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Nucleotide Sequence of the *fruA* Gene, Encoding the Fructose Permease of the *Rhodobacter capsulatus* Phosphotransferase System, and Analyses of the Deduced Protein Sequence

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The nucleotide sequence of the *fruA* gene, the terminal gene in the fructose operon of *Rhodobacter capsulatus*, is reported. This gene codes for the fructose permease (molecular weight, 58,575; 578 aminoacyl residues), the fructose enzyme II (II^{Fru}) of the phosphoenolpyruvate-dependent phosphotransferase system. The deduced aminoacyl sequence of the encoded gene product was found to be 55% identical throughout most of its length with the fructose enzyme II of *Escherichia coli*, with some regions strongly conserved and others weakly conserved. Sequence comparisons revealed that the first 100 aminoacyl residues of both enzymes II were homologous to the second 100 residues, suggesting that an intragenic duplication of about 300 nucleotides had occurred during the evolution of II^{Fru} prior to divergence of the *E. coli* and *R. capsulatus* genes. The protein contains only two cysteyl residues, and only one of these residues is conserved between the two proteins. This residue is therefore presumed to provide the active-site thiol group which may serve as the phosphorylation site. II^{Fru} was found to exhibit regions of homology with sequenced enzymes II from other bacteria, including those specific for sucrose, β-glucosides, mannitol, glucose, *N*-acetylglucosamine, and lactose. The degree of evolutionary divergence differed for different parts of the proteins, with certain transmembrane segments exhibiting high degrees of conservation. The hydrophobic domain of II^{Fru} was also found to be similar to several uniport and antiport transporters of animals, including the human and mouse insulin-responsive glucose facilitators. These observations suggest that the mechanism of transmembrane transport may be similar for permeases catalyzing group translocation and facilitated diffusion.

The integral membrane permeases of the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS), also called enzymes II, catalyze the unusual process of group translocation in which transmembrane transport and phosphorylation of the sugar substrates are coupled in a single, concomitant process (43, 48, 49; M. H. Saier, Jr., *in* P. L. Yeagle, ed., *The Structure and Function of Biological Membranes*, in press). The genes encoding more than dozen of these permeases have been sequenced, and their deduced aminoacyl sequences have been analyzed in some detail (4, 5, 12, 56). Sequence comparisons have established that at least several of the PTS permeases are evolutionarily related, but the evolutionary relationships of others have not yet been demonstrable (12, 52, 56).

Recently, the sequence of the fructose-specific enzyme II (II^{Fru}) of *Escherichia coli* was published (44), as was that of the fructose-specific enzyme III (III^{Fru}) protein of *Salmonella typhimurium* (21). The published analyses revealed some homology with other PTS proteins, but anomalies were also noted. Thus, the fructose permease appears to be substantially larger than other sequenced PTS permeases, and unlike most other PTS permeases, a large hydrophilic region of over 200 residues precedes the first presumed transmembrane segment (44). On the basis of sequence comparisons, the postulated phosphorylation site within the *E. coli* protein is suggested to be histidyl residue 381 (44).

Species of the genus *Rhodobacter* (previously designated *Rhodopseudomonas*) have long been known to possess an unusual, two-component, fructose-specific PTS (47, 51). The system has been subjected to kinetic and biochemical analyses (31–33). Recently, Daniels et al. (10) cloned the fructose (*fru*) operon from *Rhodobacter capsulatus* in preparation for sequence analyses. We had maintained that a fructose PTS might have been the primordial PTS and that analysis of the unusual fructose-specific PTS from these photosynthetic bacteria might provide insight into the evolution of the PTS (47, 52).

In this paper, we report the complete nucleotide sequence of the *R. capsulatus fruA* gene which encodes the fructose enzyme II of the PTS in this organism. We show that the gene encodes a 578-residue protein which exhibits striking sequence identity throughout most of its length with the corresponding *E. coli* protein. Sequence analyses revealed that the N-terminal 100 amino acids of the primordial protein had been duplicated during its evolution and that it therefore possessed a 100-residue repeat unit at its N terminus. Further sequence analyses revealed that the same must have been true of the *E. coli* protein, but these two homologous domains in the *E. coli* II^{Fru} have diverged almost to the point of obscurity. Of further interest was the fact that the histidyl residue in the *E. coli* II^{Fru} (II^{Fru}_{Ec}), postulated to be the phosphorylation site, was not conserved in the *R. capsulatus* II^{Fru} (II^{Fru}_{Rc}). A single cysteyl residue was found to be conserved between the two proteins, and consequently this residue is presumed to supply the catalytic thiol group in the enzyme (31).

Sequence comparisons with other PTS permeases have shown that II^{Fru}_{Rc} is homologous throughout its length with some of these permeases and that specific regions of the hydrophobic domain of this protein exhibit a significant degree of sequence identity with most sequenced PTS permeases. It therefore shares a common ancestry with them. Of equal significance, however, is the surprising finding that

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  L  G  E  I  G  P  H  L  P  P  P  E  R  L  A  A  L  A  R  T
GCTGGGCGAGATCGGCCCGCATCTGCCGCCGCCGGAGCGCCTTGCCGCGCTGGCCCGCAC   60

  V  T  V  K  T  L  P  P  V  *                    M  S  K  I    4
CGTCACCGTCAAAACGCTGCCGCCCGTCTGAGCCGCAGGGGAGGAACCCATGTCGAAGAT  120
                                    SD

  V  A  V  T  A  G  A  K  G  V  A  H  T  H  L  A  A  E  A  L   24
CGTTGCCGTGACCGCCGGGGCCAAGGGCGTCGCGCATACCCATCTGGCCGCCGAGGCGCT  180

  S  A  T  A  Q  A  L  G  H  Q  I  R  V  E  R  H  S  A  E  G   44
GAGCGCCACCGCGCAGGCGCTGGGCCATCAGATCCGTGTCGAACGCCACTCTGCCGAGGG  240

  V  E  A  P  L  Q  G  A  E  I  A  A  A  D  V  V  L  I  A  A   64
CGTCGAGGCGCCGCTGCAGGGGGCCGAGATCGCCGCCGCCGATGTGGTGCTGATCGCCGC  300

  D  L  R  I  E  D  V  R  F  V  T  K  P  V  Y  R  T  S  T  A   84
CGACCTCCGCATCGAGGATGTGCGCTTCGTCACGAAACCCGTTTACCGGACCTCGACTGC  360

  R  A  V  T  Q  T  A  A  V  L  A  E  A  L  A  L  T  G  E  E  104
CCGCGCCGTCACCCAGACGGCGGCGGTTCTGGCCGAGGCCCTTGCCCTGACCGGAGAGGA  420

  T  P  Q  M  T  T  D  T  G  Q  R  P  L  R  V  V  A  I  T  S  124
GACCCCGCAAATGACGACAGATACAGGCCAAAGGCCGCTCAGGGTGGTGGCGATCACCTC  480

  C  P  T  G  I  A  H  T  F  M  A  A  D  A  L  K  K  T  A  A  144
CTGTCCGACCGGCATTGCCCATACGTTCATGGCGGCCGATGCGCTGAAAAAGACCGCCGC  540

  A  R  G  W  E  I  A  V  E  T  Q  G  S  V  G  S  Q  N  A  L  164
CGCCCGCGGCTGGGAAATCGCGGTCGAGACGCAGGGCTCGGTGGGCTCGCAAAACGCGCT  600

  S  A  A  Q  I  Q  A  A  D  L  V  V  I  A  A  D  T  H  V  D  184
GAGCGCGGCGCAGATCCAGGCCGCCGATCTGGTGGTGATCGCGGCCGACACCCATGTCGA  660

  D  S  R  F  A  G  K  K  V  Y  K  T  S  V  G  A  A  V  K  G  204
TGACAGCCGCTTTGCCGGGAAAAAGGTCTACAAGACCTCGGTCGGCGCGGCGGTGAAGGG  720

  A  A  K  V  L  D  A  A  L  A  E  G  V  V  L  G  T  N  L  A  224
CGCGGCCAAGGTGCTCGATGCGGCCCTGGCCGAAGGCGTCGTGCTGGGCACCAACCTGGC  780

  D  T  V  D  A  L  K  A  Q  R  A  A  T  R  S  G  P  Y  M  H  244
CGACACGGTCGATGCGCTCAAGGCGCAACGCGCCGCGACCCGCTCGGGCCCCTATATGCA  840

  L  L  T  G  V  S  Y  M  L  P  L  V  V  A  G  G  L  L  I  A  264
CCTGCTGACGGGCGTGTCCTACATGCTGCCGCTGGTGGTGGCGGGCGGTCTCTTGATCGC  900

  L  S  F  V  F  G  I  K  A  F  E  V  E  G  T  L  P  A  A  L  284
GCTTTCGTTCGTCTTCGGGATCAAGGCCTTCGAGGTCGAGGGCACGCTGCCCGCGGCGCT  960

  M  A  I  G  G  G  A  A  F  K  L  M  V  P  V  L  A  G  F  I  304
GATGGCGATCGGTGGCGGCGCCGCCTTCAAGCTGATGGTGCCGGTTCTGGCGGGCTTCAT 1020

  A  Y  S  I  A  D  R  P  G  L  T  P  G  L  I  G  G  M  L  A  324
CGCCTATTCGATCGCCGACCGTCCCGGCCTGACCCCGGGTCTGATCGGTGGCATGCTGGC 1080

  V  N  L  N  A  G  F  L  G  G  I  V  A  G  F  L  A  G  Y  V  344
GGTGAACCTGAATGCCGGGTTCCTAGGCGGCATCGTCGCGGGCTTCCTGGCGGGCTATGT 1140
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the hydrophobic domain of the *R. capsulatus* fructose enzyme II shows sequence identity with transport proteins of animal cells (67). The latter proteins include mammalian insulin-responsive glucose facilitators (20, 26) and the chicken erythroid anion transporter (9). The statistical analyses presented suggest that PTS group translocators and the facilitators of animal cells, lower eucaryotes, and procaryotes (24, 65) may share a common ancestry. These observations suggest the possibility that these two classes of permeases share a common translocation mechanism.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were obtained from Bethesda Research Laboratories, Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Inc. The T7 polymerase sequencing kit was from Pharmacia. The deoxyadenosine 5′-([α-^{35}S]thio)triphosphate (1,335 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Isopropyl-β-D-thiogalactopyranoside, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and lysozyme were purchased from Boehringer Mannheim. Acrylamide, bisacrylamide, and *N,N,N′,N′*-tetramethylethylenediamine were from Bio-Rad Laboratories, while agarose was from Bethesda Research Laboratories. Deoxynucleotides and dideoxynucleotides were obtained from Pharmacia and P-L Biochemicals, Inc. All other chemicals and enzymes used were of the highest quality available commercially.

