Novel ligands for the extracellular solute receptors of two bacterial TRAP transporters

Gavin H. Thomas,[†] Thomas Southworth, Maria Rocio León-Kempis, Andrew Leech[‡] and David J. Kelly

Correspondence

David J. Kelly
d.kelly@sheffield.ac.uk

Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK

Tripartite ATP-independent periplasmic (TRAP) transporters are relatively common prokaryotic secondary transporters which comprise an extracytoplasmic solute receptor (ESR) protein and two dissimilar membrane proteins or domains, yet the substrates and physiological functions of only a few of these systems are so far known. In this study, a biophysical approach was used to identify the ligands for the purified *Rhodobacter capsulatus* RRC01191 and *Escherichia coli* YiaO proteins, which are members of two phylogenetically distinct families of TRAP-ESRs found in diverse bacteria. In contrast to previous indirect evidence pointing to RRC01191 orthologues being involved in polyol uptake, it was shown that RRC01191 binds pyruvate, 2-oxobutyrate and a broad range of aliphatic monocarboxylic 2-oxoacid anions with varying affinities (K_d values 0.08–3 μ M), consistent with a predicted role in monocarboxylate transport related to branched-chain amino-acid biosynthesis. The *E. coli* YiaMNO TRAP transporter has previously been proposed to be an L-xylulose uptake system [Plantinga *et al.* (2004) *Mol Membr Biol* **21**, 51–57], but purified YiaO did not bind L- or D-xylulose as judged by fluorescence spectroscopy, circular dichroism or mass spectrometry. Instead, these techniques showed that a breakdown product of L-ascorbate, 2,3-diketo-L-gulonate (2,3-DKG), binds by a simple one-step mechanism with sub-micromolar affinity. The data provide the first evidence for the existence of ESR-dependent transporters for 2-oxoacids and 2,3-DKG, homologues of which appear to be widespread amongst prokaryotes. The results also underline the utility of direct ESR ligand-binding studies for TRAP transporter characterization

Received 8 July 2005

Revised 27 September 2005

Accepted 7 October 2005

INTRODUCTION

Extracytoplasmic solute receptors (ESRs) are soluble or membrane-anchored proteins which recognize and bind specific ligands with high affinity, and have an important function in prokaryotic solute transport systems and in chemotaxis (Tam & Saier, 1993; Quijcho & Ledvina, 1996). ESRs (commonly called periplasmic binding-proteins in Gram-negative bacteria) are invariably found in bacterial or archaeal solute uptake systems in the well-characterized ABC transporter superfamily, which utilize the free energy of ATP hydrolysis to drive solute uptake via a highly conserved energy-coupling subunit, the ATP-binding cassette or ABC protein (Higgins, 2001). Until recently, no bacterial

secondary solute transporters were known which possessed an ESR protein. However, there is now extensive evidence that a novel class of secondary transport system exists, the tripartite ATP-independent periplasmic ('TRAP') transporters, which use an ESR in the transport mechanism, but do not possess an ABC protein (Forward *et al.*, 1997; Kelly & Thomas, 2001). Biochemical evidence indicates that solute accumulation by such systems is driven not by ATP hydrolysis but by an electrochemical ion gradient. The discovery of TRAP transporters (Forward *et al.*, 1997) was important in demonstrating that the use of an ESR in solute transport is not exclusive to ABC systems, and indeed there now appear to be other families of secondary transporters like the 'tripartite tricarboxylate transporters' (TTT), which also use ESR proteins in the transport mechanism (Winnen *et al.*, 2003).

The first TRAP transporter to be identified and fully characterized was the Dct system from *Rhodobacter capsulatus*, which mediates uptake of the C₄-dicarboxylates malate, succinate and fumarate (Forward *et al.*, 1997). In this system, DctP is the ESR, DctQ is a four-helix membrane

[†]Present address: Department of Biology (Area 10), University of York, PO Box 373, York YO10 5YW, UK.

[‡]Present address: Technology Facility, Department of Biology (Area 15), University of York, PO Box 373, York YO10 5YW, UK.

Abbreviations: ABC, ATP-binding cassette; CD, circular dichroism; 2,3-DKG, 2,3-diketo-L-gulonate; ESR, extracytoplasmic solute receptor; TRAP, tripartite ATP-independent periplasmic.

protein of unknown function (Wyborn *et al.*, 2001) and DctM is a twelve-helix membrane protein which is believed to mediate solute transport across the cytoplasmic membrane. Microbial genome sequencing has now revealed the presence of hundreds of TRAP transport systems in a wide range of bacteria and archaea but not eukaryotes (Kelly & Thomas, 2001). Like the DctPQM system, all TRAP transporters contain two integral membrane domains, which can be on separate proteins or fused into a single polypeptide, in addition to the ESR component (Kelly & Thomas, 2001). The DctM homologues have been shown to be members of the ion-transport superfamily (Rabus *et al.*, 1999; Prakash *et al.*, 2003), which contains many families of well-characterized secondary transporters, and it has been proposed that TRAP transporters evolved by the recruitment of an ESR to an ancestral secondary transport protein (Rabus *et al.*, 1999).

