Genes for D-arabinitol and ribitol catabolism from *Klebsiella pneumoniae*

H. Heuel, A. Shakeri-Garakani, S. Turgut and J. W. Lengeler

Author for correspondence: J. W. Lengeler. Tel: +49 541 969 2290. Fax: +49 541 969 2293.
e-mail: lengeler@sfbbio1.biologie.uni-osnabrueck.de

Universität Osnabrück,
Fachbereich
Biologie/Chemie, D-49069
Osnabrück, Germany

The enzymes for catabolism of the pentitols D-arabinitol (Dal) and ribitol (Rbt) and the corresponding genes from *Klebsiella pneumoniae* (*dal* and *rbt*) and *Escherichia coli* (*atl* and *rtl*) have been used intensively in experimental evolutionary studies. Four *dal* and four *rbt* genes from the chromosome of *K. pneumoniae* 1033-5P14 were cloned and sequenced. These genes are clustered in two adjacent but divergently transcribed operons and separated by two convergently transcribed repressor genes, *dalR* and *rbtR*. Each operon encodes an NAD-dependent pentose dehydrogenase (*dalD* and *rbtD*), an ATP-dependent pentulose kinase (*dalK* and *rbtK*) and a pentose-specific ion symporter (*dalT* and *rbtT*). Although the biochemical reactions which they catalyse are highly similar, the enzymes showed interesting deviations. Thus, DalR (313 aa) and RbtR (270 aa) belong to different repressor families, and DalD (455 aa) and RbtD (248 aa), which are active as a monomer or as tetramers, respectively, belong to different dehydrogenase families. Of the two kinases (19.3% identity), DalK (487 aa) belongs to the subfamily of short D-xylulokinases and RbtK (D-ribulokinase; 535 aa) to the subfamily of long kinases. The repressor, dehydrogenase and kinase genes did not show extensive similarity beyond local motifs. This contrasts with the ion symporters (86.6% identity) and their genes (82.7% identity). Due to their unusually high similarity, parts of *dalT* and *rbtT* have previously been claimed erroneously to correspond to 'inverted repeats' and possible remnants of a 'metabolic transposon' comprising the *dal* and *rbt* genes. Other characteristic structures, e.g. a secondary *attλ* site and *chi*-like sites, as well as the conservation of this gene group in *E. coli* C are also discussed.

Keywords: D-arabinitol, ribitol, *dal*, *rbt*, *Klebsiella pneumoniae*, enteric bacteria

INTRODUCTION

The pentitols ribitol (adonitol; Rbt) and D-arabinitol (D-arabitol; Dal) are used by 85% of *Klebsiella* strains and by 10% of *Escherichia coli* strains, e.g. *E. coli* C, but not *E. coli* K-12 and B (Reiner, 1975). Their metabolism in both organisms comprises similar catabolic steps but separate and inducible genes and enzymes. Metabolism starts with an H⁺ symporter for transport and proceeds as summarized in Fig. 1. The corresponding genes are named *dal* and *rbt* in *Klebsiella*, but *atl* and *rtl* in *E. coli*. They are controlled by repressors DalR and RbtR and the inducers D-arabinitol and D-ribulose, respectively (references in Hartley, 1984b; Mortlock, 1984).

The GenBank accession numbers for the sequences reported in this paper are AF045245 (*dal*) and AF045244 (*rbt*).

The genetics of pentitol catabolism in enteric bacteria is of interest for several reasons. (i) The *dal* and *rbt* genes are adjacent and oppositely oriented, perhaps indicating a gene duplication and inversion which occurred during their evolution (Charnetzky & Mortlock, 1974c; Neuberger & Hartley, 1979; Scangos & Reiner, 1978). (ii) The *dal* and *rbt* genes are flanked by long (about 1400 bp) inverted repeats and are perhaps part of a 'metabolic transposon' (Link & Reiner, 1982). (iii) The *dal* and *rbt* genes are replaced in Dal[−] Rbt[−] strains by the non-homologous *gat* genes for galactitol metabolism (genotypic exclusion), i.e. these genes map at identical positions but appear to be mutually exclusive (Link & Reiner, 1983; Reiner, 1975; Woodward & Charles, 1983). (iv) Pentitol catabolism has been used extensively (for a review see Mortlock, 1982) as a model for studying the acquisition of new metabolic capacities, in particular

Fig. 1. Metabolic pathways for D-arabinitol and ribitol in enteric bacteria. The metabolic pathways for D-arabinitol (Dal) and ribitol (Rbt) as found in enteric bacteria are shown together with the enzymes and transporters, repressors and inducers, and the corresponding genes. The genes are called *dal* and *rbt* in *Klebsiella pneumoniae*, but *atl* and *rtl* in *E. coli*. *rpeA* encoding D-ribulose-5-phosphate epimerase is not one of the *dal* or *rbt* genes.

experimental evolution in chemostats involving gene duplications and amplifications (Hartley, 1984a; Mortlock, 1984; Wu *et al.*, 1968).