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  A  R  W  L  R  D  A  I  K  L  P  R  T  L  E  G  L  K  P  V  364
CGCGCGCTGGCTGCGCGATGCGATCAAGCTGCCGCGCACGCTGGAAGGGCTCAAGCCCGT 1200

  L  I  L  P  L  L  S  T  A  I  T  G  L  I  M  V  Y  V  V  G  384
CCTGATCCTGCCGCTGCTCTCGACCGCGATCACCGGGCTGATCATGGTCTATGTCGTCGG 1260

  T  P  V  A  A  I  L  A  A  M  T  A  F  L  Q  G  L  G  T  T  404
CACGCCGGTGGCGGCGATCCTGGCCGCGATGACCGCCTTCCTGCAGGGGCTGGGCACCAC 1320

  N  A  V  V  L  G  L  I  L  G  G  M  M  A  V  D  M  G  G  P  424
CAATGCCGTCGTTCTGGGCCTGATCCTTGGCGGCATGATGGCCGTCGACATGGGGGGGCC 1380

  I  N  K  A  A  Y  T  F  A  V  G  L  L  T  S  S  T  Y  A  P  444
GATCAACAAGGCCGCCTATACTTTCGCCGTCGGCCTGCTGACCTCGAGCACCTATGCGCC 1440

  M  A  A  V  M  A  A  G  M  T  P  P  L  G  L  A  L  A  T  L  464
GATGGCCGCCGTGATGGCCGCGGGCATGACGCCGCCGCTGGGTCTGGCGCTGGCGACGCT 1500

  V  A  K  N  R  F  T  A  E  E  R  E  A  G  G  A  A  A  V  L  484
GGTTGCGAAGAACCGCTTCACCGCGGAAGAACGTGAAGCCGGGGGCGCCGCCGCGGTGCT 1560

  G  L  S  F  I  T  E  G  A  I  P  F  A  A  K  D  P  A  R  V  504
GGGCCTGTCCTTCATCACCGAAGGCGCCATTCCCTTTGCCGCGAAAGACCCGGCCCGGGT 1620

  I  P  S  I  I  V  G  S  A  I  T  G  A  L  S  M  A  L  G  C  524
GATCCCCTCGATCATCGTCGGCTCGGCGATCACCGGGGCGCTGTCGATGGCGCTGGGTTG 1680

  L  L  V  A  P  H  G  G  I  F  V  L  A  I  P  H  A  V  T  N  544
CCTGCTGGTCGCGCCGCATGGCGGGATCTTCGTGCTGGCGATCCCGCATGCGGTGACGAA 1740

  L  G  L  Y  A  L  S  I  V  V  G  T  L  V  T  T  G  L  L  I  564
CCTCGGGCTTTATGCGCTTTCGATCGTTGTCGGCACGCTGGTGACGACGGGTCTGCTGAT 1800

  A  L  K  K  P  I  P  A  E  E  R  A  R  S  *                 578
CGCGCTGAAAAAGCCGATCCCGGCTGAAGAGCGCGCCCGCAGTTGATCGGGCAAGGCCGC 1860

GAACTCAAGGCCGACGGTCCCCGGATCGTCGGCCTTTGGCTGTCCGCCGGGGCAGAAA   1918
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FIG. 1. Nucleotide sequence of the *fruA* gene of *R. capsulatus* and the deduced amino acid sequence of the encoded fructose-specific enzyme II of the PTS. In addition to the *fruA* open reading frame, the Shine-Dalgarno sequence (labeled SD) (GGAGG) is underlined, as is the inverted repeat following the *fruA* gene. The initial 29 codons depicted encode the C-terminal 29 amino acids of the *fruK* gene product, fructose-1-phosphate kinase.

Bacterial strains and plasmids. *E. coli* TG1 was provided by T. Gibson of the Laboratory of Molecular Biology, Medical Research Council, Cambridge, United Kingdom (7). Strain XL1-Blue was obtained from Stratagene. The pBluescript SK(+) and KS(+) plasmids as well as helper phage VCS-M13 were obtained from Stratagene. Phage M13mp18 and M13mp19 were from Bio-Rad.

Synthesis of oligodeoxynucleotides. Oligodeoxynucleotides were synthesized from β-cyanoethyl phosphoramidite precursors on a model 380B DNA synthesizer (Applied Biosystems). The oligonucleotides were synthesized as trityl-on derivatives which were deblocked at 58°C for 12 h in concentrated ammonium hydroxide. These solutions were then applied to Applied Biosystems OPC oligonucleotide purification columns. The oligonucleotides were eluted as described by the manufacturer. These solutions were taken to dryness under vacuum.

Growth media and selection conditions. Transformants were selected on Luria-Bertani (LB) plates (Difco Laboratories) (37) containing ampicillin (50 μg/ml). For the subcloning of fragments into pBluescript SK(+) or KS(+) or into phage M13mp18 or M13mp19, LB plates containing 1 mM isopropylthio-β-galactoside and 30 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were used. Ampicillin (50 μg/ml) was included when required.

DNA procedures. *E. coli* XL1-Blue and TG1 carrying the Bluescript plasmids were grown at 37°C in LB medium containing ampicillin (50 μg/ml). Single-stranded M13mp18 and M13mp19 DNA were prepared from *E. coli* TG1 cultures infected with M13 phage as described by Sanger et al. (57). Single-stranded DNA from the Bluescript plasmids was prepared with helper phage VCS-M13 as described in the Stratagene manual. Small- and large-scale preparation of phage replicative-form and plasmid DNAs was accomplished by using the procedure of Birnboim and Doly (3). Competent cells of *E. coli* TG1 and XL1-Blue were prepared by the $CaCl_2$ method (36).

Nucleotide sequencing was conducted with the T7 sequencing kit of Pharmacia with Deaza ^{35}Sequencing Mixes as described previously to overcome G+C compression resulting from the high G+C content of *Rhodobacter* DNA (70% for the *fru* operon) (68). We reported previously the location of the *fruA* and *fruK* genes on an *Eco*RI-*Hin*dIII fragment of 4.5 kb. Three smaller fragments (*Eco*RI-*Pst*I, 2.0 kb; *Pst*I-*Pst*I, 1.0 kb; and *Pst*I-*Pst*I, 1.1 kb) were subcloned from this fragment into phage M13mp18/19 or plasmid Bluescript SK(+) or KS(+). An *Sma*I fragment of 1.2 kb which partially overlapped with the *Pst*I-*Pst*I fragment of 1.1 kb was also subcloned into phage M13mp19. Universal and synthesized primers were used for the sequencing.

The large *Eco*RI-*Pst*I fragment of 4.1 kb was sequenced in both directions at least twice. This fragment included the entire coding region of the *fruA* gene (Fig. 1). The average number of nucleotides determined per reaction was about 250 bp.

Computer analyses. The Staden programs were used for statistical analyses and structural prediction studies (63). For homology screening, the Fasta programs with the combined

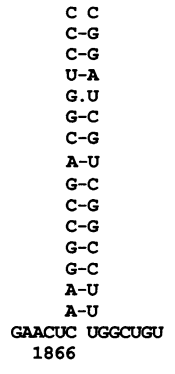

FIG. 2. Depiction of the hairpin structure following the *fruA* gene which is believed to function as a rho-dependent transcriptional terminator. The number, 1866, corresponds to the numbering in Fig. 1, and the stem-loop structure corresponds to the sequence underlined in Fig. 1 following the *fruA* gene. The program SECSTRUC was used for searching the stem-loop structures. The Gibbs free energy for the H-bonds in the stem region was calculated by using Tinoco free energy values. ΔG is equal to -35.4 kcal.

PIR and GenBank banks were used. Multialignment was conducted by using the Newat program (18). The significance of homology was calculated by using the Los Alamos program (27). The programs are available through the University of California, San Diego, Computer Center.

RESULTS

Nucleotide sequence of the *fruA* gene encoding the II^{Fru} of *R. capsulatus*. Figure 1 presents the nucleotide sequence of the *R. capsulatus fruA* gene and flanking regions. Preceding the start codon of the *fruA* gene is a Shine-Dalgarno sequence, GGAGG, located 5 bp upstream of the ATG initiation codon within a short intercistronic region of 18 bp between *fruK* (encoding fructose-1-phosphate kinase) and *fruA*. The corresponding intercistronic region in *E. coli* is 20 bp. Ninety nucleotides of the *fruK* gene are also presented. The *fruA* open reading frame is 1,737 bp long, corresponding to 579 codons (578 amino acids in the protein; molecular weight, 58,575). This molecular weight is similar to that determined experimentally for II^{Fru} from *R. capsulatus* (68) and is slightly larger than that of the *E. coli* II^{Fru} calculated from the nucleotide sequence of the *fruA* gene of this organism (molecular weight, 57,500) (44). The *Rhodobacter fruA* open reading frame is present within the 1,918-bp chromosomal fragment, the sequence of which is shown in Fig. 1.

Rho-dependent transcriptional termination signal. Twenty nucleotide bases following the UGA translational stop codon (nucleotide bases 1844 to 1846; indicated by the asterisk in Fig. 1) is a stable stem-loop structure with a calculated free energy of stabilization of -35.4 kcal (ca.-148 kJ) (underlined in Fig. 1 and shown in Fig. 2). This structure is likely to correspond to a rho-dependent transcriptional termination signal (2). The *fruBKA* operon of *E. coli* terminates with a rho-independent terminator (44). Only 10 bp separates the transcriptional terminator of the *E. coli fru* operon from the *fruA* termination codon (44). Codon usage within the *fru* operon will be described in a separate publication concerning the *fru* operon in general.

