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Substrate Recognition Domains As Revealed by Active Hybrids between the D-Arabinitol and Ribitol Transporters from *Klebsiella pneumoniae*

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Two new genes, *dalT* and *rbtT*, have been cloned from the *dal* operon for D-arabinitol and the *rbt* operon for ribitol uptake and degradation, respectively, in *Klebsiella pneumoniae* 1033-5P14, derivative KAY2026. Each gene codes for a specific transporter which, based on sequence data, belongs to a large family of carbohydrate transporters which constitutes 12 transmembrane helices. DalT and RbtT show an unusually high similarity (86.2% identical residues for totals of 425 and 427 amino acids, respectively). This allowed the construction of DalT′-Rbt′T and RbtT′-Dal′T crossover hybrids by using a natural restriction site overlapping Met202. This site is located within the large cytoplasmic loop which connects the putative helices 6 and 7 and in particular the amino- and the carboxy-terminal halves of the transporters. Both hybrids have close to normal transport activities but essentially the substrate specificities and kinetic properties of the amino-terminal half. This result localizes essential substrate binding and recognition sites to the amino-terminal halves of the proteins in this important class of carbohydrate transporters.

Membrane transport systems from prokaryotic and eukaryotic organisms form a large superfamily of facilitators (MFS) that catalyzes uniport, symport, and antiport (11, 19). Members of the MFS superfamily share conserved amino acid sequence motifs and structures. These transporters include in particular facilitators and H^+-symporters for pentoses, hexoses, and polyhydric alcohols. The presence of conserved sequences and structural similarities implies common mechanisms of action. Replacement by localized mutagenesis of defined amino acids which impair transport properties has been used to identify putative catalytic centers. Alternatively, hybrids have been constructed in which a part of a transporter is replaced by the corresponding part of a related transporter having, e.g., different substrate specificity. At present, active hybrids only have been obtained when small parts have been exchanged or when very closely related transporters, as often found in one eukaryotic organism, have been used, e.g., four human glucose transporters (4, 35) or the glucose and galactose transporters from yeast (23). The results from such studies with 12 transmembrane helix facilitators and H^+-symporters tend to localize a region involved in substrate recognition to the carboxy-terminal half or they indicate the presence of a second site in the middle part of these transporters.

In this paper we describe hybrids between a transporter (DalT) for the polyhydric alcohol (pentitol) D-arabinitol and one (RbtT) for its isomer ribitol. The corresponding genes, *dalT* and *rbtT*, respectively, have been located within the *dal* and the *rbt* operons of *Klebsiella pneumoniae* 1033-5P14 and have been cloned from the chromosome of its derivative KAY2026. Direct (15) and indirect (9, 13, 21, 27, 30, 34) evidence for the existence of a transporter for each pentitol in this organism has been given. The corresponding genes, however, have not been mapped or identified, nor has it been shown conclusively which type of transporter is involved in the uptake of the two isomeric pentitols. We describe, furthermore, some properties derived from sequence alignments and kinetic studies for DalT and RbtT and for two crossover hybrids in which the amino- and carboxy-terminal halves have been successfully exchanged. The results localize a region involved in substrate recognition to the amino-terminal half of this ubiquitous class of carbohydrate transporters.

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MATERIALS AND METHODS

Chemicals. D-[1-^{3}H]arabinitol and [1-^{3}H]ribitol were synthesized by Amersham Life Science (Buckinghamshire, United Kingdom). All other chemicals were of commercial origin.

Bacterial strains and plasmids. *K. pneumoniae* KAY2026 (32) and *Escherichia coli* HB101 (7) have been previously described. *E. coli* K-12 strain LGS323 is a D-arabinitol-resistant mutant from LGS322 (12) with a mutation in gene *crr*, while LJ140 Δ(*ptsHIcrr*)::Kan^r is a derivative of *E. coli* K-12 (strain W3110) which lacks enzyme I, histidine protein HPr, and enzyme IIA^{Crr} of the phosphotransferase system. Plasmids pHEX3 and pHEX5 are two low-copy-number derivatives of pSU18 (28) in which the *Hae*II box of pSU18 has been exchanged by the 645-bp *Hae*II box from plasmids pBluescript KS$^+$ (pHEX3) and pBluescript SK$^+$ (pHEX5), respectively. This box includes the multicloning site, the *lacZ*α fragment, the *lacZp-lacZo* region, and the T7 promoter.