Despite their importance in evolutionary studies, for most *dal* and *rbt* genes only partial sequences are available; neither their exact number nor their nature are known, nor have comparisons for all genes and gene products been presented. Furthermore, contradictory results concerning gene location within the operons of *Klebsiella* have been published (Charnetzky & Mortlock, 1974c; Wu *et al.*, 1985). Different nomenclature for the genes of different *Klebsiella* strains has added to the confusion. In this study we present the complete DNA sequence of the *dal* and *rbt* genes from *K. pneumoniae* and describe their location within two operons. We also show that the *dal* and *rbt* genes, although encoding enzymes catalysing similar enzymic reactions, are non-homologous, except for the transport systems, and are not flanked by long inverted repeats. Finally, we propose an amended genetic nomenclature for both systems. *rbtB* and *dalB* (Charnetzky & Mortlock, 1974c) are renamed *rbtR* and *dalR* (for repressor), *rbtC* and *dalC* (Charnetzky & Mortlock, 1974c) are renamed *rbtDp* and *dalDp* (for promoter) and *rtlABC* and *atlABC* (Reiner, 1975) now correspond to *rtlDKR* and *atlDKR* to comply with official genetic nomenclature.

METHODS

Bacterial strains, plasmids and culture conditions. Strain KAY2026 (Sprenger & Lengeler 1984) used in this study as the source of the *dal* and *rbt* genes is a phage P1-sensitive Arg⁻

Gua⁻ derivative of *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) 1033-5P14 (Tanaka *et al.*, 1967). *Escherichia coli* C (*atl rtl*), HB101, mutants BL21(λDE3) and JM109 of *E. coli* K-12, and plasmids pSU18, pBluescript II SK(+) (Ausubel *et al.*, 1990), and pHEX3 and pHEX5 (Heuel *et al.*, 1997) have all been described. Minimal and complex media, and MacConkey indicator plates containing 1% carbon source have been described previously (Lengeler, 1975).

Transport and enzyme tests. Transport tests using [¹⁴C]-labelled D-arabinitol, D-mannitol and ribitol were done as described previously taking samples between 0 and 60 s (Heuel *et al.*, 1997). D-Arabinitol and ribitol dehydrogenases were tested in cell extracts also as described previously (Lengeler & Lin, 1972). To test the inducibility of the *dal* and *rbt* genes, cells were grown exponentially in minimal medium with glycerol as carbon source. For induction, D-arabinitol or ribitol (0.2%) were added and the cells were harvested after one further generation. They were washed twice in minimal medium before being tested.

Isolation of plasmid DNA, restriction analysis and cloning procedures. All manipulations with recombinant DNA were carried out by standard procedures as described by Ausubel *et al.* (1990). Preparation of plasmid DNA was done by phenol extraction (Sambrook *et al.*, 1989) or by using the JetStar DNA purification system (Genomed), while the isolation of chromosomal DNA from KAY2026 was according to Neumann *et al.* (1992). Restriction enzymes from various commercial sources were used according to the recommendations of the suppliers. Oligonucleotides for sequencing or PCR were purchased either from TIB Molbiol Syntheselabor or from Life Technologies. DNA amplification by PCR was done according to Saiki *et al.* (1988) with *Taq* polymerase from Boehringer Mannheim and an Air Thermo-Cycler 1605 from Idaho Technology.

DNA sequencing and sequence analysis. To sequence the *dal* genes cloned into pHHL104 and the *rbt* genes cloned into pFCK1 (Fig. 2), known restriction sites were used to obtain smaller clones, the ends of which could be sequenced after subcloning. Where needed, longer fragments were shortened by exonuclease III deletion. Finally, specific DNA primers were used to complete the sequences flanking restriction sites. All DNA sequences were determined on both strands by the dideoxy chain-termination method with the T7 sequencing kit from Pharmacia. Computer analysis was done with the GenMon 4.3 program from the Gesellschaft für Biotechnologische Forschung (Braunschweig, Germany) and the BLAST programs and database services provided by the National Center for Biotechnology Information (Bethesda, MD, USA).

