Melibiose Permease and α-Galactosidase of *Escherichia coli*: Identification by Selective Labeling Using a T7 RNA Polymerase/Promoter Expression System†

Thierry Pourcher,‡ Martine Bassilana,‡ Hemanta K. Sarkar,§ H. Ronald Kaback,§ and Gérard Leblanc*,‡

Laboratoire J. Maetz, Département de Biologie du Commissariat à l'Energie Atomique, 06230 Villefranche-sur-Mer, France, and Department of Biochemistry, Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110

Received May 23, 1989; Revised Manuscript Received August 30, 1989

ABSTRACT: Identification and selective labeling of the melibiose permease and α-galactosidase in *Escherichia coli*, which are encoded by the melB and melA genes, respectively, have been accomplished by selectively labeling the two gene products with a T7 RNA polymerase expression system [Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074]. Following generation of a novel *Eco*RI restriction site in the intergenic sequence between the two genes of the mel operon by oligonucleotide-directed, site-specific mutagenesis, melA and melB were separately inserted into plasmid pT7-6 of the T7 expression system. Expression of melB was markedly enhanced by placing a strong, synthetic ribosome binding site at an optimal distance upstream from the initiation codon of melB. Expression of cloned gene products was characterized functionally and by performing autoradiographic analysis on total cell, inner membrane, and cytoplasmic proteins from cells pulse labeled with (^{35}S)methionine in the presence of rifampicin and resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The results first confirm that α-galactosidase is a cytoplasmic protein with an M_r of 50K; in contrast, the membrane-bound melibiose permease is identified as a protein with an apparent M_r of 39K, a value significantly higher than that of 30K previously suggested [Hanatani et al. (1984) *J. Biol. Chem. 259*, 1807].

Bacteria accumulate a variety of solutes by means of cation-coupled symport (cotransport) systems. A wealth of studies on the *lac* permease of *Escherichia coli*, in particular, and on permeases or carriers that transport other sugars or amino acids have convincingly established that these transporters catalyze the obligatory coupled translocation of solute with a cation in a 1:1 stoichiometry [cf. Kaback (1983), Wright et al. (1986), Henderson (1988), and Anraku et al. (1988)]. Importantly, however, the chemical identity of the coupling cation may differ from one symporter to another. Thus, while the lactose, arabinose, and xylose permeases, for example, catalyze the coupled translocation of sugar and H^+, proline is cotransported with Na^+. In this regard, the melibiose permease is of particular interest, since it catalyzes symport with H^+, Na^+, or Li^+, depending on the substrate (Tsuchiya & Wilson, 1978).

The diversity of cations used by the melibiose permease makes this transport system particularly well suited for studies on the role of cations in energy coupling by symporters. It has been reported (Damiano-Forano et al., 1986; Bassilana et al., 1985, 1987, 1988; Leblanc et al., 1988) that, depending on the coupling cation used, membrane potential ($\Delta\psi$)[1] driven active transport, sugar influx and efflux down a concentration gradient, equilibrium exchange, and sugar binding are altered differentially. These observations led to the suggestion that the coupling cation enhances the affinity of the permease for the cotransported sugar, contributes to the stabilization of the ternary complex between the cation, the sugar, and the permease, and limits the rate of permease turnover under physiological conditions because of the slow rate of cation release into the cytoplasm. In addition, it has been suggested that $\Delta\psi$ (interior negative) destabilizes the ternary complex, resulting in an increase in the rate of release of the cotransported species into the cytoplasm. Melibiose permeases with altered cation specificities have also been isolated by genetic means (Yazyu et al., 1985; Botfield & Wilson, 1988; Kawakami et al., 1988). This class of mutants should prove useful in identifying specific amino acid residues or protein domains involved in either cation recognition or the coupling mechanism.

Introduction of DNA recombinant technology has opened broad avenues for the study of symporters and their mechanism of action. Genes encoding permeases have been cloned into plasmid vectors, and DNA sequencing has led to deduction of primary amino acid sequences and hydropathy profiling as a working model for secondary structure [cf. Büchel et al. (1980), Foster et al. (1983), Yazyu et al. (1984), Nakao et al. (1987), and Maiden et al. (1988)]. Furthermore, overexpression of permeases from cloned genes has facilitated biochemical identification of the proteins involved and in some instances has led to their purification in a functional state (Kaback, 1983; Wright et al., 1986; Maiden et al., 1988; Hanada et al., 1985, 1988). Finally, use of oligonucleotide-directed, site-specific mutagenesis has led to the hypothesis that a charge relay type mechanism may be involved in lactose-coupled H^+ translocation via the *lac* permease (Kaback, 1988).

Molecular studies on the mechanism of the melibiose permease require biochemical identification of the symporter. By studying maxicells transformed with a recombinant form of pBR322 encoding melA and melB, Hanatani et al. (1984)

†This work has been supported in part by grants from NATO (0760/87) and from the Centre National de la Recherche Scientifique (associated with CEA, DO 638).

‡Département de Biologie du Commissariat à l'Energie Atomique.

§Roche Research Center.

[1] Abbreviations: $\Delta\psi$, transmembrane electrical gradient; TMG, 1-thio-β-D-galactopyranoside; RF, replicative form of M13 phage DNA; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; NPG, *p*-nitrophenyl α-D-galactopyranoside.

0006-2960/90/0429-690$02.50/0

demonstrated by complementation that melA encodes α-galactosidase and melB encodes melibiose permease. In addition, evidences were presented suggesting that the melA gene product has an M_r of 50K and the melB gene product has an apparent M_r of 30K on the basis of their electrophoretic mobilities in sodium dodecyl sulfate/polyacrylamide gels. Identification of melB gene product was unclear, however, because of the in tandem cloning procedure used and because of the presence of β-lactamase at 31K which is heavily labeled with (^{35}S)methionine. Subsequently, Yazyu et al. (1984) sequenced melB and determined a molecular weight of 52K from the deduced amino acid sequence. We now describe a procedure utilizing the T7 RNA polymerase/promoter system of Tabor and Richardson (1985) that allows unambiguous identification of the melibiose permease as the product of melB.