Amino acid composition of II^{Fru}. Table 1 presents the amino acid compositions of the II^{Fru} proteins of *R. capsulatus* and *E. coli*. A striking difference between the two proteins was the low C and W contents of the *R. capsulatus* II^{Fru} compared with those of the *E. coli* II^{Fru} (two versus six C's and two versus four W's). Additionally, V, T, and R showed increased frequencies relative to the *E. coli* protein, while I, M, P, and K showed decreased frequencies. Regardless of the compositional differences, about 56% of the amino acids in both proteins were hydrophobic, 28% were semipolar, 7% were acidic, and 9% were basic. These values are similar to those of other enzymes II of the PTS (55).

TABLE 1. Amino acid composition of the fructose enzyme II from *R. capsulatus* compared with that from *E. coli*[a]

Amino acid	*R. capsulatus* II^{Fru}		*E. coli* II^{Fru}	
	No. of amino acids	% of total residues	No. of amino acids	% of total residues
A	109	18.8	100	17.7
L	70	12.1	62	11.0
G	60	10.4	64	11.3
U	56	9.7	40	7.1
T	45	7.8	32	5.7
I	35	6.0	40	7.1
P	26	4.5	32	5.7
S	23	4.0	22	3.9
E	21	3.6	23	4.1
R	21	3.6	14	2.5
K	20	3.5	31	5.5
M	17	2.9	24	4.3
F	16	2.8	16	2.8
D	16	2.8	15	2.7
Q	12	2.1	10	1.8
Y	10	1.7	13	2.3
H	9	1.6	6	1.1
N	8	1.4	9	1.6
C	2	0.3	6	1.1
W	2	0.3	4	0.7
Hydrophobic (PAUMILF)		56.8		55.7
Semipolar (NTSQGCYW)		28.0		28.4
Acidic (DE)		6.9		6.8
Basic (KRH)		8.7		9.1

[a] The amino acid composition was calculated by using the ANALYSEP program. Amino acids are listed in order of frequency in the *R. capsulatus* II^{Fru}. The relative abundance of the four groups of residues (hydrophobic, semipolar, acidic, and basic) is provided for both proteins.

Alignment of the fructose enzymes II from *R. capsulatus* and *E. coli*. Figure 3 shows the alignment of the *R. capsulatus* II^{Fru} (II^{Fru}_{Rc}) with the *E. coli* II^{Fru} (II^{Fru}_{Ec}). II^{Fru}_{Rc} is 578 residues long, whereas II^{Fru}_{Ec} is 15 residues shorter. The two proteins show 55% overall sequence identity. However, almost no sequence identity is observed in the first 56 residues of the *R. capsulatus* protein, corresponding to the first 40 residues of the *E. coli* protein, and relatively little sequence identity is observed in the first 100 residues of these two proteins. Also, in the C-terminal 20 residues, little sequence identity is observed. Consequently, the percent sequence identity in remaining portions of the two proteins is substantially higher (nearly 70%). Optimal alignment resulted in the generation of only two gaps, a three-residue gap at position 112 of the *R. capsulatus* protein and a two-residue gap at position 209 of the *E. coli* protein (Fig. 3).

Detection of an internal repeat of 100 residues in the two fructose-specific enzymes II. Computer analyses revealed that residues 1 to 100 of II^{Fru}_{Rc} align with residues 116 to 215 of the same protein with 46% identity. Residues 103 to 202 of II^{Fru}_{Ec} exhibit 40% sequence identity with residues 1 to 100 and 57% identity with residues 116 to 215 of II^{Fru}_{Rc} (Fig 4 and Table 2).

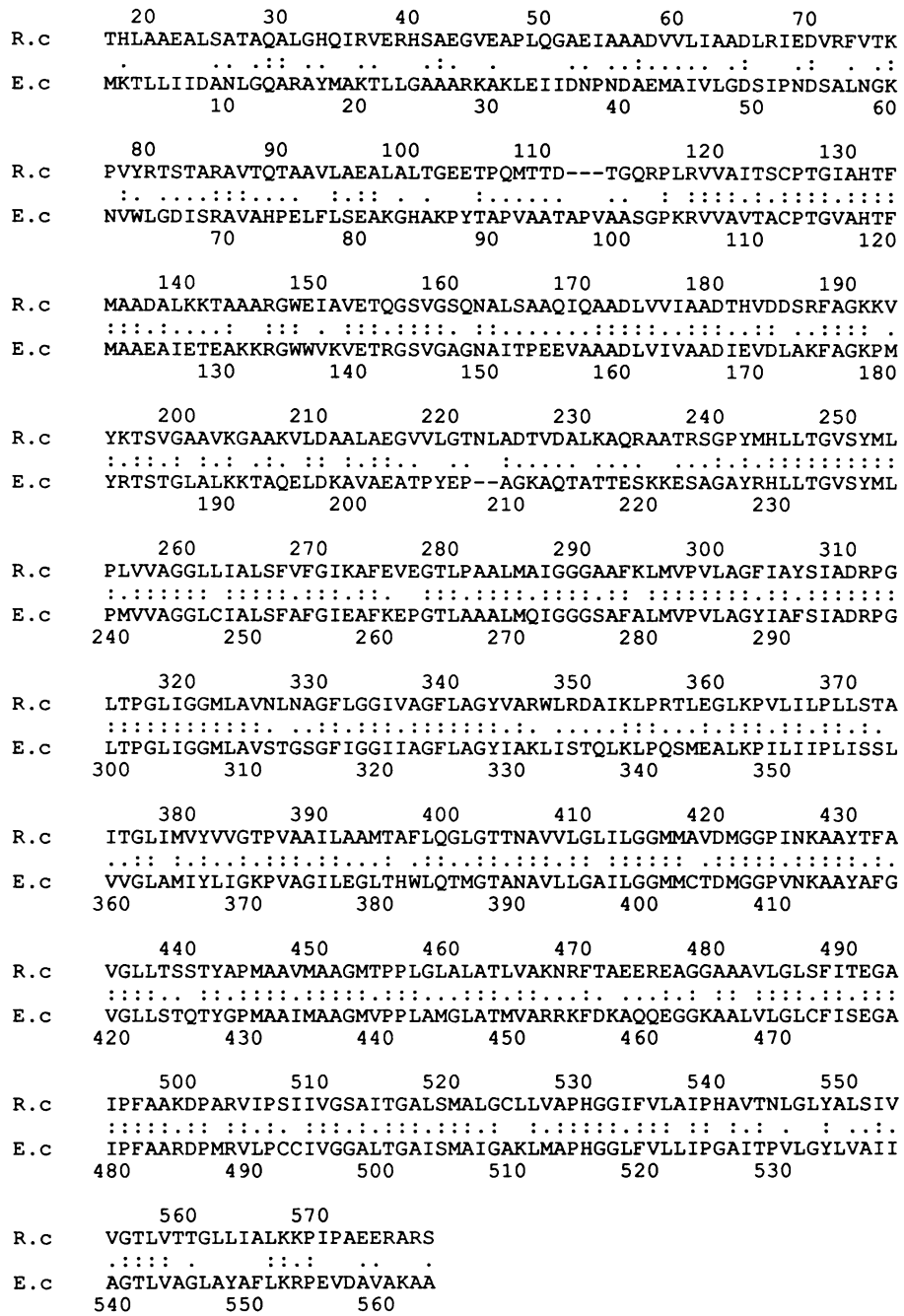

FIG. 3. Alignment of II^{Fru} from *R. capsulatus* (R.c) with II^{Fru} from *E. coli* (E.c). A colon indicates an identity, while a single dot reveals a conservative substitution. Dashes represent gaps in the aligned sequences. All residues of the 563-residue *E. coli* protein and the best alignment of the 578-residue *R. capsulatus* protein (beginning at residue 17) are shown.

Moreover, residues 41 to 84 of II^{Fru}_{Ec} show 25% identity with both segments of II^{Fru}_{Rc} and 21% identity with residues 103 to 202 of II^{Fru}_{Ec} in the region of overlap (Table 2). It therefore appears that the N termini of these proteins arose by internal gene duplication prior to their evolutionary divergence. The conserved cysteyl and histidyl residues in the second repeat domains of the two proteins (discussed below) are not conserved in the first domains.

Connecting the repeated segments of both proteins is a region predicted to serve as a flexible linker. In II^{Fru}_{Ec}, the sequence between residues 85 and 105 inclusive included seven A's, four P's, and one G consistent with the composition of an A-P-rich linker (see reference 68 for a discussion of these linkers). Surprisingly, this region contains a direct repeat of the hexa aminoacyl residue sequence, TAPVAA (residues 89 to 94 and residues 95 to 100). This region exhibits sequence identity with a previously identified linker present twice in the multiphosphoryl transfer protein of *R. capsulatus* (68). The corresponding region in II^{Fru}_{Rc} shows the characteristics of a flexible Q linker (two Q's, two E's, two R's, and two P's) (66). One of the only two gaps in the alignment between the aminoacyl sequences of these two