T7 RNA polymerase-dependent overexpression and primer extension analysis. T7 RNA polymerase-dependent overexpression of the *dal* gene products and of *rbtT* was done according to a protocol described previously (Nobelman & Lengeler, 1996) using derivatives of medium-copy-number plasmids pHEX3 and pHEX5 (Heuel *et al.*, 1997) and strain BL21(λDE3). The original high-copy-number expression vectors of Tabor & Richardson (1985) or pBluescript II SK(+) (Ausubel *et al.*, 1990) were exceedingly unstable and could not be used. Primer extension experiments were performed according to Ausubel *et al.* (1990). To determine the transcription initiation sites of *dalDp* and *dalRp*, total cellular RNAs were prepared from HB101(pHHL101) (*dalDKT*) and from HB101(pHHL102) (*dalR*). The cells were inoculated into 50 ml of 2 × TY plus chloramphenicol (25 µg l⁻¹) to an OD₆₅₀ of 0.1 and harvested when the cultures reached an

Fig. 2. Structure of the *dal* and *rbt* operons from *K. pneumoniae*. The operons with genes *D* (dehydrogenase), *K* (kinase), *T* (transporter) and *R* (repressor) were cloned from the chromosome of strain 1033-5P14. The genes are arranged and transcribed (direction of arrows) as indicated. They are flanked by *dhnaA*, also found in *E. coli* K-12, and an ORF, *orf1*, of unknown function not found in *E. coli*. *dhnaA*, *orf1* and *rbtRD* sequenced by Hartley (1984b) are indicated by broken arrows. The secondary *attλ* site, the area defined as inverted repeats (IR), chi-like recombination sites (chi), the G + C content (mol %) of the ORFs, a simplified restriction map and essential clones discussed in the text are also shown. The *dal* genes were cloned in the direction of the *lacZp* promoter of the vector in pPHL101 but in the opposite direction in pPHL103. Circled restriction sites are conserved between *Klebsiella* and *E. coli* C. B, *Bam*HI; C, *Clal*; H, *Hind*III; K, *Kpn*I; N, *Nde*I; P, *Pst*I; S, *Sma*I; Sp, *Sph*I; X, *Xho*I.

OD₆₅₀ of 0.5. After centrifugation, the cells were washed, resuspended in 400 µl RNA buffer, lysed and treated as described by Ausubel *et al.* (1990) to isolate total RNAs. Primer extension reactions were also performed according to Ausubel *et al.* (1990) using a 31 bp primer (see Fig. 4) for both strands. Primers (50–100 ng) were labelled with [γ -³²P]ATP and T4 polynucleotide kinase. To remove labelled ATP, a 'MicroSpin Sephadryl' column (Pharmacia) was used. A total of 10–50 µg RNA was mixed with 17.5 ng labelled primer and annealed at 65 °C in hybridization buffer for 90 min. The oligonucleotide primers were extended in the reaction mixture (45 µl) containing 5 U avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) by incubation for 1 h at 42 °C. After extraction with phenol, precipitation with ethanol and resuspension in 5 µl stop solution, according to Ausubel *et al.* (1990), the cDNAs synthesized were separated by electrophoresis on a 6% sequencing gel. The size markers were generated by running a dideoxy nucleotide chain-termination reaction with the same primer and plasmid DNA template, including either *dalDKT* or *dalR* DNA fragments.

RESULTS AND DISCUSSION

Cloning of the *dal* and *rbt* genes from the chromosome of *K. pneumoniae*

Previous studies with genes and DNA from two different strains of *Klebsiella* (Charnetzky & Mortlock, 1974a, b, c; Hartley, 1984b) and from *E. coli* C (Link & Reiner, 1983) indicated that the *dal* and *rbt* (*atl* and *rtl*) genes were contiguous. Hartley and co-workers originally transferred the genes from *K. pneumoniae* 1033-5P14, by means of P1-transduction, into the chromosome of *E.*

coli K-12 where the genes inserted at a similar place (near 46 min) as in *E. coli* C (Rigby *et al.*, 1976). Using a secondary *attλ* site near the pentitol-specific genes and a phage λ derivative, Neuberger & Hartley (1979) cloned the *dal* and *rbt* genes onto phage λ *p rbt dal*. The phage contained *rbtDK* and *dalDK* on a 10.9 kbp insert as identified by growth and enzyme tests; it probably also contained *rbtR* and *dalR* because the clones conferred control properties (inducibility) identical to those of parental strain 1033-5P14. The gene order was reported to be *dalKD-dalR-rbtR-rbtDK* (Fig. 2). This contradicts the order reported by Charnetzky & Mortlock (1974c) given as *dalKD-rbtB-dalB-rbtDK*. It is, however, equivalent to the *atl rtl* gene order as determined by P1-transduction for *E. coli* C (Scangos & Reiner, 1978).