Culture media, growth conditions, and transport assays. Lennox broth, phosphate-buffered minimal medium, and MacConkey agar plates containing 1% (wt/vol) of the carbohydrate to be tested have been described before (16). In minimal medium the following were added to the concentrations indicated: L-amino acids and nucleosides, 20 mg/liter; carbohydrates, 10 mM; and vitamins, 5 mg/liter. Sterile-filtered antibiotics were used at the following final concentrations (milligrams per liter): chloramphenicol and kanamycin, 25; tetracycline, 10; streptomycin, 50; ampicillin and spectinomycin, 100; and rifampin, 400.

For transport assays, bacteria were grown at 37°C to about 5×10^8 cells/ml, harvested, washed three times by centrifugation at room temperature, and resuspended in minimal medium lacking substrate to a final density of 5×10^8 cells/ml. Routinely, 0.9 ml of cell suspension was added to 100 μl of the labeled substrate (D-arabinitol, ribitol, galactitol, or mannitol) to the final concentrations indicated, and the solutions were rapidly mixed and then incubated at 25°C. Samples (0.2 ml) were taken after 20, 40, and 60 s, collected on cellulose nitrate discs (pore size, 0.6 μm) by filtration, and washed twice with 1 ml of minimal medium. As a control, cells carrying vectors without the *dalT* or *rbtT* gene for the transporter were used and tested in an analogous way. The filters were dried, and the radioactivity was determined as previously described (31).

Genetic and cloning techniques. Cloning and isolation of DNA, as well as the isolation of plasmids, were done by standard methods (3, 28). The nucleotide sequences of genes *dalT* and *rbtT* were determined by the dideoxy chain termination method of Sanger et al. (29) with the T7 sequencing kit from Pharmacia Biotech, Freiburg, Germany. Computer analysis was done with the GenMon 4.3 program package from the Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, Germany, and by using the BLAST programs and database services provided by the National Center for Biotechnology Information Bethesda,

Md. The T7-dependent overexpression of gene products (33) was done according to a protocol described before (24).

RESULTS

Cloning, sequencing, and identification of the *dalT* and *rbtT* genes from the chromosome of *K. pneumoniae*. Chromosomal DNA was isolated from *K. pneumoniae* KAY2026 (32), a derivative of 1033-5P14 (34), and fragmented with various restriction endonucleases before being cloned into pSU18. To select for D-arabinitol-positive (Dal^+) clones, plasmids were cloned into the mannitol-negative (Mtl^-) strain HB101, which due to a *mtlA1* mutation lacks the mannitol-specific phosphotransferase system enzyme II^{Mtl}. II^{Mtl}-negative strains can be suppressed to a Mtl^+ Dal^+ phenotype by a constitutively expressed DalT transporter which also accepts mannitol and by a D-arabinitol dehydrogenase (gene *dalD*) which converts D-arabinitol to D-xylulose and D-mannitol to D-fructose (2). Among several thousand transformants containing *Hin*dIII-derived DNA fragments, one Mtl^+ Dal^+ colony was found on a MacConkey Mtl Cam plate. The corresponding *dal* genes were located by *Hin*dIII subcloning on a 4.8-kb fragment (pHHL101). The *dal* gene cluster contained, besides the known and promoter proximal genes *dalD* and *dalK* (for a D-xylulose kinase), a new and distantly located gene, *dalT*, but no intact *dalR* gene for a repressor. This is in agreement with previous mapping data (13, 17, 22, 36) which had located *dalR* upstream of *dalD* and had indicated that promoters *dalRp* and *dalDp* are transcribed divergently. Plasmid pHHL101, upon reisolation and transformation into the Δ*mtl* Δ*gut* strain LGS323, conferred a Mtl^+ Dal^+ phenotype again, thus confirming that a Dal or Mtl transporter gene had been cloned and was expressed from this plasmid in large (constitutive) amounts. The transformants retained their Gut^- phenotype, further corroborating the conclusion that the uptake of mannitol and arabinitol were through DalT and not through II^{Gut}, which also accepts mannitol (15). All transformants retained their Rbt^- phenotype.

In a parallel approach, the *rbt* genes for ribitol uptake and degradation were isolated on *Bam*HI-derived DNA fragments, cloned into pHEX3, and transformed into HB101. Among several thousand transformants plated on MacConkey ribitol Cam plates was one Rbt^+ Dal^- Mtl^- colony. The corresponding plasmid pFCK1 contained a 7.2-kb *Bam*HI fragment. The clone as ascertained by DNA sequencing carries a complete *rbtR* gene for the repressor, the known (13, 21, 36) genes for a ribitol dehydrogenase (*rbtD*) and a ribulokinase (*rbtK*), plus as the most distal gene of the *rbt* operon, gene *rbtT* for a transporter (Fig. 1).