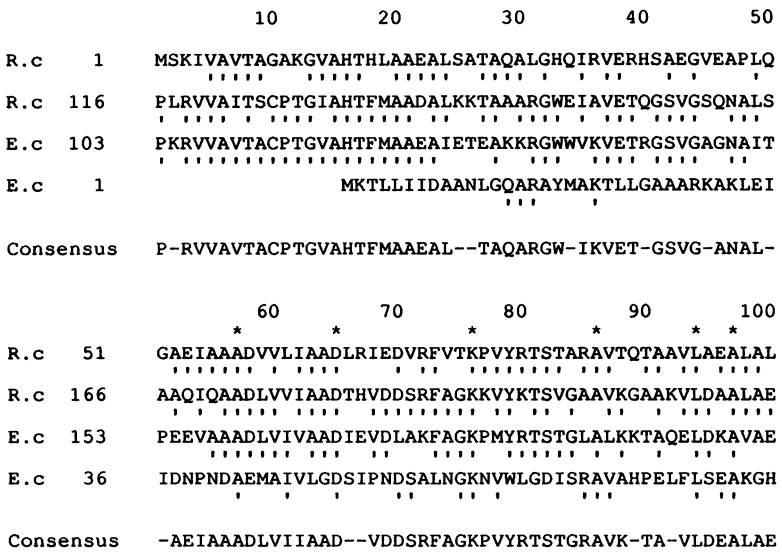

FIG. 4. Alignment of the two 100-residue internal repeats at the N-terminus of the *R. capsulatus* (R.c) II^{Fru} with those from the *E. coli* (E.c) II^{Fru}. The symbols are as indicated in the legend to Fig. 3 except that a dot under a sequence indicates identity with the consensus sequence. The top line shows the sequence of residues 1 to 100 of the *R. capsulatus* II^{Fru}, the second line shows residues 116 to 215 in the same protein, the third line shows residues 103 to 202 in the *E. coli* II^{Fru}, and the bottom line shows residues 1 to 84 in the *E. coli* protein. Only the C-terminal part of the first repeat unit in the *E. coli* II^{Fru} shows significant identity with the other three domains.

proteins was observed in this region (Fig. 3). These results suggest that residues 1 to 100 and 116 to 215 in II^{Fru}_{Rc} as well as residues 1 to 85 and 103 to 202 in II^{Fru}_{Ec} form relatively autonomous domains within these two proteins. The second of these repeated domains exhibits striking sequence identity in the two proteins (including conservation of possibly functional cysteyl and histidyl residues), suggesting conservation of structure and function. On the other hand, the first of these repeated domains has clearly diverged more than the second, with residues 1 to 85 of the *E. coli* protein diverging the most.

Residues 1 to 48 of the *E. coli* protein were computer screened for sequence identity with the protein bank. Only one protein, the Ca^{2+}-transporting ATPase of rat brain (61) (residues 487 to 535), showed appreciable sequence identity with this segment (19.1% identity). The function(s) of the first segment in these two proteins remains obscure. It is interesting to note, however, that both segments of II^{Fru}_{Ec} diverged more than the respective segments in II^{Fru}_{Rc} (19% identity versus 60% identity with the consensus sequence for the segments 1; 64% identity versus 71% identity for the segments 2).

Hydropathy, amphiphilicity, and secondary structural analyses of II^{Fru} proteins. A hydropathy analysis of II^{Fru}_{Ec} has been published (44). A comparison of this plot with that for the *R. capsulatus* protein is shown in Fig. 5A and C. These plots are very similar, particularly in the region beginning at residue 245 in the *R. capsulatus* protein and residue 230 in the *E. coli* protein. These proteins are strongly hydrophobic, suggesting an integral membrane topology, possibly with eight transmembrane helical segments as suggested previously (44). The hydropathy plots of the N-terminal repeated domains show greater divergence. Interestingly, in the *R. capsulatus* protein, each of the two repeated domains appeared by secondary structural analysis (ANALYSEP program) (63) to consist of three α-helical regions of about equal

TABLE 2. Percent identity between the N-terminal-repeated segments of the II^{Fru} proteins from *R. capsulatus* and *E. coli*[a]

Segments of II^{Fru} proteins	% Identity[b] with:			
	R. capsulatus		*E. coli*	
	1-100	116-215	103-202	41-84
R. capsulatus 1-100		46 (24.5)	40 (21.3)	25 (4.6)
R. capsulatus 116-215			57 (32.2)	25 (6.5)
E. coli 103-202				21 (1.7)

[a] The alignments of these segments are shown in Fig. 4.
[b] Percent identity was calculated as described by Feng and Doolittle (18). The Los Alamos comparison scores are given in parentheses in SDs (27).

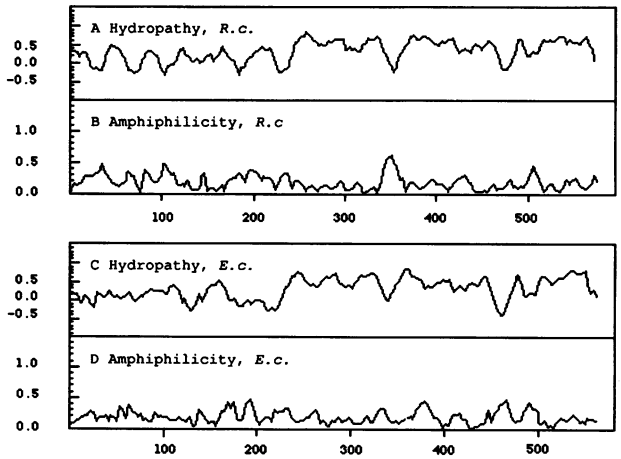

FIG. 5. Hydropathy and amphiphilicity analyses of II^{Fru} of *R. capsulatus* (A and B, respectively) and *E. coli* (C and D, respectively). The ANALYSEP programs were used with a window length of 18 and an angle of 100° for the amphiphilicity plots. Values are expressed in terms of the hydrophobicity values of Kyte and Doolittle (28) (A and C) or the hydrophobic moment per residue values of Eisenberg (14).

length connected by random coil or β-turns (data not shown). The *E. coli* protein showed deviation from this pattern only in its first, more divergent domain.

The amphiphilicity plots of these two proteins are shown in Fig. 5B and D. Marked differences between the two proteins are apparent. Thus, regions which when α-helical possess large hydrophobic moments in one protein do not necessarily exhibit large hydrophobic moments in the other protein.

In the *E. coli* II^{Fru}, two strongly amphipathic, potentially α-helical regions precede the first transmembrane segment (residues 188 to 203 and 211 to 228) (54, 55). We examined the *Rhodobacter* protein in the homologous region and found that within this region there was low sequence conservation (25% identity compared to 55% overall identity) and that neither the amphipathic character of the two helical regions nor the prolines which separate them were conserved. The presence of a two-residue gap in this region further suggests that structural features, and consequently the function of this region in the *E. coli* protein, had not been retained in the *R. capsulatus* protein. These major structural differences suggest that this region may comprise a flexible region separating domains in both proteins.

Conservation of residues of potential catalytic significance in the two II^{Fru} proteins. Comparisons of the aligned sequences of the II^{Fru} proteins from *R. capsulatus* and *E. coli* revealed that in contrast to the *E. coli* protein which contains six cysteyl residues, the *R. capsulatus* protein contains only two, and of these, only one (Cys-125 in the *R. capsulatus* protein; Cys-112 in the *E. coli* protein) is conserved. Since it is known that II^{Fru} from *Rhodobacter spheroides* contains an essential cysteyl residue (as do all well-characterized enzymes II of the PTS), Cys-125 is likely to be the essential cysteyl residue in this protein. On the basis of the work of Lolkema and Robillard (31) and Pas and Robillard (40), it is a candidate for the phosphorylation site within this protein.

Figure 6 shows the aminoacyl sequences around the cysteyl residues of the two II^{Fru} proteins and compares them with the sequences surrounding essential cysteyl residues in II^{Mtl}, II^{Glc}, and II^{Bgl}, as well as those surrounding established or postulated essential cysteyl residues in other enzymes II. Four points are worthy of note. (i) The sequence surrounding the conserved Cys in the two II^{Fru} proteins is strongly conserved (70% identity), but it is not conserved in II^{Mtl}, II^{Glc}, or II^{Bgl} (20 to 30% identity). (ii) These sequences are also poorly conserved between II^{Mtl} and either II^{Glc} or II^{Bgl} (10 to 20% identity). (iii) Conservation of sequence is observed to the right of the cysteyl residue in II^{Nag}, II^{Glc}, II^{Bgl}, and II^{Scr} and partially with II^{Gut}, but not in the other proteins. (iv) II^{Mtl} and II^{Man} show a startling degree of homology (80% identity for this 10-residue stretch).

Three histidyl residues in the *E. coli* II^{Fru} are conserved in the *R. capsulatus* II^{Fru}: H131, H244, and H530 in the *R. capsulatus* protein, which correspond to H118, H229, and H515 in the *E. coli* protein, respectively. All of these histidyl residues occur in strongly conserved regions of the proteins. While the first pair of these His residues is six residues to the right of the conserved cysteyl residues and therefore serves as a candidate for an active-site residue, the second pair immediately precedes the membrane-embedded region, and the third pair is within a hydrophilic loop which, according to the model of Prior and Kornberg (44), is localized to the periplasmic side of the membrane. If this model is correct, only the first two of these histidyl residues in each protein may function in energy coupling. Histidyl residue 381 in II^{Fru}_{Ec} has been postulated to be the phosphorylation site (44), but

Fru-Rc	125	A	T	T	S	C	P	T	G	I	A
Fru-Ec	112	A	V	T	A	C	P	T	G	V	A
Gut-Ec	252	A	V	I	D	C	G	G	T	L	R
Mtl-Ec	380	I	I	V	A	C	D	A	G	M	G
Man-Ec	16	F	I	V	A	C	I	A	G	M	G
Nag-Ec	412	A	I	D	A	C	I	T	R	L	R
Glc-Ec	421	N	L	D	A	C	I	T	R	L	R
Bgl-Ec	24	S	L	M	H	C	A	T	R	L	R
Scr-Bs	26	S	A	A	H	C	A	T	R	L	R
Scr-St	26	S	A	A	L	C	A	T	R	L	R
Scr-Sm	25	A	A	A	H	C	A	T	R	L	R
Lac-Gp	441	V	L	V	L	C	A	G	G	G	T

FIG. 6. Alignment of the single conserved cysteyl (C) residues in the *R. capsulatus* and *E. coli* II^{Fru} proteins (Fru-Rc and Fru-Ec, respectively) with the known, essential cysteyl residues in II^{Mtl} (Mtl-Ec) (30, 40), II^{Glc} (Glc-Ec) (15, 39), II^{Bgl} (Bgl-Ec) (4, 59, 60), and II^{Lac} (Lac-Gp) of three gram-positive bacteria (*S. aureus*, *S. lactis*, and *L. casei*) (6; DeVos et al., unpublished data; Alpert and Chassey, unpublished data) as well as with presumed essential cysteyl residues in other enzymes II of the PTS. The 10-residue segment depicted was chosen so that the cysteine (the number of which is given on the left) would always align in position 5. Except for the Man-Ec sequence, which is derived from the II^{Man}-P protein (16, 17), all of these segments are present in hydrophilic portions of the proteins. Additional abbreviations, referring to the PTS enzymes II, are as follows: Fru-Rc or Fru-Ec, fructose (*R. capsulatus* or *E. coli*) (44; this paper); Gut-Ec, glucitol (*E. coli*) (69); Nag-Ec, *N*-acetylglucosamine (*E. coli*) (41, 46); Glc-Ec, glucose (*E. coli*); Bgl-Ec, β-glucoside (*E. coli*); Scr-Bs, sucrose (*Bacillus subtilis*) (19); Scr-Sm, sucrose (*S. mutans*) (58); and Scr-St, sucrose (*S. typhimurium*) (12; modified according to J. Lengeler, personal communication).

this residue is not conserved in the *Rhodobacter* II^{Fru} and is replaced by an alanyl residue (position 397). Many basic, acidic, and polar residues are conserved between the two proteins, and some of these are within regions which are conserved with other enzymes II (55, 56).

