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Molecular Cloning of the *Escherichia coli* Glutamine Permease Operon, *glnP*, Using Mini-F Plasmid

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ABSTRACT. The genes encoding the *Escherichia coli* high-affinity transport system for glutamine, glutamine permease operon, were cloned by means of phenotypic complementation using a mini-F cloning vehicle. Plasmid pTN101 contains the structural gene for the periplasmic glutamine binding protein, *glnH*, and at least one other gene, *glnP*, also required for the permease activity. It is difficult to reclone the entire operon into high-copy number plasmids pBR322 or pUC18: The resultant plasmids are extremely unstable and are rapidly lost from the host cell. Subcloning of the operon in pBR322 resulted in the loss of Gln^+ phenotype, an ability to utilize glutamine as a sole source of carbon. Direct cloning of the permease operon by using pBR322 was unsuccessful; although three types of Gln^+ growers were obtained, they do not contain DNA in the *glnP* locus.

Key words : Glutamine permease — Glutamine binding protein — *glnP* — *glnH* — *Escherichia coli*

Glutamine is actively transported into *Escherichia coli* by a single highly specific system called glutamine permease.[1-3] This system belongs to the so-called osmotic shock-sensitive transport system characterized by high affinity for transport solute, multicomponents consisting of a periplasmic binding protein and membrane-bound proteins, and high-energy phosphate bond-driven energetics.[4-6]

Reconstitution experiments in spheroplasts and membrane vesicle system demonstrated that periplasmic glutamine binding protein and other macromolecular components are required for glutamine transport.[6-8] Genetic studies have localized glutamine permease operon *glnP* at 18 min on the *E. coli* genetic map between *bioB* and *chlE*.[3]

In this paper, we describe cloning of *glnP* by using mini-F-derived plasmid. Attempts to clone *glnP* by means of phenotypic complementation or chromosome walking by using high-copy plasmid pBR322 were unsuccessful. This inability can be attributable to the *glnH* gene whose amplification apparently is harmful to the cell.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli K-12 strains used are listed in Table 1. Plasmids used were pBR322, pUC18, pACYC184, pDF41[9] and pGHY3760

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TABLE 1. Bacterial strain

Strain	Genotype	Source or reference
JSH210	F⁻ *thi metC*	Ref. 3)
PSM2	F⁻ *thi metC glnPo*	Ref. 3)
PSM3	F⁻ *thi metC glnPo glnP*	Ref. 3)
PSM116	F⁻ *thi metC glnPo glnH*[a]	Ref. 3)
PSM219	F⁻ *thi metC* Δ(*glnP-chlE*)	Ref. 3)
PSM220	F⁻ *thi metC* Δ(*glnP-chlE*)	Ref. 3)
PSM221	F⁻ *thi metC* Δ(*glnP-chlE*)	Ref. 3)
PSM222	F⁻ *thi metC* Δ(*galKTE-glnP*)	Ref. 3)
PSM223	F⁻ *thi metC* Δ(*nadA-glnP*)	Ref. 3)
BK9MDG	F⁻ *thi metC hsdS*(r^- m^-) *endB* Δ(*glnP-chlE*)	Ref. 23)
DF1311	HfrC *pgi pgl relA tonA spoT pit*	CGSC#4877, from B. Bachmann
LCB382	F⁻ *thi argH hisG pro leuB thr purE chlA mtl xyl malA gal lacY rpsL tonA supE*	CGSC#4442, from B. Bachmann

[a]Mutation in PSM116, formerly called *glnP*,[3] is in the structural gene of glutamine binding protein and, therefore, termed *glnH* in this paper to distinguish from the mutation in PSM3.