Because these discrepancies could be due to chromosomal rearrangements that might have occurred during cloning into phage λ and because neither all *dal* (*atl*) nor all *rbt* (*rtl*) genes encoding D-arabinitol and ribitol transport and catabolism had been cloned and identified, we wanted to reclone all genes and map them completely. Hence, chromosomal DNA was isolated from *K. pneumoniae* KAY2026 (Sprenger & Lengeler, 1984), one of the strains used by Lin, Hartley and others in their evolutionary studies (references in Mortlock, 1982). The DNA was treated with *Hind*III before being cloned into the medium-copy-number plasmid pSU18 and transformed into *E. coli* HB101. This *mtlA1* mutant lacks the mannitol-specific phosphotransferase system (II^{Mtl}) as well as the *atl* and *rtl* genes and hence the capacity to grow on D-mannitol, D-arabinitol and ribitol. Transformants were first plated on MacConkey indicator plates containing D-arabinitol or D-mannitol. II^{Mtl}-negative strains can be suppressed to a Dal⁺ Mtl⁺ phenotype provided a DalT transporter (*dalT* or *atlT*), which also transports free D-mannitol, and a D-arabinitol dehydrogenase (*dalD* or *atlD*), which also converts D-mannitol to D-fructose, are expressed constitutively (Tanaka *et al.*, 1967; Lengeler, 1975; Aulkemeyer *et al.*, 1991; Heuel *et al.*, 1997). Among several thousand transformants containing *Hind*III DNA fragments, one Dal⁺ (strong) Mtl⁺ (weak) but Rtl⁻ colony was found. *dalT* and *dalD* were subcloned and located on a 4.73 kbp fragment. The fragment was reisolated and religated into the polylinker site of pSU18. The subclones were transformed into strain JM109 and the transformants plated on LB₆-Cam plates (Lengeler, 1975), which were supplemented with 1 mM IPTG and 0.02% X-Gal. From white colonies, plasmids pPHL101 and pPHL103 (Fig. 2) were obtained. Based on their restriction maps, which could be compared to the known maps of *dal* genes (Hartley, 1984b; Wu *et al.*, 1985; Heuel *et al.*, 1997), it could be concluded that on both plasmids *dalD*, *dalK* (encoding a D-xylulose kinase) and *dalT* had been cloned, but in the opposite direction. After transformation into HB101, all transformants from both plasmids had the same Dal⁺ Mtl⁺ phenotype. This result implies that the *dal* genes are expressed constitutively in both clones, i.e. that no DalR-dependent repression occurs. Because in pPHL103 the *dal*

Table 1. Gene products of the *dal* and *rbt* operons

Gene product	Function	No. of aa	Observed molecular mass (kDa)	Calculated molecular mass (Da)	Inducer or major substrate	Type or family	Identical residues (%) [*]
DalR	Repressor	313	34.9†	34940	D-Arabinitol	DeoR	None
RbtR		270	—	29700	D-Ribulose	LacI/GalR	
DalD	NAD dehydrogenase	455	46.5†	50050	D-Arabinitol	Long (monomer)‖	None
RbtD		248	27.0†	27280	Ribitol	Short (tetramer)‖	
DalK	ATP kinase	487	54.0 (110)‡	53570	D-Xylulose	Hexokinase	19.3
RbtK		535	60.0 (112)‡	58850	D-Ribulose	Hexokinase	
DalT	Transporter	425	—	46750	D-Arabinitol	Ion symporter	86.6
RbtT		427	42§	46970	Ribitol	Ion symporter	

^{*} Local similarities, e.g. for helix–turn–helix motifs or NAD- and ATP-binding motifs, are not considered.

† Purified monomer.

‖ Long and short dehydrogenases, according to Persson *et al.* (1991a, b), active as monomers or as tetramers, respectively.

‡ Purified monomer and dimer in parentheses.

§ Data from Heuel *et al.* (1997).

genes have been cloned against the *lacZp* promoter of pSU18, the result also implies that the fragment should contain a *dalDp* promoter (Fig. 2).

In a parallel approach, the *rbt* genes were isolated after cloning *Bam*HI fragments into the newly constructed medium-copy-number vector pHEX3 (Heuel *et al.*, 1997). Among several thousand HB101 transformants plated on MacConkey ribitol plates was one Rbt⁺ Dal[−] colony. Based on restriction analysis of the new plasmid, pFCK1, a 7.23 kb *Bam*HI fragment had been cloned (Fig. 2) which, analogous to previous studies (Wu *et al.*, 1985), should contain *rbtR* (repressor), *rbtD* (ribitol dehydrogenase) and *rbtK* (D-ribulose kinase). Again, as for the *dal* genes, a new promoter-distal gene, *rbtT*, encoding a ribitol-specific ion symporter was also found (Heuel *et al.*, 1997). To clone the missing gene, *dalR*, which should map between *rbtR* and *dalD* (Fig. 2), the terminal sequences of the *Bam*HI fragment from pFCK1 and of the *Hind*III fragment from pHHL101 were determined. These were used to devise PCR primers which allowed the synthesis of a DNA fragment carrying *dalR* flanked by *Hind*III and *Bam*HI sites using chromosomal DNA as a template. The primers used were primer 1 (5′ GCCATAGATCACAAAAGAGCAATT-ACCC 3′) starting 31 bp before the *Hind*III restriction site and primer 2 (5′ GGGTTGTGCGCTGGCTGTC-GCAGG 3′) starting 50 bp after the *Bam*HI restriction site. PCR yielded a 1.2 kb *Hind*III–*Bam*HI fragment which was cloned into pHEX5 to yield pHHL102. The 4.7 kb *Hind*III fragment of pHHL101 was isolated next and cloned into the *Hind*III site of pHHL102. The new plasmid, pHHL104, carried *dalRDKT* on a 5.9 kb *Hind*III–*Bam*HI insert (Fig. 2). The correct insertion of the *dal* genes on pHHL104 was ascertained by restriction analysis using *Nde*I, *Sph*I and *Xho*I, three restriction enzymes with single, asymmetrically located cutting sites within the *dal* genes. Cells of HB101 transformed