Despite the wealth of bioinformatic evidence for the widespread distribution of TRAP transporters in prokaryotes, the substrates and thus physiological role of only a few of these systems have been elucidated. One other C_4 -dicarboxylate transporter that has a similar function to the *R. capsulatus* DctPQM system in the uptake of succinate as a carbon source for growth has been characterized in *Wolinella succinogenes* (Ullmann *et al.*, 2000). A TRAP transporter involved in movement of the compatible solute ectoine into cells of *Halomonas elongata* has been characterized in some detail (Grammann *et al.*, 2002). The ESR component, TeaA, has been purified and shown to bind ectoine and hydroxyectoine (Tetsch & Kunte, 2002). A TRAP transporter in *Synechocystis* has been shown unequivocally to be a glutamate uptake system (Quintero *et al.*, 2001) and is of interest as the ESR is a homologue of GlnH, a glutamine-binding protein from an ABC system. Recently, two studies have shown sialic acid to be a substrate for a TRAP transporter from the pathogen *Haemophilus influenzae* (Allen *et al.*, 2005; Severi *et al.*, 2005). Biochemical evidence suggests that an ESR-dependent secondary glutamate transporter exists in *Rhodobacter sphaeroides* (Jacobs *et al.*, 1996), although this has not yet been confirmed by sequence analysis to be a member of the TRAP transporter family. Finally, the substrate for the single TRAP transporter in *Escherichia coli* (YiaMNO) has been reported to be L-xylulose (Plantinga *et al.*, 2004), although we show here that this is likely to be incorrect.

Ligand-binding kinetics have been determined for several ESRs from ABC systems, and the majority of those studied appear to operate by a simple association of protein and ligand, involving the closure of a binding cleft by the so-called 'Venus fly trap' mechanism (Quiocho & Ledvina, 1996). The transition from the open-unliganded to closed-liganded forms of several of these ESRs has been observed by stopped-flow fluorescence spectroscopy (Miller *et al.*, 1980, 1983), and in all cases a linear concentration dependence of the association rate constant has been found. However, the *R. capsulatus* DctP protein is kinetically distinct: the rate of

ligand-induced fluorescence change was found to decrease in a hyperbolic fashion as the concentration of ligand was increased (Walmsley *et al.*, 1992a, b). This is consistent with a slow isomerization of the protein, with the ligand binding to only one form of an equilibrium mixture of two pre-existing protein conformations:

CU represents the non-binding (closed) unliganded conformation, OU the binding (open) unliganded form, L the ligand and CL the closed liganded form. As the rate of ligand binding is much faster than the rate of the conformational change, the rate constant for the fluorescence change decreases with ligand concentration in a hyperbolic manner (Walmsley *et al.*, 1992a). In the absence of ligand, the position of the CU/OU equilibrium strongly favours the CU form of DctP, while with those other ESRs studied the position of this equilibrium appears to strongly favour the OU form.

We have selected two distinct TRAP-ESR proteins for purification and ligand-binding analysis by intrinsic tryptophan fluorescence spectroscopy, circular dichroism and mass spectrometry. New ligands have been identified from this analysis, which has allowed insights into the physiological function of the cognate TRAP transporters.

METHODS

Bacterial strains and culture conditions. TRAP transporter genes were cloned from *R. capsulatus* strain PAS100 and *E. coli* strain W3110. All cloning procedures utilized *E. coli* strain DH5 α , while strains BL21(DE3) pLysS and 'Tuner' (Novagen) were used for expression of pET21 derivatives. *E. coli* was routinely grown aerobically in Luria medium (broth or agar), containing antibiotics when required. *R. capsulatus* was grown anaerobically in RCV minimal salts medium (Weaver *et al.*, 1975) with the modifications of Hillmer & Gest (1977). Cultures (30 ml) were grown in McCartney bottles filled to capacity with RCV medium and incubated at 30 °C for 48 h, 30 cm from a 60 W light bulb. Antibiotics were used at the following final concentrations: ampicillin 100 μ g ml⁻¹, chloramphenicol 30 μ g ml⁻¹.

Preparation of periplasmic extracts. Periplasmic proteins were released using a modified osmotic shock procedure. Cells were harvested at 4700 g for 15 min at room temperature; the cell pellet was resuspended in 5 ml STE (0.5 M sucrose, 10 mM Tris/HCl, pH 8.0, and 2 mM EDTA, pH 8.0). Lysozyme was added to 1 mg ml⁻¹ and the suspension was incubated for 30 min on ice, after which time 15 ml distilled water was added and the suspension was spun at 18 000 g for 20 min at 4 °C. The supernatants were kept at -20 °C until needed.