The newly discovered genes *dalT* and *rbtT*, which encode a putative D-arabinitol and a ribitol transporter, respectively, were sequenced completely, covering both DNA strands. Gene *dalT* corresponds to an open reading frame containing 1,275 bp and 425 amino acids (calculated M_r, 46,800). It starts with an ATG located 69 bp downstream of *dalK* and is preceded by a typical ribosome binding site (ACGAGG) located 5 bp upstream of the ATG initiation codon. Attempts to visualize the corresponding peptide have failed thus far in a T7 overexpression system. Gene *dalT* is part of the *dal* operon (Fig. 1) and is expressed from promoter *dalDp* located upstream of genes *dalD* and *dalK* (unpublished data).

Gene *rbtT* corresponds to an open reading frame containing 1,281 bp and 427 amino acids (calculated M_r, 46,970). The gene starts with a typical ribosome binding site (TCGAGG) located 5 bp upstream of an initiator codon ATG. It is preceded by gene *rbtK* and is expressed as part of the *rbt* operon (Fig. 1) from promoter *rbtDp* located upstream of gene *rbtD* (reference 36 and unpublished data). In a T7 expression system a diffuse peptide band with an apparent molecular mass of about 42 kDa on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel could be identified as RbtT (data not shown). Running on such gels as a diffuse band and with an apparently decreased molecular mass appears to be typical for integral membrane-bound proteins and numerous carbohydrate transporters (1).

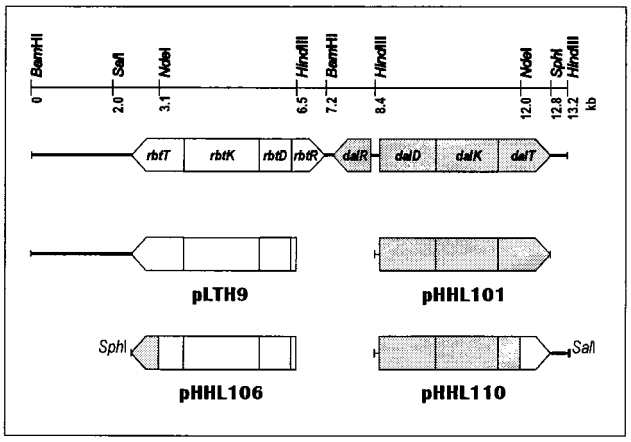

FIG. 1. *dal* and *rbt* gene cloning and construction of hybrid transporters. The genes *dal* for D-arabinitol utilization and *rbt* for ribitol utilization were cloned from the chromosome of *K. pneumoniae* where they are located side by side (accession no. U97126, DalT; U97127, RbtT). Plasmid pHHL101 contains genes $dalR'D^+K^+T^+$ on a 4.8-kb *Hin*dIII fragment (pHHL103 in opposite orientation) cloned into pSU18, and pLTH9 contains the $rbtR'D^+K^+T^+$ genes on a 6.5-kb *Bam*HI-*Hin*dIII fragment cloned into the pSU18 derivative pHEX3. The two crossover hybrids on pHHL106 and pHHL110 were constructed with the restriction sites indicated. They are expressed from promoters *dalDp* or *rbtDp*. Within the vectors, *dal* genes are in shaded boxes and *rbt* genes are in open boxes, and thin lines indicate adjacent chromosomal DNA also present on the clones.

In comparing the DalT and RbtT sequences (Fig. 2), it became immediately apparent that they have an unusually high similarity (425 versus 427 residues in length; 86.2% identical residues; 44 exchanges, excluding the C-terminal loop, among them 26 nonconservative exchanges). This high similarity is also reflected in their almost identical hydrophobicity plots (Fig. 3). According to these plots both proteins correspond to the class of integral membrane-bound proteins and in particular to those proteins which constitute two times six transmembrane structures connected through a large hydrophilic loop. The calculated two-dimensional model for both transporters closely resembles those proposed for other members of the MFS superfamily (Fig. 4) and more precisely those for several bacterial H^+-symporters for carbohydrates such as the L-arabinose (AraE), D-xylose (XylE), lactose (LacY), sucrose (CscB), and sn-glycerolphosphate (GlpT) permeases. This fits the physiological data (see below) which support the notion that DalT and RbtT correspond to two pentitol-specific H^+-symporters.

