Cloning and nucleotide sequence of the \textit{brnQ} gene, the structural gene for a membrane-associated component of the LIV-II transport system for branched-chain amino acids in \textit{Salmonella typhimurium}

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ABSTRACT

The genetically defined \textit{gleR-brnQ} region responsible for the branched-chain amino acid transport in \textit{Salmonella typhimurium} was mapped in the 3.3-kilobase \textit{SalI-PstI} segment of plasmid pOH56 by complementation analysis. By subcloning and genetic recombination analysis, the \textit{gleR} and \textit{brnQ3} mutational sites were localized within the 0.85-kilobase \textit{SalI-EcoRV} segment, and \textit{brnQ4} within the 0.8-kilobase \textit{EcoRV-HindIII} segment. The nucleotide sequence of the \textit{brnQ} gene and its flanking regions was determined. The \textit{brnQ} gene is encoded by the sequence starting 24 base pairs upstream from the \textit{EcoRV} site. Transcription of the \textit{brnQ} gene starts at three sites separated by 171, 173 and 174 nucleotides, respectively, from the initiation codon. The promoter sequences can be seen in the immediate upstream region of the transcription initiation sites. There is a long silent region between the transcription initiation sites and a potential Shine-Dalgarno nucleotide sequence. The coding sequence of the \textit{brnQ} gene, which is 1317 base pairs long, specifies a very hydrophobic protein of 439 amino acid residues.

1. INTRODUCTION

Active transport of branched-chain amino acids in \textit{Salmonella typhimurium} is mediated by three distinct general systems, high-affinity (LIV-I), low-affinity-(1) (LIV-II), and low-affinity-(2) (LIV-III) systems (Kiritani and Ohnishi, 1978). The LIV-I system contains a periplasmic binding protein binding to leucine, isoleucine, valine, and threonine (Ohnishi and Kiritani, 1983). A structural gene, \textit{livA}, controlling the LIV-I system is located at 76–77 min on the \textit{Salmonella} genetic map (Matsubara et al., 1987). The LIV-II system appears to be the major transport system as judged by kinetic and genetic analyses (Matsubara et al., 1988), and is governed by the \textit{brnQ} gene, located at 8 min on the map (Kiritani, 1974; Sanderson and Roth, 1983). Transport activity of the LIV-III
system is concealed by that of the LIV-II system in strain Wild-type, but is
detectable in brnQ mutants lacking the LIV-II activity. These transport systems
are repressible by excess glycy-I-leucine added to the bacterial culture, and as a
consequence growth of an isoleucine-valine requiring mutant (ilvC) is almost
completely inhibited under the repressed conditions (Kiritani and Ohnishi, 1977).
A glycyIeucine-resistant (Gle) mutant, which was isolated from the ilvC strain,
carries a regulatory mutation affecting expression of the LIV-II transport activity
(Ohnishi and Kiritani, 1978). The mutational site, designated gleR1, is closely
linked to the brnQ locus (Ohnishi and Kiritani, 1980).

In this report, we describe cloning of the gleR-brnQ region by using plasmid
pBR322 as a vector, and the physical locations of gleR and brnQ mutational sites
determined by complementation and recombination analyses. We also describe
the nucleotide sequence of the brnQ gene and its flanking regions, and the
predicted amino acid sequence of BrnQ protein.