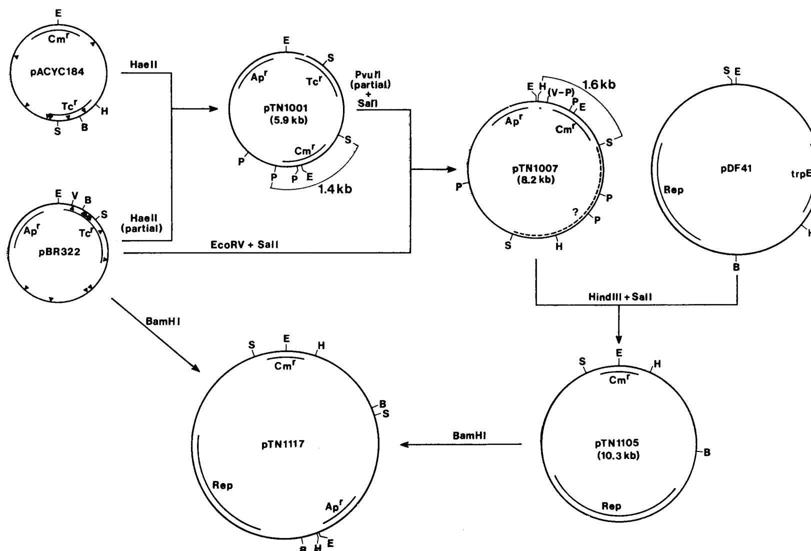

Fig. 1. Construction of pTN1105 cloning vehicle. The following abbreviations are used : E, *Eco*RI ; H, *Hind*III ; B, *Bam*HI ; S, *Sal*I ; V, *Eco*RV ; P, *Pvu*II Apʳ, ampicillin resistance ; Cmʳ chloramphenicol resistance ; Tcʳ, tetracycline resistance ; Rep, replicon of mini-F plasmid. The following symbols are used : ▲, *Hae*II site ;······, DNA from unknown origin. Plasmid pTN1001 was obtained by ligating partial *Hae*II digest of pBR322 with complete *Hae*II digest of pACYC184 and selecting Apʳ, Tcʳ and Cmʳ plasmids. This plasmid had additional *Sal*I site presumably derived from small *Hae*II fragment of pACYC184 containing *Sal*I site. The 1.4 kb *Pvu*II-*Sal*I fragment of pTN1001 containing Cmʳ gene was ligated with the 3.9 kb *Eco*RV-*Sal*I fragment of pBR322 to obtain pTN1007 which carried additional 2.9 kb *Sal*I fragment from unknown origin at *Sal*I site. The *trpE* gene of pDF41 was replaced by Cmʳ gene (1.6 kb) from pTN1007 after digestion with *Hind*III and *Sal*I to obtain pTN1105. Plasmid pTN1117 was constructed by joining pTN1105 and pBR322 at *Bam*HI site.

(from Lawrence Grossman). Plasmid pTN1105 was constructed from pDF41 by replacing *trpE*$^+$ gene with *cat* gene from pACYC184 (Fig. 1). To facilitate preparation of large quantities of DNA for cloning, pTN1117 was constructed.

Media. Minimal media contained minimal salts medium E[10] with 0.5% carbon source. When required, amino acids and thymine were added to 0.4 mM; thiamine, niacinamide and uracil to 0.05 mM; and biotin to 20 nM. Nutrient broth medium contained 8 g nutrient broth (Difco) and 5 g NaCl per liter. Luria-Bertani broth was prepared as described by Miller.[11] Solid media for plates contained 1.5% agar. When required, ampicillin, chloramphenicol and tetracycline were added to the medium to final concentrations of 25, 10 and 12.5 μg/ml, respectively. MacConkey galactose plates contained 4% MacConkey agar base (Difco) and 0.5% galactose, and EMB maltose plates contained 2.75% EMB agar base (Difco) and 0.5% maltose. Selection of Chl$^+$ transformants was performed on the lactate-nitrate medium[12] by incubating at 37°C anaerobically. Chlorate resistance (Chl$^-$) was determined on the nutrient broth medium containing 0.2% $KClO_3$ by anaerobic incubation.

Genetic procedure. Conventional genetic procedure was as described by Miller.[11] Competent cells were prepared by $CaCl_2$ treatment and transformation with plasmid DNA was carried out as described.[13]

Manipulation of DNA. Plasmid DNA was prepared from cleared lysate by CsCl-ethidium bromide centrifugation as described by Davis *et al.*[14] Rapid isolation method of plasmid DNA from 5 ml overnight culture in Luria-Bertani medium was also employed.[14] Chromosomal DNA was prepared according to Marmur.[15] The standard methods for the analysis of DNA, such as digestion with restriction enzymes, treatment with alkaline phosphatase, ligation with T4 DNA ligase, gel electrophoresis, isolation of DNA fragment from low melting point agarose gel, preparation of ^{35}S-labeled DNA probe for hybridization, etc., were adopted from Maniatis *et al.*[16]

Southern hybridization. Chromosomal DNA was digested to completion with restriction endonucleases. The digested DNA was subjected to 0.7% agarose gel electrophoresis and transferred to nitrocellulose filter for hybridization against ^{35}S-labeled DNA probe as described by Southern.[17] The plasmid DNA insert homologous with probe was used as a marker after digestion with appropriate restriction enzymes.

Colony hybridization. This was performed as described by Maniatis *et al.*[16] Plasmids were amplified by incubating colonies grown on the nitrocellulose filter paper on nutrient broth plates containing 200 μg/ml chloramphenicol before cell lysis. Chromosome walking was done by repeating colony hybridization and plasmid preparation.

Transport assay. Transport of glutamine by the whole cells was assayed as described[3] using 2 μM [^{14}C]glutamine.

Analysis of glutamine binding protein in osmotic-shock fluid. Osmotic-shock fluid was prepared from the cells grown in minimal succinate medium containing appropriate antibiotics according to the method of Willis *et al.*[18] Glutamine binding protein was partially purified by pH 4.5 treatment and $(NH_4)_2SO_4$ fractionation at 65–100% saturation. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli.[19] Protein bands were stained with Coomassie brilliant blue R-250. Presence of glutamine binding protein in the shock fluid was also examined by Ouchterlony double

diffusion test with antibody against purified glutamine binding protein.