Materials and Methods

Materials

Restriction enzymes *Pst*I, *Eco*RI, *Hin*dIII, *Eco*RV, *Sma*I, and *Bsm*I, as well as bacteriophage T4 DNA ligase and mung bean nuclease, were from Appligene or New England Biolabs; Klenow fragment of DNA polymerase I, T4 DNA polynucleotide kinase, and calf intestinal alkaline phosphatase were from Pharmacia. Plasmids pT7-6 and pGP1-2 were gifts from S. Tabor and C. C. Richardson (Harvard Medical School), and plasmids pRC23 and pRK248 were gifts from R. Crowl (Hoffmann-La Roche, Inc.). (^{35}S)Methionine (1000 Ci/mmol), [α-^{35}S]ATP, and [γ-^{32}P]ATP (5000 Ci/mmol) were from Amersham (U.K.), and (^{14}C)methyl 1-thio-β-D-galactopyranoside (TMG) was from the Commissariat â l'-Energie Atomique (Saclay, France). All other materials were reagent grade and were obtained from commercial sources.

Bacterial Strains. The following strains of *E. coli* were used: JM 101 [supE, thi, Δ(lac-proAB)/traD36, proA$^+$B$^+$, lacI^{q}ZΔM15] (Zoller & Smith, 1983); BMH 71-18 [Δ(lac-pro)supE, thi/proA$^+$B$^+$, lacI^{q}ZΔM15/mutL:Tn10] (Kramer et al., 1984); DW2 (ΔlacZY, melA$^+$B$^-$) (Botfield & Wilson, 1988), RA11 (ΔlacZY, melA$^-$B$^+$), and JA200/pLC25-33 [gifts from T. H. Wilson (Harvard Medical School)].

Methods

Deoxyoligonucleotide Synthesis. Deoxyoligonucleotides were synthesized on an Applied Biosystems Model 381B DNA synthesizer (Foster City, CA) and purified by polyacrylamide gel electrophoresis according to the protocol described in the Applied Biosystems manual.

Recombinant DNA Techniques and Site-Directed Mutagenesis. Recombinant DNA techniques were carried out according to Maniatis et al. (1982), and mung bean nuclease treatment was performed according to the assay conditions described by New England Biolabs.

Oligonucleotide-directed, site-specific mutagenesis was performed as described (Zoller & Smith, 1983; Sarkar et al., 1985). A 3 kbp DNA fragment encoding melA and melB was excised from the Clarke and Carbon colony bank plasmid pLC25-33 (Clarke & Carbon, 1976) by using restriction enzymes *Pst*I (upstream from melA) and *Eco*RI (downstream from melB). The DNA fragment was then ligated into the replicative form (RF) of M13mp18 DNA that had been linearized with the same enzymes. An aliquot of the ligation mixture was used to transfect *E. coli* JM101, and recombinant single-stranded (ss) DNA containing the sense strand of melA and melB was isolated and used as a template for mutagenesis. A novel *Eco*RI site was created by modifying the wild-type sequence from CATTTC to GAATTC a few bases upstream from the initiation codon of melB. A 32-base synthetic deoxyribonucleotide (5′-ATACCCTATGAGG*AA*TTCA-**ATG**ACTACAAAAC-3′) complementary to the sense strand except for two mismatches (*) was used as a mutagenic primer (initiation codon in boldface). Closed circular heteroduplex DNA with the desired mutation was synthesized in vitro and used to transfect *E. coli* BMH 71-18 in order to minimize mismatch repair (Kramer et al., 1984). Phage harboring the mutation were identified initially by colony-blot hybridization using ^{32}P-labeled mutagenic primer (Carter et al., 1984), and the mutation was verified by subjecting M13mp18 recombinant RF DNA to *Eco*RI digestion and by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) using Sequenase (USB).

Selective Cloning of melA and melB into the T7 Polymerase/Promoter System. An *Eco*RI DNA fragment (1.5 kbp) containing melB and a *Pst*I–*Eco*RI DNA fragment (1.5 kbp) containing melA were successively isolated following sequential digestion of the mutated M13mp18 RF recombinant DNA with *Eco*RI and *Pst*I. The DNA fragments were ligated separately into pT7-6 plasmids that had been digested respectively with *Pst*I and *Eco*RI (for cloning melA) or linearized with *Eco*RI (for cloning melB), thereby giving rise to plasmids pT7-A and pT7-B, respectively. Orientation of melB with respect to the T7 promoter was determined by making use of a unique *Eco*RV site in the 3′ portion of melB and *Hin*dIII. For control experiments, the *Pst*I–*Eco*RI DNA fragment encompassing the wild-type mel operon (3 kbp) and containing both melA and melB was also inserted into pT7-6 (plasmid pT7-AB).

melA and melB Complementation Studies. Plasmid pT7-A, pT7-B, pT7-AB, or pT7-(SD/B) [plasmid pT7-6 containing melB with an engineered ribosome binding site (i.e., Shine–Dalgarno sequence; Shine & Dalgarno, 1974) upstream from the initiation codon; cf. Results and Discussion] was used to transform *E. coli* DW2 (melA$^+$B$^-$). Ampicillin-resistant colonies were isolated and transformed with pGP1-2 which contains the gene for T7 polymerase under the control of the heat-inducible P_L promoter of phage λ and a kanamycin resistance gene (Tabor & Richardson, 1985). The resultant strains designated DW2/pT7-A, DW2/pT7-B, and DW2/pT7-(SD/B) were grown in Luria broth at 30 °C. DW2/pT7-(SD/B) was grown at 25 °C and then shifted to 30 °C. Complementation of melB activity in these strains was estimated by measuring the time course of (^{14}C)TMG (3 mCi/mmol) accumulation in intact cells by using a rapid filtration method (Kaback, 1971). Complementation tests for α-galactosidase activity were performed in *E. coli* RA11 (melA$^-$B$^+$) transformed with pGP1-2 and one of the recombinant pT7-6 plasmids. Sugar hydrolysis was detected qualitatively on McConkey plates (Miller, 1972) or measured quantitatively by following α-galactosidase activity in cells at 37 °C according to Burstein and Kepes (1971).