with pHHL104 exhibited a Dal⁺ (strong) phenotype but remained Mtl[−]. This is the expected phenotype for a *dalR*⁺ strain because D-mannitol is not an inducer for the D-arabinitol-specific DalR repressor. The restriction map of *λp rbt dal* (Hartley, 1984b) corresponds almost exactly to our map of the 10.93 kbp *Hind*III–*Bam*HI fragment covering the *rbt* and *dal* genes (Fig. 2). Because we isolated the fragment directly from the chromosome of the *Klebsiella* strain used by these authors, this map should correspond to the genuine map.

Sequencing of the *dal* and *rbt* genes from *K. pneumoniae* and identification of their gene products

We sequenced the entire 10.93 kbp fragment covering the region from *orf1* to *dhnA* completely on both strands, except for *rbtR* and *rbtD*. First, the sequence data revealed the new genes *dalT* and *rbtT* encoding a D-arabinitol- and a ribitol-specific transporter (Heuel *et al.*, 1997), respectively, and second, proved the gene order to be as proposed by Hartley (1984b) and as summarized in Fig. 2. In particular, the *dal* and *rbt* genes are adjacent, arranged in a symmetrical but divergent way. The operons are separated by a control region (2.2 kbp) encoding the DalR and RbtR repressors. Because *dalR* and *dalDKT* are transcribed from opposite strands, and correspondingly for the *rbt* genes, *dalR* and *rbtR* are transcribed convergently as indicated.

Many of the gene products from the *dal* and *rbt* genes have been purified and characterized (references in Hartley, 1984b and Wu *et al.*, 1985). The corresponding properties are summarized in Table 1. The complete peptide sequence for RbtD, and the partial peptide sequences, in particular the amino-terminal sequences, for DalD, DalK and RbtK are available. Furthermore, RbtT (42 kDa) has been made visible in the T7 RNA

polymerase-dependent overexpression system (Heuel *et al.*, 1997).

DNA sequence analysis and protein comparison

After complete sequencing of the *dal* and *rbt* genes, including the regulatory genes, sequence alignment and comparisons at the DNA and peptide level could be done. Due to previously unresolved DNA compressions, the published sequences had to be corrected in numerous places. These corrections, however, do not alter previous major conclusions. On the other hand, the comparisons revealed some new and unexpected results (Fig. 2 and Table 1).

dalR and *rbtR* encoding repressors DalR and RbtR

dalR and *rbtR* are transcribed convergently from promoters *dalRp* and *rbtRp*, respectively (Fig. 2). The secondary *attλ* site (nt 5777–5921), used by Hartley and co-workers (Neuberger & Hartley, 1979) to clone the *dal* and *rbt* genes onto phage λ, is located within the intergenic sequence of 209 bp. The RbtR repressor (270 aa), which recognizes the molecular inducer D-ribulose, belongs to the LacI/GalR family. It contains the typical amino-terminal helix–turn–helix motif (aa5–25) and other characteristic motifs in the central and carboxy-terminal parts (Weickert & Adhya, 1992). The DalR repressor (313 aa), on the other hand, binds D-arabinitol as the molecular inducer and belongs to the DeoR repressor family (van Rooijen & de Vos, 1990). Characteristic members of this group are DeoR (deoxy-ribose; about 32% identical residues), GatR (galactitol), GutR (D-glucitol) and SorC (L-sorbose metabolism) in enteric bacteria. All contain a common inducer-binding motif near the carboxy-terminal end and an amino-terminal helix–turn–helix motif (26–45). Neither *dalR* and *rbtR* at the DNA level, nor DalR and RbtR at the peptide level showed any significant similarity.

dalD and *rbtD* encoding D-arabinitol (DalD) and ribitol dehydrogenase (RbtD)

