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Amplification and characterization of the proline transport carrier of *Escherichia coli* K-12 by using *proT*$^+$ hybrid plasmids

(*Escherichia coli* proline transport/gene amplification/restriction mapping/binding assay)

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ABSTRACT **A previous report [Motojima, K., Yamato, I. & Anraku, Y. (1978) *J. Bacteriol.* 136, 5–9] described the characteristics of mutants (*proT*$^-$) of *Escherichia coli* K-12 that are defective in proline transport carrier activity. Two hybrid plasmids from the Clarke and Carbon colony bank were found to complement the mutation by conjugation and transformation. Analysis with restriction endonucleases showed that both plasmid DNAs carried the same fragment of the *E. coli* chromosome. A polypeptide with a molecular weight of 24,000, specifically coded for by the *proT*$^+$ plasmid, was identified in the cytoplasmic membrane by using a double-isotope labeling method in a minicell system. The strain harboring the *proT*$^+$ plasmid has 6 times as much proline transport carrier as the wild strain. This amplification of the carrier makes it possible to measure proline binding to the carrier by microequilibrium dialysis. Detailed analysis of binding indicated that the maximal amount of proline bound to the carrier is 0.70 nmol/mg of protein of the cytoplasmic membrane of the amplified strain. From this value and the assumption that the carrier has one binding site per minimal molecular weight of 24,000, the content of the proline transport carrier in the cytoplasmic membrane was estimated to be 1.7%.**

During the last decade, basic principles of the molecular mechanisms of active solute transport across bacterial membranes have been recognized biochemically and bioenergetically (1–4). Regarding the unique ability of a carrier to couple with a proton motive force or chemical energy (4), it now seems attractive to find out what the intrinsic attributes are that make a carrier function as a chemosmotic pump. Recent findings of a protonation-dependent mechanism of substrate binding by carrier (5) and possible cooperative control of the uptake rate by sodium ion (6), as well as improved methods for purifying alanine translocating membrane proteins (7, 8), may offer important clues to answer this question.

Of the many transport carriers that have been well-characterized kinetically or genetically, only the glucose carrier of human erythrocytes has been purified to near homogeneity in a reconstitutively active form (9). A preliminary estimation indicated that the concentration of the lactose carrier in membranes was relatively low (10), and it is still difficult to determine the exact amount of the carriers in membranes. Attempts to purify transport carriers from the membranes of *Escherichia coli* have been hampered by many difficulties, such as the instability of the carriers in the presence of detergents, the small amounts of carriers in membranes, and the absence of convenient biochemical assay methods. To overcome these difficulties and to obtain more biochemical information on the carrier's function, we attempted to amplify the concentration of a proline carrier in the cytoplasmic membrane because we have known that it was extractable from the membranes as a reconstitutively active form (11). Recently, Weiner *et al.* (12) identified plasmids that can complement a defect in the activity of glycerol-3-phosphate transport and described a 26,000-dalton polypeptide as a possible carrier protein.

This paper reports the identification of hybrid plasmids from the Clarke and Carbon colony bank (13) that complement a defect of proline transport activity (14). Using these plasmids, we identified a 24,000-dalton polypeptide in the membranes as a *proT*$^+$-specific product. We have also established a reliable method for measuring the binding activity of proline carrier in the membrane. By application of this method, we estimated that the cytoplasmic membrane of an amplified strain contains 1.7% of this proline carrier protein, which is 6-fold more than that in the wild strain. Distinct effects of the pH of the reaction medium on the binding activity of the proline carrier are described briefly, giving additional support to previous observations (5).

MATERIALS AND METHODS

Bacterial Strains and Screening of Hybrid Plasmids. The strains of *E. coli* K-12 used are listed in Table 1. Strain P678-58 min (15) was obtained from Y. Sakakibara and was used as the parent of MinS. All of the 2000 F$^+$ strains harboring the ColE1 hybrid plasmids in the Clarke and Carbon colony bank (13) were screened by conjugation with strains PT19, PT21, and PT22 as recipients according to the published method (16), and the strains that complemented *proT* and *proA* were isolated. JA200/pLC10-15 was isolated as a strain carrying the *xyl*$^+$ plasmid as reported (13). Cells were grown as described (14), and the *ProT*$^-$ phenotype was defined as the requirement for a high concentration of L-proline (400 μg/ml) for growth (14).