Construction of DalT′-Rbt′T and RbtT′-Dal′T hybrid transporters. Active hybrids between two transporters with different substrate specificities have thus far only been obtained in eukaryotes in which many closely related systems often occur in one organism. The analysis of such eukaryotic hybrids, however, is often hampered by the lack of adequate expression systems or of tester strains lacking all transporters for the substrate under consideration or by close overlapping substrate specificities of the two parent systems. Because the highly similar DalT and RbtT transporters can be distinguished based on their substrate specificities and affinities, because *E. coli*

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                10         20         30         40         50
DalT    1  MSINNKQWLG LPLNLLWGYI AIAVFMTGDG FELAFLSHYI KALGFSPAEA
           || ||||| | ||||| ||| ||||||||||| |||||||||| |||| || |
RbtT    1  MSVNNKQWYG LPLNLIWGYV AIAVFMTGDG FELAFLSHYI KALGFTPAQA

                60         70         80         90        100
DalT   51  SFAFTLYGLA AALSAWISGV VAEIITPLKT MMIGFVLWCV FHVLFLVFGL
           |||||||||| |||||| ||| ||||||| | | |||||||| |||||||||
RbtT   51  SFAFTLYGLA AALSAWVSGV VAEIITPRKA MLIGFVLWCV FHVLFLVFGL

               110        120        130        140        150
DalT  101  GHANYALILL FYGIRGFAYP LFLYSFIVAI VHNVKSDNAS SAIGWFWAVY
           | |||||||| |||||| ||| |||||||||| ||| || | || |||||||
RbtT  101  GRANYALILL FYGIRGLAYP LFLYSFIVAI IHNVRSDSSS SALGWFWAVY

               160        170        180        190        200
DalT  151  SIGIGVFGSY IPSFTIPHIG EMGTLWLALA FCLTGGVIAL VSLRHIQTPQ
           | |||||||| |||||||||| ||||||||| || ||| ||| || || ||
RbtT  151  SVGIGVFGSY IPSFTIPHIG EMGTLWLALL FCATGGIIAL VSMRHTETPR

               210        220        230        240        250
DalT  201  HMQNLTTREK FSELGRAATL LYTNRNILLS SMVRIINTLS LFGFAVIMPM
           |||||||||| | |||||||| ||||| || | | |||||||| ||||||||||
RbtT  201  HMQNLTTREK FAELGRAATL LYTNRSILFS SIVRIINTLS LFGFAVIMPM

               260        270        280        290        300
DalT  251  MFVDELGFST SEWLQVWAVF FFTTIFSNVL WGILGEKLGW MKVVRWFGCI
           |||||||| | |||||||| | ||||||||| ||| || || ||| ||||||
RbtT  251  MFVDELGFTT SEWLQVWAAF FFTTIFSNVF WGIVAEKMGW MKVIRWFGCI

               310        320        330        340        350
DalT  301  GMALSSLAFY YIPQHFGHSF AMALIPAIAL GIFVAAFVPL AAVFPALEPK
           |||||||||| | |||||| | |||| ||||| ||||||||| |||||||||
RbtT  301  GMALSSLAFY YLPQHFGHNF AMALVPAIAL GIFVAAFVPM AAVFPALEPN

               360        370        380        390        400
DalT  351  HKGAAISVYN LSAGMSNFLA PAIAVVLLPF FSTIGVVIAY TALYVVAFFL
           |||||||||| |||| ||||| ||||||||| |||||||||| |||| ||||
RbtT  351  HKGAAISVYN LSAGLSNFLA PAIAVVLLPY FSTIGVVIAY TALYILAFFL

               410
DalT  401  CAFIRVEQPG F-S--H-KEA TAREQVEFS    425
           | |||||||| | | | |   || |  |
RbtT  401  CPLIRVEQPG FTSDQHAKPF TANAA-E-S    427
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FIG. 2. Amino acid sequence alignment for DalT and RbtT. Both sequences are given in single-letter code with identical residues indicated by vertical lines.

K-12 strains lacking the corresponding transport activities are available, and because both systems can be expressed at physiological levels, they should provide optimal hybrids to map essential catalytic domains. By using a natural *Nde*I restriction site located in both transporters at an identical site (Met202) (Fig. 4), two crossover hybrids (DalT′-Rbt′T and RbtT′-Dal′T) were constructed. Because these sites are located within the cytoplasmic loop which connects the amino- and the carboxy-terminal halves of the molecule, these major domains have been shuffled in the hybrids.

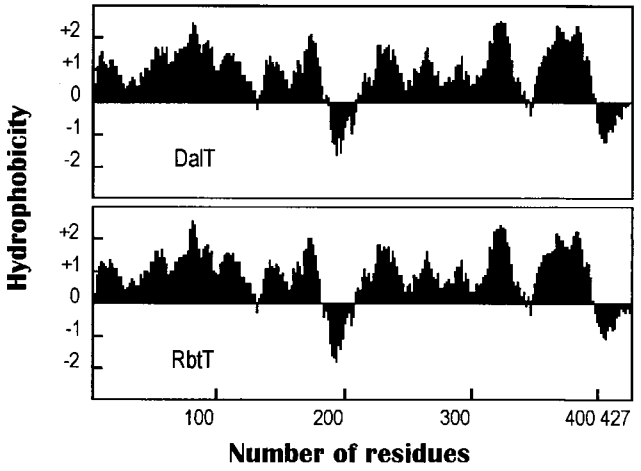

FIG. 3. Hydropathy plots of DalT and RbtT. The plots, which are almost identical, identify both transporters as integral membrane proteins with up to 12 transmembrane structures (plots were generated by using an active windows size of 20).