RESULTS

Attempts to clone glnP using pBR322 as a vector. Cloning of *glnP* was first attempted by phenotypic complementation using pBR322 as vector and the *glnP–chlE* deletion strain, BK9MDG, as host. Chromosomal DNAs prepared from JSH210 and PSM2 were digested with *Pst*I and ligated to pBR322 at *Pst*I site. BK9MDG was transformed with the ligated DNA and transformants capable of growth on glutamine as a carbon source (Gln$^+$ phenotype) were selected. Several strong glutamine growers were obtained and plasmids prepared from two representatives were characterized. Plasmid pJH1 carried a 4.0 kb insert and was capable of conferring active glutamine transport to BK9MDG. Plasmid pJH90 carried a 7.5 kb insert and was incapable of conferring active glutamine transport to BK9MDG. Southern hybridization showed, however, that both inserts in pJH1 and pJH90 were present in the *glnP* deletion strains, PSM222, PSM223 and BK9MDG, indicating that these inserts do not carry *glnP.*

To facilitate cloning *glnP* it became necessary to first eliminate non-*glnP* Gln$^+$ plasmids, such as pJH1 and pH90, by screening Gln$^+$ plasmids for those carrying DNA present in wild type but absent in the deletion strains. Large scale transformation was carried out using 10–20 kb partial *Sau*3AI fragments of chromosomal DNAs prepared from JSH210 and PSM2 and ligated to *Bam*HI-cut pBR322. Glutamine growers were selected and then screened by *in situ* hybridization using the inserts of plasmid pJH1 and pJH90 as probes. Several hundred rapid glutamine growers were examined; one did not hybridize with the probes used. This plasmid pTN24 contained 13.7 kb insert DNA derived from JSH210. Fig. 2 shows the restriction maps of plasmid pTN24 and its subclones. Subclones pTN41, 42, 48 and 49 were as active as pTN24 in the ability to transform *glnP* deletion strains to Gln$^+$. However, plasmid pTN24 or the subclone was incapable of conferring the active glutamine transport. Furthermore, no glutamine binding protein could be detected in the osmotic shock fluid of PSM223/pTN41 by SDS-polyacrylamide gel electrophoresis and Ouchterlony double diffusion test.

pTN24 carried biotin operon. Plasmid pTN24 harbors *bio* operon as it confers on PSM222 and PSM223 (*Δbio*) the ability to grow without biotin in the presence of avidin (Bio$^+$). The *bio* operon is localized to the left-hand region of pTN24 (Fig. 2) since pTN41 was Bio$^-$. The proximity to *bio* operon of a gene(s), designated *glnX*, capable of conferring glutamine growth indicates that the *glnX* cannot be part of the *glnP* since *glnP* is localized at least 0.6 min away from *bio*. Furthermore, *glnX* was localized in the region between *pgl* gene and *bio* operon by chromosome walking (cf. Fig. 8). Thus, *glnX* represents yet another locus potentially involved in glutamine utilization.

Cloning glnP by chromosome walking technique. Since the use of phenotypic complementation method failed to clone *glnP*, we decided to clone *glnP* by chromosome walking technique from converging directions. The starting points were *uvrB* and *chlE* genes between which *glnP* resides (cf. Fig. 8). Hybridization probes for the *uvrB* and *chlE* genes were prepared from plasmids pGHY3760 and pTN68, respectively. Figs. 3 and 4 summarize the results of chromosome walking experiments. None of the plasmid obtained was capable

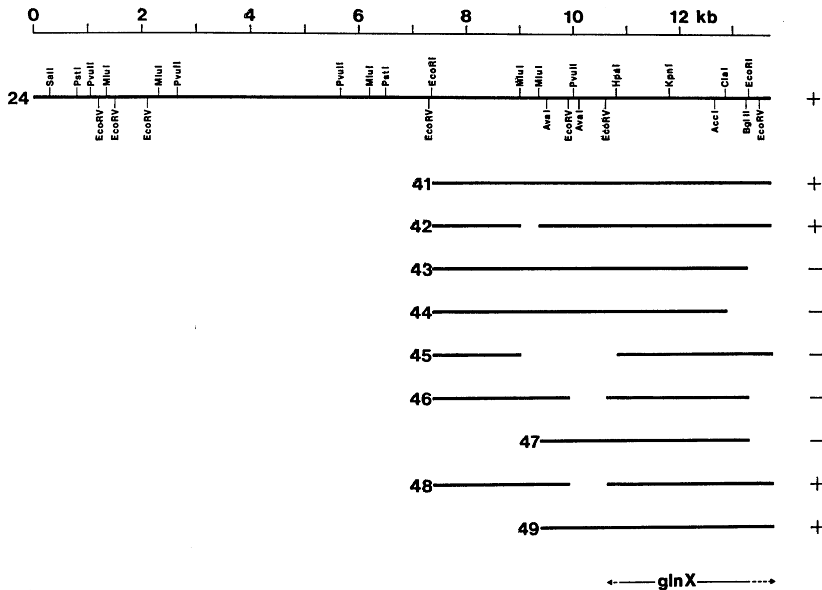

Fig. 2. Restriction map of pTN24 and its derivatives and activity to transform *glnP* deletion mutants to Gln^+. Vector pBR322 DNA is not shown. Restriction sites in the DNA insert are indicated. *Ava*I, *Hpa*I, *Kpn*I and *Acc*I sites were determined only in the region of pTN41, which carried *Eco*RI–*Sau*3AI fragment (7.3–13.7 kb) of pTN24. Plasmids pTN42 to pTN49 are deletion derivatives of pTN41. The ability of the plasmids to transform *glnP* deletion strains, BK9MDG and PSM223, to Gln^+ are shown on the right.