Labeling of Cells with (^{35}S)Methionine and Preparation of Inner Membrane and Cytoplasmic Fractions. DW2/pT7-A, DW2/pT7-B, or DW2/pT7-AB was grown at 30 °C and DW2/pT7-(SD/B) was grown at 24 °C in 10 mL of Luria broth containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) to an optical density of about 0.5 at 600 nm. Cells were harvested by centrifugation and washed in 1× M9 salt medium. Washed cells were resuspended in 1 mL of sterile M9 medium (Miller, 1972) supplemented with Difco Methionine Assay Medium (1/5 v/v). Phenylmethanesulfonyl fluoride (PMSF, 1 mM final concentration) was added and kept constant throughout the experiment. Suspensions (1 mL each) were first incubated for 2 h at 30 °C with shaking to

minimize the cellular methionine pool and then transferred to 42 °C for 15 min to induce synthesis of T7 RNA polymerase. Rifampicin (200 μg/mL) was added, and the incubation was continued for an additional 20 min at 30 °C. An aliquot of radioactive methionine was finally added (20 nM, 1000 Ci/mmol), and after 20 min, reactions were terminated by adding an excess of unlabeled methionine (10 mM final concentration). The cells were then extensively washed with salt medium (1× M9) and frozen in liquid nitrogen until use. Cytoplasmic membranes were isolated by osmotic lysis after treatment with lysozyme/ethylenediaminetetraacetic acid (EDTA) (Kaback, 1971). Soluble proteins obtained from lysed cells were concentrated by precipitation with 10% trichloroacetic acid (TCA), followed by neutralization with sodium hydroxyde. Samples were then solubilized in 1% sodium dodecyl sulfate ($NaDodSO_4$) and incubated for 1 h at 37 °C. Proteins were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis (Laemmli, 1971) using acrylamide and bis(acrylamide) at final concentrations of 12% and 0.32%, respectively. Gels were silver stained, dried, and autoradiographed.

Protein Determinations. Protein was measured according to Lowry et al. (1951) with bovine serum albumin as standard.

Results and Discussion

The strategy used to selectively express and label the products of melA (α-galactosidase) and melB (melibiose permease) involves three operations: (i) introduction of a novel *Eco*RI restriction site in the intergenic sequence between melA and melB to enable separate handling of DNA fragments encoding each gene; (ii) independent cloning of melA and melB into pT7-6 in order to identify gene products; and (iii) improvement of melB expression by insertion of a synthetic ribosome binding site [i.e., Shine–Dalgarno sequence (Shine & Dalgarno, 1974)] in the 5′ flanking region of melB.

Creation of a Novel EcoRI Site in the Intergenic Region of melA and melB. Hanatani et al. (1984) demonstrated by complementation tests that a *Pst*I–*Eco*RI DNA fragment from one recombinant plasmid in the Clarke and Carbon colony bank (pLC25-33; Clarke & Carbon, 1976) contains the two structural genes of the mel operon, melA encoding α-galactosidase and melB encoding the melibiose permease. This 3 kbp DNA fragment, delimited by a *Pst*I site in the 5′ flanking region of melA and by an *Eco*RI site in the 3′ flanking region of melB, was isolated and ligated into the RF of M13mp18 DNA digested with the same enzymes. *E. coli* JM101 transfected with the recombinant M13mp18 DNA produced ss phage DNA containing the sense strand of melA and melB in reverse orientation with respect to the *lac* promoter. Figure 1 illustrates the modifications of the inserted sequence leading to the creation of a novel *Eco*RI site. The wild-type CATTTC sequence located a few bases upstream from the ATG initiation codon (in boldface) of melB (Yazyu et al., 1984) was mutated to GAATTC by using a 32-base synthetic deoxyribonucleotide primer complementary to the sequence of the recombinant ss DNA except for two mismatches. Recombinant M13mp18 RF DNA harboring the mutation was first digested with *Eco*RI, and the 1.5 kbp fragment containing melB (with *Eco*RI ends) and the larger 8.6 kbp DNA fragment were purified. The 8.6 kbp fragment was further digested with *Pst*I, and a second 1.5 kbp DNA fragment containing melA (with *Pst*I and *Eco*RI ends) was isolated. The DNA fragment containing melB was ligated into pT7-6, which contains the T7 promoter, after linearization with *Eco*RI, thereby giving rise to plasmid pT7-B. Orientation of melB with respect to the T7 promoter was determined by making use of the asymmetric location of a unique *Eco*RV site within melB and a *Hin*dIII site adjacent to the 3′ end of the insert. Plasmid pT7-A was constructed by ligating the DNA fragment containing melA into pT7-6 after digestion with *Eco*RI and *Pst*I. Since the ends of the melA fragment differ, forced ligation was achieved such that melA was in the correct orientation with respect to the T7 promoter. In addition, the wild-type *Pst*I–*Eco*RI 3 kbp DNA fragment containing both melA and melB was ligated into pT7-6 restricted with *Pst*I and *Eco*RI, giving rise to plasmid pT7-AB.

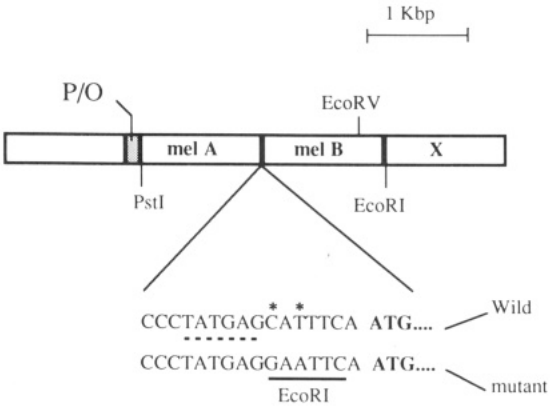

FIGURE 1: Schematic representation of the melibiose operon and positioning of the newly created *Eco*RI site in the melA–melB intergenic region. melA, melB, and possibly melX (Hanatani et al., 1984) are three successive genes composing the melibiose operon. P/O: promoter/operator of the melibiose operon. ATG (in bold): initiation codon of the melB gene. The two bases of the wild-type antisense strand which were mutagenized to give rise to the novel *Eco*RI site (thick underline) are indicated by (*). The possible Shine–Dalgarno sequence of the melB gene in the wild-type DNA sequence is indicated by the dashed underline.