2. MATERIALS AND METHODS

Bacterial strains, plasmids, and phage

The bacterial strains used in this study are all derivatives of S. typhimurium
LT2, and are listed in Table 1. Strain OH563 is a galE (defective in galactose-4-
epimerase) derivative of CE5, with a high transformation frequency. pBR322
carrying ampicillin- and tetracycline-resistance genes was used as a cloning
vector. Derivatives of pBR322 are illustrated in Fig 1. Phage Felix O-1, which
can infect the smooth strains but not the semirough galE strain (Lindberg and
Hellerqvist, 1971), was kindly supplied by Dr. Kutsukake of the University of
Tokyo.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td>Kiritani, 1974</td>
</tr>
<tr>
<td>KA981</td>
<td>ilvC8</td>
<td>Kiritani, 1974</td>
</tr>
<tr>
<td>CE4</td>
<td>ilvC8, brnQ4</td>
<td>Kiritani, 1974</td>
</tr>
<tr>
<td>CE5</td>
<td>ilvC8, brnQ4</td>
<td>Kiritani, 1974</td>
</tr>
<tr>
<td>KA204</td>
<td>brnQ4</td>
<td>Kiritani and Ohnishi, 1977</td>
</tr>
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<td>KA224</td>
<td>ilvC8, gleR1</td>
<td>Ohnishi and Kiritani, 1978</td>
</tr>
<tr>
<td>KA2241</td>
<td>gleR1</td>
<td>Ohnishi et al., 1980</td>
</tr>
<tr>
<td>OH563</td>
<td>ilvC8, brnQ4, galE</td>
<td>galE mutant of CE5 mutagenized with EMS</td>
</tr>
<tr>
<td>SL4213</td>
<td>metA22, metE551, ilv</td>
<td>K. Kutsukake</td>
</tr>
<tr>
<td></td>
<td>xyl-104, strA120, hspLT6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hspS29, galE496</td>
<td></td>
</tr>
</tbody>
</table>
Cloning and Nucleotide sequence of the brnQ gene

Media

Nutrient broth (Difco Lab.), LB-broth (Maniatis et al., 1982), L-broth (Ohnishi and Kiritani, 1978), and 2×YT-broth (Messing, 1983) were used as complete media. The composition of a minimal medium was described previously (Kiritani, 1974). Unless otherwise mentioned, supplements to the minimal medium were used at the following concentrations (in μg/ml): L-isoleucine, 10; L-valine, 20; glycyl-L-leucine, 400; Ca-pantothenate, 1. When needed, ampicillin (50 μg/ml) or tetracycline (15 μg/ml) was added to these media. For an agar medium, 1.5% agar was added.

Isolation of a galE derivative from CE5

Since galE mutants are known as efficient transformation recipients (MacLachlan and Sanderson, 1985), a galE mutant, OH563, was isolated from CE5 using the direct selection method of Wilkinson et al. (1972). Cells of CE5 were mutagenized with ethyl methanesulfonate (EMS) by the method described previously (Kiritani, 1974). The mutagenized cells were infected with phage Felix O-1, and grown in nutrient broth to select for phage-resistant cells. The cells were then spread on nutrient agar, and colonies grown on the plate were replica-plated on minimal agar supplemented with large amounts of isoleucine (100 μg/ml) and valine (200 μg/ml), and on the agar where the glucose was substituted with galactose (5 μg/ml). After 24 h of incubation at 37°C, galactose-nonusers (Gal-) were isolated and purified. Among these Gal- mutants, OH563 showed the "galactose-induced-bacteriolysis" phenotype due to a galE mutation (Fukasawa and Nikaido, 1961). The activity of galactose-4-epimerase in OH563 was not measured.

Transport assay

Transport activity of bacteria for branched-chain amino acids was measured as described previously (Ohnishi et al., 1980).

Enzymes and chemicals

Restriction endonucleases, T4 ligase, Klenow fragment of DNA polymerase I, reverse transcriptase, S1 nuclease, mung-bean nuclease, and T4 polynucleotide kinase were purchased from either Takara Shuzo Co. or Nippon Gene Co., calf thymus alkaline phosphatase from Boehringer Mannheim Biochemicals Co., and [γ-32P]ATP (>5,000 Ci/mmol), [α-32P]dCTP (>400 Ci/mmol), [α-35S]dCTP (>400 Ci/mmol), L-[14C]isoleucine and L-[14C]leucine from Amersham Co. Agarose was the product of Bethesda Research Lab. All other chemicals were commercial materials of analytical grade.
Isolation of chromosomal and plasmid DNAs

To obtain a chromosomal DNA preparation from KA224, the method of Smith (1967) was slightly modified. Cells grown in LB-broth to an optical density at 560 nm = 1 (about 5 x 10^8 cells/ml) were collected, and washed once with a half volume of ice-cold 0.03 M Tris-HCl buffer (pH 8.3) containing 0.01 M ethylenediaminetetraacetic acid (EDTA). The cells were treated with lysozyme (final concentration: 100 μg/ml) for 3 min at 37°C and lysed by addition of sodium dodecyl sulfate (SDS) (final concentration: 0.5%). The viscous lysate was extracted once with the same volume of phenol-chloroform (1:1, v/v), and DNA was precipitated with 3 volumes of 95% ethanol. The DNA was spooled out on a glass rod and dissolved in a small volume of 0.01 M Tris-HCl buffer (pH 8.0) containing 1 mM EDTA (TE buffer). The procedure of extraction and precipitation was repeated once again, and the DNA was redissolved in TE buffer.