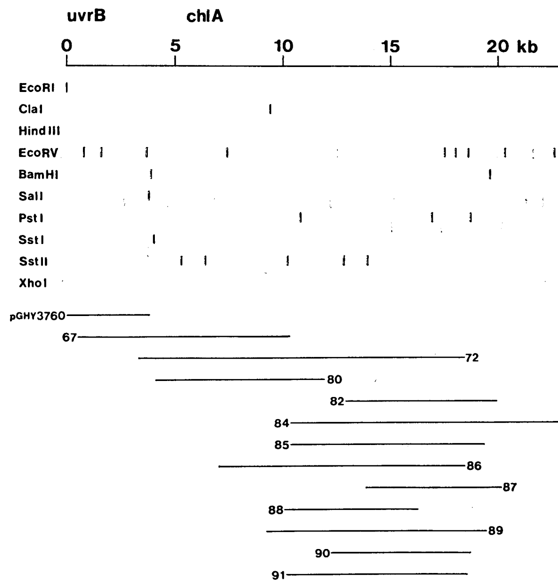

Fig. 3. Chromosome walking in the *uvrB-chlA* region and restriction map. Plasmids pTN67, pTN72 and pTN80 to pTN91 were obtained from partial *Sau*3AI digest of JSH210 chromosomal DNA ligated with pBR322 at *Bam*HI site. Vertical bars represent locations of restriction sites recognized by the enzymes shown on the left. Solid lines indicate DNA covered by the plasmids.

of transforming BK9MDG or PSM223 to Gln^+.

Plasmid pTN67 transformed LCB382 to Chl^+, indicating the presence of *chlA* gene in the insert. The *chlA* gene is known to have two *Sst*II sites 1.1 kb apart;[20] these sites are present in the plasmids, pTN67, 72 and 80. Attempts were made to clone DNA to the right of plasmid pTN84, but were unsuccessful. More than 10^4 transformants with 10–15 kb insert in pBR322 were screened by the probe prepared from 2.3 kb *Eco*RV fragment of pTN84 (corresponding to the 20.3–22.6 kb in Fig. 3), but none of the plasmids prepared from the positive colonies carried DNA insert extending beyond the region already covered by pTN84. Attempts were also made to clone DNA to the left of pTN93 and the right of pTN76 (Fig. 4) using as probes 2.4 kb *Bam*HI–*Eco*RV fragment of pTN93 (0.0–2.4 kb in Fig. 4) and 2.8 kb *Eco*RI–*Hind*III fragment of pTN76 (23.3–26.1 kb in Fig. 4), respectively. None was obtained.

The directionality of the cloned DNA in the *chlE* region relative to *glnP* and *uvrB* was determined by using probes prepared from pTN76 and pTN93 and chromosomal DNAs prepared from *glnP-chlE* deletion strains. The pTN76 probe hybridize with chromosomal DNA obtained from BK9MDG, whereas the pTN93 probe did not hybridize with the DNAs from BK9MDG, PSM219, PSM220, or PSM221. The results demonstrate that *glnP* genes, and therefore *uvrB* gene, were localized to the left-hand region of pTN93 in Fig. 4. The results of Southern hybridization analyses using the pTN93 and pTN84 probes demonstrate that (i) the *gal-bio-glnP* deletions in PSM222 and PSM223 do not extend into the *chlE* region covered by pTN93; (ii) the *glnP-chlE* deletions in BK9MDG, PSM219 and PSM220 do not extend into the *chlA* region covered by pTN84; (iii) the starting point of the deletion in PSM221 is in the region between two *Eco*RV sites at 20.3 and 22.6 kb in Fig. 3; and (iv) this 2.3 kb

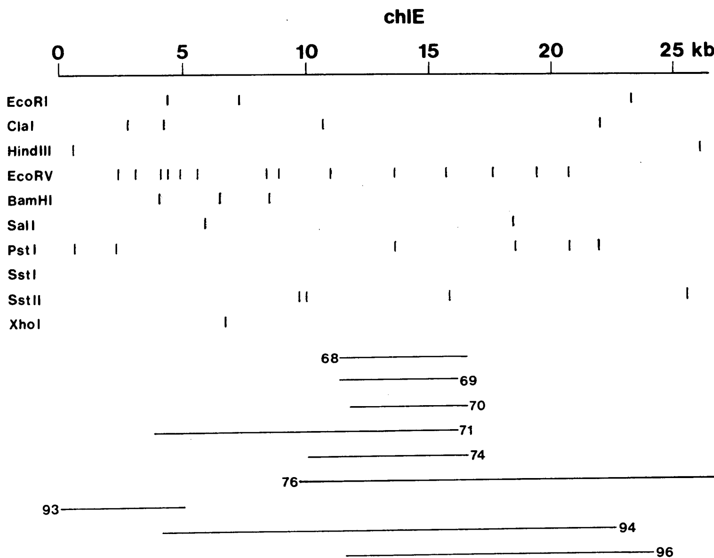

Fig. 4. Chromosome walking in the *chlE* region and restriction map. Plasmids pTN68 to pTN71, pTN74, pTN76 and pTN93 to pTN96 were obtained as in Fig. 3. Vertical bars indicate locations of restriction sites recognized by the enzymes shown on the left. Solid lines indicate DNA covered by the plasmids.

*Eco*RV sequence is absent in PSM222 and PSM223 (cf. Fig. 8).