Complementation Tests and (^{35}S)Methionine Labeling. E. coli DW2 ($melA^+B^-$) or RA11 ($melA^-B^+$) were transformed with pT7-A, pT7-B, or pT7-AB, and ampicillin-resistant clones were isolated and transformed with pGP1-2, which encodes T7 RNA polymerase under the control of the heat-inducible P_L promoter (Tabor & Richardson, 1985). The resulting strains are termed DW2/pT7-A, DW2/pT7-B, and DW2/pT7-AB or RA11/pT7-A, RA11/pT7-B, and RA11/pT7-AB. The T7 RNA polymerase expression system involves heat-induced derepression of the T7 polymerase gene under control of the P_L promoter (plasmid pGP1-2), followed by T7 polymerase catalyzed transcription of the gene under control of the T7 promoter (plasmid pT7-6). Moreover, since *E. coli* RNA polymerase is inhibited by rifampicin, but T7 polymerase is resistent, the system allows specific expression of the gene under control of the T7 promoter.

The ability of each plasmid construct to confer either melibiose transport or α-galactosidase activity to strains DW2 ($melA^+B^-$) or RA11 ($melA^-B^+$), respectively, was analyzed first (Table I). The results demonstrate clearly that only those RA11 transformants harboring melA or melA and melB, but not those harboring melB alone, exhibit α-galactosidase activity. In contrast, the rate and extent of TMG transport is considerably enhanced in *E. coli* DW2 harboring pT7-AB, but not pT7-A. Surprisingly, however, the transport activity of DW2/pT7-B is only about 3-fold better than that observed in DW2/pT7-6, which has no melB insert. Limited but significant melB expression in DW2/pT7-B is confirmed by the observation that the cells appear as light red colonies on McConkey plates containing melibiose after 36 h.

Expression of polypeptides encoded by melA and melB in the DW2 transformants was determined by studying (^{35}S)-

Table I: Genetic Complementation Tests[a]

plasmids	α-galactosidase activity in RA11 transformants [nmol of NPG hydrolyzed/ (mg of protein·min)]	(^{14}C)TMG accumulation in DW2 transformants [nmol/(mg of protein· 10 min)]
pT7-6	0.1	0.5
pT7-A	1.3	0.5
pT7-B	0.2	1.5
pT7-AB	0.7	40.5

[a] *E. coli* RA11 (melA$^-$B$^+$) or DW2 (melA$^+$B$^-$) cells were first transformed with each recombinant plasmid or with plasmid pT7-6 and next transformed with plasmid pGP1-2 harboring the T7 RNA polymerase gene. Cells were grown at 30 °C, washed, and resuspended in 100 mM potassium phosphate (pH 6.6) containing chloramphenicol. α-Galactosidase activity was assessed spectrophotometrically by measuring the rate of *p*-nitrophenyl α-D-galactopyranoside (NPG) hydrolysis in RA11 transformants resuspended in 100 mM potassium phosphate/10 mM $MgSO_4$ (pH 6.6) and 1.5 mM NPG at 37 °C (Burstein & Kepes, 1971). Complementation for transport activity was measured in DW2 transformants. Aliquots (50 μL) of washed cells containing 100 μg of protein in 100 mM potassium phosphate (pH 6.6)/10 mM $MgSO_4$ were assayed for (^{14}C)TMG transport at 25 °C in the presence of 0.2 mM labeled sugar (2.9 mCi/mmol), 10 mM NaCl, and 20 mM ascorbate/1 mM phenazine methosulfate (PMS) under oxygen. Accumulation is expressed as nmol/mg dry weight in 10 min. Recombinant plasmids pT7-A, pT7-B, and pT7-AB contains melA. melB, and melAB inserts, respectively; pT7-6 is the original vector without insert.

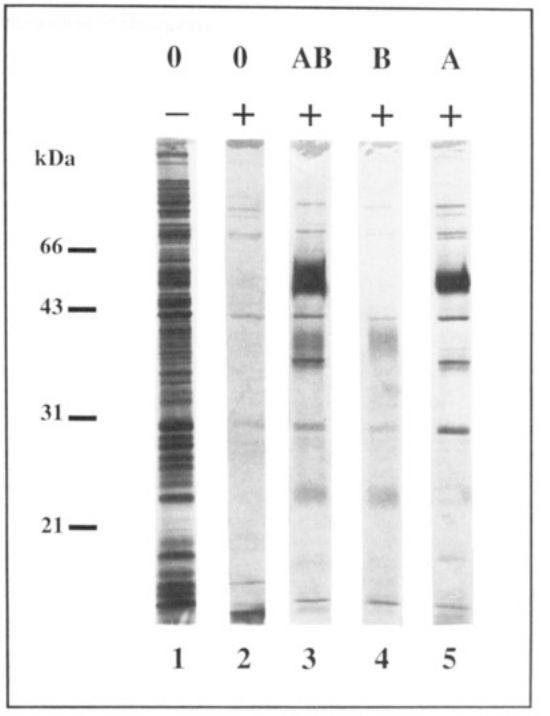

FIGURE 2: Autoradiographic analysis of the gene products encoded by plasmids pT7-A, pT7-B, and pT7-AB. pT7-6 derivatives (constructed as described under Methods and in Figure 1) and plasmid pGP1-2 were used to cotransform *E. coli* DW2. Forty minutes after heat induction, transformants were pulse-labeled with (^{35}S)methionine in the absence (−) (lane 1) or the presence (+) of rifampicin (lanes 2–5) as described under Methods. The total cell proteins were resolved on $NaDodSO_4$/polyacrylamide gel electrophoresis and identified by autoradiography. Plasmids tested were pT7-6 (0) devoid of insert (lane 1, in the absence of rifampicin; lane 2, in the presence of rifampicin); pT7-AB (AB, lane 3); pT7-B (B, lane 4); and pT7-A (A, lane 5).