Characterization of Plasmid DNAs. Amplification of ColE1 and ColE1-hybrid plasmids was carried out as described by Clewell (17). The DNAs were prepared by CsCl density gradient centrifugation by the method of Sakakibara and Tomizawa (18). For electron microscopic examination the plasmid DNAs were treated by the method of Kleinschmidt and Zahn (19) with some modifications as described by Nishimura *et al.* (16). The specimens were examined and photographed with a JEM100B electron microscope at a magnification of ×10,000. The contour length of the DNA molecules was measured as described (16). Transformation with the plasmid DNAs was carried out as described by Wensink *et al.* (20). Digestion of the DNAs by restriction endonucleases was performed at 37 °C for 60 min, and reactions were terminated by heating at 65 °C for 10 min as described (16). The restriction endonucleases used were purchased from Miles Laboratories or New England

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Table 1. Strains of *E. coli* K-12 used

Strain	Relevant Genotype	Source
PT19	*proA proT xyl*	Motojima *et al.* (14)
PT21	*proA proT xyl*	Motojima *et al.* (14)
PT22	*proA proT xyl*	Motojima *et al.* (14)
JA200/pLC4-45	ColE1-*proT*$^+$	Clarke and Carbon (13)
JA200/pLC35-38	ColE1-*proT*$^+$	Clarke and Carbon (13)
JA200/pLC10-15	ColE1-*xyl*$^+$	Clarke and Carbon (13)
P678-58min	*min thr leu xyl*	Adler *et al.* (15)
MinS	*min thr leu xyl str*	This study
MisS/pLC4-45	*min thr leu xyl str*/ColE1-*proT*$^+$	This study
MinS/pLC35-38	*min thr leu xyl str*/ColE1-*proT*$^+$	This study
MinS/pLC10-15	*min thr leu xyl str*/ColE1-*xyl*$^+$	This study

BioLabs. The linear polydeoxynucleotide fragments obtained by endonuclease digestion were analyzed electrophoretically in 0.5 or 0.7% agarose gel by the method of Sharp *et al.* (21).

Identification of *proT* Product. Minicells were purified from culture medium by a modification of the procedure of Roozen *et al.* (22), with three cycles of centrifugation on 15% (wt/vol) sucrose solution in place of a linear sucrose gradient. The purified minicells, which contained no more than 1 viable cell per 10^7 cells, were suspended (8 mg of cell protein per ml) in Davis minimal salts medium (23) supplemented with 0.5% glucose, 2 μg of vitamin B-1 per ml and 20 μg each of 19 amino acids other than leucine per ml. The mixtures were preincubated for 30 min at 37°C and then the proteins synthesized under these conditions were labeled for 90 min at 37°C by adding either 10 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]-leucine or 2 μCi of [^{14}C]leucine per ml of medium. During this incubation period, labeled leucines were incorporated linearly into the hot trichloroactic acid-insoluble fraction. After incubation, the minicells were collected and the membrane fraction was obtained by three cycles of sonication and washing with 10 mM Tris·HCl at pH 7.2. The membranes were then subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (24) and the distribution of radioactivities was determined with purified *E. coli* H^+-ATPase (25) as an internal standard of molecular weight.