Cloning glnP using mini-F derived plasmid as a vector. The failure to clone *glnP* by either phenotypic complementation or chromosome walking could have resulted from the use of high-copy number plasmid vector pBR322. Consequently, cloning was attempted by using a low-copy number vector. This approach should be feasible in light of the fact that Gln^- phenotype of *glnP* deletion strains could be made to Gln^+ by the transfer of F' factors covering *bio-chlE* region.[3] To facilitate cloning, the mini-F plasmid pDF41,[9] which is present in 1 or 2 copies per cell and has *trpE* marker but no antibiotic marker, was modified to suit our need. The *trpE* gene in pDF41 was replaced by the *cat* gene from pACYC184 after introduction of *Sal*I and *Hind*III sites at the ends, yielding plasmid pTN1105 (Fig. 1). Transformation of BK9MDG to chloramphenicol resistant and Gln^+ was carried out with ligated DNA of *Bam*HI-cut pTN1105 and 5-6 kb *Sau*3AI fragments of PSM2 DNA. A few colonies appeared as rapid glutamine growers out of 3×10^4 transformants. Plasmid pTN101 was one of the rapid grewers.

Plasmid pTN101 confers the ability to transport glutamine to PSM221 and BK9MDG. Furthermore, plasmid pTN101, but not pTN1105, restores the sensitivity to toxic glutamine analogs, γ-glutamylhydrazide and D-glutamine, to PSM221 and BK9MDG. This is characteristic of $glnP^+$; *glnP* mutants are known to be resistant to these toxic analogs and more than 90% of the resistant mutants were glutamine transport defective.[3]

Genetic and physical location of pTN101 insert. Fig. 5 shows the restriction maps of plasmid pTN101 and its subclones, which were constructed in pBR322 or pUC18. To examine whether the cloned DNA covered the deleted regions

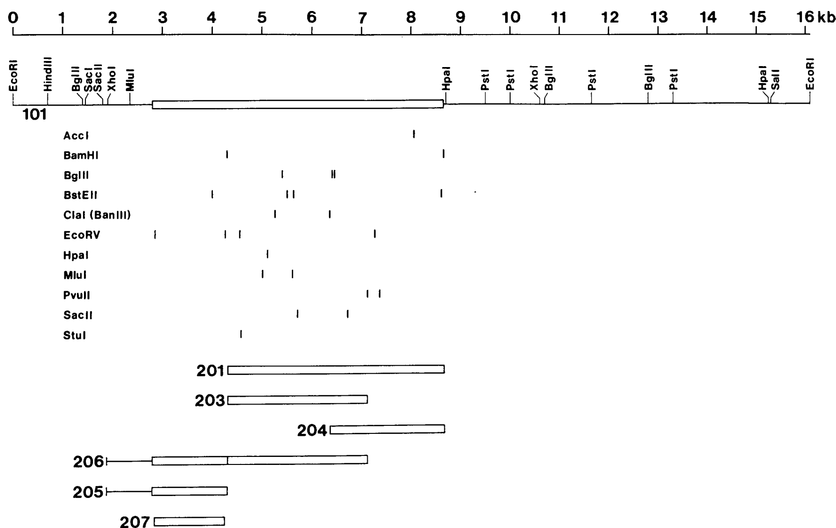

Fig. 5. Restriction map of pTN101 and its subclones. PSM2 chromosomal DNA cloned is shown by open bar. Solid line denotes DNA from pTN1105 mini-F vector. Vertical bars in the 2.8-8.8 kb region represent the locations of restriction sites recognized by the enzymes shown on the left. Plasmids pTN201 to pTN207 are deletion derivatives of pTN101 constructed in pBR322 or pUC18 vector.

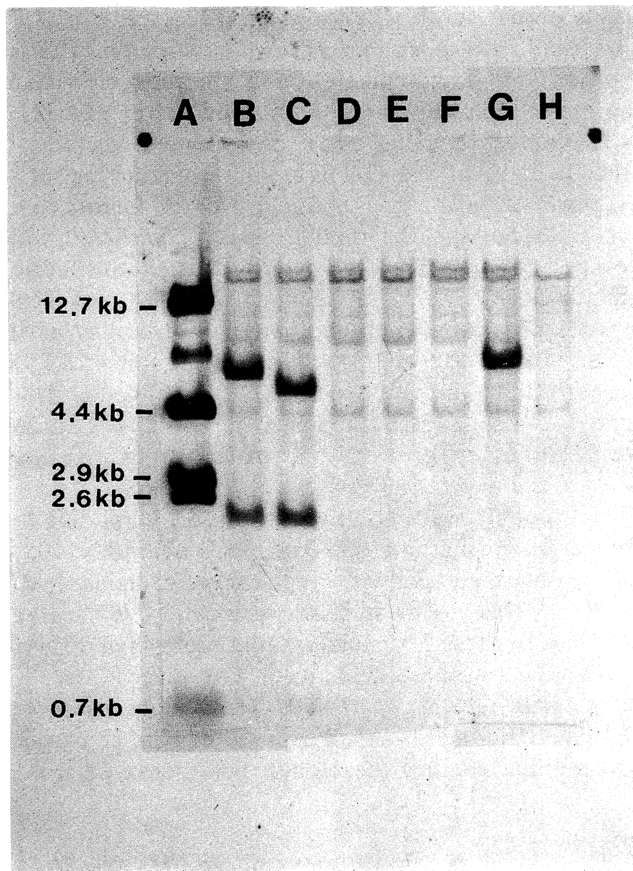

Fig. 6. Blotting hybridization of *Mlu*I digests of chromosomal DNAs with *Eco*RV fragment of pTN201 (4.5-7.3 kb region in Fig. 5) as a probe. Chromosomal DNAs were prepared from JSH210 (lane B), PSM219 (lane C), PSM220 (lane D), PSM221 (lane E), PSM222 (lane F), PSM223 (lane G) and BK9MDG (lane H). Size markers (lane A) were a mixture of digests of pTN101 with either *Bam*HI, *Eco*RV, or *Mlu*I.