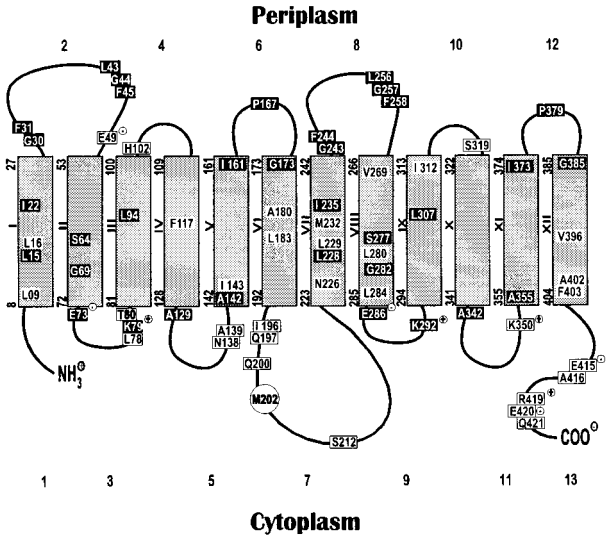

FIG. 4. Calculated two-dimensional model for the DalT and RbtT transporters. An identical two-dimensional model deduced from the hydropathy plots and by analogy to local similarities with other members of the MFS superfamily (see text) has been derived for both transporters. Care was taken to place identical residues and equidistant motifs (boxed in black) conserved between the amino- and carboxy-terminal halves at (nearly) identical positions. Also indicated are nonconservative exchanges between DalT and RbtT (open boxes) and the conserved M202 residue which indicates the *Nde*I restriction site used to construct the DalR′-Rbt′T and Rbt′T-Dal′T crossover hybrids. Transmembrane structures are indicated by roman letters, and hydrophilic loops are indicated by arabic numbers.

To construct a *rbtT′-dal′T* hybrid, the 4.8-kb *Hin*dIII fragment from pHHL103 (Fig. 1) with genes *dalR′D*$^+$*K*$^+$*T*$^+$ was cut with *Sph*I, treated with Klenow enzyme to generate 3′ blunt ends, and then cut with *Nde*I to obtain the 660-bp carboxy-terminal half of gene *dalT*. In parallel, pLTH9 (Fig. 1) with genes *rbtR′D*$^+$*K*$^+$*T*$^+$ was cut with *Bam*HI, treated with Klenow enzyme to generate 5′ blunt ends, and then cut with *Nde*I. The carboxy-terminal half of *dalT* was finally fused to the *rbtT′* half of pLTH9 deleted of its *rbt′T* half. The *dalT* fragment ends 10 bp after the stop codon, and the *rbtT′-dal′T* hybrid is expressed from its natural promoter *rbtDp* together with genes *rbtD* and *rbtK* (pHHL106) (Fig. 1).

To construct the crossover *dalT′-rbt′T* hybrid, the *Hin*dIII-*Nde*I fragment with genes *dalR′D*$^+$*K*$^+$*T*$^+$ obtained as before was fused at the *Nde*I site with the carboxy-terminal half of *rbtT*. This construct, which ends 751 bp downstream of the *rbtT* stop codon, was cloned into the same low-copy-number vector as the previous constructs to yield pHHL110 (Fig. 1). In both hybrids, the sequences surrounding the *Nde*I fusion sites were determined on both strands from plasmids which had been transformed and replicated repeatedly in HB101. No unexpected mutations were detected (data not shown).

Physiological characterization of the new clones and hybrid permeases. In contrast to *E. coli* or *K. pneumoniae* KAY2026, which both have a Dal$^+$ Rbt$^+$ phenotype, *E. coli* K-12 lacks the corresponding genes and enzymes and is unable to metabolize these isomeric pentitols (13, 26, 30). After transformation with plasmid pLTH9 carrying the *rbt* genes or pHHL101 carrying the *dal* genes from KAY2026, the K-12 mutant HB101 (*mtlA1 lacY1*) shows a Rbt$^+$ or a Dal$^+$ phenotype, respectively. As

TABLE 1. Phenotype of *E. coli* HB101 transformed with different plasmids[a]