Assays and Chemicals. Cytoplasmic membrane vesicles were prepared by the method of Yamato *et al.* (26). Specific binding of proline or serine to carriers of the cytoplasmic membrane vesicles was measured by equilibrium dialysis with microdialysis cells as described (27). Standard assay conditions were as follows. Membrane vesicles (50–200 μg of protein per 30 μl) and 1 μM uncoupler SF6847 were placed in one compartment of the dialysis cell and dialyzed for >8 hr at 4°C against the buffer in the other, which contained 1–2 μM isotope-labeled amino acid. The buffers used were 100 mM sodium acetate and 100 mM sodium phosphate at the pH values indicated in the legends to figures. Under these conditions, equilibrium of binding was achieved after 6 hr of dialysis and >98% of the bound substrate was recovered in the dialyzed solution as an unmodified amino acid upon further dialysis for 2 hr with 200-fold excess nonlabeled substrate. The activities of D-lactate-driven amino acid uptake by the vesicles (28) and of D-lactate and succinate oxidases of the vesicles (29) were measured by published methods. Protein was determined by the method of Lowry *et al.* (30).

L-[*U*-^{14}C]proline (163 mCi/mmol), L-[*U*-^{14}C]serine (130 mCi/mmol), L-[*U*-^{14}C]leucine (298 mCi/mmol), and L-[3,4,5-^{3}H]leucine (110 Ci/mmol) were purchased from Daiichi Chemicals, Tokyo. The other reagents used were standard commercial products of analytical grade.

Table 2. Screening of hybrid plasmids in Clarke and Carbon colony bank: Conjugation

Recipient	Selection	pLC*
PT19, PT21, PT22 (*proA proT*)	ProT$^+$	4-45
		35-38
PT19 (*proA proT*)	Pro$^+$	28-33
		44-11
MinS (*xyl*)	Xyl$^+$	10-15

* Plasmids of L. Clarke; see ref. 13.

RESULTS

Hybrid Plasmids. Two strains, JA200/pLC4-45 and JA200/pLC35-38, specifically complemented the *proT* defect of recipient cells by conjugation and transformation (Tables 2 and 3). Considering the properties of the *proT* mutants used as recipients (14), we assume that both the hybrid plasmids carried the structural gene for the proline transport carrier. In the course of this screening we also confirmed the previous result (13) that pLC28-33 and pLC44-11 complemented *proA* and pLC10-15 complemented *xyl*.

Characterization of Hybrid Plasmid DNAs. Table 4 and Fig. 1 summarize the physicochemical structure of the hybrid plasmid DNAs studied by electron microscopy and by digestion with restriction endonucleases. An open circle plasmid DNA was prepared by γ-irradiation of purified, covalently closed circular DNA of each plasmid. Electron micrographs (not shown) were taken with open circular ColE1 DNA as an internal standard, and the contour lengths of the plasmid DNAs were measured (Table 4). The molecular weights of the inserted chromosomal DNAs in the hybrid plasmid in strains JA200/pLC4-45 and JA200/pLC35-38 were calculated as 4.4 and 4.1 $\times 10^6$, respectively.

Partial restriction maps of these plasmid DNAs were obtained by using several restriction endonucleases. The chromosomal segments both had no site for *Bam*HI or *Xho* I, one for *Xba* I and *Bgl* II, and two for *Hpa* I (data not shown), although the relative order of these sites was not determined. Differences in the numbers of restriction sites were observed only when the plasmid DNAs were digested with *Sal* I, *Bgl* I, or *Xma* I (see Fig. 1). On the basis of these and other results, partial restriction maps of the plasmid DNAs were constructed as shown in Fig. 1. About 3.5 megadalton of the chromosomal segments of *E.*

Table 3. Screening of hybrid plasmids in Clarke and Carbon colony bank: Transformation

Plasmid DNA	Recipient	Selection	Transformants per μg DNA
pLC4-45	PT21	ProT$^+$	1.3×10^4
pLC35-38	PT21	ProT$^+$	2.5×10^4

Table 4. Size of the hybrid plasmids

Plasmid	Contour length,* nm	Molecular weight,* $\times 10^{-6}$	Molecular weight of chromosome moiety, $\times 10^{-6}$
pLC4-45	3.88 ± 0.16	8.58 ± 0.35	4.4
(ColE1)	(1.88 ± 0.11)	(4.20 ± 0.10)†	—
pLC35-38	3.91 ± 0.16	8.29 ± 0.34	4.1
(ColE1)	(1.98 ± 0.10)	(4.20 ± 0.10)†	—

* Mean ± SD.
† From Clewell and Helinski (31).

coli are common to both hybrid plasmids, and these common segments contain one restriction site for *Pst* I and *Hin*dIII.