Alignment of selected PTS proteins with the fructose permeases. Computer analyses indicated that the two II^{Fru} proteins aligned throughout their lengths with about 20% sequence identity with the sucrose permeases of *S. typhimurium* and *B. subtilis* (Fig. 7, ScrSt and ScrBs, respectively) as well as the β-glucoside permease of *E. coli* (Fig. 7, BglEc) (Table 3). The region encompassed by the sequence depicted in Fig. 7 includes all of the membrane-embedded regions of the fructose permeases, a stretch 323 residues long. Careful scrutiny of the sequence alignments in Fig. 7 reveals that all but one of the fully conserved residues are prolyl, glycyl, alanyl, and hydrophobic residues, and consequently they are likely to be of structural rather than catalytic significance. Of the 23 fully conserved residues, 11 are G's, 4 are A's, and 3 are P's. Thus, it appears that conservation of structure is more important than conservation of some specific catalytic function within the hydrophobic domains of these proteins.

The fact that structural residues are largely conserved in these hydrophobic domains is consistent with the suggestion that the membrane-embedded domain functions as a sugar-binding channel while the hydrophilic portions possess the catalytic domains of these proteins (see Discussion). It is interesting to note that the essential histidine in II^{Bgl}_{Ec} (His-306) is replaced by asparagine with an adjacent lysine in both II^{Fru} proteins.

The one exception to the generalization that structural

```
                !    !  !* *!!  !           ^^     ^      !!*    * !
FruRc 246 LTGVSYMLPLVVAGGLLIALSFVFGIKAFEVEGTLPAALMAIGGGAAFKLMVPVL 300
FruEc 231 LTGVSYMLPMVVAGGLCIALSFAFGIEAFKEPGTLAAALMQIGGGSAFALMVPVL 285
ScrSt 111 NIFVP IIPAIVASGLLMGLLGMVKTYGWVDPGNAIYIMLDMCSSAAF IILPIL 163
ScrBs 111 NIFVP IIPAIVASGLLMGLLGMINAFHWMSKDSALLQLLDMFSSAAF IFLPIL 163
BglEc 106 GIFTP LIGLMAATGILKGMLALALTFQWTTEQSGTYLILFSASDALF WFFPII 158

           *^ *       * !^  ^!** !          ^ ^  ^^^^ ^  !^   ! ^
FruRc 301 AGFIA YSIADRPGLTPGLIGGMLAV       NLNA  GFLGGIVAGF   LAG 342
FruEc 286 AGYIA FSIADRPGLTPGLIGGMLAV       STGS  GFIGGIIAGF   LAG 327
ScrSt 164 IGFTAAREFGGNPYLGATL GGILTHPALTNAWGVAA  GFHTMNFFGFEIAMIG 215
ScrBs 164 IGVSASKEFGSNPYLGA VIGGIMIHPNLLNPWGLAE EQLITCIFSDL ISLFS 215
BglEc 159 LGYTAGKRFGGNPF TAMVIGGALVHPLILTAFENGQKADALGLDFLGIPVTLLN 212

          !         ^ ^^   ^   ^ ^ ^      ^  *^^   ^^! ^    ^*
FruRc 343 Y        VARWLRDAI  KLPRTLEGLKPVLILPLLSTAITGLIMVYVVGTPV 387
FruEc 328 Y        IAKLISTQL  KLPQSMEALKPILIIPLISSLVVGLAMIYLIGKPV 372
ScrSt 216 YQGTVFPVLLAVWFMSIVEKQLRRAIPDALDLILTPFLTVIISGFIALLIIGPAG 270
ScrBs 216 ATGNCYPCPACGVCDEQGREMDEKSGSTCGDLLVTPFVTVIVTGFVAFIAIGPLG 270
BglEc 213 YSSSVIPIIFSAWLCSILERRLNAWLPSAIKNFFTPLLCLMVITPVTFLLVGPLS 267

           ^^ ^^ ^^ ! ^^  ^   ^ *^^ **   ^     ^          !!^  ^^
FruRc 388 AAILAAMTAFLQGLGTTNAVVLGLILGGMMAVDMGGPINKAAYTFAVGLL  TSS 440
FruEc 373 AGILEGLTHWLQTMGTANAVLLGAILGGMMCTDMGGPVNKAAYAFGVGLL  STQ 425
ScrSt 271 RALGDGISFVLSTLISHAGWLAGLLFGGLYSVIVITGIHHSFHAVEAGLLGNPSI 323
ScrBs 271 RALGSGITVALTYVYDHAGFVAGLIFGGTYSLIVLTGVHHSFHAIEAGLI  ADI 323
BglEc 268 TWISELIAAGYLWLYQAVPAFAGAVMGGFWQIFVMFGLHWGLVPLCINNF  TVL 320

           ^  ^  ^ ^   ^   ! ^*!  ^ ^^   ^!^        ^ !*   *!* !!
FruRc 441 TYAPMAAVMAAGMTPPLGLALAT LVAKNRFTAEEREAGGAAAVLGLSFITEGAI 494
FruEc 426 TYGPMAAIMAAGMVPPLAMGLAT MVARRKFDKAQQEGGKAALVLGLCFISEGAI 479
ScrSt 324 GVNFLLPIWAMANVAQGGACLAVWFKTKDAKIKAITLPSAFSAMLG   ITEAAI 375
ScrBs 324 GKNYLLPIWSMANVAQGGAGLAVFFMAKKAKTKEIALPAAFSAFLG   ITEPVI 375
BglEc 321 GYDTMIPLLMPAIMAQVGAALGVFLCERDAQKKVVAGSAALTSLFG   ITEPAV 372

           !    ^          !!*^ **         ^!   *!    *!^
FruRc 495 PFAAKDP ARVIPSIIVGSAITGALSMALGCLLVAPHGGIFVLAIPHAVTNLGLY 548
FruEc 480 PFAARDP MRVLPCCIVGGALTGAISMAIGAKLMAPHGGLFVLLIPGAITPVLGY 533
ScrSt 376  FGINLRFVKPFIAALIGGAAGGAWVVSVHVYMTAV  GLTA  IPGMAIVGASS 425
ScrBs 376  FGVNLRYRKPFIAAMIGGALGGAYVVFTHYAANAY  GLTG  IPMIAIAAPFG 425
BglEc 373  YGVNLPRKYPFVIACISGAL GATIIGYAQTKVYSF GLPS  IFTFMQTIPST 423

          ^^  ^^^  ^ ^^^ ^
FruRc 549 ALSIVVGTLVTTGLLIALKK 568
FruEc 534 LVAIIAGTLVAGLAYAFLKR 553
ScrSt 426 LLNYIIGMVMPLASPLRCSL 445
ScrBs 426 FSNLIHYLIGMAIAAVSAFI 445
BglEc 424 GIDFTVWASVIGGVIAIGCA 444
```

FIG. 7. Alignment of the entire hydrophobic domains of the two fructose PTS permeases with those of three other PTS permeases, those specific for sucrose (Scr) from *S. typhimurium* (St) and *B. subtilis* (Bs) and that specific for β-glucosides (Bgl) from *E. coli*. The alignment was performed by using the progressive multiple alignment program of Feng and Doolittle (18). Abbreviations are as described in the legend to Fig. 6. Symbols: *, all five residues at this position are conserved; !, four of the five residues at this position are conserved; ^, at least one identity is observed at this position between a fructose enzyme II and one of the other three aligned proteins.

residues are conserved is the glutamyl residue (E491 in II^{Rc}_{Fru}; E479 in II^{Fru}_{Ec}). Recognition of the conservation of this residue facilitated the computer alignment of 12 of the 14 sequenced PTS permeases which establishes a common origin for these proteins.

Evidence for a common ancestry of 12 PTS permeases. Figure 8 shows the alignment of 12 of the 14 currently sequenced PTS permeases within a restricted region of their hydrophobic domains surrounding the conserved glutamyl residue noted in the previous section. This glutamyl residue was found to be conserved in the following enzymes II: II^{Mtl}_{Ec}, II^{Glc}_{Ec}, and II^{Nag}_{Ec}; II^{Scr} of *Streptococcus mutans* (58); and the three II^{Lac} proteins of *Staphylococcus aureus* (6), *Lactobacillus casei* (C.-A. Alpert and B. M. Chassy, unpublished data), and *Streptococcus lactis* (W. M. DeVos, R. J. Van Rooijen, I. Boerrigter, B. Reiche, and W. Hengstenberg, unpublished data), in addition to those shown in Fig. 7 suggesting functional significance. Interestingly, the model of Prior and Kornberg (44) places this glutamyl residue directly in the center of a transmembrane helix.

