>
<td>100</td>
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</table>
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**Fig. 6.** Multiple alignment of the deduced amino acid sequences of the GltT, GltTb, and GltPc-EK-12 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 and GltTb proteins. GltPc-EK-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 GltTb and GltTc 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; Deguchi 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 *B. stearothermophilus*, which is similar to $\sigma^{43}$ in *B. subtilis* and $\sigma^{70}$ in *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 codon (Fig. 5A). The RBS shows extensive complementarity to the 3' end of *B. stearothermophilus* 16S rRNA (Douthwaite et al., 1983). The stop codon (TAA at position 1373–1375) is followed by an inverted repeat ($\Delta G^0 = -114.2$ 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 *gltT* are identical to those of *gltT* of *B. stearothermophilus*. The putative ribosomal binding site (Fig. 5B), however, shows major differences and is probably much weaker than the one upstream of *gltT* of *B. stearothermophilus*. Also, the transcription terminator sequence is much weaker when compared with the one in the *B. stearothermophilus* glutamate transport gene ($\Delta G^0 = -75.6$ kJ mol$^{-1}$, calculated according to Tinoco et al., 1973). The differences in the expression signals of *gltT*.

**Table 4. Codon usage of the glutamate transport gene of *B. stearothermophilus* (Bs) and *B. caledonenar* (Be).**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino Acid</th>
<th>No. of times used (Bs)</th>
<th></th>
<th>Codon</th>
<th>Amino Acid</th>
<th>No. of times used (Be)</th>
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</thead>
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<td>TTT</td>
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<td>(20)</td>
<td></td>
<td>TAT</td>
<td>Tyr 8</td>
<td>(10)</td>
</tr>
<tr>
<td>TAC</td>
<td>Phe 9</td>
<td>(8)</td>
<td></td>
<td>TAC</td>
<td>Tyr 4</td>
<td>(2)</td>
</tr>
<tr>
<td>TTA</td>
<td>Leu 17</td>
<td>(17)</td>
<td></td>
<td>TAA</td>
<td>---</td>
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<tr>
<td>TGG</td>
<td>Leu 7</td>
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<td>TAG</td>
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</tr>
<tr>
<td>CTT</td>
<td>Leu 11</td>
<td>(12)</td>
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<td>CAT</td>
<td>His 3</td>
<td>(3)</td>
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<td>CTC</td>
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<tr>
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<td>(0)</td>
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<td>CAA</td>
<td>Gin 11</td>
<td>(10)</td>
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<tr>
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<td>(3)</td>
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<td>(6)</td>
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<tr>
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<td>(6)</td>
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<tr>
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<td>GAA</td>
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</tr>
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<tr>
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<td>(0)</td>
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<td>Glt 1</td>
<td>(2)</td>
</tr>
<tr>
<td>CCC</td>
<td>Pro 0</td>
<td>(0)</td>
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<td>CCC</td>
<td>Arg 1</td>
<td>(0)</td>
</tr>
<tr>
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<td>Pro 4</td>
<td>(5)</td>
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<td>Arg 0</td>
<td>(0)</td>
</tr>
<tr>
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<td>CGG</td>
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<td>(0)</td>
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<td>GCT</td>
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<td>(10)</td>
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<td></td>
<td>GCC</td>
<td>Gly 10</td>
<td>(10)</td>
</tr>
<tr>
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<td>Ala 8</td>
<td>(11)</td>
<td></td>
<td>GCA</td>
<td>Gly 12</td>
<td>(11)</td>
</tr>
<tr>
<td>GCG</td>
<td>Ala 14</td>
<td>(12)</td>
<td></td>
<td>GCG</td>
<td>Gly 8</td>
<td>(9)</td>
</tr>
</tbody>
</table>
and \textit{gltTBc} are not reflected in the expression levels of the proteins both in \textit{E. coli} and the thermophilic bacilli (Table 1). Since the expression levels of \textit{GltT} in \textit{B. stearothermophilus} and \textit{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 \textit{B. stearothermophilus} and \textit{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 \textit{B. stearothermophilus} and \textit{B. caldotenax} are very similar (Table 1). However, \textit{B. caldotenax} can grow approximately 10-fold faster than \textit{B. stearothermophilus} in media with 50 mM glutamate as sole source of energy, carbon and nitrogen (data not shown). Therefore it is unlikely that the \textit{GltTbs} transport protein is limiting the growth of \textit{B. stearothermophilus} on glutamate as sole source of energy, carbon and nitrogen.