Identification of *proT* Product. Judging from the size of the inserted segments of the *E. coli* chromosome, either plasmid should be able to code for as many as 10 polypeptides, one of which may be a proline carrier protein. A minicell system of strain MinS/pLC4-45 for protein synthesis was used to identify the *proT* product specifically. As a control plasmid, pLC10-15 which complements *xyl* but not *proT* (Table 2) was chosen. Minicells containing the *xyl*$^+$ plasmid were labeled with [^{3}H]leucine, and those containing the *proT*$^+$ plasmid were labeled with [^{14}C]leucine. Samples of membrane fractions from minicells containing these plasmids were mixed and subjected to electrophoresis in a sodium dodecyl sulfate/polyacrylamide gel. Then the gel was sectioned and the radioactivity was analyzed. When the ratio of the radioactivity of ^{14}C to that of ^{3}H was plotted, only the *proT* product was detectable as a peak with a high ratio. The proline transport carrier protein was demonstrated on the gel as a sharp, single peak and its molecular weight was estimated as 24,000 (Fig. 2).

Characteristics of Proline Transport in Strain MinS/pLC4-45. The activities of D-lactate and succinate oxidases and of serine and leucine uptakes were essentially similar in membrane vesicles of strains MinS/pLC4-45 and MinS, but proline transport activity was higher in those of MinS/pLC4-45 (Table 5). This is consistent with the aforementioned conclusion that integration of the *proT*$^+$ plasmid into strain MinS results in specific enrichment of the proline carrier.

The most reliable method for quantitative assay of the gene-dosage effect and of the content of proline carrier in the

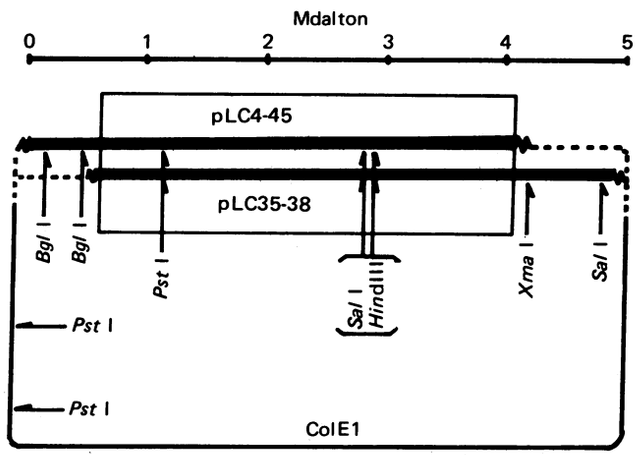

FIG. 1. Partial restriction maps of plasmid pLC4-45 and pLC35-38. The thick lines show the chromosomal segments of *E. coli* and their orientation in the ColE1 DNA, which is drawn with a thin line and with an expanded scale. The box indicates the common region of the chromosomal segments, and wavy lines show terminal dA/dT regions that are connected to ColE1 DNA as indicated by dotted lines. The bracket around *Sal* I and *Hin*dIII indicates that the relative order of restriction sites has not been determined.