The statistical analyses of this region are presented in Table 4. The 12 permease segments fell into five groups of closely related segments (Table 4, footnote *a*). Comparison scores for segments of permeases in distinct groups revealed that they are all homologous (Table 4, footnote *a*). Particularly significant is the similarity of the three lactose permeases (which are all similar to one another) with the sucrose permease of *S. mutans* (comparison score of 8.6 standard

TABLE 3. Statistical analyses of sequence comparisons between various enzymes II of the PTS[a]

First enzyme II and region compared	Second enzyme II and region compared	% Identity	SD[b]	No. of gaps
Fru-Rc 17-578	Fru-Ec 1-563	54.8	71.0	2
Fru-Rc 155-553	Scr-St 41-435	18.3	7.5	4
Fru-Rc 365-557	Scr-Bs 248-433	19.7	4.5	8
Fru-Rc 251-561	Bgl-Ec 141-440	20.1	7.8	8
Fru-Rc 158-414	Glc-Ec 77-334	19.3	4.3	7
Fru-Ec 142-538	Scr-St 41-435	15.6	5.3	4
Fru-Ec 350-538	Scr-Bs 248-429	21.7	4.5	3
Fru-Ec 235-520	Bgl-Ec 141-426	15.0	4.7	6
Fru-Ec 318-399	Glc-Ec 281-361	22.4	4.0	4
Scr-St 41-435	Scr-Bs 41-435	45.0	73.0	3
Scr-St 201-440	Bgl-Ec 201-440	25.0	16.2	4
Scr-St 5-66	Glc-Ec 400-461	38.1	9.6	2
Scr-Bs 201-440	Bgl-Ec 201-440	27.1	17.6	4
Scr-Bs 5-67	Glc-Ec 400-461	44.4	15.6	1
Bgl-Ec 2-70	Glc-Ec 399-466	36.2	12.4	1

[a] Selected regions of the five PTS permeases whose hydrophobic sequences are depicted in Fig. 7 as well as the glucose permease of *E. coli* are compared. Segments analyzed correspond to those which showed the highest comparison score for each of these pairs of proteins. For abbreviations of PTS enzymes II, see legend to Fig. 6.

[b] The Los Alamos comparison scores were determined as described previously (27). Values are based on the weighted frequencies of identities, conservative substitutions, and gaps.

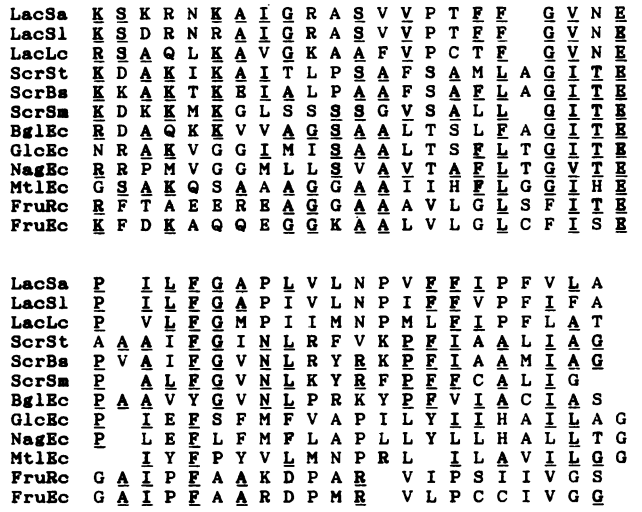

FIG. 8. Alignment of 45 residue segments of 12 of the 14 sequenced PTS permeases. The alignment was performed by using the progressive multiple alignment program of Feng and Doolittle (18). Residues which occur at least four times in any one column (position) are underlined and boldfaced. Abbreviations are the same as those described in the legend to Fig. 7 and as follows: Lac, lactose; Glc, glucose; Nag, *N*-acetylglucosamine; Mtl, mannitol; Sa, *S. aureus*; Sl, *S. lactis*; Lc, *L. casei*; Sm, *S. mutans*. The statistical analyses of these alignments are presented in Table 4, which gives the percent identities between the pairs of segments as well as the comparison scores in SDs for representative comparisons (Table 4, footnote *a*).

deviations [SDs] when the *S. aureus* lactose permease is used in the comparison (Table 4). No evidence was previously available, suggesting that the lactose enzymes II are evolutionarily related to other sequenced PTS permeases (8). Our results strongly support a common evolutionary origin for these proteins. These four proteins exhibited significant similarity throughout their hydrophobic domains (data not shown). Since the sucrose permease of *S. mutans* is clearly homologous to the other sucrose PTS permeases (comparison scores between 15 and 30 standard deviations), this relationship establishes that all 12 permeases are evolutionarily related.

Homology between the bacterial fructose permeases and the human and mouse insulin-responsive glucose facilitators. When the sequence of the *R. capsulatus* enzyme II^{Fru} was screened versus the data base, several proteins were found to exhibit significant identity with it. All of these potentially homologous proteins proved to be transport proteins (uniporters, antiporters, and symporters), and those exhibiting the highest degree of sequence identity with II^{Fru}_{Rc} were glucose facilitators of animals. The alignments of two mammalian glucose facilitators with the two bacterial fructose PTS permeases were published in a preliminary communication (67). The *Rhodobacter* II^{Fru} and the human insulin-responsive glucose facilitator (HirGT) had the highest percent identity (20% identity over a stretch of 178 aminoacyl residues). The average Los Alamos comparison score was 6.1 SDs higher than that obtained with 10×100 comparisons of randomized sequences of these proteins. This fact suggests that II^{Fru} of *R. capsulatus* and the human glucose

TABLE 4. Percent identities between the homologous segments of the 12 PTS permeases shown in Fig. 8[a]

PTS permease	% Identity with:										
	Lac-Sl	Lac-Lc	Scr-St	Scr-Bs	Scr-Sm	Bgl-Ec	Glc-Ec	Nag-Ec	Mtl-Ec	Fru-Rc	Fru-Ec
Lac-Sa	84	53	26	26	33	21	26	19	22	14	19
Lac-Sl		51	23	23	29	16	23	16	20	10	17
Lac-Lc			19	21	21	30	16	21	17	10	14
Scr-St				73	52	42	27	18	33	16	20
Scr-Bs					55	47	34	23	38	20	23
Scr-Sm						43	26	24	23	17	22
Bgl-Ec							32	18	26	25	18
Glc-Ec								60	40	21	21
Nag-Ec									26	16	9
Mtl-Ec										24	24
Fru-Rc											61

[a] The abbreviations are as described in the legends to Fig. 6 through 8. Comparison scores (27) between permease segments within a coherent group ([1] Lac-Sa, Lac-Sl, Lac-Lc; [2] Scr-St, Scr-Bs, Scr-Sm, Bgl-Ec; [3] Glc-Ec, Nag-Ec; [4] Mtl-Ec; [5] Fru-Rc, Fru-Ec) were greater than 15 SDs. The probability (*P*) of getting a score of 15 SD by chance is less than 10^{-40}. Comparison scores obtained for representative segments between groups which establish homology for all 12 segments include: Lac-Sa:Scr-Sm, 8.6 SD ($P < 10^{-17}$); Lac-Sa:Mtl-Ec, 5.8 SD ($P < 10^{-8}$); Scr-Bs:Glc-Ec, 9.2 SD ($P = 10^{-20}$); Bgl-Ec:Fru-Rc, 6.3 SD ($P = 10^{-10}$); Glc-Ec:Mtl-Ec, 9.7 SD ($P < 10^{-20}$); and Mtl-Ec:Fru-Ec, 6.0 SD ($P = 10^{-9}$).

TABLE 5. Percent identities between the segments of two mammalian glucose facilitators and the two fructose enzymes II[a]

Glucose facilitator or enzyme II	% Identity[b] with:			
	MirGT	HirGT	Fru-Rc	Fru-Ec
MirGT		93 (64)	19 (5.7)	16 (2.9)
HirGT			20 (6.1)	17 (3.6)
Fru-Rc				61 (48)

[a] Abbreviations: MirGT and HirGT, insulin-responsive glucose transporters of mice and humans, respectively; Fru-Rc and Fru-Ec, fructose-specific enzymes II of *R. capsulatus* and *E. coli*, respectively.

[b] The program of Feng and Doolittle (18) was used to calculate percent identities. The Los Alamos comparison scores are given in parentheses in SDs (27).

facilitator are homologous, i.e., derived from a common ancestor. The probability of getting such a score by chance is less than 10^{-9}. The Los Alamos comparison score for II^{Fru}_{Rc} with the mouse glucose transporter was 5.7 SDs (19% identity over a stretch of 166 aminoacyl residues), a value which also suggests common ancestry ($P = 10^{-8}$) (Table 5). Corresponding values obtained when the II^{Fru}_{Ec} was compared with these two glucose facilitators were 3.6 and 2.9 SDs, respectively (P values of about 10^{-4} and 10^{-3}, respectively), values which are below the level of reliability for establishing homology. However, the obvious relatedness of the two glucose facilitators with each other and of the two fructose PTS permeases with each other clearly suggests that these four proteins are related. The lower values obtained when II^{Fru}_{Ec} was compared suggest that evolutionary divergence had been more rapid in *E. coli* than in *R. capsulatus*, a conclusion which agrees with the greater degree of sequence divergence of the N-terminal-repeated domain of II^{Fru}_{Ec} relative to those of II^{Fru}_{Rc}.

Examination of the alignments between the two II^{Fru} proteins and the two glucose facilitators (67) revealed that all of the aligned residues may play a structural rather than a catalytic role. Thus, five G's, three P's, two A's, seven L's, and one F are conserved among the four proteins.

Of the 21 residues which aligned in these four permeases, 4 also align with the five PTS permeases shown in Fig. 7. Furthermore, 20 of the 39 residues which show identity between at least one of the bacterial fructose permeases and at least one of the glucose facilitators also show alignment with at least one other PTS permease. These statistics suggest that residues which are conserved between the fructose PTS permeases and the glucose facilitators also tend to be conserved among the PTS permeases. This observation supports the conclusion that these residues serve an important structural role in these transmembrane proteins and that they all may share secondary and tertiary structural features.