Table II: Subcloning *Eco*RI–*Eco*RI melB DNA Fragment into Plasmid pRC23: DNA Sequence of the Upstream Region from the Insert (A) and Effect on melB Gene Expression (B)[a]

plasmids	Part A DNA sequence
	mel B
pT7-B	5′- AGAGGATCCCCGGGTACCGAGCTCGAATTCA ATG... -3′
pRC1E	5′- TAAGGAGGAATTCA ATG... -3′

plasmids	Part B (^{14}C)TMG accumulation [nmol/(mg of protein·10 min)]
pRC23	0.5
pRC1E	40

[a] (Part A) Upper DNA sequence: 5′ flanking region of melB gene inserted into plasmid pT7-B showing the 26 bp spacing between the initiation codon of melB (ATG in bold) and a sequence possibly acting as a Shine–Dalgarno (SD) sequence (thick underline). Lower DNA sequence: 5′ flanking region of melB gene inserted downstream from the synthetic SD sequence (thick underline) in plasmid pRC1E illustrating the reduction of spacing between ATG of melB and the synthetic SD sequence. (Part B) (^{14}C)TMG accumulation in DW2 cells cotransformed with plasmid pRK248cIts and either pRC1E (plasmid with melB insert containing the synthetic SD sequence) or pRC23 (control plasmid without insert). Cellular (^{14}C)TMG accumulation at 10 min was determined as described in Table I.

methionine incorporation. Cells were first depleted of their endogenous methionine pool and heat-shocked to induce synthesis of T7 RNA polymerase. Rifampicin was added to inhibit host cell polymerase activity, and the cells were pulse-labeled with (^{35}S)methionine for 20 min. Labeled cell proteins were then resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis and vizualized by autoradiography (Figure 2). Comparison of lanes 1 and 2 demonstrates that rifampicin almost completely inhibits (^{35}S)methionine labeling in DW2/pT7-6 except for slight incorporation into proteins migrating with M_rs of about 30K (probably β-lactamase), 43K, and 80K which contribute to the background labeling observed in other lanes. In DW2/pT7-AB, which contains melA and melB in tandem, three distinct proteins are expressed (lane 3). One band centered at about 50K is intensively labeled, while the other two at about 39K and 24K are relatively faint. More definitive identification of the products of melA and melB is obtained when the genes are expressed independently in DW2/pT7-A and DW2/pT7-B. The 50K protein appears as the major labeled band in DW2/pT7-A (lane 5) and is totaly absent in DW2/pT7-B (lane 4). When considered in conjunction with Table I, the data provide a clear demonstration that the 50K band encoded by melA is α-galactosidase. Parenthetically, the labeled material that migrates at about 35K (lane 5) is probably a degradation product of α-galactosidase; the intensity of the band increases with a concomitant decrease in the 50K band when the protease inhibitor PMSF is omitted (data not shown).

In contradistinction to the 50K band, the two less intensely labeled polypeptides at 39K and at 24K (lane 3) are detected in DW2/pT7-B (lane 4), but not in DW2/pT7-A (lane 5). Therefore, expression of these proteins appears to be correlated exclusively with the presence of melB.

Enhancement of melB Expression. TMG transport and (^{35}S)methionine labeling experiments in DW2/pT7-B indicate that melB expression is poor. Examination of the DNA sequence between the T7 promoter and the initiation codon of melB in pT7-B reveals that the initiation codon is 25 bases downstream from an AGAGG sequence which is the only purine-rich sequence that could serve as a ribosome binding site (Table II). Since 25 bases is far beyond the optimal spacing between ribosome binding sites and initiation codons in prokaryotic genes (Gold, 1988), a ribosome binding site was introduced into the 5′ flanking region of melB and its effect on functional expression and (^{35}S)methionine labeling was examined.

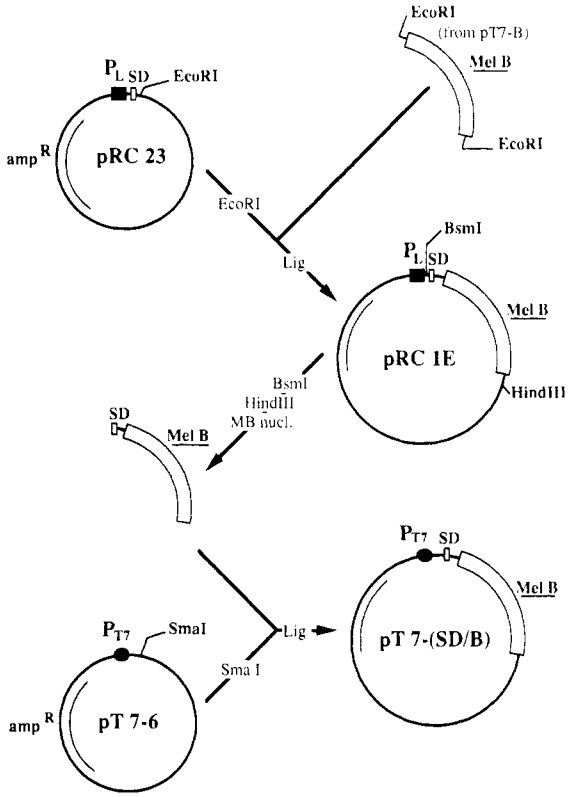

FIGURE 3: Construction of a recombinant plasmid pT7-(SD/B) containing the melB gene positioned downstream from the synthetic SD sequence of pRC23. Double-stranded melB DNA isolated from *Eco*RI-digested pT7-B plasmid was ligated (Lig) into the *Eco*RI site of plasmid pRC23. The resulting pRC1E plasmid was digested with restriction enzymes *Bsm*I and *Hin*dIII, and a 1611 bp DNA fragment containing both melB and SD was isolated. This DNA fragment was incubated with mung bean nuclease (MB nucl) to remove protruding termini and ligated into the plasmid pT7-6, which was previously restricted with restriction enzyme *Sma*I. Recombinant plasmid with the right orientation of the melB gene was selected as described under Methods. P_L and P_{T7} are λ and T7 phage promoters, respectively; SD is the synthetic Shine–Dalgarno sequence as shown in Table IIA; amp^R denotes ampicillin resistance.