The vector plasmid, pBR322, was prepared from strain SL4213 harboring the plasmid by alkaline lysis method of Maniatis et al. (1982). Recombinant plasmid DNAs were prepared by the rapid, small-scale isolation method of Maniatis et al. (1982) after copy numbers of the plasmid in host cells were amplified with chloramphenicol (200 μg/ml).

Isolation of glycylleucine-resistant (Gle⁺) transformants by shot-gun cloning

pBR322 DNA (1 μg) and chromosomal DNA (4 μg) of KA224 were mixed and digested with PstI endonuclease (Takara Shuzo Co.) by the supplier's instructions. After phenol-chloroform extraction and ethanol precipitation, the DNA fragments were ligated with excess T4 DNA ligase at 10°C overnight, and the ligase was inactivated by heating at 65°C for 15 min. Transformation of competent OH563 cells with the recombinant plasmid DNAs was carried out by the method of MacLachlan and Sanderson (1985). The transformed cells were grown on minimal agar containing isoleucine, valine, glycylleucine, and tetracycline, and colonies formed on the plate were isolated, purified, and used as Gle⁺ transformants.

DNA sequencing

DNA sequencing was carried out by the method of Sanger (1981) using an universal primer or chemically synthesized primers. The sequences of the primers were 5’CTGGGATTGCGATTGTA3’ for the primer A, 5’AAGCAAGGTGGGATCTA3’ for the primer B, 5’ATCGCGCGATTATTAACGTGATG3’ for the primer C, and 5’CGTTGCTGATTTACA3’ for the primer D. These primers were synthesized with a DNA synthesizer apparatus from Bechman Co., and purified by chromatography using a Shim-pack CLC-ODS column attached to a Bechman 332 HPLC system. The 2.1-kb SalI-EcoRI fragment of pOH70 was digested with an appropriate restriction endonuclease, and the resultant fragments were cloned into phages M13mp18 and M13mp19.
Cloning and Nucleotide sequence of the *brnQ* gene

To analyze the DNA sequence of the 1.2-kb *Bst*EII-*Pst*I region of *pOH56*, the kilo-sequencing method of Henikoff (1984) was used. The 1.65-kb *Hind*III-*Pst*I fragment of *pOH56* was cloned into M13mp19. The RF (replicative form) phage DNA was extracted from the infected cells, purified, and cleaved at the multi-cloning site with *Sal*I and *Kpn*I endonucleases. The *Sal*I-*Kpn*I fragments were digested with exonuclease III and then with mung-bean nuclease to produce various lengths of deletions from the *Sal*I site. Both ends of the fragments were repaired with the Klenow fragment and self-ligated with T4 ligase.

Template DNA for the dideoxy chain-termination sequencing reaction was isolated by the method of Messing (1983). A Deaza-sequencing kit of Takara Shuzo Co. and a Sequencing kit of Nippon Gene Co. were used in most sequencing analyses. The complete DNA sequence was analyzed using the DNA analysis program from Software Development Co.

**Preparation of RNA**

Strains used for in vivo RNA preparations were Wild-type, KA224, and KA224 harboring *pOH56*. Strain Wild-type was grown in a minimal medium, and the other strains in the medium supplemented with isoleucine, valine, and pantothenate. Ampicillin was also added to the medium for KA224 harboring *pOH56* for its selected growth. Cells in exponential phase (OD_{660nm} = 0.3 to 0.4) in a 250 ml culture were harvested, and washed once with 50 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and 1 mM EDTA. RNAs were prepared from these cells by the method of Aiba et al. (1981).