Preparation and sequencing of DNA. Plasmid DNA for screening clones and sequencing was routinely isolated using anion-exchange resin spin-columns (QIAGEN), according to the manufacturer's instructions. Double-stranded sequencing of plasmid DNA was carried out using Applied Biosystems Taq DyeDeoxy terminator cycle sequencing reactions analysed on an ABI model 373A automated DNA sequencer. Primers were obtained commercially. Total genomic DNA was extracted using a modified SDS lysis procedure (Marmur,

1961). Restriction endonucleases, T4 DNA ligase and *Taq* polymerases were purchased from Promega, and *Pfu* or *Pwo* polymerase from Stratagene, and used according to the manufacturer's instructions.

Amplification and cloning of genes encoding ESRs. DNA sequences of the relevant ESR genes were obtained from publicly available databases. The *R. capsulatus* sequences were obtained from Integrated Genomics (<http://ergo.integratedgenomics.com/ERGO/>). The genes encoding the ESR proteins were amplified from genomic DNA by PCR using primers that introduced *Nde*I and *Bam*HI or *Eco*RI restriction sites at the 5' and 3' end of the gene, respectively. The primers used to amplify the RRC01191 gene were 1191-F (5'-GGAATTCCATATGGATCGTTCATTCTGACC-3') and 1191-R (5'-CGGGATCCTCAGATCTTGCCGTGCG-3'). Restriction sites for *Nde*I and *Bam*HI are underlined. The primers used to amplify *yiaO* were *yiaO*-F (5'-GAGACATATGAAATTACGCTCTGTAACT-ACG-3') (*Nde*I site underlined) and *yiaO*-R (5'-TTGAATTCCTAT-TGCACCTCATCCACGG-3'), the latter introducing an *Eco*RI site (underlined). PCR was performed with *Pwo* or *Pfu* polymerase and the products cloned into pET21b(+). The sequences of the genes cloned in this manner were determined by automated methods and found to be identical to those reported in the published genome sequences.

Overexpression and purification of ESR proteins. Cells were grown overnight in 2 litre batches of LB medium with ampicillin, and induced with 1 mM IPTG when cells had reached an OD₆₅₀ of 0.5. After overnight growth at 25 °C, cells were harvested by centrifugation, resuspended in 20 ml 50 mM Tris/HCl pH 8, broken by two passages through a French press and centrifuged at 12 000 g for 15 min at 4 °C to remove cell debris and unbroken cells. Aliquots (10 ml) of this extract were applied to a 50 ml DEAE-Sepharose column that had been equilibrated with 50 mM Tris/HCl pH 8 and connected to a BioLogic HP protein purification system (Bio-Rad). The bound protein was eluted using a linear 0–500 mM NaCl gradient and 7 ml fractions were collected. Both proteins required further purification on a Q-Sepharose column, which was done under the same conditions. If necessary, proteins were subjected to a final Superdex S75 gel filtration step using a Pharmacia FPLC system. The column was equilibrated and eluted with 50 mM Tris/HCl pH 8 containing 200 mM NaCl. Pure proteins were dialysed extensively against 50 mM Tris/HCl pH 8, concentrated by treatment with Aquacide (Calbiochem) and stored in aliquots at –20 °C. N-terminal sequences were determined by the automated Edman degradation method, carried out by Dr A. J. G. Moir, University of Sheffield, UK.

Protein analysis by mass spectrometry. Protein samples were first dialysed into 5 mM Tris pH 8 or water before dilution into solvents for analysis. Electrospray mass spectrometry was performed on an API Qstar mass spectrometer using an ionspray source. For mass determinations, samples were made up in 0.1% formic acid. To study non-covalent protein–ligand interactions, samples were made up in 20% (v/v) methanol/water by dilution from stock solutions of protein and ligand in water, and infused at between 1 and 5 µl min^{–1}. They were run within 1 h of preparation. Multiply charged protein signals were observed between 1800 and 3500 *m/z* under these conditions (signals at much lower *m/z* values were observed for protein in 1:1:0.001 acetonitrile/water/formic acid mixtures). Attempts to use 100% aqueous solutions gave very weak signals. Errors in the mass determinations were approximately 1 in 10⁴ over the range used; calibration was with caesium iodide and pheromone inhibitor peptide (sequence: ALILTLVS; Bachem). Raw *m/z* data were deconvoluted to mass spectra using the Bayesian Protein Reconstruction routine in the manufacturer's BioAnalyst software. Sodium adducts seen in the spectra were attributed to carry-over from the protein solution.

Steady-state and stopped-flow fluorescence spectroscopy.