Plasmid	Genotype	Phenotype			
		Rbt	Dal	Mtl	Lac
pSU18		w	w	w	w
pLTH9	*rbtD*$^+$*K*$^+$*T*$^+$	2	w	w	w
pHHL106	*rbtD′K*$^+$*T′dal′T*	2	s	w	w
pHHL101	*dalD*$^+$*K*$^+$*T*$^+$	w	2	+	+
pHHL110	*dalD*$^+$*K*$^+$*T*$^+$*rbt′T*	w	2	+	+

[a] Strain HB101 transformed with the plasmids indicated was tested on MacConkey plates containing (1% wt/vol) ribitol (Rbt), D-arabinitol (Dal), D-mannitol (Mtl), or lactose (Lac). The reactions ranged from positive (strongly red [2+] and red [+]) to negative (white [w]) and sensitive (s).

stated before, the pHH101 clone which expresses the *dal* gene constitutively due to the deletion of the DalR repressor gene also causes in HB101 an Mtl$^+$ phenotype. This confirms previous observations (8, 34) according to which the D-arabinitol transporter also transports free D-mannitol (15). Curiously, pHHL101 causes a weak Lac$^+$ phenotype.

After transformation, the *dalT′-rbt′T* hybrid on pHHL110 confers to the cells the same (Dal$^+$ Mtl$^+$ Lac$^+$ Rbt$^-$) phenotype as the *dalT*$^+$ clone, i.e., D-arabinitol and D-mannitol transport and metabolism. Due to the lack of the ribitol-specific metabolic enzymes the Rbt$^-$ phenotype does not necessarily indicate a lack of ribitol transport in the transformed cells. Transformation with the *rbtT′-dal′T* hybrid on pHHL106, however, causes a Rbt$^+$ phenotype indicating ribitol uptake and fermentation. Transformed cells remain Mtl$^-$ and become Dals (Table 1). This indicates D-arabinitol uptake which, due to the lack of D-arabinitol-degrading enzymes, should cause a sensitive phenotype (30). In summary, the results with the hybrid permeases indicate that both are active and seem to have the specificity of the amino-terminal half (Table 1). This includes the Lac$^+$ phenotype of cells transformed with the DalT′-Rbt′T hybrid.

To test the various transport activities in a more quantitative way, direct transport tests with radioactively labeled D-arabinitol, galactitol, and ribitol were done. No direct tests for ribitol uptake had thus far been possible in enteric bacteria, and direct DalT tests had only been possible with the low-affinity analog D-mannitol (15). Based primarily on growth tests (references 8, 9, 27, 30, 34 and unpublished data), the following substrates (with decreasing efficiency) have been proposed for DalT: D-arabinitol, galactitol, D-mannitol, xylitol, ribitol, and D-xylose. For RbtT, however, ribitol, D-arabinitol, galactitol, and L-arabinitol have been postulated as substrates.

The direct tests (Table 2) corroborate these conclusions for Rbt (pLTH9) although they were hampered by a high endogenous ribitol uptake activity present in HB101. The ribitol tests were thus done in a Δ(*ptsHIcrr*)::Kan^r derivative of *E. coli* K-12 (LJ140) which lacks this activity. In contrast to HB101, however, all plasmids were unstable in LJ140. Thus, only freshly transformed cultures containing less than 50% cured cells could be used. These conditions and the lowered expression of the cyclic AMP receptor protein-dependent *dal* and *rbt* genes in the Crr$^-$ mutant LJ140 might cause decreased V_{max} values but they should not alter the apparent K_m values (K_m^{app} values). Because no labeled xylitol was available, a 500-fold excess of cold xylitol (10 mM) was added together with labeled ribitol (20 μM). No inhibition was found, thus excluding xylitol as a substrate for RbtT.

For DalT, the direct tests identified D-arabinitol and galactitol as high-affinity substrates (Table 2). This contrasts with D-mannitol (K_m^{app}, approximately 5 mM; V_{max}, 125 nmol per min per mg of protein) and xylitol (inhibition constant [K_i] around 10 mM), which, in agreement with previous data (15), appear to be low-affinity substrates. Cells carrying DalT (pHHL101) showed ribitol uptake activities, but these activities were too low to be measured accurately (data not shown).

Most interesting, however, were the data obtained with the two hybrid permeases (Table 2). First, both showed near-normal or normal transport activities compared to the intact transporters expressed in the same strain from the same plasmid and from their natural promoter. Second, both showed almost exactly the same substrate affinities (based on apparent K_m values) and specificities as those of the intact transporters. In particular, the RbtT′-Dal′T hybrid retained the lower affinity for D-arabinitol compared to ribitol, while the DalT′-Rbt′T hybrid did not gain any detectable ribitol uptake activity. These results seem to localize essential substrate binding and recognition sites to the amino-terminal halves of these transporters.