in PSM219, PSM220, PSM222 and PSM223, hybridization probe was prepared from 2.8 kb *Eco*RV fragment of pTN201 (4.5–7.3 kb in Fig. 5), and hybridized with chromosomal DNAs cut with *Mlu*I. No positive band was detected with DNAs from PSM220, PSM221, PSM222, and BK9MDG (Fig. 6), indicating that this 2.8 kb sequence was totally deleted in these strains. Positive bands were observed at 6.2, 2.3 and 0.7 kb with DNA of JSH210; at 5.0, 2.3 and 0.7 kb with DNA of PSM219; and at 6.8 kb with DNA of PSM223. Because the order of these *Mlu*I fragments in the wild type must be 2.3 kb, 0.7 kb and 6.2 kb, the absence of 2.3 kb and 0.7 kb bands observed with PSM223 indicates that the deletion in this strain begins within the sequence between *Mlu*I site at 5.6 kb and *Eco*RV site at 7.3 kb in Fig. 5 and extends leftwards. The presence of 5.0 kb band instead of 6.2 kb in PSM219 indicates that the deletion in PSM219 also begins within the 2.8 kb *Eco*RV region and extends rightwards.

The starting point of the deletion in PSM219 could be mapped more precisely using DNAs digested with *Bgl*II and *Sac*II. The results localize the

starting point in PSM219 at the sequence between *Bgl*II site at 6.4 kb and *Sac*II site at 6.7 kb in Fig. 5. Since both PSM219 and PSM223 were completely devoid of periplasmic glutamine binding protein,[3,7] part of the structural gene of the binding protein should be in the region between *Bgl*II site at 6.4 kb and *Sac*II site at 6.7 kb.

Hybridization analysis using pTN207 and pTN84 probes also suggests that the gap between the inserts in plasmids, pTN207 and pTN84, was about 0.8 kb. The restriction sites on the chromosomal DNA of JSH210 which matched using these probes were *Bam*HI, *Bgl*II, *Cla*I and *Sac*II (*Sst*II) sites. On the other hand, the gap between the inserts in pTN201 and pTN93 was at least about 17 kb, since there was not matching restriction site except *Pst*I site. Therefore, chromosomal deletions in PSM220 and BK9MDG are considered to begin at a point very close to the left end of cloned DNA in pTN101.

Characterization of pTN101-derived subclones. Several subclones were constructed in either pBR322 or pUC18 (Fig. 5). Table 2 summarizes the ability of pTN101 and its subclones to complement *glnP* and *glnH* mutations.

TABLE 2. Complementation of glutamine permease mutations by pTN101-derived plasmids

Strain		Plasmid							
		pTN201	pTN203	pTN204	pTN205	pTN207	pBR322	pTN101	pTN1105
PSM2	*glnPo*	+/+[a]	N.D.[b]	N.D.	+/+	±/±	+/+	++/++	+/+
PSM3	*glnPo glnP*	±/+	±/+	−/−	−/−	N.D.	−/−	+/+	−/−
PSM116	*glnPo glnH*	+/+	+/+	+/−	±/−	±/−	±/−	++/+	±/−
PSM219	Δ(*glnP-chlE*)	±/±	±/±	−/−	N.D.	N.D.	−/−	N.D.	N.D.
PSM221	Δ(*glnP-chlE*)	−/±	−/±	N.D.	−/−	N.D.	−/−	+/+	−/−
BK9MDG	Δ(*glnP-chlE*)	−/±	−/±	−/−	−/−	−/−	−/−	+/+	−/−

[a]The first and second symbols separated by a slash indicate abilities to grow on minimal medium utilizing glutamine and glutamate, respectively, as sole carbon source. ++, rapid; +, medium; ±, slow; −, no growth after 2-3 days incubation at 37°C. [b]N.D., not determined.

Plasmids pTN201 and pTN203 complemented *glnP* deletion in PSM219, although the growth rate of PSM219 carrying these plasmids on glutamine and glutamate was lower than that of PSM2/pBR322 or PSM221/pTN101. Rapidly growing colonies were found to appear in PSM219/pTN201 and PSM219/pTN203 cell streaks, but not in the control, PSM219/pBR322. These rapid growers apparently resulted from recombination between plasmid and chromosomal DNAs. The growth rate of these rapid growers approached that of PSM221/pTN101. Since the right-hand end of the cloned DNA in pTN101, that is from *Pvu*II site at 7.1 kb to *Bam*HI site at 8.8 kb (Fig. 5), is deleted in PSM219 and pTN203, we conclude that this sequence is nonessential for *glnP* activity.