Slow-timescale fluorescence spectroscopy was performed using a Hitachi F-2500 spectrofluorimeter with an excitation wavelength of 295 nm (slit width 5 nm) and an emission wavelength of 330 nm (slit width 10 nm). The assay mixture consisted of 0.1 µM protein in 1.5 ml 50 mM Tris/HCl pH 8. The sample cuvette was maintained at 25 °C in the spectrofluorimeter housing and the assay mixture was continuously stirred. Fluorescence changes upon the addition of ligands were monitored until the fluorescence change stabilized. In some experiments, protein photobleaching, which caused baseline drift, was noted. Hence, ligand titrations were performed by removing the cuvette between additions of ligand, which minimized this effect. All fluorescence values were corrected for dilution. For calculation of *K_d* values, data were averaged from at least three titrations and the hyperbolic curve-fitting algorithms in SigmaPlot used to analyse the data. Rapid reactions were followed using an Applied-Photophysics SX.18MV stopped-flow spectrofluorimeter, routinely operated at 23 °C. For work with *YiaO*, the temperature was lowered to 10 °C. Tryptophan fluorescence was excited at 295 nm, selected by focusing a 150 W xenon arc lamp onto a *f*/3.4 monochromator, and the emitted light selected with a WG320 Schott filter, positioned in front of the observation photomultiplier tube. Data were recorded with oversampling enabled and analysed by non-linear regression using the Applied Photophysics software. For the RRC01191 protein, a linear phase that was due to photobleaching was removed from the analysis. At least three stopped-flow traces were averaged prior to non-linear regression analysis using the program KaleidaGraph (Synergy Software).

Circular dichroism. CD spectra were obtained using a Jasco J810 instrument operating at 20 °C. A 1 cm path-length cuvette was used with a 2 ml volume of purified protein (1 µM final concentration in 50 mM Tris/HCl buffer pH 8). Ligands were added in small volumes and the CD change recorded and corrected for dilution.

Analysis of protein sequences and homologies. Sequences of TRAP ESR genes were obtained from the ERGO (Overbeek *et al.*, 2003) and UniProt databases. The contigs from the unfinished *R. capsulatus* genome sequence were analysed using the Artemis package (Berriman & Rutherford, 2003). Sequences for ESR proteins associated with other genes for TRAP transporters and obvious homologues of these were collected from the NCBI database. For phylogenetic analyses, sequences were aligned in CLUSTAL X (Thompson *et al.*, 1997) and the output file used in PHYLIP (Felsenstein, 1989) to produce a distance matrix tree, which was viewed in TREEVIEW (Page, 1996). Signal sequences were identified using SignalP (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1999).

RESULTS

Phylogenetic relationships of TRAP ESR proteins

A phylogenetic tree was generated from a selection of 59 sequences of TRAP ESR proteins (Fig. 1a). All these proteins show a statistically significant degree of sequence similarity to the archetypal DctP protein from *R. capsulatus*, which is contained in cluster 10 along with some other known C₄-dicarboxylate binding proteins. The majority of the proteins in the other clusters are uncharacterized with respect to ligand binding. The clustering observed in Fig. 1 is similar to that obtained in a recent phylogenetic analysis (Prakash *et al.*, 2003) of the large membrane components (DctM homologues) of TRAP transporters. We chose to study two proteins (shown boxed in Fig. 1), from clusters 8 and 12, for

Fig. 1. (a) Phylogenetic tree of selected members of the DctP family of ESR proteins associated with TRAP transporters. Proteins are identified using systematic names that can be fetched from UniProt (<http://www.ebi.uniprot.org>). The *R. capsulatus* sequences (prefix RRC; DctP in bold) can be found in ERGO Light (<http://www.ergo-light.com/>). Boxed numbers are the clades identified for the DctM components of the same systems (Prakash *et al.*, 2003). The boxed sequences are those chosen for analysis in this study. (b) Schematic representation of the gene organization of a selection of orthologues of *Rsph1195* (*smoM*). The image is centred on the structural genes for the small and large membrane subunits of the cognate TRAP transporter (shaded solid black). The *Rsph1195* orthologues are shaded with diagonal lines. A set of two-component regulatory genes associated with this type of transporter are shaded with vertical lines. The genes for acetolactate synthase in *R. capsulatus* are shaded in checkerboard, while the other known *smo* genes from *R. sphaeroides* are unshaded.

which there were some previous suggestions of possible ligands. These were the *R. capsulatus* RRC01191 protein, closely related (81% amino-acid identity) to the *R. sphaeroides* SmoM protein (Rsph1195) that has been predicted to bind sorbitol and mannitol (Stein *et al.*, 1997), and the *E. coli* YiaO protein, which has been suggested to be part of an L-xylulose TRAP transporter (Plantinga *et al.*, 2004, 2005).

The *R. capsulatus* RRC01191 protein binds pyruvate and 2-oxobutyrate but not sorbitol or mannitol

RRC01191 was overexpressed and purified from *E. coli* cell-free extracts using two anion-exchange column steps, resulting in a single band on SDS-PAGE. The N-terminal sequence of the purified protein was determined as MDRRSFLT, which corresponds to the first eight residues of the translated protein. The mass determined from mass spectrum analysis was 39 823.7 Da, which is identical to the calculated mass of the full-length protein (39 823.3 Da). Hence, the signal peptide on this protein is not recognized in *E. coli* and the unprocessed form has been purified. Previous studies have shown that the retention of the signal peptide does not alter the specificity or ligand-binding characteristics of ESR proteins (Rohrbach *et al.*, 1995; Horlacher *et al.*, 1998).