DISCUSSION

Two genes, *dalT* and *rbtT*, located downstream of the known *dal* genes for D-arabinitol metabolism and *rbt* genes for ribitol metabolism, respectively, have been cloned from the chromosome of *K. pneumoniae* and sequenced. The corresponding genes are also present within the *atl* (for D-arabinitol) and the *rtl* (for ribitol) operons from *E. coli* C (unpublished data). Previous studies had indicated that these systems in enteric bacteria are neither phosphoenolpyruvate-dependent phosphotransferase systems (34) nor osmotic-shock-sensitive (binding-protein-dependent) systems and thus are most likely secondary transport systems (15). This notion is supported by the sequencing data which indicate that both systems belong to the large MFS superfamily as defined by Marger and Saier (19). They do not, however, clearly belong to one specific family of the established five families and instead have properties in common with several of these, in particular with those of the first family of Griffith et al. (11). Based on hydropathy plots (Fig. 3), they resemble other H$^+$-symporters which constitute two times six transmembrane helices. The amino- and carboxy-terminal halves of DalT resemble each other (about 11% identical residues, no gaps), and the two halves of RbtT have a similar resemblance. A total of 58% of these identical residues are also conserved between both transporters (Fig. 4 and 5). Based on similar observations, an ancient gene duplication has been proposed as an explanation for the amino acid conserva-

TABLE 2. Transport activities of the various pentitol transporters[a]

Plasmid	Genotype	K_m^{app} (μM)			V_{max} (nmol/mg of protein)		
		Dal	Gat	Rbt	Dal	Gat	Rbt
pSU18		ND	ND	ND	≤0.1	≤0.1	≤0.1
pLTH9	*rbtD*$^+$*K*$^+$*T*$^+$	150	500	45	21.0	100.0	4.0
pHHL106	*rbtD*$^+$*K*$^+$*T′dal′T*	200	400	54	22.0	45.0	8.0
pHHL101	*dalD*$^+$*K*$^+$*T*$^+$	55	230	ND	16.0	90.0	Yes[c]
pHHL110	*dalD*$^+$*K*$^+$*T′rbt′T*	50	170	ND	16.0	83.0	Yes

[a] Cells of strain HB101 growing exponentially on minimal glycerol medium with the appropriate antibiotic for the various plasmids were tested for transport activity by using D-[1-^{3}H]arabinitol (Dal), [1-^{3}H]galactitol (Gat), and [1-^{3}H]ribitol (Rbt). For Rbt tests, a Δ(*ptsHIcrr*)::Kan^r derivative LT140 was used. Ribitol transport activities have lowered V_{max} values because of a lowered expression of the *dal* and *rbt* genes in the Crr$^-$ mutant LJ140 and plasmid curing.

[b] ND, not determined.

[c] Activities with cells containing plasmids pHHL101 and pHHL110 were too low to be determined accurately.

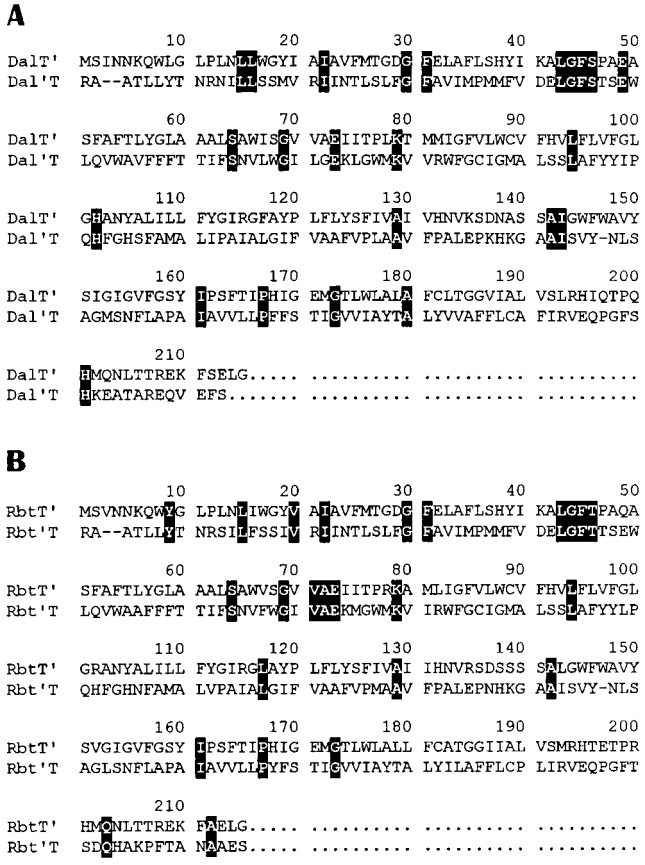

FIG. 5. Alignments of the amino- and carboxy-terminal halves of DalT and RbtT. Alignments of the amino-terminal half (residues 1 to 215) and the carboxy-terminal half (residues 216 to 425) of DalT (A) and RbtT (B) are shown. Identical residues located at identical positions in both halves are boxed.

tion between both halves of the two times six transmembrane helix transporters (18).