In both operons, the promoter-proximal genes *dalD* and *rbtD* encode pentose-specific and NAD-dependent dehydrogenases. Against expectation, however, neither *dalD* and *rbtD* nor the corresponding proteins showed any similarity at the DNA and peptide level beyond local motifs which identified the gene products as members of the dehydrogenase family. Thus, DalD (455 aa) with affinity to D-arabinitol (K_M^{app} 24 mM) and D-mannitol (K_M^{app} 70 mM), but not to ribitol, xylitol and D-glucitol, belongs to the subfamily of long (≥ 450 aa) dehydrogenases (Persson *et al.*, 1991a, b). These contain a typical NAD-binding (aa 9–17) and substrate-binding (205–211) motif also conserved in the D-mannitol-1-phosphate dehydrogenase of, e.g. *E. coli* K-12. Long dehydrogenases are active as monomers as has also been shown for DalD (Hartley, 1984b). RbtD (248 aa), by contrast, belongs to the subfamily of short dehydrogenases and acts as a tetramer. Substrates are (in decreasing order)

		***** Pho-1 *****	
RbtK	1	EHNDTQNIIT	VVYSGSRR
DalK	1	Y-----YL	ISUTSEKKA
		***** Sub *****	
RbtK	51	SREIMQVCH	CIKIVANAG
DalK	45	PELMWETET	LMALREKCA
		***** Con-1 *****	
RbtK	101	PSOQADNII	VKTHATGQ
DalK	90	---KATIEPA	LAMTHCAAE
		***** Con-2 *****	
RbtK	151	LKENRHHY	QARHFDLA
DalK	137	VRRHEDH	RTATVLLPK
		***** Hel *****	
RbtK	201	EGYFRQIGL	EELADEFVR
DalK	172	AGATLWLDV	AKRDNSALL
		***** Adeno- *****	
RbtK	251	PVAVGMIDH	AGIGTSCIL
DalK	216	---PQVARR	WELNASTAA
		***** Con-2 *****	
RbtK	301	WGPYYSAVF	GYWLSEGGQS
DalK	262	LFVVTDAYR	APQSA---VH
		***** Hel *****	
RbtK	351	PVWLADRVIT	QVASEPSEAVT
DalK	309	GTTE--VAL	AEIAELSEED
		***** Adeno- *****	
RbtK	401	GMRDLNLT	AMVAGGCSI
DalK	357	---THASLRA	QGYAMUEPV
		***** Con-2 *****	
RbtK	451	ELVROLADA	CGVSAYSTAS
DalK	404	EFWADILALI	LAMPATHKG
		***** Con-2 *****	
RbtK	501	QVDATYHSET	AFSPLHQRRY
DalK	454	WQTRADPIR	HHTLMQRYAQ

Fig. 3. Comparison of D-xylulose kinase (DalK) and D-ribulose kinase (RbtK). Identical residues (19.3%) are boxed. The phosphate-binding motifs 1 and 2 (Pho-1/2), the linker or connect motifs 1 and 2 (Con-1/2), the adenosine-binding motif (Adenosine), a helix motif (Hel) and a putative substrate binding area (Sub) are also indicated.

ribitol (K_M^{app} 11 mM), L-arabinitol (267 mM) and xylitol (1000 mM), but not D-arabinitol and D-mannitol (Hartley, 1984b). An NAD-binding motif (21–27) was found as expected.

dalK and *rbtK* encoding D-xylulose (DalK) and D-ribulose kinase (RbtK)

dalK and *rbtK* encode ATP-dependent kinases for D-xylulose and D-ribulose, respectively (Fig. 3). As for the dehydrogenase genes, *dalK* and *rbtK* did not show extended similarities. The D-xylulose kinase DalK (487 aa) is active as a dimer (about 110 000 Da) and resembles the *xylB*-encoded D-xylulokinase from *K. pneumoniae* (484 aa; 52.9% identical residues). For comparison, the *XylB* kinases from *K. pneumoniae* and *E. coli* share 81.3% identical residues. Among the many pentoses, pentitols and hexitols, only D-xylulose [K_M^{app} 0.8 mM; V_{max} 150 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$] is a substrate for DalK (Hartley, 1984b). It has been claimed that *XylB* accepts D-arabinitol and produces D-arabinitol 5-phosphate (Scangos & Reiner, 1979). The three kinases belong to the prokaryotic D-xylulokinase group of the hexokinase family as defined by Bork *et al.* (1993). Based on three-dimensional crystal structures, hexokinases comprise characteristic structures: in DalK

Fig. 4. Mapping of the transcription initiation sites of *dalR* (a) and *dalD* (b) by primer extension analysis. Radiolabelled primers complementary to the 5' end of the non-coding strand of *dalR* (5' CCATGCGGCACGCACCTTCTGATCCAACCGG 3') or *dalD* (5' CCACGCCTGATGCGCGCATGAAAAGAACCC 3') were used to direct cDNA synthesis by reverse transcription from total RNAs of cells of strain HB101 containing either pPHL101 (*dalDKT*) or pPHL102 (*dalR*). The respective extended primers (+1) are shown in the right-hand lanes. The sequence of the complementary DNA is also given. This was derived from lanes A, C, G and T showing the sequencing ladders with primers labelled at 5' positions by T4 polynucleotide kinase. An asterisk indicates the initiation site, namely an A residue, 50 bp upstream of the putative ATG codon of *dalR* and 21 bp upstream of the putative ATG codon of *dalD*.