The tryptophan fluorescence emission maximum of RRC01191 was centred at 331.5 nm. Addition of 200 μ M sorbitol or mannitol to 0.1 μ M protein resulted in no change in fluorescence intensity at the emission maximum, and thus we found no evidence that either sorbitol or mannitol could bind to the protein. Closer examination of the sequence information deposited to support the data in Stein *et al.* (1997) revealed that the evidence for the function of the *R. sphaeroides* SmoM protein as a sorbitol/mannitol-binding protein is indirect. There is a 515 bp gap between the end of the confirmed *smo* operon, encoding known sorbitol/mannitol catabolic genes, and the downstream *smoM* gene. The region immediately downstream of the *smoM* gene has been sequenced during the *R. sphaeroides* genome sequencing project, revealing two genes, transcribed convergently with the *smoM* gene, that are homologous to *dctQ* and *dctM* (Choudhary *et al.*, 1999). Hence these three genes are likely to form a functional TRAP-transport system, but not necessarily related to either sorbitol or mannitol uptake.

The apparent lack of binding of sorbitol and mannitol to RRC01191 prompted a bioinformatic approach to derive hypotheses about other potential ligands. The organization of the genes encoding several SmoM-type TRAP transporters is shown in Fig. 1(b). We identified pyruvate as a potential ligand from two observations. First, the genes that are divergently transcribed from the *dctQ/dctM* homologues in *R. capsulatus* encode an acetolactate synthase. This heterodimeric enzyme catalyses the first step in valine biosynthesis and condenses two molecules of pyruvate to synthesize α -acetolactate (Dailey & Cronan, 1986). It can

also combine pyruvate and 2-oxobutyrate to form 2-aceto-2-hydroxybutyrate. Secondly, in several bacteria including *Brucella melitensis*, *Vibrio cholerae* and *Agrobacterium tumefaciens*, the *smoM* orthologues are genetically linked to two-component sensor-regulator genes (Fig. 1b) which encode homologues of the MctRS proteins from *Rhizobium leguminosarum* encoded by the *mctPRS* operon (Hosie *et al.*, 2002). MctP is a secondary transport protein that mediates the transport of monocarboxylates, including pyruvate. Therefore, the ability of pyruvate and 2-oxobutyrate to bind to RRC01191 was determined, using tryptophan fluorescence. Addition of 200 μ M pyruvate to 0.1 μ M protein caused a large quench of the fluorescence of around 23%, which could be titrated to give a K_d value of 3.4 μ M (Table 1). Addition of excess 2-oxobutyrate also caused a large quench (about 28%) in the fluorescence of the protein, and titration with this ligand revealed a much higher affinity, with a K_d value of 0.32 μ M (Table 1).

Structural requirements for 2-oxoacid binding to the *R. capsulatus* RRC01191 protein

The identification of the monocarboxylate 2-oxoacids pyruvate and 2-oxobutyrate as RRC01191 ligands which have significantly different K_d values prompted the determination of the specificity of binding by fluorescence titration experiments, using a range of structurally related 2-oxoacids and other compounds. Lactate, formate, alanine, valine, isoleucine, DL-3-hydroxybutyrate, tartrate, malonate, acetate, glutamate, glutamine, malate, fumarate, succinate or aspartate did not cause any fluorescence change in the protein. Compounds tested that caused a fluorescence quench in the protein are listed in Table 1 with their respective dissociation constants. Only a monocarboxylate 2-oxoacid structure with an aliphatic backbone allows binding to the protein with physiologically relevant K_d values. The 2-oxo and 1-carboxyl groups are essential, as compounds lacking either or both of these functional groups did not give any fluorescence change. A methyl

Table 1. Ligand-binding affinities of the *R. capsulatus* RRC01191 protein

Kinetic data were calculated from multiple titrations (*n*) of the fluorescence change with increasing ligand concentrations, which were then averaged.

Ligand	K_d (μ M)	SD
Pyruvate	3.4	± 2.0 (<i>n</i> =6)
Hydroxypyruvate	105	± 40 (<i>n</i> =5)
2-Oxo-3-methylvalerate	0.78	± 0.19 (<i>n</i> =3)
2-Oxoisovalerate	0.27	± 0.18 (<i>n</i> =3)
2-Oxobutyrate	0.32	± 0.09 (<i>n</i> =6)
2-Oxoisocaproic acid	0.40	± 0.05 (<i>n</i> =5)
2-Oxovalerate	0.085	± 0.008 (<i>n</i> =5)

group bonded to the carbonyl function, as in pyruvate, allows binding, while a hydrogen in this position (as in glyoxylate) does not. Dicarboxylic 2-oxoacids like oxaloacetate and 2-oxoglutarate did not interact with the protein. However, a progressive increase in the chain length of the aliphatic backbone of the monocarboxylate 2-oxoacids increased the ligand-binding affinity, with 2-oxovalerate binding with a K_d value almost an order of magnitude lower than 2-oxobutyrate. Branched-chain aliphatic 2-oxoacids like 2-oxoisovalerate, 2-oxoisocaproate and 2-oxo-3-methylvalerate also bound to the protein, with intermediate K_d values (Table 1).