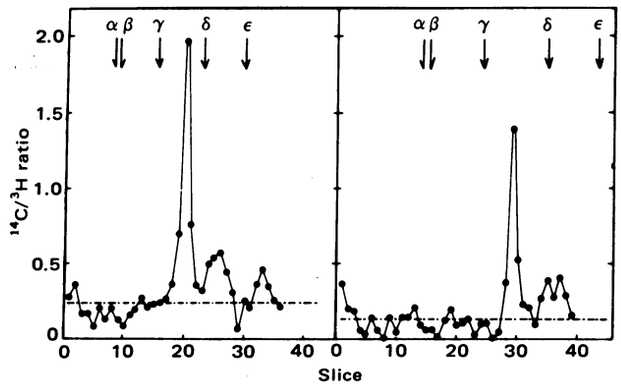

FIG. 2. Identification of a specific gene product of pLC4-45 plasmid. The polypeptides coded for by pLC4-45 DNA were labeled with [^{14}C]leucine, and those coded for by pLC10-15 DNA, with [^{3}H]leucine by using a minicell system. Sonically disrupted membranes of the two types of minicells were mixed and subjected to electrophoresis on 12% (*Left*) and 7.5% (*Right*) sodium dodecyl sulfate/polyacrylamide gels. Gels were cut into slices, and ^{3}H and ^{14}C were measured separately after treatment with an oxidizer. The broken lines indicate the ratio of the total counts recovered. Five subunits, α, β, γ, δ, and ϵ, of *E. coli* H$^+$-ATPase were used as internal standards of molecular weight; their locations are indicated by arrows.

cytoplasmic membrane is direct assay of the maximal binding sites by measuring the binding of proline to carrier (5). We have established a standard microdialysis method for specific measurement of proline binding to carriers, but not transport of the bound substrate (see *Materials and Methods*). The binding activity detected under standard conditions is stereospecific for L-proline, is inhibited completely by 200 μM *p*-chloromercuribenzoate, and is unaffected by 1–30 μM SF6847, confirming previously described properties of proline carrier (5, 11).

Results of analysis of proline binding by cytoplasmic membranes from MinS/pLC4-45 at various pH values are shown in Fig. 3. The BS_{max} for proline (0.70 nmol/mg of protein of cytoplasmic membrane) was constant between pH 5.3 and 6.5, whereas the apparent K_d values changed with change in the pH of the medium. A protonation-dependent binding mechanism, with the characteristics shown in Fig. 3, has been analyzed theoretically in a previous paper (5). Using the BS_{max} value and assuming that the proline carrier has a minimal molecular weight of 24,000 and one binding site per molecule, we calculated the content of proline carrier in the cytoplasmic membrane of the amplified strain as 1.7%.

The gene-dosage effect in strain MinS/pLC4-45 was determined by equilibrium dialysis (Table 6). We conclude that the proline carrier is amplified >6-fold in this strain by a *proT*$^+$-hybrid plasmid.

Table 5. Effect of the hybrid plasmid pLC4-45 on activities of oxidases and transport in the cytoplasmic membranes

Assay	Substrate	Strain		Ratio
		MinS/pLC4-45	MinS	
Oxidase*	D-Lactate	122.0	111.7	1.09
	Succinate	19.5	20.0	0.98
Transport†	Proline	7.30	1.60	4.56
	Serine	0.17	0.19	0.89
	Leucine	0.053	0.060	0.88

* As nmol of O_2 consumed per min/mg of protein. The concentrations of substrate used were 20 mM.
† As nmol of amino acid taken up per min/mg of protein. The concentrations of substrate used were 1 μM.