To localize the glutamine binding protein gene (*glnH*) on pTN101, complementation tests were performed with PSM116, a binding protein mutant. The results of tests performed on minimal glutamine medium were, however, somewhat equivocal due to the fact that PSM116 still grows rather well on glutamine despite the mutation.[3] Nevertheless, the ability to complement the *glnH* mutation in PSM116 can be assessed on glutamate. PSM116 has been

shown to lose concomitantly the ability to utilize glutamate as a sole carbon source.[3] As shown in Table 2, pTN201, pTN203 and pTN204, but not pTN205 and pTN207, complemented *glnH* mutation in PSM116. Another *glnP* point mutant, PSM3, which had a defect in a component other than glutamine binding protein, could be comlemented by pTN201 and pTN203 but not by pTN204 and pTN205. Thus chromosomal *glnH* and *glnP* mutations in PSM116 and PSM3 are located in the region between *Bam*HI site at 4.3 kb and *Pvu*II site at 7.1 kb in pTN101 (Fig. 5). The inability of subclones pTN201 and pTN203 to complement PSM221 and BK9MDG as tested on glutamine seemed to result from the use of high copy plasmid vector. In fact, when the DNA covered by pTN201 was cloned into pTN1105 vector, the resultant plasmid, pTN106, conferred Gln$^+$ phenotype to PSM221.

Putative destabilizer gene, glnH. Among the subclones, pTN201, pTN203, pTN204 and, especially, pTN206 were found unstable when the cells were

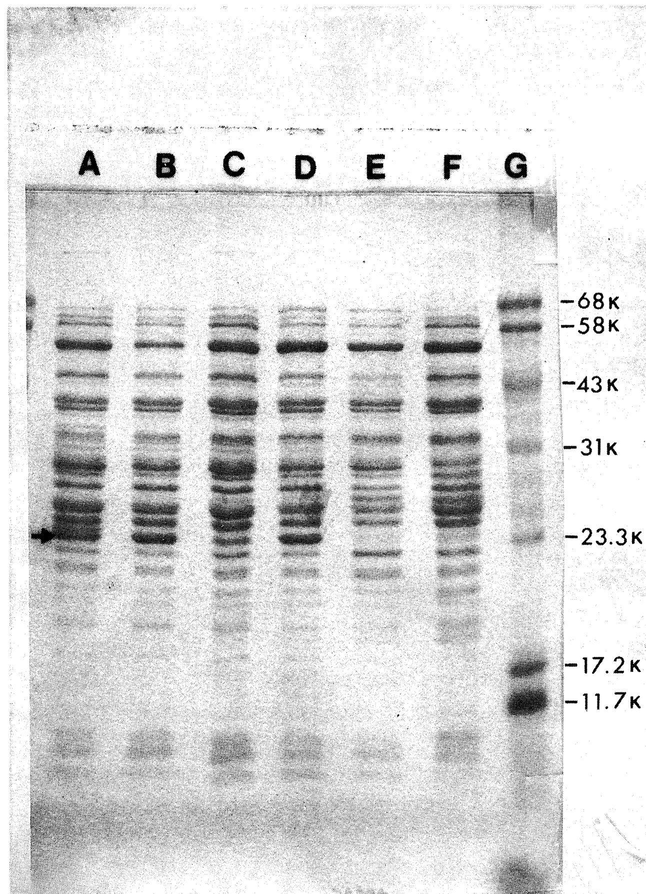

Fig. 7. SDS-polyacrylamide gel electrophoresis of partially purified osmotic-shock fluid proteins prepared from PSM221 host harboring plasmids. Shock fluid proteins from PSM2/pTN1105 (lane A, as control), PSM221/pTN101 (lane B), PSM221/pTN1105 (lane C), PSM221/pTN201 (lane D), PSM221/pTN205 (lane E) and PSM221/pBR322 (lane F) were prepared as described in MATERIALS AND METHODS. The arrow indicates glutamine binding protein. Positions of size markers (lane G) are shown on the right.

cultured in rich medium containing ampicillin. Growth of the cells harboring these plasmids was very poor, and the amount of plasmid DNA was extremely low, usually less than 10% of pBR322, pTN205 and pTN207. The DNA segment between *Cla*I site at 6.3 kb and *Pvu*II site at 7.1 kb should be responsible for destabilizing the plasmids constructed in pBR322 or pUC18, and overlaps the *glnH* gene as demonstrated above. These findings could account for the observation that the entire *glnP* operon could not be cloned in pBR322 vector by phenotypic complementation as well as chromosome walking method.

pTN101 encodes glutamine binding protein. The presence of the structural gene of glutamine binding protein in pTN101 and pTN201 was further determined by examining whether glutamine binding protein was present in the osmotic-shock fluids prepared from PSM221 host. SDS-polyacrylamide gel electrophoresis (Fig. 7) of the osmotic-shock fluids detected a strong protein band corresponding to glutamine binding protein only when the fluids were made from PSM221/pTN101 (lane B) and PSM221/pTN201 (lane D) as in the control, PSM2/pTN1105 (lane A). The apparent molecular weight of this band was estimated to be about 24,000 which is consistent with the reported value.[2] The faint band at the similar position observed with PSM221/pTN1105 (lane C) is considered to be chloramphenicol acetyltransferase produced by *cat* gene.[21,22] Presence of glutamine binding protein in the osmotic-shock fluid of PSM221/pTN101 was confirmed by Ouchterlony double diffusion test using antibodies raised against purified glutamine binding protein.