**Preparation of end-labeled DNA fragments for S1 mapping analysis**

Plasmid *pOH61* was linearized with *EcoRV* and purified by agarose gel electrophoresis. The DNA was dissolved in 55 μl of 50 mM Tris-HCl buffer (pH 9.0) containing 1 mM MgCl$_2$, 0.1 mM ZnCl$_2$, and 1 mM spermidine, and treated with 0.7 U of calf intestinal alkaline phosphatase at 37°C for 30 min. The alkaline phosphatase in the reaction mixture was inactivated by heating at 68°C for 15 min. After phenol-chloroform extraction and ethanol precipitation, the DNA was digested with *Sal*I, and the 0.8-kb *Sal*I-*EcoRV* fragments were purified, then dissolved in 50 μl of 65 mM Tris-HCl buffer (pH 9.5) containing 10 mM MgCl$_2$, 5 mM dithiothreitol, 5% glycerol, and [γ-32P]ATP (100 μCi). The *Sal*I-*EcoRV* fragment was treated with 20 U of T4 polynucleotide kinase at 37°C for 30 min, and the reaction was terminated by the addition of 20 mM EDTA. The DNA fragment, 32P-labeled at its *EcoRV* 5' end, was separated from unreacted [γ-32P]ATP by passing the reaction mixture through a small column of Sephadex G-50 by centrifugation.

**S1 mapping**

The method described by Aiba et al. (1981) was slightly modified. In general,
the end-labeled fragment (1.1 × 10^6 cpm/110 ng) and 300 μg of RNA were lyophilized and dissolved in 30 μl of hybridization buffer, containing 20 mM N-(2-hydroxyl)piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 6.5), 80% formamide, and 0.4 M NaCl. The mixture was heated at 80°C for 5 min and gradually cooled for about 4 h until it reached 37°C, and incubation was continued for another 2 h. After adding 240 μl of H2O and 30 μl of 0.3 M sodium acetate buffer (pH 4.6) containing 0.5 M NaCl, 10 mM ZnCl2, and 50% glycerol, the hybrid fragments were digested with 400 U of S1 nuclease at 37°C for 30 min, and then 20 μg of tRNA molecules were added. After phenol-chloroform extraction and ethanol precipitation, the fragments were dissolved in an appropriate amount of 80% formamide containing 50 mM Tris-HCl borate (pH 8.3), 1 mM EDTA, and 0.1% bromophenol blue, and were analyzed by electrophoresis on a 8% acrylamide-8 M urea sequencing gel.

Reverse transcriptase mapping

The annealing of primer C to template RNA and the elongation reaction were carried out as follows: RNA (4 μg) prepared from KA224 and the primer C (3.6 ng) were mixed in 6 μl of distilled water, heated at 60°C for 20 min, and gradually cooled to 25°C. The primer extension reaction was performed at 42°C for 30 min in total 10 μl of a reaction mixture containing the hybrid solution, 25 mM Tris-HCl (pH 8.3), 150 mM KCl, 10 mM MgCl2, 5 mM dithiothreitol, 0.5 mM dNTPs (dATP, dGTP, dTTP, and 20 μCi of [α-35S]dCTP) and 20 U of reverse transcriptase. To terminate the reaction, 1 μl of 5 mM dCTP was added to the mixture and incubation was continued for 30 min. The size of extended products was analyzed by electrophoresis on a 6% acrylamide-8 M urea sequencing gel.

3. RESULTS

Cloning of the brnQ gene

A GleR clone containing plasmid pOH14, which requires isoleucine and valine for growth, was isolated from OH563 (ilvC, brnQ) as a recipient (Fig. 1). By shot-gun cloning, GleR clones were found among transformants having the ampicillin-sensitive and tetracycline-resistant phenotypes at a frequency of 1.7 × 10^-3. As shown in Table 2, isoleucine uptake mediated by the LIV-II transport system was about 20- and 4-fold higher in OH563 harboring pOH14 than in KA204 and Wild-type, respectively, and comparable to that in KA2241. The isoleucine uptake by the LIV-I system, however, stayed at a low level. Binding activity to isoleucine found in osmotic-shock fluids of Wild-type cells harboring pOH14 was similar to that of the plasmid-free cells (data not shown). These lines of evidence suggest that the gleR-brnQ+ genetic region was cloned on pOH14.
Cloning and Nucleotide sequence of the brnQ gene