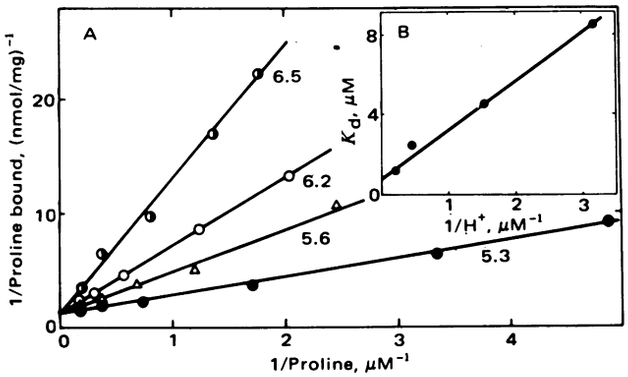

FIG. 3. Binding of proline to carrier at various pH values (*A*) and linear relationship between the observed dissociation constants, K_d, of the proline–carrier complex and the reciprocals of the proton concentration (*B*). The initial concentrations of proline used were 0.5–5.0 μM, and the concentrations of free proline after dialysis (abscissa) were calculated by assaying the radioactivity of the dialyzed solution in the compartment of the dialysis cell containing no membrane vesicles. The dissociation constants K_1 and K_2 (carrier + H$^+$ $\overset{K_1}{\rightleftharpoons}$ carrier–H$^+$; carrier–H$^+$ + proline $\overset{K_2}{\rightleftharpoons}$ carrier–H$^+$–proline, respectively) were determined to be 3.3 and 0.75 μM, respectively (see ref. 5). The buffers used were 100 mM sodium acetate at pH 5.3 or 5.6 and 100 mM sodium phosphate at pH 6.2 or 6.5.

DISCUSSION

A "bank" of *E. coli* strains that carry hybrid ColE1 plasmids, prepared by Clarke and Carbon (13), is known to have distinct advantages for amplification of particular genes, including those that code specific membrane proteins (12, 16). Hirota and coworkers (16) have demonstrated that strain JA200/pLC26-6 carrying the *ftsI* region of the *E. coli* chromosome on the plasmid overproduced the penicillin-binding protein 3 by about 10-fold more than a control cell. Weiner *et al.* (12) have shown that glycerol 3-phosphate transport activity and the protein components of this system were specifically amplified by the plasmids of this colony bank.

Two hybrid plasmids from the Clarke and Carbon colony bank were found to complement the *proT* defect of proline transport mutants (14) and to contain a common segment of *E. coli* chromosome with a molecular weight of 3.5×10^6 (Fig. 1). As expected, only the proline transport activity was appreciably enhanced in the strain carrying the plasmid (Table 5). A polypeptide with a molecular weight of 24,000 was identified in the membrane fraction of MinS/pLC4-45 as a specific product of the *proT*$^+$ plasmid (Fig. 2). Therefore, we think that this polypeptide may be the proline transport carrier protein. For conclusive evidence of this, we must examine the effect of its absence, using a mutant with a *proT* deletion from the *proT*$^+$ plasmid. The partial restriction maps of *proT*$^+$-hybrid plasmids (Fig. 1) may offer a primary information for further manipulation of the gene *proT*.

Table 6. Gene-dosage effect of the hybrid plasmid pLC4-45 on the amounts of proline transport carrier in membranes

Strain	Membrane	Binding activity,* pmol/mg protein (%)	
		Proline	Serine
MinS	Cytoplasmic	4.60 (100)	1.51 (100)
MinS/pLC4-45	Cytoplasmic	28.85 (627)	1.41 (93)
MinS/pLC4-45	Outer	0.10 (2)	ND
PT21	Cytoplasmic	0.10 (2)	ND

* Proline and serine bindings were measured at pH 5.3 (100 mM sodium acetate) and at pH 7.5 (100 mM Tris·HCl), respectively. The initial concentrations of substrate used were 1 μM proline and 2 μM serine. ND, not determined.

For biochemical studies of transport it is essential to determine the amount of transport carrier in membranes or its molecular activity during coupling with an energy source. We have previously described a procedure for measuring the binding of substrate to carriers in membranes (5), but this method requires a large amount of membranes for each assay. Specific enrichment of cytoplasmic membranes with proline transport carrier (Table 6) enabled us to measure the amount of proline bound to the carrier quantitatively by equilibrium dialysis on a micro scale. Several control experiments indicated that only the binding of substrate to carriers was measurable under standard conditions. In addition, the proline carrier was stable for a day or two, judging from the steady-state level of accumulation and the reversibility of the binding affinity upon change in the pH of the reaction medium. The binding activity was irreversibly lost when cytoplasmic membranes were exposed to a pH below 5.0. We suggest that cytoplasmic membranes (26) should be used for assay because sonically disrupted membranes contain an unidentified proline oxidase. Recently, similar, but semimicro, equilibrium dialysis has been successfully used in measurement of binding of [^{3}H]thiodigalactoside to vesicles (32) and in study of the binding properties of the lactose carrier (33).

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