Initially, the *Eco*RI DNA fragment containing melB was cloned into the *Eco*RI site of pRC23 which is downstream from a thermally inducible λ P_L promoter and a synthetic ribosome binding site (Crowl et al., 1985). In the recombinant plasmid, designated pRC1E, the initiation codon of melB is 6 bases downstream from the synthetic ribosome binding site. Expression of melB was studied in *E. coli* DW2 harboring pRK248cIts (cIts is a thermosensitive repressor of the P_L promoter). When grown on McConkey plates containing melibiose, *E. coli* DW2/pRK248cIts/pRC1E appears as white colonies at 30 °C, and the colonies become red when the growth temperature is shifted to 37 °C. In contrast, DW2/pRK248cIts/pRC23, which does not have the melB insert, grows as white colonies at both 30 and 37 °C. Furthermore, DW2 cells harboring pRC1E accumulate TMG 40–80 times better than DW2 cells harboring pRC23 (Table II). Clearly, therefore, melB is expressed more efficiently when a functional ribosome binding site is present at an appropriate distance upstream of the initiation codon.

In order to bring melB with the synthetic ribosome binding site under the control of the T7 promoter in pT7-6, pRC1E was digested with *Bsm*I and *Hin*dIII (Figure 3). The 1.6 kbp DNA fragment from pRC1E containing the ribosome binding site and melB was isolated and digested with mung bean nuclease to produce blunt ends. The modified DNA fragment was then ligated into the *Sma*I site of the polylinker region

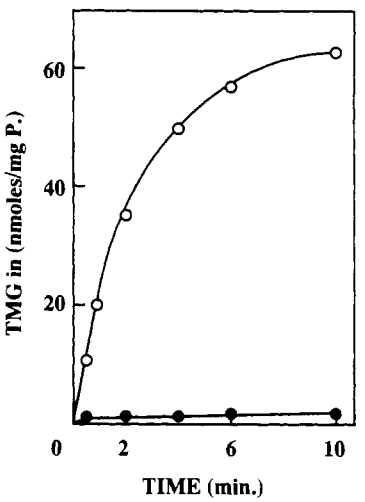

FIGURE 4: Time course of (^{14}C)TMG transport in DW2/pT7-(SD/B) and DW2/pT7-6 cells. Cells were initially grown in Luria broth at 25 °C and then shifted to 30 °C for 1 h. TMG transport was measured in the presence of 0.2 mM (^{14}C)TMG and 10 mM NaCl as described in Table I. (○) DW2/pT7-(SD/B) cells; (●) DW2/pT7-6 cells. Accumulation is expressed as nmol/mg dry weight of cells.

of pT7-6 (Tabor & Richardson, 1985). The resulting plasmid pT7-(SD/B), which contains the ribosome binding site/melB construction correctly oriented with respect to the T7 promoter, was selected by making use of the unique *Eco*RV site in the 3′ end of the melB and *Hin*dIII (Figure 1). *E. coli* DW2 transformed successively with pT7-(SD/B) and pGP1-2 are designated DW2/pT7-(SD/B).

Efficient expression of melB in DW2/pT7-(SD/B) is suggested by the growth characteristics of the cells as a function of temperature. Optimal growth is observed at 25 or 30 °C, and raising the temperature to 37 or 42 °C immediately blocks cell division. In addition, cells grown on McConkey/melibiose plates appear as white colonies at 25 °C, deep red at 30 °C, and pink when the cells are grown first at 25 °C and shifted to 37 or 42 °C. The phenomena are best explained by the temperature sensitivity of melB expression when it is controlled by λ P_L/T7 RNA polymerase and by the lethal effect of overexpression of integral membrane proteins, permeases in particular (Padan et al., 1983). In any event, efficient expression of melB in DW2/pT7-(SD/B) is demonstrated by the data presented in Figure 4. As shown, both the rate and steady-state level of TMG accumulation in DW2/pT7-(SD/B) grown at 25 °C and shifted to 30 °C for 1 h are dramatically increased relative to DW2/pT7-6 treated in the same manner. Furthermore, the specific permease content of DW2/pT7-(SD/B), as determined by measuring Na^+-dependent binding of *p*-nitro(^{3}H)phenyl α-D-galactopyranoside in right-side-out membrane vesicles (Cohn & Kaback, 1980; Damiano-Forano et al., 1986), is 0.6 nmol/mg of membrane protein. This value is 3–4 times higher than that observed in *E. coli* RA11 (0.15–0.2 nmol/mg of membrane protein), which contains a single chromosomal copy of melB.