(Fig. 3) these correspond to two phosphate-binding sites (aa 2–22 and 251–266) and an adenosine binding site (388–417), all three involved in ATP binding, two connect or linker structures (230–248 and 421–441) and a putative substrate-binding site (68–86).

The kinase encoded by *rbtK* (535 aa) also acts as a dimer (about 112 000 Da). Substrates are D-ribulose [K_M^{app} 0.4 mM; V_{max} 71 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$] and to a lesser extent D-arabinitol and ribitol (K_M^{app} 140 and 220 mM, respectively) but not D-xylulose (Hartley, 1984b). Based on sequence alignments, RbtK corresponds to a typical prokaryotic hexokinase, in particular to the group of L-ribulose-, D-gluconate- and glycerol kinases (Bork *et al.*, 1993). Curiously, its closest relative is the hydrophilic part of a large (715 aa) and allegedly membrane-bound protein from *Saccharomyces cerevisiae* (accession no. Z48785). RbtK shares 25–53% identical residues with the kinases, the characteristic phosphate 1 and 2 sites (aa 8–28 and 290–305), the connect 1 and 2 motifs (269–287 and 468–488), and the adenosine-binding site (435–464), the latter preceded by a helix motif (411–423) (Fig. 3). The total DalK and RbtK molecules share 19.3% identical residues.

Operons *dal* and *rbt* are not flanked by inverted repeats

It has been claimed repeatedly that *dal* and *rbt* genes are sandwiched between rather long (0.4–1.4 kbp) inverted repeats. It has been speculated that these could be the remnants of a metabolic transposon (Hartley, 1984b)

and perhaps could be involved in genotypic exclusion between the *atl* and *rtl* genes and the *gat* genes in *E. coli* (Link & Reiner, 1982). We have shown recently (Heuel *et al.*, 1997) that the *dal* and *rbt* operons of *K. pneumoniae* contain previously unidentified genes, *dalT* and *rbtT* that are located proximal to genes *dalk* and *rbtK*, respectively (Fig. 2). The genes encode D-arabinitol- and ribitol-specific transporters of unusually high similarity (86.6% identical amino acids) that is reflected at the DNA level (82.7% identical bases). The postulated inverted repeats correspond to parts of both genes (see IR in Fig. 2), but not to short inverted repeats as they are characteristic of transposable elements. The pronounced similarity starts exactly 6 bp before the ribosome-binding site of *dalT* and *rbtT* within intergenic sequences which otherwise did not show any similarity. It ends abruptly within a TTC codon for *dalT* and a TTT codon for *rbtT* located 42 and 48 bp before the corresponding stop codons, hence 14 and 16 aa before the end of the peptides, respectively. These residues are parts of the terminal cytoplasmic loop of the permeases. At 58 bp downstream from *rbtT*, *dhnA* begins (Fig. 2), a gene found in all *atl rtl (dal rbt)* and *gat* strains of *E. coli* and *K. pneumoniae* tested thus far (unpublished results). *dalT* on the other side is followed by an ORF not present on the chromosome of *E. coli* K-12.

Regulatory sequences involved in transcription control and size of the complete *dal* and *rbt* operons

Sequence alignments have revealed putative promoter, CRP-binding and ribosome-binding motifs as well as putative initiation and stop codons. DalD, DalK, RbtK and RbtD have been purified to homogeneity. Their amino-terminal sequences have been used to define putative ribosome-binding sites and initiation codons (references in Hartley, 1984b; Wu *et al.*, 1985).

In the case of the *dalRp* and the *dalDp* promoters, the transcription initiation bases were determined directly by primer extension analysis as described in Methods and in the legend to Fig. 4. These are an A residue located 50 bp upstream of the *dalR* initiation codon and an A residue located 21 bp upstream of the *dalD* initiation codon. Promoter *dalDp* contains typical –10 (TACAGT) and –35 (TTATTT) sequences, the latter preceded immediately by a CRP-binding consensus motif (TGTGA N₆ GCTCT) with an imperfect symmetry (Fig. 5). Four short palindromes (AATTA) interspersed between these sequences are possibly involved in DalR binding as operators. Similar structures are not found in front of *dalR* and in *dalRp*. Consistent with these results it was observed that the *dal* operon in KAY2026 was inducible by external D-arabinitol as tested by DalT activity [1 and 46 nmol transport min^{-1} (mg protein) $^{-1}$ for uninduced and induced cells, respectively] and that deletion of *dalR* produced fully constitutive clones [16 nmol transport min^{-1} (mg protein) $^{-1}$ for uninduced cells]. Induction was repressed in the presence of glucose (Knott, 1982).