Localization of the gleR and brnQ mutational sites

We sought to localize the gleR and brnQ mutational sites within the chromosomal insert contained on pOH14. The physical map of pOH14 was constructed by determining the cleavage sites of restriction endonucleases, and its derivative plasmids were illustrated in Fig. 1. Plasmids pOH39 and pOH56 were constructed as recombinants between pOH14 and pBR322; a fragment of pOH14 cleaved with HindIII or SalI was ligated to the respective restriction site of pBR322. The other plasmids, except pOH70, were produced by cleaving a plasmid with an appropriate endonuclease and ligating its own ends. To construct pOH70, pOH56 was digested with Clal and BstEII, and the Clal-BstEII fragment was treated with the Klenow fragment to create blunt ends, which were then self-ligated. Among these seven derivative plasmids, pOH56 and pOH70 complemented the brnQ mutation of OH563 (Fig. 1). The OH563/pOH56 transformant took up isoleucine as efficiently as the OH563/pOH14 (Table 2).

The presence of plasmid pOH59, pOH60, or pOH61 did not complement the brnQ mutation of the host cell, and neither gave the gleR property to the cell. As a result of intragenic recombination, however, gleR and/or brnQ+ recombinants could be isolated from brnQ mutants harboring these plasmids. As shown in Table 3, Gle+ and BrnQ+ recombinants appeared from both CE4 and CE5 harboring pOH60, though the frequencies of Gle+ were lower than those of BrnQ+. These BrnQ+ recombinants were indistinguishable from KA931 with respect to growth on isoleucine and valine, as well as transport activity for isoleucine and leucine (Table 4). These results indicate that the 1.65-kb SalI-HindIII segment of pOH60 contains gleR, brnQ3+, and brnQ4+ sites. Growth of Gle+ recombinants obtained from KA931/pOH61 transformants was not affected by the presence of excess glycyleucine, and its transport activity for isoleucine at 115
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Table 2. Apparent Km and Vmax for transport systems of OH563 harboring plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>LIV-I</th>
<th>Isoleucine</th>
<th>LIV-II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>Vmax</td>
<td>Km</td>
</tr>
<tr>
<td>OH563/pOH14</td>
<td>0.3</td>
<td>0.3</td>
<td>25.0</td>
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<td>OH563/pOH56</td>
<td>0.2</td>
<td>0.1</td>
<td>13.8</td>
</tr>
<tr>
<td>KA204</td>
<td>0.6</td>
<td>1.5</td>
<td>9.6</td>
</tr>
<tr>
<td>KA2241</td>
<td>0.8</td>
<td>3.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.7</td>
<td>3.7</td>
<td>8.1</td>
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</table>

a) KA204, KA2241 and Wild-type were grown in minimal medium, and OH563 harboring plasmids in IVP medium.

b) Km, μM; Vmax, μmol/min/g of cells. Specific activities of L-[14C] isoleucine in the concentration ranges of 0.15 to 0.2 μM and 0.3 to 5.5 μM were 4.1×10^6 cpm/μmol and 2.6×10^6 cpm/μmol, respectively, and the concentrations above 6 μM 2.3×10^7 cpm/μmol.

μM was markedly increased (Tables 3 and 4). This indicated that the gleR site is localized within the 0.85-kb SalI-EcoRV segment, and governs the brnQ gene function in cis-acting manner. Both BrnQ+ and Gle+ recombinants were produced from CE4/pOH61 transformants at a frequency of 10^-5, though their growth on the selective media was poor (Table 3). In the Gle+ cell, isoleucine transport activity at 115 μM was low, but it was partial resistance to repression

Table 3. Occurrence of BrnQ+ and Gle+ recombinants from brnQ transport mutants harboring various plasmids