(^{35}S)Methionine Labeling of the Melibiose Permease in DW2/pT7-(SD/B). *E. coli* DW2/pT7-(SD/B) were pulse-labeled with (^{35}S)methionine in the presence of rifampicin as described, and total cell proteins, membrane proteins, and soluble cytoplasmic proteins were resolved by NaDodSO$_4$/polyacrylamide gel electrophoresis (Figure 5). Autoradiography of total cell protein (lane 2) shows that at least 50% of the radioactivity incorporated is found in a broad band migrating at an apparent M_r of approximately 39K (36–42K). Surprisingly, the enhancement in the extent of labeling of the

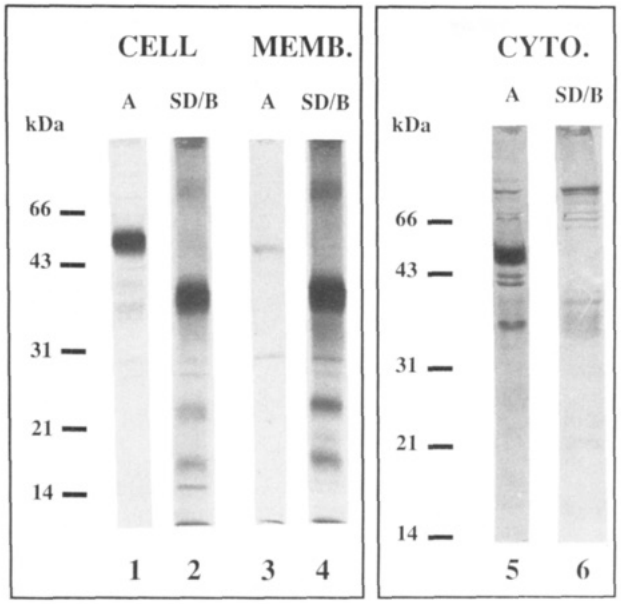

FIGURE 5: Identification and subcellular localization of the gene product encoded by plasmid pT7-(SD/B). Plasmid pT7-(SD/B), constructed as described in Figure 3, and plasmid pGP1-2 were used to cotransform *E. coli* DW2. Transformants were pulse-labeled with (^{35}S)methionine, and inner membrane protein and soluble cytoplasmic protein fractions were prepared by subjecting labeled cells to osmotic shock as described under Methods. Comparative experiments were performed on cells transformed with plasmid pT7-A containing the melA gene. Autoradiographic analysis of total cell proteins (cell, lanes 1 and 2) or proteins from either the inner membrane (memb., lanes 3 and 4) or soluble cytoplasmic (cyto., lanes 5 and 6) fractions prepared from cells transformed with either pT7-A (A) or pT7-(SD/B) was carried out as described in Figure 2.

39K polypeptide in cells harboring plasmid pT7-(SD/B) (Figure 5, lane 2), as compared to that observed in cells transformed with plasmid pT7-AB (Figure 2, lane 3), is proportionally much higher than that expected from the corresponding increase in transport rates (compare Table I and Figure 4). Since the apparent transport constants (K_t) are similar in these two strains (data not shown), one can conclude that a fraction of labeled melibiose permeases overproduced in DW2/pT7-(SD/B) cells have impaired catalytic activity. In direct relation with this remark is a recent report by Roepe and Kaback (unpublished information) which mentions that a substantial amount of ^{35}S-labeled melibiose carriers overproduced by T184 cells transformed with pT7-(SD/B) is apparently adsorbed on the inner surface of the cytoplasmic membrane rather than inserted into the membrane core, as they can be urea-extracted in a manner similar to peripheral membrane proteins. In any instance, the labeled material is almost exclusively associated with the cytoplasmic membrane (Figure 5, lane 4) and is only a minor contaminant in the soluble cytoplasmic fraction (Figure 5, lane 6). Moreover, the apparent M_r of the 39K polypeptide varies as a function of acrylamide concentration, decreasing from about 40K to about 37K as the acrylamide concentration is decreased from 15% to 8% (data not shown).

The relative migration of melB gene product is faster than that expected from the theoretical M_r value of 52K deduced from melB DNA sequence by Yazyu et al. (1984). This finding is not surprising as anomalous migration of prokaryotic permease has been repeatedly reported (Ehring et al., 1980; Padan et al., 1983; MacPherson et al., 1981; Hanada et al., 1985) and is believed to result from excessive binding of negatively charged SDS molecules on these hydrophobic proteins. Limited proteolysis of the transport protein leading to quite large deletion of the NH_2 or COOH termini (about 100 amino acids) would also account for the discrepancy between the observed apparent M_r of 39K and the theoretical value of 52K. Two observations indicate that this explanation is unlikely. First, we observed that the labeled 39K polypeptide is also specifically recognized by antibodies [anti-MBct10, Botfield and Wilson (1989)] directed against the last nine amino acid residues of the mel carrier COOH terminus (M. Bassilana, M. Deckert, and G. Leblanc, unpublished results). On the other hand, carrier proteins deleted of the first 75 amino acids of the NH_2 terminus are no longer active (T. Pourcher and G. Leblanc, unpublished observation). This finding is in contradistinction to the observed increase of permease activity reported in our experiments. Besides these remarks, it should be finally noted that in no instance did we observe that expression of mel B gave rise to a labeled product with an apparent M_r of 30K, as tentatively claimed by Hanatani et al. (1984). Although no direct explanation can be offered for this discrepancy, it should be mentioned that expression of product of genes inserted together with melB gene or even expression of fusion proteins (for example, mel X-COOH terminus of the β-lactamase?) has not been excluded in the case of the plasmids used by these authors to identify melB product. Such events are entirely eliminated in the case of the recombinant plasmid pT7-(SD/B), which only contains melB gene as an insert.

Beside the 39K band, three other labeled bands at approximately 88K, 24K, and 17K are evident in the total cell protein and the membrane fraction from DW2/pT7-(SD/B) (lanes 2 and 4, respectively). The 88K band probably represents a dimeric aggregate of melibiose permease, as similar aggregates have been observed with both *lac* permease (Ehring et al., 1980; Carrasco et al., 1982) and the arabinose permease (McPherson et al., 1981). The 24K and 17K bands are either proteolytic fragments derived from the 39K band or arise as the result of incorrect transcription and/or translation of melB.

Finally, when *E. coli* DW2/pT7-A is subjected to the same procedures (lanes 1, 3, and 5), the labeled polypeptide corresponding to α-galactosidase which migrates at 50K (lane 1) is recovered primarily in the soluble cytoplasmic fraction (compare lanes 3 and 5); the observed M_r value of α-galactosidase corresponds to the theoretical molecular weight deduced from the DNA sequence (Liljestrom & Liljestrom, 1987).