Fig. 5. Regulatory sequences involved in *dal* gene control. The intergenic sequences located between *dalD* and *dalR* are shown. Underlined (under the non-coding strand) and in bold type are the initiation codons, transcription initiation sites (+1), as well as putative -10 and -35 binding sites for promoters *dalDp* (labelled D) and *dalRp* (labelled R). For *dalDp*, a putative CRP-binding site (CRP) and short palindromes (asterisks), which may correspond to operator sites, are also shown.

Fig. 6. Intergenic sequences separating the *dal* and *rbt* genes. The ends of *dalR* and *rbtR* are shown together with the deduced peptide sequences and stop codons. *dalR* and *rbtR* are separated by 209 bp. This includes a secondary attachment site for phage λ (underlined sequence, *att λ*), the core of which is indicated (asterisks).

dalT is followed by a short intergenic sequence (48 bp) and an ORF (*orf1*) of about 1 kbp length. *orf1* did not show similarity to any sequence in the sequence databases. Deletion of *orf1*, as a *HindIII*–*SphI* fragment from pPHL101 (Fig. 2), caused no visible change in the Dal

phenotype of transformants and preliminary Northern hybridization studies gave no indication for the presence of *orf1*-specific mRNA in pPHL101 transformants. These results seem to rule out *orf1* as a member of the *dalDKT* operon.

Repressors DalR and RbtR showed no similarity. In accordance with this result, there was no sequence similarity between the intergenic sequences separating *dalR* and *dalD*, and *rbtR* and *rbtD*, respectively, that carry the corresponding repressor-binding sites. Putative -10 and -35 consensus sequences for promoters *rbtDp* and *rbtRp* have been postulated (Wu *et al.*, 1985). Neither these nor a putative CRP-binding sequence(s) in front of *rbtDp* have been identified and characterized by direct tests. The expression of the *rbt* genes is, however, strongly reduced during growth on glucose (Neuberger & Hartley, 1979). Early attempts to follow *rbtD*-specific mRNA synthesis by identifying polysomes engaged in RbtD synthesis through RbtD-specific antibodies, indicated a dramatic shift in the mid-exponential phase of growth, i.e. a fivefold increase in RbtD activity paralleled by an increase in corresponding polysomes (Hartley, 1984b). It remains to be shown which mechanism underlies this pattern.

Conclusions

As stated above, the *dal* and *rbt* genes have been considered as models for the natural evolution of new operons and metabolic pathways by gene duplication and for metabolic transposons. The present results corroborate previous doubts voiced by Hartley (1984b) on this too simplistic view. Thus, *dalR*, *D* and *K* do not share extensive similarity with *rbtR*, *D* and *K*, respectively, and the corresponding repressors, dehydrogenases and kinases belong to different protein families. This argues against evolution by gene or operon duplication followed by diversification and argues for modular evolution ('gene swapping') in which the various genes have been assembled in a mosaic-like way into their present positions. The only area of extensive similarity covers *dalT* and *rbtT*. Similarity is so high (82.7% identical bases and 86.6% identical amino acid residues) over the entire length of the genes/peptides that it almost certainly indicates a recent origin from a common ancestor, i.e. true homology. It is these DNAs (Fig. 2) which have been considered previously as inverted repeats and the remnants of a putative metabolic transposon (Link & Reiner, 1982, 1983; Neuberger & Hartley, 1979).

The evolution of the catabolic pathways for D-arabinitol and ribitol must have been closely linked among the enteric bacteria. Thus, based on biochemical studies, both catabolic pathways are highly similar in *Klebsiella* (Fig. 1) and in *E. coli* C (Reiner, 1975), and based on genetic studies the operons are very similar in both organisms and are located at a similar place in the chromosome (Scangos & Reiner, 1979). We cloned the *atl* and *rhl* genes from *E. coli* C and were able to show that they map in both organisms at an identical location,

that is between the *attP2I* site and the *dhnA* gene (Fig. 2). This location may explain why Rigby *et al.* (1976) were able to transduce the *dal* and *rht* genes from *K. pneumoniae* to *E. coli* K-12 by means of phage P1. Partial sequencing of the *atl* and *rtl* genes revealed the expected high similarity with the *dal* and *rht* genes (about 82% identical bases) and the same operon structure (unpublished results). We were also able to confirm the presence of a secondary attachment site for phage λ located between the *dal* and *rht* genes (Fig. 6), which allowed the original cloning of these genes into phage λ derivatives (Neuberger & Hartley, 1979). Finally, we were able to confirm the presence of chi-like recombination sites (GCCTGCC) within *dalk* (nt 1921–1927) and *rhtK* (7974–7980) (see also Fig. 2). These apparently became active in the selection of strains overproducing RbtD during continuous culture on xylitol and caused amplification of *dalDR* and *rhtRD* (Neuberger & Hartley, 1979). These results strongly indicate the presence of a natural mechanism for interspecies horizontal gene transfer of the pentitol-specific genes and enzymes within enteric bacteria.

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