The deduced amino acid sequence of the Na\(^+\)/H\(^+\)/glutamate symport proteins of \textit{B. stearothermophilus} and \textit{B. caldotenax} were initially compared with the H\(^+\)/glutamate symport protein of \textit{E. coli} K-12 (Wallace \textit{et al.}, 1990). This did reveal regions of homology while other regions differed completely. By translating the nucleotide sequence of \textit{gltPEcK-12} in different reading frames, and by comparing the translated sequences with those of \textit{GltTbs} and \textit{GltTBc}, it became apparent that the sequence divergence between \textit{GltPEcK-12} and \textit{GltTbs} and \textit{GltTBc} was probably caused by sequencing errors, i.e. base substitutions, deletions and insertions, in the L-glutamate transport gene of \textit{E. coli}. The \textit{gltPEcK-12} sequence, as published by Wallace \textit{et al.} (1990), was therefore resequenced and revised (Tolner \textit{et al.}, 1992). Sequence comparisons revealed extensive similarity between the \textit{GltTbs} and \textit{GltTBc} and the revised \textit{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 \(\ldots\text{G}_{45}\ldots\text{A}_{69}\ldots\text{A}_{74}\ldots\text{G}_{78}\text{R}_{79}\ldots\); see also Fig. 6). However, apart from one mismatch, the SOB motif can also be found in the \textit{GltPEC} protein \(\ldots\text{G}_{45}\ldots\text{A}_{69}\ldots\text{A}_{74}\ldots\text{G}_{78}\text{R}_{79}\ldots\); see also Fig. 6). Indeed, this SOB motif is essential for Na\(^+\) binding this mismatch could explain the inability of \textit{GltPEC} to use Na\(^+\) as coupling ion despite the extensive similarity between \textit{GltTbs} and \textit{GltPEC}. On the other hand, the SOB motif of \textit{GltTbs} and \textit{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. \textit{B. stearothermophilus} and \textit{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. \textit{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 \textit{et al.}, 1989). The mineral media were supplemented with essential nutrients as indicated by the auxotropic markers. When needed, carbenicillin and IPTG were added to a final concentration of 100 \(\mu\text{g}\) ml\(^{-1}\) and 100 \(\mu\text{M}\), 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 \textit{et al.}, 1990), except that mutanolysine was omitted. The strains were transformed by the rubidium chloride (Sambrook \textit{et al.}, 1989) or by the electroporation (Dower \textit{et al.}, 1998) method. Other DNA techniques were performed as described previously (Sambrook \textit{et al.}, 1989).

**Cloning of the glutamate transport gene**

The strategy for cloning the \textit{GltTbs} and \textit{GltTBc} genes is based on the complementation of \textit{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 \textit{B. stearothermophilus} or \textit{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 \textit{E. coli} JC5412 by electroporation. 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 \textit{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 pGMBT102 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><em>B. stearothermophilus</em></td>
<td></td>
<td>ATCC7954</td>
</tr>
<tr>
<td><em>B. caldopeptolyticus</em></td>
<td></td>
<td>Heinen and Heinen (1972)</td>
</tr>
<tr>
<td>E. coli</td>
<td>∆(lac-proAB) (F' lacIΔM15) 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>Willetts 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 <em>B. stearothermophilus</em> on a 2500 bp EcoR I-EcoR I fragment</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pGBT102</td>
<td>pUC18, carrying gltT of <em>B. stearothermophilus</em> on a 1537 bp EcoR I-EcoR I fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT112</td>
<td>pUC18, carrying gltT of <em>B. stearothermophilus</em> on a 1537 bp EcoR I-EcoR I fragment (in reverse orientation relative to pGBT102)</td>
<td>This work</td>
</tr>
<tr>
<td>pGBT231</td>
<td>pUC18, carrying gltT of <em>B. caldopeptolyticus</em> on a 1535 bp EcoR I-EcoR I 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 (R<sub>sal</sub>, Sau3A, HpaI, HindIII or HincII fragments), were determined by using the dideoxy-chain termination method (Sanger <i>et al.</i>, 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 whole cells**

Cells (15 ml) of strain JC5412 harbouring plasmid pUC18, pGBT102, or pGBT231, grown for 10 h in LB (supplemented with carbenicillin and IPTG), were harvested, washed three times in 50 mM potassium phosphate, pH 6.0, and 5 mM MgSO<sub>4</sub> and resuspended to a final A<sub>660</sub> of approximately 300 in the same buffer. Uptake of L-[14C]-glutamate or L-[14C]- aspartate was assayed at 37°C, upon 100-fold dilution of the cells into buffer consisting of 50 mM potassium phosphate, pH 6.0, 5 mM MgSO<sub>4</sub> and 10 mM glucose, with or without the addition of 20 mM NaCl or 20 mM KCl (see legends for details). This mixture was incubated for 1 min at 37°C under continuous aeration. To initiate the uptake experiment L-[14C]-glutamate or L-[14C]-aspartate was added to a final concentration of 1.75 or 2.23 µM, respectively. The uptake reactions were terminated by adding a 20-fold excess of ice-cold potassium chloride, followed by immediate filtration over cellulose nitrate filters (0.45 µm, pore size). The filters were washed once with 2 ml of ice-cold potassium chloride.

**Transport assays with membrane vesicles**

For transport studies in membrane vesicles, cells 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-[14C]-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)-quinoxaline-4,5-dione (PQQ)/glucose was used to generate a Δp<sub>mem</sub> (van Schie <i>et al.</i>, 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-[14C]-glutamate. The electron donor system 2,7,9-tricarboxy-1-H-pyrrolo-(2,3)-quinoxaline-4,5-dione (PQQ)/glucose was used to generate a Δp<sub>mem</sub> (van Schie <i>et al.</i>, 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-[14C]-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 Km and Vmax, 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. steatothermophilus 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 subscripts Bs, Bc and Ec (B or K-12) were added, for B. steatothermophilus, B. caldotenax or K-12, respectively.

References