In conclusion, the results reported above demonstrate that separate cloning of melA and melB genes into one of the plasmids of T7 expression system led to unambiguous identification of the two gene products: α-galactosidase, encoded by melA gene, is detected as a cytoplasmic protein with an M_r of 50K; the melibiose permease, encoded by melB gene, is a membrane-bound protein with an apparent M_r of 39K. Importantly, the high level of (^{35}S)methionine incorporation into the melibiose carrier protein provides a mean to monitor its purification.

ACKNOWLEDGMENTS

We thank R. Lemonnier and P. Lahitette for excellent technical assistance. We also thank Dr. T. H. Wilson for a gift of anti MBct-10 antibodies.

Registry No. melB, 9055-24-7; melA, 9025-35-8.

REFERENCES

Anraku, Y., Hanada, K., & Yamato, I. (1988) in *Molecular Basis of Biomembrane Transport* (Palmieri, F., & Qua-

gliariello, E., Eds.) pp 25–32, Elsevier Science Publishers, Amsterdam.
Bassilana, M., Damiano-Forano, E., & Leblanc, G. (1985) *Biochem. Biophys. Res. Commun. 129*, 626–631.
Bassilana, M., Pourcher, T., & Leblanc, G. (1987) *J. Biol. Chem. 262*, 16865–16870.
Bassilana, M., Pourcher, T., & Leblanc, G. (1988) *J. Biol. Chem. 263*, 9663–9667.
Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., & Boyer, H. W. (1977) *Gene 2*, 95–113.
Botfield, M. C., & Wilson, T. H. (1988) *J. Biol. Chem. 263*, 12909–12915.
Botfield, M. C., & Wilson, T. H. (1989) *J. Biol. Chem. 264*, 11649–11652.
Büchel, D. E., Gronenborn, B., & Muller-Hill, B. (1980) *Nature 283*, 541–545.
Burstein, C., & Kepes, A. (1971) *Biochim. Biophys. Acta 230*, 52–63.
Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6894–6898.
Carter, P. J., Winter, C., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835–840.
Clarke, L., & Carbon, J. (1976) *Cell 9*, 91–99.
Cohn, D. E., & Kaback, H. R. (1980) *Biochemistry 19*, 4237–4243.
Crowl, R., Seamans, C., Lomedico, P., & McAndrew, S. (1985) *Gene 38*, 31–38.
Damiano-Forano, E., Bassilana, M., & Leblanc, G. (1986) *J. Biol. Chem. 261*, 6893–6899.
Ehring, R., Beyreuther, K., Wright, J. K., & Overath, P. (1980) *Nature 283*, 537–544.
Foster, D. L., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31–34.
Gold, L. (1988) *Annu. Rev. Biochem. 57*, 199–233.
Hanada, K., Yamato, I., & Anraku, Y. (1985) *FEBS Lett. 191*, 278–282.
Hanada, K., Yamato, I., & Anraku, Y. (1988) *J. Biol. Chem. 263*, 7181–7185.
Hanatani, M., Yazyu, H., Shiota-Niiya, S., Moriyama, Y., Kanazawa, H., Futai, M., & Tsuchiya, T. (1984) *J. Biol. Chem. 259*, 1807–1812.
Henderson, P. J. F. (1988) in *Molecular Basis of Biomembrane Transport* (Palmieri, F., & Quagliariello, E., Eds.) pp 1–13, Elsevier Science Publishers, Amsterdam.
Kaback, H. R. (1971) *Methods Enzymol. 31*, 698–709.
Kaback, H. R. (1983) *J. Membr. Biol. 76*, 95–118.
Kaback, H. R. (1988) *Annu. Rev. Physiol. 50*, 243–256.
Kawakami, T., Akizawa, Y., Ishikawa, T., Shimamoto, T., Tsuda, M., & Tsuchiya, T. (1988) *J. Biol. Chem. 263*, 14276–14280.
Kramer, B., Kramer, W., & Fritz, H. J. (1984) *Cell 38*, 879–887.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Leblanc, G., Bassilana, M., & Pourcher, T. (1988) in *Molecular Basis of Biomembrane Transport* (Palmieri, F., & Quagliariello, E., Eds.) pp 53–62, Elsevier Science Publishers, Amsterdam.
Liljestrom, P. L., & Liljestrom, P. (1987) *Nucleic Acids Res. 15*, 2213–2220.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
MacPherson, A. J. S., Jones-Mortimer, C. J., & Henderson, P. J. F. (1981) *Biochem. J. 196*, 269–283.
Maiden, M. C. J., Jones-Mortimer, C. J., & Henderson, P. J. F. (1988) *J. Biol. Chem. 263*, 8003–8010.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miller, J. H. (1972) in *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Nakao, T., Yamato, I., & Anraku, Y. (1987) *Mol. Gen. Genet. 208*, 70–75.
Padan, E., Arbel, T., Rimon, A., Bar Shira, A., & Cohen, A. (1983) *J. Biol. Chem. 258*, 5666–5673.
Sanger, F., & Coulson, A. R. (1978) *FEBS Lett. 87*, 107–110.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Sarker, H. K., Viitanen, P. V., Padan, E., Trumble, W. R., Poonian, M. S., McComas, W., & Kaback, H. R. (1985) *Methods Enzymol. 125*, 214–230.
Shine, J., & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1342–1346.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074–1078.
Tsuchiya, T., & Wilson, T. H. (1978) *Membr. Biochem. 2*, 63–79.
Wright, J. K., Sekler, R., & Overath, P. (1986) *Annu. Rev. Biochem. 55*, 225–248.
Yazyu, H., Shiota-Niiya, T., Shimamoto, T., Kanazawa, H., Futai, M., & Tsuchiya, T. (1984) *J. Biol. Chem. 259*, 4320–4326.
Yazyu, H., Shiota, S., Futai, M., & Tsuchiya, T. (1985) *J. Bacteriol. 162*, 933–937.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 469–500.