<table>
<thead>
<tr>
<th>Transformed strain</th>
<th>No. of colonies on IVP</th>
<th>Frequency of BrnQ+ recombinants</th>
<th>No. of colonies on IVgL</th>
<th>Frequency of Gle+ recombinants</th>
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</thead>
<tbody>
<tr>
<td>CE4/pOH60</td>
<td>6490</td>
<td>5.0×10^-4</td>
<td>650</td>
<td>5.1×10^-5</td>
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<td>CE4/pOH61</td>
<td>608^a)</td>
<td>2.8×10^-5</td>
<td>833^b)</td>
<td>4.6×10^-5</td>
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<tr>
<td>CE4/pOH62^b)</td>
<td>11^a)</td>
<td>5.2×10^-7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CE4/pOH59</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CE4/pOH63</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CE5/pOH60</td>
<td>7290</td>
<td>1.0×10^-3</td>
<td>4670</td>
<td>6.5×10^-4</td>
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<td>1</td>
<td>9.3×10^-3</td>
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<td>0</td>
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<tr>
<td>CE5/pOH59</td>
<td>560^a)</td>
<td>5.5×10^-5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CE5/pOH63</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>-</td>
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<td>-</td>
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<td>0</td>
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<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Number of recombinants was scored on IVP or IVgL agar, and the number of viable cells was counted on LB-agar containing an appropriate antibiotic.

a) Minute colonies. b) Plasmid pOH62 was prepared from Wild-type cells; the cloned DNA region is identical to that of pOH61.
Cloning and Nucleotide sequence of the brnQ gene

The sequence of the brnQ gene and its flanking regions was determined. The sequencing strategy is summarized in Fig. 2. Both strands of the DNA fragments or only one of the strands but with different overlapping regions were used, so that they can cover each region at least twice. In this gleR-brnQ region, we

Table 4. Generation time and transport activity of recombinants

<table>
<thead>
<tr>
<th>Recombinant or strain</th>
<th>Marker selected (Relevant genotype)</th>
<th>gL (5 mM)</th>
<th>Generation time (min) in IVP</th>
<th>Transport activity (μmol/min/g cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Isoleucine</td>
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<td>0.19 μM</td>
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<td>−</td>
<td>70</td>
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<td>(brnQ⁴⁺)</td>
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<td>+</td>
<td>225</td>
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<td>CE4/pOH61a)</td>
<td>Gle⁺</td>
<td>−</td>
<td>−</td>
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<td>(gleR brnQ)</td>
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<td>−</td>
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<td>60</td>
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<tr>
<td>(gleR brnQ)</td>
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<td>65</td>
<td>0.99</td>
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<td>65</td>
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<td>(brnQ⁴⁻)</td>
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<td>360</td>
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<tr>
<td>CE4⁴</td>
<td>(brnQ)</td>
<td>−</td>
<td>−</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>−</td>
<td>NM²⁰</td>
</tr>
</tbody>
</table>

The values of generation time and transport activity were the average of those obtained with 3 to 6 recombinants.

a) Cells were grown in a minimal medium supplemented with glycerol-L-isoleucine (20 μg/ml), glycerol-L-valine (40 μg/ml) and pantothenate, or with these substances plus glycerol-L-valine as indicated.
b) NM; not measurable.

by glycyllleucine; i.e., the residual LIV-II activity was enhanced (Table 4). The CE4/pOH61 transformant, which carries the wild-type SalI-EcoRV segment on the plasmid, yielded only BrnQ⁺ colonies at a frequency of 10⁻⁷. These results imply that 1) the SalI-EcoRV segment of pOH61 contains the brnQ⁴⁺ site as well as gleR, and 2) most of the BrnQ⁺ recombinants from the CE4/pOH61 transformants carry the gleR-brnQ genotype instead of gleR-brnQ⁺. BrnQ⁺ recombinants were produced from the CE5/pOH59 transformants, so that the brnQ⁴ site can be localized within the EcoRV-HindIII segment (Table 3). A BrnQ⁺ colony from the CE5/pOH61 transformants might be due to a brnQ⁺ reverse mutation.