DISCUSSION

We have succeeded in cloning the *glnP* operon using the mini-F vector. Several lines of evidence indicate the presence of complete *glnP* operon in pTN101 : (i) The plasmid pTN101 confers on *glnP* deletion strains, PSM221 and BK9MDG, the ability to transport glutamine, the ability to utilize glutamine and glutamate as sole carbon source and the sensitivity to toxic glutamine analogs exhibited by wild type ; (ii) pTN101-derived subclones pTN203 complemented mutations in two representative *glnH* and *glnP* point mutants; (iii) The structural gene of glutamine binding protein, *glnH*, was shown to be present in pTN101; (iv) Plasmid pTN101 contains cloned DNA within which two chromosomal deletions with respect to *glnP*, PSM219 which carries a deletion in *glnP-chlE* region and PSM223 which carries a deletion *nadA-uvrB-chlA-glnP* region, diverge outwards.

Complementation tests presented (Table 2) indicate that at least two genes are contained in the pTN101. There are the binding protein gene designated *glnH*, the gene encoding the essential membrane component impaired in PSM3, designated *glnP*. The *glnH* and *glnP* genes are localized in the region between the *Bam*HI site at 4.3 kb and the *Pvu*II site at 7.1 kb (Fig. 5). If the glutamine transport system is typical of the osmotic-shock sensitive high affinity transport systems, then it is expected that one more gene will be found in the cloned DNA. Data on gene product identification and DNA sequencing to be published elsewhere indicate that this is indeed the case.[23]

Our inability to clone the entire glutamine permease operon by using pBR322 could have resulted from the use of such vector. It seems possible that overproduction of some proteins encoded by genes in the *glnP* region in

the high-copy number plasmid may be harmful to the cells. In fact, one of the genes constituted *glnP* operon, *glnH*, appears to be one such gene. Cells carrying *glnH*-containing plasmids pTN201, pTN203, pTN204 and pTN206 grow poorly in the presence of ampicillin and lose the plasmids rapidly. This could explain our failure to clone the *glnP* operon directly by phenotypic complementation and chromosome walking to the right of plasmid pTN84 (Fig. 3).

Existence of other "lethal" genes could account for our inability to clone DNA to the left beyond pTN93 as well as the region between *bioD* and *uvrB* by chromosome walking (Fig. 8). In the case of the latter region, Taylor *et al.*

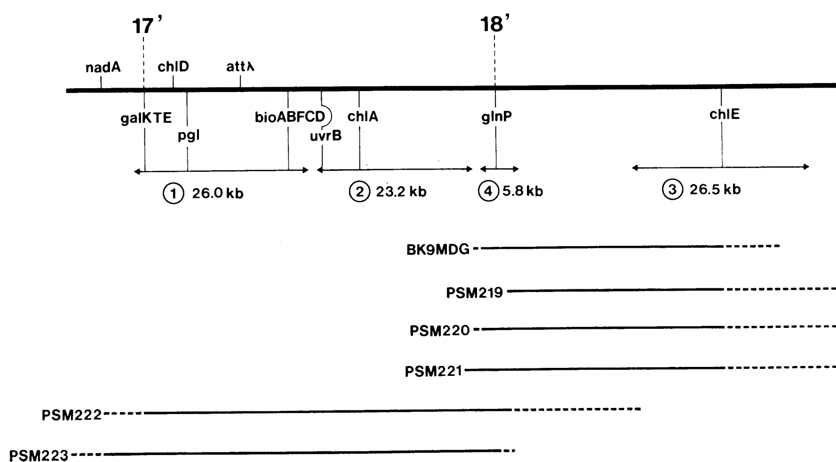

Fig. 8. Summary of mapping results of molecular cloning. A portion of the *E. coli* linkage map is shown. The arrows indicate the regions cloned in the present study: (1), from Fig. 2 plus data not presented; (2), from Fig. 3; (3) from Fig. 4; (4), from Fig. 5. The vertical bars on the arrow show genes identified on the cloned DNA. Chromosomal deletions of *glnP* deletion strains are indicated below. Solid lines denote the known extent of deletions, and broken lines represent uncertainty in the endpoints of deletions.

reported that their clone, pJT1, which covered *chlD-bioABFCD-chlA* region, was unstable whereas its subclone, pJT13, which carried *chlA* gene alone, was not.[20] Mukai *et al.* also encountered difficulty in cloning *bio* operon in ColE1 plasmid.[24] Thus some unidentified gene present in the *bioD-uvrB* region may also be responsible for destabilization of the plasmid as in the case of *glnH*.

The nature of Gln$^+$ clones obtained with pBR322 vector is unclear. Since these plasmids all carry DNA present in *glnP* deletion strains, the genes conferring the ability to utilize glutamine are apparently genes distinct from *glnP* and located elsewhere. However, it is clear that they are capable of glutamine utilization only if they are present in high gene dosage because the ability to confer Gln$^+$ is lost when the cloned DNA is recloned into pTN1105 (data not shown). The fact that BK9MDG/pJH90 is insensitive to toxic glutamine analogs indicates a transport system entirely different from the *glnP*. The transport system cloned in plasmid pJH1 represents another system, as it confers some sensitivity to the glutamine analogs. It is possible that the cloned DNA in pJH1 may contain a gene encoding a low-affinity and low-specific glutamine transport system such as glutamate-aspartate transport system[25] and methionine

transport system.[26]

The clone pTN24 appears to be an artifact of *in vitro* ligation. Preliminary results suggest this glutamine clone, *glnX*, was produced by fusion of DNA fragment derived from *pgl–chlA* region and an unknown origin, and both DNAs are essential for Gln^+ activity based on the deletion study (Fig. 2).

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