Mechanism of ligand binding to RRC01191

The mechanism of ligand binding to RRC01191 was investigated using stopped-flow fluorescence spectroscopy with pyruvate. Over the range of concentrations tested, the stopped-flow data could be fitted to a double exponential process, indicative of two optically active phases in the binding mechanism. The first phase had a strong concentration dependence (Fig. 2), with a similar inverted hyperbola as observed previously for DctP (Walmsley *et al.*, 1992a). The second phase was concentration independent, and is most likely due to an optically active but ligand-independent isomerization process subsequent to ligand binding, as has been observed in other systems (e.g. Walmsley *et al.*, 2001). For phase one the k_1 and k_{-1} parameters were $0.4 \pm 1.3 \text{ s}^{-1}$ and $32.9 \pm 6.6 \text{ s}^{-1}$, respectively. Two phases were also consistently observed using 2-oxobutyrate as the ligand in stopped-flow experiments. The rate of the first phase showed a similar inverse concentration dependence to that seen with pyruvate, but the variation in replicates was too large to yield reliable rate constants (data not shown).

Fig. 2. Concentration dependence of the pyruvate-induced fluorescence change of 1 μM RRC01191, measured by stopped-flow fluorescence spectroscopy. Stopped-flow traces were fitted to a double exponential process and rates for the first (●) and second (□) phase are plotted as a function of pyruvate concentration.

The *E. coli* YiaO protein binds 2,3-diketo-L-gulonate but not L-xylulose

YiaO is the ESR of the sole TRAP transporter in *E. coli*, which is formed with the products of the *yiaM* and *yiaN* genes, encoding the large and small transmembrane proteins, respectively. The *yiaMNO* genes are part of the *yiaK–yiaS* gene cluster that encodes enzymes implicated in the utilization of the pentose L-lyxose (Badia *et al.*, 1998; Ibanez *et al.*, 2000). YiaP (LyxK) has been shown to be a kinase that can phosphorylate L-xylulose (Sanchez *et al.*, 1994) and some evidence has been published that YiaMNO encodes an L-xylulose transporter (Plantinga *et al.*, 2004). However, in a study of L-ascorbate fermentation by *E. coli*, Yew & Gerlt (2002) showed that purified LyxK could also phosphorylate 3-keto-L-gulonate (an intermediate of L-ascorbate catabolism) with kinetic constants comparable to those with L-xylulose. Moreover, they demonstrated that the enzyme encoded by *yiaK* is a reductase that can form 3-keto-L-gulonate from 2,3-diketo-L-gulonate (2,3-DKG), the latter produced from L-ascorbate via L-dehydroascorbate. Yew & Gerlt (2002) thus suggested that YiaMNO might form a 2,3-DKG transporter that could supply YiaK with its substrate, which would be reduced to 3-DKG, the substrate for LyxK.

We sought to clarify the conflicting data on the function of the YiaMNO TRAP transporter, by determination of the ligand-binding properties of YiaO. The protein was overexpressed and purified to homogeneity by two anion-exchange steps followed by gel filtration. The molecular mass of the pure protein was determined by mass spectrometry to be 33 468.8 Da, which is identical (within the error of the instrument, ± 2 Da), to that calculated for the deduced processed form of the protein (33 469.1). When excited at 295 nm, YiaO showed a strong fluorescence emission maximum at 330 nm. Fig. 3 shows a series of experiments in which the change in the steady-state tryptophan fluorescence at 330 nm was measured after the addition of various potential ligands for this protein. The addition of excess (200 μM) L- or D-xylulose did not result in any discernible change in fluorescence emission from the protein (Fig. 3a), while the addition of even a low (5 μM) concentration of 2,3-DKG resulted in an easily measurable fluorescence quench (Fig. 3b). The maximal fluorescence change observed was about 7% at 200 μM 2,3-DKG. The 2,3-DKG concentration dependence of the fluorescence change is shown in Fig. 3(c). The calculated K_d value was $0.77 \pm 0.19 \text{ }\mu\text{M}$, indicating that 2,3-DKG binds to YiaO with reasonably high affinity.