The SalI-HindIII fragment of pOH60 contains a promoter sequence of the brnQ gene, since the fragment can promote transcription of a promoter-deficient tet-gene (data not shown). This notion agreed well the results obtained from S1 mapping analysis as described later.
found a single open reading frame, which is considered to be the \textit{brnQ} gene. The complete nucleotide sequence of the \textit{brnQ} gene and the predicted amino acid sequence of the gene product are shown in Fig. 3. The translational initiation codon is preceded at a distance of 8 nucleotides by a potential Shine-Dalgarno sequence (AGGCAGT). The translation terminates at position 1317. A transcription termination sequence is seen at the region lying between 1341 and 1365, which is followed by a polythymidylate sequence. The region of transcription termination was confirmed by S1 mapping analysis (data not shown). A possible secondary stem-and-loop structure of mRNA can be generated with 25 nucleotides of the transcribed terminator sequence, and the free energy of the hairpin, calculated by the estimation method of Tinoco et al. (1973), is $-28.8 \text{ kcal/mol}$. The DNA sequence of \textit{brnQ} gene was estimated to encode a 439-amino-acid protein (BrnQ) with a calculated molecular weight of 46,534. The BrnQ protein appears to be a membrane-associated transport protein as it is hydrophobic. The hydropathic profile of BrnQ protein, calculated with a window of 9 residues using the method described by Kyte and Doolittle (1982), is shown in Fig. 4. The BrnQ protein is very hydrophobic, and only 13% of the total amino acid residues is polar.

\textbf{Transcription initiation sites of the \textit{brnQ} gene}

The transcription initiation sites were determined by S1 nuclease protection analysis. RNA prepared from Wild-type, KA224, or a KA224/pOH56 transformant was hybridized to the 5'-end of \textit{BrnQ} gene and the predicted amino acid sequence of the gene product are shown in Fig. 3. The translational initiation codon is preceded at a distance of 8 nucleotides by a potential Shine-Dalgarno sequence (AGGCAGT). The translation terminates at position 1317. A transcription termination sequence is seen at the region lying between 1341 and 1365, which is followed by a polythymidylate sequence. The region of transcription termination was confirmed by S1 mapping analysis (data not shown). A possible secondary stem-and-loop structure of mRNA can be generated with 25 nucleotides of the transcribed terminator sequence, and the free energy of the hairpin, calculated by the estimation method of Tinoco et al. (1973), is $-28.8 \text{ kcal/mol}$. The DNA sequence of \textit{brnQ} gene was estimated to encode a 439-amino-acid protein (BrnQ) with a calculated molecular weight of 46,534. The BrnQ protein appears to be a membrane-associated transport protein as it is hydrophobic. The hydropathic profile of BrnQ protein, calculated with a window of 9 residues using the method described by Kyte and Doolittle (1982), is shown in Fig. 4. The BrnQ protein is very hydrophobic, and only 13% of the total amino acid residues is polar.

\textit{Transcription initiation sites of the \textit{brnQ} gene}

The transcription initiation sites were determined by S1 nuclease protection analysis. RNA prepared from Wild-type, KA224, or a KA224/pOH56 transformant was hybridized to the \textit{SalI-EcoRV} DNA fragment labeled at the 5' end of \textit{EcoRV} site. S1 nuclease-protected fragments were analyzed on a sequence gel by coelectrophoresis with chemically cleaved products of the same labeled DNA fragment, and the results are shown in Fig. 5A. Three bands at positions of 169, 171, and 172 nucleotides upstream from the initiation codon were detected with
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Fig. 3. DNA nucleotide sequence of the brnQ gene and its flanking regions. The nucleotide positions are numbered from the first A residue of the ATG initiation colon. The predicted amino acid sequence of the BrnQ protein is shown below the DNA sequence. The open circles indicate the transcription start sites of the brnQ gene. The putative promoter and Shine-Dalgarno sequences are boxed. The termination colon TAA is marked with asterisks. The inverted arrows represent complementary sequences of the transcription terminator.
these hybrid fragments. Intensities of these bands from KA224/pOH56 transformant were stronger than those from the other strains. Since microheterogeneity was reported to be inherent in the S1 mapping analysis (Green and Roeder, 1980; Grosschedl and Birnstiel, 1980), reverse transcriptase mapping was also carried

![Hydrophobicity profile of the BrnQ protein.](image)

**Fig. 4.** Hydrophobicity profile of the BrnQ protein. The average hydrophobicity values were calculated at a span setting 9. The numbering is relative to the N-terminal amino acid (Met) of the predicted amino acid sequence of BrnQ protein.