Low but possibly significant alignment was observed between the fructose enzymes II and both the chicken erythroid anion transporter (9) and the glycerol phosphate permease (*glpT* gene product) of *E. coli* (13) (23.7% identity over a 131-residue stretch with six gaps, and 22.2% identity over a 99-residue stretch with three gaps, respectively). The comparison scores were too low to establish homology.

DISCUSSION

Numerous recent studies have confronted the problem of the nature of energy coupling to active transmembrane solute transport. (For up-to-date discussions of this subject as it applies to several classes of bacterial permeases including the PTS, see reference 50.) Several such studies applied to the PTS have suggested that although uptake of sugar via a PTS permease is usually coupled to sugar phosphorylation, transport of free sugar in the inward direction via an enzyme II can occur at low rates (62) while efflux of free sugar from the bacterial cell via an enzyme II can occur at accelerated rates under certain physiological conditions (23, 45, 64). Additionally, enzymes II can be specifically mutated so that they lose the capacity to phosphorylate their sugar substrates but retain the capacity to transport them across the membrane (39, 42, 60) or vice versa (35). Recent studies have convincingly demonstrated that for the mannitol-specific enzyme II of *E. coli*, the energy coupling moieties of the permease, which are themselves phosphorylated, are the hydrophilic domains localized to the cytoplasmic side of the membrane while the integral membrane hydrophobic domains bear the sugar binding site (22). The transmembrane channel through which the sugar passes is also presumed to be localized to this region of the protein. Thus, the PTS permeases can be thought of as protein mosaics with distinct functions relegated to distinct protein domains. The hydrophobic, membrane-embedded parts function in transport while the hydrophilic parts, localized to the cytoplasmic side of the membrane, function to couple energy expenditure to solute accumulation. If the PTS proteins are structural mosaics, they may represent evolutionary mosaics with different moieties derived from entirely different ancestral protein domains.

The present study provides substantiation for this prediction. Thus, we recently showed that enzyme I of the *E. coli* and *R. capsulatus* PTSs exhibit homology with pyruvate: phosphate dikinases of plants and bacteria (68), and in this paper we show that the hydrophobic domains of the fructose enzymes II of *R. capsulatus* and *E. coli* are probably homologous with corresponding domains of 10 other PTS permeases as well as with several facilitators of animal cells. The latter proteins do not possess hydrophilic domains and do not couple the expenditure of chemical energy to transport.

Recent evidence has led several investigators to argue that symporters, antiporters, and uniporters (all subclasses of facilitators) are functionally and evolutionarily related (24, 25, 34, 38, 49, 50, 65). We here suggest that this large group of related transport proteins apparently includes the group translocating PTS permeases. Moreover, in a recent report we showed that the pore-forming glycerol facilitator of *E. coli* shares a common ancestry with several proteins of higher organisms which probably possess transport functions (1). These pore-type permeases share structural features with the carrier-type facilitators (1). It is therefore possible that the many pore-type and carrier-type transporters found throughout the living world are derived from one or a few pore-forming, transmembrane, primordial proteins.

Sequencing of the *R. capsulatus* fructose permease and sequence comparisons led to important mechanistic advances related specifically to energy coupling. Thus, the proposed phosphorylation site in the *E. coli* fructose enzyme II (44) seems likely to be incorrect, but we have identified the probable catalytic cysteyl residue in the two proteins since the *R. capsulatus* II^{Fru} possesses only two cysteyl residues and only one of these is conserved between this protein and the *E. coli* homolog. Surprisingly, residues surrounding this cysteyl residue are not well conserved with those surrounding the identified or suspected essential cysteyl residues of other PTS permeases.

We have shown that almost all PTS permeases possess

amphiphilic, potentially helical segments which are believed to function in membrane targeting and initiation of insertion into the membrane (54, 55). Two such structures precede the first presumed transmembrane hydrophobic segment of the *E. coli* II^{Fru} (55). Surprisingly, neither of these amphipathic segments is well conserved in the *Rhodobacter* II^{Fru}. Since *R. capsulatus* is a photosynthetic bacterium with two distinct membranes of different protein compositions (11, 28) and since the *R. capsulatus* II^{Fru} has not yet been successfully expressed in *E. coli* in a functional state (G. A. Daniels, L.-F. Wu, and M. H. Saier, Jr., unpublished results), it is possible that different membrane protein biogenic signals are operative in the two organisms.

The present study emphasizes the universality of fundamental biological principles and serves to suggest rational avenues of scientific investigation. It is becoming increasingly clear that basic molecular mechanisms operative in one organism are applicable to distantly related organisms. These studies thus provide further evidence for the theorem that "the basic life-endowing molecular processes had to exist prior to extensive evolutionary divergence" (53).

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LITERATURE CITED

1. **Baker, M. E., and M. H. Saier, Jr.** 1990. A common ancestor for bovine lens fiber major intrinsic protein, soybean nodulin-26 protein, and *Escherichia coli* glycerol facilitator. Cell **60**:185–186.
2. **Bear, D. G., and D. S. Peabody.** 1988. The *E. coli* Rho protein: an ATPase that terminates transcription. Trends Biochem. Sci. **13**:343–347.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Bramley, H. F., and H. L. Kornberg.** 1987. Nucleotide sequence of *bglC*, the gene specifying enzyme II^{bgl} of the PEP:sugar phosphotransferase system in *Escherichia coli* K12 and overexpression of the gene product. J. Gen. Microbiol. **133**:563–573.
5. **Bramley, H. F., and H. L. Kornberg.** 1987. Sequence homologies between proteins of bacterial phosphoenolpyruvate-dependent sugar phosphotransferase systems: identification of possible phosphate-carrying histidine residues. Proc. Natl. Acad. Sci. USA **84**:4777–4780.
6. **Breidt, F., Jr., W. Hengstenberg, U. Finkeldei, and G. C. Stewart.** 1987. Identification of the genes for the lactose-specific components of the phosphotransferase system in the lac operon of *Staphylococcus aureus*. J. Biol. Chem. **262**:16444–16449.
7. **Carter, P., H. Bedouelle, and G. Winter.** 1985. Improved oligonucleotide site-directed mutagenesis using M13 vector. Nucleic Acids Res. **13**:4431–4443.
8. **Chassy, B. M., and C.-A. Alpert.** 1989. Molecular characterization of the plasmid-encoded lactose-PTS of *Lactobacillus casei*. FEMS Microbiol. Rev. **63**:157–166.
9. **Cox, J. V., and E. Lazarides.** 1988. Alternative primary structures in the transmembrane domain of the chicken erythroid anion transporter. Mol. Cell. Biol. **8**:1327–1335.
10. **Daniels, G. A., G. Drews, and M. H. Saier, Jr.** 1988. Properties of a Tn*5* insertion mutant defective in the structural gene (*fruA*) of the fructose-specific phosphotransferase system of *Rhodobacter capsulatus* and the cloning of the *fru* regulon. J. Bacteriol. **170**:1698–1703.
11. **Drews, G., and J. Oelze.** 1986. Photosynthese bei phototrophen Bakterien. Biol. Unserer Zeit **16**:113–123.
12. **Ebner, R., and J. W. Lengeler.** 1988. DNA sequence of the gene *scrA* encoding the sucrose transport protein Enzyme II^{scr} of the phosphotransferase system from enteric bacteria: homology of the enzyme II^{scr} and enzyme II^{bgl} proteins. Mol. Microbiol. **2**:9–17.
13. **Eiglmeier, K., W. Boos, and S. T. Cole.** 1987. Nucleotide sequence and transcriptional startpoint of the *glpT* gene of *Escherichia coli*: extensive sequence homology of the glycerol-3-phosphate transport protein with components of the hexose-6-phosphate transport system. Mol. Microbiol. **1**:251–258.
14. **Eisenberg, D.** 1984. 3-Dimensional structure of membrane and surface proteins. Annu. Rev. Biochem. **53**:595–623.
15. **Erni, B., and B. Zanolari.** 1986. Glucose-permease of the bacterial phosphotransferase system. Gene cloning, overproduction, and amino acid sequence of enzyme II^{glc}. J. Biol. Chem. **261**:16398–16403.
16. **Erni, B., B. Zanolari, P. Graff, and H. P. Kocher.** 1989. Mannose permease of *Escherichia coli*. Domain structure and function of the phosphorylating subunit. J. Biol. Chem. **264**: 18733–18741.
17. **Erni, B., B. Zanolari, and H. P. Kocher.** 1987. The mannose permease of *Escherichia coli* consists of three different proteins. Amino acid sequence and function in sugar transport, sugar phosphorylation, and penetration of phage lambda DNA. J. Biol. Chem. **262**:5238–5247.
18. **Feng, D. F., and R. F. Doolittle.** 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evol. **25**:351–360.
19. **Fouet, A., M. Arnaud, A. Klier, and G. Rapport.** 1987. *Bacillus subtilis* sucrose-specific enzyme II of the phosphotransferase system: expression in *Escherichia coli* and homology to enzyme II from enteric bacteria. Proc. Natl. Acad. Sci. USA **84**:8773–8777.
20. **Fukumoto, H., T. Kayano, J. B. Buse, Y. Edwards, P. F. Pilch, G. I. Bell, and S. Seino.** 1989. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. J. Biol. Chem. **264**:7776–7779.
21. **Geerse, R. H., F. Izzo, and P. W. Postma.** 1989. The PEP: fructose phosphotransferase system in *Salmonella typhimurium*: FPr combines enzyme III^{fru} and pseudo-HPr activities. Mol. Gen. Genet. **216**:517–525.
22. **Grisafi, P. L., A. Scholle, J. Sugiyama, C. Briggs, G. R. Jacobson, and J. W. Lengeler.** 1989. Deletion mutants of the *Escherichia coli* K-12 mannitol permease: dissection of transport-phosphorylation, phospho-exchange, and mannitol-binding activities. J. Bacteriol. **171**:2719–2727.
23. **Haguenauer, R., and A. Kepes.** 1971. The cycle of renewal of intracellular α-methyl glucoside accumulated by the glucose permease *E. coli*. Biochimie **53**:99–107.
24. **Henderson, P. J. F.** 1990. The homologous glucose transport proteins of prokaryotes and eukaryotes. Res. Microbiol. **141**: 316–328.
25. **Higgins, C. F.** 1990. The role of ATP in binding-protein-dependent transport systems. Res. Microbiol. **141**:353–360.
26. **Kaestner, K. H., R. J. Christy, J. C. McLenithan, L. T. Braiterman, P. Cornelius, P. H. Pekala, and M. D. Lane.** 1989. Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. Proc. Natl. Acad. Sci. USA **86**:3150–3154.
27. **Kanehisa, M.** 1982. Los Alamos sequence analysis package for nucleic acids and proteins. Nucleic Acids Res. **10**:183–196.
28. **Kiley, P. J., and S. Kaplan.** 1988. Molecular genetics of photosynthetic membrane biosynthesis in *Rhodobacter sphaeroides*. Microbiol. Rev. **52**:50–69.
29. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
30. **Lee, C. A., and M. H. Saier, Jr.** 1983. Mannitol-specific enzyme II of the bacterial phosphotransferase system. II. Reconstitution