Sequence conservation within residues 1 to 130 and 230 to 340 is highest between the two halves of one molecule and between various members of the MFS superfamily. These conserved sequences most likely indicate essential two-dimensional (or three-dimensional) structures. All residues conserved between the amino- and the carboxy-terminal halves of DalT and RbtT are located at identical places (Fig. 4), further corroborating this conclusion. Local similarities (from 20 to 50 residues) can be found with other members of the MFS family, some of which are between the amino- and the carboxy-terminal halves of a single transporter. Thus, based on hydropathy plots and sequence similarities, transmembrane helices TM I to IV, VI, and VII (and probably V) of the pentitol transporters are located in a configuration which is similar to that for the sn-glycerolphosphate transporter GlpT from *E. coli* (20% identical residues, no gap), for which the topology has been analyzed through PhoA and LacZ fusions (10). For the remaining part of GlpT, only three fusions were available and the exact topology is not known. Regions of extensive similarity (24 to 35% identical residues, no gaps) were also found for the quinolone resistance protein NorA (25, 37) at residues 19 to 97 (TM I to III), 228 to 258 (TM VII to loop 8), and 352 to 377 (TM XI). In contrast, TM VIII (residues 256 to 296) of the pentitol transporters shows similarity (36% identical residues) to TM II of members of the LacY family, e.g., the sucrose H^+-symporter of *E. coli* (CscB, residues 41 to 81) (6), and TM III to V (residues 81 to 173) of the pentitol transporters show similarity (21% identical residues) to TM IX to XI of a xanthosine permease of *E. coli* (accession no. 1788745 [5]). The results from the hydropathy plots (Fig. 3), as well as from the GlpT (10) and LacY fusion studies (14), were used to construct a two-dimensional model (Fig. 4). In this model identical motifs found in the amino- and in the carboxy-terminal halves (Fig. 5) are placed in an analogous way in or out of the membrane. TM II, III, and VI correspond almost exactly to TM VIII, IX, and XII, while TM IV-V and TMX-XI deviate only slightly in their location (length). However, a clear deviation is found between residues 9 to 51 and 222 to 264. These 43 residues, which show the highest conservation between the amino- and the carboxy-terminal halves (24% identical residues, no gaps), form TM I and the hydrophilic loop 2 in the amino-terminal half. In the carboxy-terminal half they are predicted to form part of hydrophilic loop 7 and part of TM VII. It remains to be shown whether this deviation is a computer artifact and the symmetric model shown in Fig. 4 is the correct one or whether this puzzling deviation in otherwise highly symmetric molecules represents an essential structure. A certain asymmetry is also seen in the distribution of the six charged and hydrophilic residues found in the central parts of the 12 transmembrane structures. These are N4 in TM I, H92 in TM II, R115 in TM III, R237 and N237 in TM VII, and N278 in TM VIII.

The results obtained from the two crossover hybrids used in this study seem to locate essential residues responsible for the different substrate specificities and affinities between DalT and RbtT within the amino-terminal half of these pentitol specific transporters. These differences should be caused by one to several of the 20 peculiar amino acid exchanges found between the amino-terminal halves of the two molecules. Most previous studies with hybrids and with mutants obtained through localized mutagenesis and exchange of a particular amino acid have tended to localize catalytic sites to the carboxy-terminal halves and regulatory and structural motifs to the amino-terminal halves (references 4, 14, 20, 23, and 35 and references therein). Very often, however, the hybrids have no or very low activities and only slightly altered binding affinities (4). Alternatively, the substrate binding sites have been determined through competition studies with nonphysiological analogs, e.g., cytochalasin B and forskolin as glucose and galactose analogs, for which direct binding to the physiological carbohydrate binding site has never been shown conclusively (20, 35). Interestingly, active hybrids between a galactose- and a glucose-specific transporter from yeast tend to localize essential binding sites to both halves (23). Finally, the observation discussed before, that structures in the amino-terminal halves of one subfamily are often found in the carboxy-terminal halves of another subfamily, could indicate that substrate recognition sites are concentrated within one subfamily in one half, and in another subfamily in the other half. Changing deviating residues of the DalT transporter one by one or in clusters into the RbtT-specific residues or selecting spontaneous mutations converting the specificity of one transporter sequentially into that of the other transporter should help to identify the essential residues involved in substrate recognition and binding. These studies are currently under way in our group.

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