![Determination of the transcription initiation sites of the brnQ gene by the S1 nuclease (A) and reverse transcriptase (B) mappings.](image)

**Fig. 5.** Determination of the transcription initiation sites of the brnQ gene by the S1 nuclease (A) and reverse transcriptase (B) mappings. A: An RNA preparation was hybridized to the end-labeled SalI-EcoRV fragment (S1 probe in Fig. 2) and digested with S1 nuclease. Lanes 2, 3, and 4; S1-protected DNA bands using RNAs from Wild-type, KA224, and KA224/pOH56 transformant, respectively. Lanes 1 and 6; products of the G+A-specific degradation reaction (Maxam and Gilbert, 1980) of the S1 probe, which provides size markers. The numbers correspond to the actual base lengths from the EcoRV site. Lane 5; labeled S1 probe. B: The primer C was annealed to RNA from KA224 and extended with reverse transcriptase. The primer C was also used for the dideoxy chain-termination to serve as size markers; the template was the SalI-EcoRV fragment. Lanes 2 through 5 represent products of the T, G, C and A dideoxy reactions, respectively. Lane 6 displays products of the reverse transcription, and lane 1 is the control (no primer). The open circles show the nucleotide residues corresponding to the bands on lane 6.
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out to determine the correct transcription initiation sites of the brnQ gene. The primer C (Fig. 2) annealed to template RNA from KA224 was extended with reverse transcriptase. The primer C was also used in the dideoxy chain termination reaction for DNA sequencing of the SalI-EcoRV fragment inserted in M13mp19. Labeled DNA fragments obtained from the two reactions were coelectrophoresed on a sequence gel. As shown in Fig. 5B, the DNA fragments, extended with reverse transcriptase, moved to positions 171, 173, and 174 nucleotides upstream from the initiation codon; the positions corresponded to A, T, and T residues of the DNA sequencing ladders, respectively.

4. DISCUSSION

The gleR-brnQ genetic region controlling the LIV-II system, responsible for branched-chain amino acid transport in S. typhimurium, has been cloned from strain KA224 (gleR) onto a vector plasmid pBR322. The 3.3-kilobase SalI-PstI fragment, cloned in the recombinant plasmid pOH56, complements the brnQ mutation of OH563 and confers a glycylleucine-resistance phenotype on the host (Fig. 1). By recombination analysis, the gleR and brnQ mutational sites can be localized within the 1.65-kilobase SalI-HindIII segment (Tables 3 and 4). The gleR site regulates the adjacent brnQ gene in cis-acting manner (Table 3), indicating that gleR is likely to be an operator-constitutive mutation.

The nucleotide sequence of the brnQ gene and its flanking regions was determined. In agreement with the data of genetic analysis, the brnQ coding sequence starts at 24 base pairs upstream from the EcoRV site, and terminates at 13 base pairs downstream from the BstEII site.

The brnQ gene product, BrnQ protein, is very hydrophobic, similar to that found in the LivH protein, which is a membrane-bound component of the LIV-I system in Escherichia coli (Nazos et al., 1986). The BrnQ protein contains several hydrophobic segments consisting of an average of 30 amino acid residues, which may span the cell membrane (Fig. 4). Comparing the amino acid sequences between the BrnQ and LivH proteins, however, no significant homology was found. Recently Uratani and Hoshino (personal communication) have determined the nucleotide sequence of the braB gene coding for a membrane component of the LIV-II system in Pseudomonas aeruginosa. The BrnQ protein of S. typhimurium shows marked homology (73%) to the BraB protein with regard to the amino acid sequence.

Transcription of the brnQ gene initiates from three distinct positions 171, 173, and 174 nucleotides upstream from the initiation codon ATG (Fig. 3). In the immediate upstream region of the transcription startpoints, we can recognize a putative promoter as a sequence consisting of the -35 hexamer (TTGCTT) and the -10 hexamer (TTAAAT or TAAATT); the -35 is separated by 17 or 18 base pairs from the -10, lying 5 or 4 base pairs upstream of the first startpoint. Since
transcription of the brnQ gene starts at multiple sites, it is also possible that RNA polymerase can recognize both the -10 sequences for its binding to DNA. At present, the meaning of the long silent region (202 to 205 base pairs long) lying between the transcription startpoints and the initiation codon is not known. The chain-growth of mRNA terminates at a putative, ρ-independent terminator sequence, which is composed of 25 nucleotides starting 20 nucleotides downstream from the termination codon TAA.

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REFERENCES


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