of vectorial transphosphorylation in phospholipid vesicles. J. Biol. Chem. **258**:10761–10767.
31. **Lolkema, J. S., and G. T. Robillard.** 1985. The coupling between transport and phosphorylation in inside-out vesicles. Eur. J. Biochem. **147**:69–75.
32. **Lolkema, J. S., R. H. ten Hoeve-Duurkens, and G. T. Robillard.** 1985. Mechanism for transfer of the phosphoryl group from phosphoenolpyruvate to fructose. Eur. J. Biochem. **149**:625–631.
33. **Lolkema, J. S., R. H. ten Hoeve-Duurkens, and G. T. Robillard.** 1986. Energetics of the phosphoryl group from phosphoenolpyruvate to fructose. Eur. J. Biochem. **154**:387–393.
34. **Maloney, P. C.** 1990. A consensus structure for membrane transport. Res. Microbiol. **141**:374–383.
35. **Manayan, R., G. Tenn, H. B. Yee, J. C. Desai, M. Yamada, and M. H. Saier, Jr.** 1988. Genetic analyses of the mannitol permease of *Escherichia coli*: isolation and characterization of a transport-negative mutant which retains phosphorylation activity. J. Bacteriol. **170**:1290–1296.
36. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
37. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Mitchell, P.** 1990. Osmochemistry of solute translocation. Res. Microbiol. **141**:286–289.
39. **Nuoffer, C., B. Zanolari, and B. Erni.** 1988. Glucose permease of *Escherichia coli*. The effect of cysteine to serine mutations on the function, stability, and regulation of transport and phosphorylation. J. Biol. Chem. **263**:6647–6655.
40. **Pas, H. H., and G. T. Robillard.** 1988. Enzyme II^{mtl} of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system: identification of the activity-linked cysteine on the mannitol carrier. Biochemistry **27**:5515–5519.
41. **Peri, K. G., and E. B. Waygood.** 1988. Sequence of cloned enzyme II N-acetylglucosamine phosphotransferase system of *Escherichia coli*. Biochemistry **27**:6054–6061.
42. **Postma, P. W.** 1981. Defective enzyme II-B^{Glc} of the phosphoenolpyruvate:sugar phosphotransferase system leading to uncoupling of transport and phosphorylation in *Salmonella typhimurium*. J. Bacteriol. **147**:382–389.
43. **Postma, P. W., and J. W. Lengeler.** 1985. Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **49**:232–269.
44. **Prior, T. I., and H. L. Kornberg.** 1988. Nucleotide sequence of *fruA*, the gene specifying Enzyme II^{fru} of the phosphoenolpyruvate-dependent sugar phosphotransferase system in *Escherichia coli* K12. J. Gen. Microbiol. **134**:2757–2768.
45. **Reizer, J., and M. H. Saier, Jr.** 1983. Involvement of lactose enzyme II of the phosphotransferase system in rapid expulsion of free galactosides from *Streptococcus pyogenes*. J. Bacteriol. **156**:236–242.
46. **Rogers, M. J., T. Ohgi, J. Plumbridge, and D. Söll.** 1988. Nucleotide sequences of the *Escherichia coli nagE* and *nagB* genes: the structural genes for the N-acetylglucosamine transport protein of the bacterial phosphoenolpyruvate:sugar phosphotransferase system and for glucosamine-6-phosphate deaminase. Gene **62**:197–207.
47. **Saier, M. H., Jr.** 1977. Bacterial phosphoenolpyruvate:sugar phosphotransferase systems: structural, functional, and evolutionary interrelationships. Bacteriol. Rev. **41**:856–871.
48. **Saier, M. H., Jr.** 1985. Mechanisms and regulation of carbohydrate transport in bacteria. Academic Press, Inc., New York.
49. **Saier, M. H., Jr.** 1990. Evolution of permease diversity and energy coupling mechanisms: an introduction to a forum on coupling of energy to transmembrane solute translocation in bacteria. Res. Microbiol. **141**:281–286.
50. **Saier, M. H., Jr. (ed.).** 1990. Sixth forum in microbiology: coupling of energy to transmembrane solute translocation in bacteria. Res. Microbiol. **141**:281–395.
51. **Saier, M. H., Jr., B. U. Feucht, and S. Roseman.** 1971. Phosphoenolpyruvate-dependent fructose phosphorylation in photosynthetic bacteria. J. Biol. Chem. **246**:7819–7821.
52. **Saier, M. H., Jr., F. C. Grenier, C. A. Lee, and E. B. Waygood.** 1985. Evidence for the evolutionary relatedness of the proteins of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. J. Cell. Biochem. **27**:43–56.
53. **Saier, M. H., Jr., and G. R. Jacobson.** 1984. The molecular basis of sex and differentiation: a comparative study of evolution, mechanism and control in microorganisms. Springer-Verlag, New York.
54. **Saier, M. H., Jr., B. Schnierow, Y. Yamada, and G. A. Daniels.** 1989. Structures, evolution and membrane insertion of the integral membrane permease proteins of the bacterial phosphotransferase system, p. 771–780. *In* A. Kotyk, J. Skoda, V. Paces, and V. Kostka (ed.), Highlights of modern biochemistry. VSP International Science Publishers, Utrecht, The Netherlands.
55. **Saier, M. H, Jr., P. Werner, and M. Müller.** 1989. Insertion of proteins into bacterial membranes: mechanism, characteristics and comparisons with eukaryotes. Microbiol. Rev. **53**:333–366.
56. **Saier, M. H., Jr., M. Yamada, B. Erni, K. Suda, J. Lengeler, R. Ebner, P. Argos, B. Rak, K. Schnetz, C. A. Lee, G. C. Stewart, F. Breidt, Jr., E. B. Waygood, K. G. Peri, and R. F. Doolittle.** 1988. Sugar permeases of the bacterial phosphoenolpyruvate-dependent phosphotransferase system: sequence comparisons. FASEB J. **2**:199–208.
57. **Sanger, F., A. R. Coulson, B. G. Barrel, A. J. H. Smith, and B. A. Roe.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161–178.
58. **Sato, Y., F. Poy, G. R. Jacobson, and H. K. Kuramitsu.** 1989. Characterization and sequence analysis of the *scrA* gene encoding enzyme II^{Scr} of the *Streptococcus mutans* phosphoenolpyruvate-dependent sucrose phosphotransferase system. J. Bacteriol. **171**:263–271.
59. **Schnetz, K., S. L. Sutrina, M. H. Saier, Jr., and B. Rak.** 1990. Identification of catalytic residues in the β-glucoside permease of *Escherichia coli* by site-specific mutagenesis and demonstration of interdomain cross reactivity between the β-glucoside and glucose systems. J. Biol. Chem. **265**:13464–13471.
60. **Schnetz, K., C. Toloczyki, and B. Rak.** 1987. β-Glucoside (*bgl*) operon of *Escherichia coli* K-12: nucleotide sequence, genetic organization, and possible evolutionary relationship to regulatory components of two *Bacillus subtilis* genes. J. Bacteriol. **169**:2579–2590.
61. **Shull, G. E., and J. Greeb.** 1988. Molecular cloning of two isoforms of the plasma membrane Ca^{2+}-transporting ATPase from rat brain: structural and functional domains exhibit similarity to Na^+, K^+- and other cation transport ATPases. J. Biol. Chem. **263**:8646–8657.
62. **Solomon, E., K. Miyal, and E. C. C. Lin.** 1973. Membrane translocation of mannitol in *Escherichia coli* without phosphorylation. J. Bacteriol. **114**:723–738.
63. **Staden, R.** 1986. The current status and portability of our sequence handling software. Summary for May 1985. Nucleic Acids Res. **14**:217–231.
64. **Sutrina, S. L., J. Reizer, and M. H. Saier, Jr.** 1988. Inducer expulsion in *Streptococcus pyogenes*: properties and mechanism of the efflux reaction. J. Bacteriol. **170**:1874–1877.
65. **Szkutnicka, K., J. F. Tschopp, L. Andrews, and V. P. Cirillo.** 1989. Sequence and structure of the yeast galactose transporter. J. Bacteriol. **171**:4486–4493.
66. **Wooton, J. C., and M. H. Drummond.** 1989. The Q-linker: a class of interdomain sequences found in bacterial multidomain regulatory proteins. Protein Eng. **2**:535–543.
67. **Wu, L.-F., and M. H. Saier, Jr.** 1990. On the evolutionary origins of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Mol. Microbiol. **4**:1219–1222.
68. **Wu, L.-F., J. M. Tomich, and M. H. Saier, Jr.** 1990. Structure and evolution of a multidomain, multiphosphoryl transfer protein: nucleotide sequence of the *fruB(HI)* gene in *Rhodobacter capsulatus* and comparisons with homologous genes from other organisms. J. Mol. Biol. **213**:687–703.
69. **Yamada, M., and M. H. Saier, Jr.** 1987. Glucitol-specific enzymes of the phosphotransferase system in *Escherichia coli*. Nucleotide sequence of the *gut* operon. J. Biol. Chem. **262**: 5455–5463.