To further confirm the inability of the protein to bind L-xylulose, we carried out a series of ligand displacement experiments. Starting with 0.1 μM protein pre-incubated with 5 μM 2,3-DKG, the addition of 200 μM D-xylulose (Fig. 3d) or L-xylulose (Fig. 3e) did not result in any fluorescence change indicative of ligand displacement. However, when 5 μM 2,3-DKG was added to 0.1 μM YiaO which had been pre-incubated with either 200 μM D-xylulose (Fig. 3f) or L-xylulose (Fig. 3g), an immediate fluorescence quench

Fig. 3. Steady-state fluorescence changes in YiaO, induced by xylulose or 2,3-DKG. In (a) the fluorescence change at 330 nm was measured after addition of 200 μM D- or L-xylulose to 0.1 μM YiaO. In (b) 5 μM 2,3-DKG was added to 0.1 μM YiaO. The concentration dependence of the resulting fluorescence change is shown in (c); the solid line is the fit to a single rectangular hyperbola. (d–g) Ligand displacement experiments (addition of ligand indicated by an arrow). In (d) and (e) 0.1 μM YiaO was preincubated with 5 μM 2,3-DKG and the fluorescence change resulting from addition of excess (200 μM) D- or L-xylulose, respectively, was measured. In (f) and (g) 0.1 μM YiaO was preincubated with 200 μM D- or L-xylulose, respectively, and the fluorescence change resulting from the addition of 5 μM 2,3-DKG was recorded.

was observed which was of a similar magnitude to that seen when the same concentration of 2,3-DKG was mixed with the protein alone (Fig. 3b). These data indicate that neither L- nor D-xylulose is a ligand for YiaO.

Mass spectrometry provides evidence that a hydrated form of 2,3-DKG is the ligand for YiaO

A non-optical method that can give additional information about ligand binding to YiaO is mass spectrometry. The purified protein was first examined under denaturing conditions to get an accurate mass estimate, which was determined to be 33 468.8, corresponding to the processed form of the protein (33 469.1). Under less denaturing conditions, in a 20 % methanol/water buffer, the signal from the protein was also still sufficient to be able to accurately and unambiguously determine its mass, even though there is less ionization (Fig. 4a). As well as the major peak, additional peaks, spaced at 23 Da intervals, were also present which probably correspond to sodium adducts on the protein. Identical conditions were then used to examine the mass of the protein after the addition of 50 μM L-xylulose. This did not change the mass spectrum from that seen with the

protein alone, indicating no binding of the ligand to the protein in these conditions (data not shown). When the experiment was repeated with the addition of 2,3-DKG, a new species was observed with a mass of 33658.6, a larger peak at a slightly greater mass of 33678.3 and a similar tail of sodium adducts of decreasing intensity (Fig. 4b). The observed mass changes of 191.1 to the first peak and 210.8 to the second are consistent with the binding of 2,3-DKG (191 mass units for the negative ion) and a hydrated *gem*-diol form of 2,3-DKG (210 mass units), respectively (Deutsch, 1998a). Upon mixing the protein with both L-xylulose and 2,3-DKG, the same mass changes as seen with 2,3-DKG alone were obtained (data not shown). Taken together, the mass spectrometry data support the fluorescence data in showing that L-xylulose is incapable of binding to YiaO, whereas 2,3-DKG does bind.

In initial experiments, we observed that the addition of either L-ascorbate or dehydroascorbate to YiaO also gave the same mass changes as those seen in Fig. 4(b) with 2,3-DKG. A number of studies have shown that 2,3-DKG is a spontaneous breakdown product of L-ascorbate and dehydroascorbate (Deutsch, 1997, 2000), which forms in

Fig. 4. Mass reconstruction of 1 μM YiaO in 20% methanol, (a) in the absence of ligand and (b) in the presence of 5 μM 2,3-DKG. In the absence of ligand the additional peaks are due to sodium adducts on the protein. In the presence of ligand the mass change to the first peak is 191.1 and that to the second, larger, peak is 210.8.

aqueous solutions at neutral pH. Consistent with this, when L-ascorbate or dehydroascorbate solutions were made fresh and used immediately in the experiment, no additional peaks in the mass spectrum were observed. We thus conclude that YiaO specifically binds 2,3-DKG and is unable to bind either L-ascorbate or dehydroascorbate.

Ligand-induced changes in YiaO studied by circular dichroism

Further evidence that YiaO specifically binds 2,3-DKG, but neither xylulose nor ascorbate, was obtained from circular dichroism studies. The room temperature CD spectrum of YiaO in the far-UV region was characterized by troughs centred at 208 nm and 220 nm typical of a protein with a large α -helical content (Fig. 5a). Addition of L- or D-xylulose did not result in any significant change in the CD molar ellipticity in this region of the spectrum. In contrast, addition of excess 2,3-DKG led to a decrease in the ellipticity of the 220 nm trough indicative of changes in the secondary structure of the protein (Fig. 5a). The 2,3-DKG-dependent change in the CD signal at 220 nm could be titrated (Fig. 5b) to give a K_d value of $1.29 \pm 0.17 \mu\text{M}$, very similar to that obtained from the fluorescence quench. CD spectroscopy proved useful to determine if L-ascorbate itself was a ligand for YiaO, as the strong absorbance of ascorbate at 260 nm interferes with fluorescence measurements. However, as with xylulose, no significant changes in the far-UV spectrum were observed (Fig. 5a), suggesting that L-ascorbate is not a ligand for the protein.

Fig. 5. Circular dichroism analysis of YiaO. (a) Far-UV CD spectrum of 1 μM YiaO in the absence of ligand (●), and in the presence of 50 μM L-xylulose (○), 50 μM sodium ascorbate (▽) or 50 μM 2,3-DKG (■). (b) Titration of the molar ellipticity at 220 nm as a function of 2,3-DKG concentration. The line through the data points is the fit to a single rectangular hyperbola.

YiaO shows simple ligand-binding kinetics with 2,3-DKG

The ligand-binding kinetics of YiaO, determined by stopped-flow fluorescence spectroscopy, proved to be very fast at room temperature. In initial experiments performed at 23 °C with 0.5 μM YiaO mixed with a wide range of 2, 3-DKG concentrations, we were unable to time-resolve the fluorescence change. Fig. 6(a) shows stopped-flow traces obtained at 10 °C with two 2,3-DKG concentrations, 1 μM (trace 2) and 10 μM (trace 3), where in each case the quench data could be fitted to a single exponential with a linear drift. The latter is due to photobleaching, which is more apparent in trace 1, where protein was pushed against buffer. Fig. 6(b) shows the dependence of k_{obs} on 2,3-DKG concentration at 10 °C. In contrast to the kinetic behaviour of RRC01191, a positive linear dependence was observed, consistent with a simple association of ligand and protein. The calculated association rate constant ($K_A[L]$) was $2.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, and the dissociation rate constant obtained from the intercept of Fig. 6(b) was 6.4 s^{-1} .

DISCUSSION

In this study we have identified several new ligands for TRAP transporter ESRs, including a range of monocarboxylate 2-oxo acids like pyruvate and 2-oxobutyrate, and the ascorbic

Fig. 6. Stopped-flow fluorescence spectroscopy of YiaO at 10 °C. (a) Stopped-flow records for YiaO mixed with (1) buffer alone, (2) 1 μ M 2,3-DKG and (3) 10 μ M 2,3-DKG. (b) Linear concentration dependence of k_{obs} on 2,3-DKG concentration.

acid derivative 2,3-DKG. These ligands and all previously characterized TRAP transporter substrates contain carboxylate groups. In addition, analysis of the *Fusobacterium nucleatum* genome sequence has revealed that one of the TRAP transporters is encoded in an operon with genes involved in sialic acid (*N*-acetylneuraminic acid) catabolism (Kapatral *et al.*, 2002), which has also been observed for the orthologous protein in *Haemophilus influenzae* (Kolker *et al.*, 2004). This ESR does indeed bind sialic acid with high affinity (Severi *et al.*, 2005) and has been shown to be part of a sialate TRAP transporter (Allen *et al.*, 2005; Severi *et al.*, 2005), extending the substrate range of the TRAP transporters to the sugar acids. Bioinformatic analyses based on gene context suggest that ligands for two additional TRAP transporters are 4-chlorobenzoate and protocatechuate (Chae *et al.*, 2000; Contzen *et al.*, 2001; Kelly & Thomas, 2001). Although these observations may suggest a common function for TRAP transporters in the uptake of carboxylic acids, the discovery of genetic linkage of the genes for a TRAP transporter with those for taurine catabolism in *R. sphaeroides* suggests a wider range of organic ligands than previously suspected (Bruggemann *et al.*, 2004), although this has yet to be confirmed experimentally.

The identification of a distinct subfamily of closely related TRAP-ESR proteins that contain the SmoM protein from *R. sphaeroides* (Fig. 1a) is significant, as SmoM has been suggested to be involved in the uptake of sorbitol and mannitol (Stein *et al.*, 1997), implying the existence of a novel type of sugar-alcohol transporter. However, we have demonstrated that the closely related *R. capsulatus* orthologue binds

2-oxoacids but neither sorbitol nor mannitol. In *R. capsulatus*, the TRAP transporter associated with this type of ESR is genetically linked to acetolactate synthase (Fig. 1b), and we suggest that the physiological role of this transporter is to supply intermediates in the synthesis of valine and isoleucine. Since *R. capsulatus* is not auxotrophic for branched-chain amino-acids, it is possible that the transporter plays a role in supplementing intracellular pools of the pathway intermediates from external sources. Determination of the most physiologically important substrate will require phenotypic analysis of transport mutants. This group of TRAP transporters (clade 12; see Fig. 1a) is currently one of the largest and may reflect a more widespread but hitherto unsuspected role for 2-oxoacid uptake in bacterial biosynthetic pathways. The ligand-binding mechanism of RRC01191 appears to be similar to that of DctP (Walmsley *et al.*, 1992a), although the rate constants for the forward transition between the closed unliganded and open unliganded form are rather lower (less than 1 s⁻¹) than for DctP. Many of the genes encoding orthologues of RRC01191 are called *smoM* and are annotated as encoding sorbitol/mannitol-binding proteins, but this now seems unlikely in the light of our data. A phylogenetic and bioinformatic analysis of the SmoM family of TRAP transporters revealed that in *Synechocystis* the gene encoding the SmoM ESR has been displaced away from the gene encoding the membrane subunits by a different ESR and is now distantly located relative to the gene encoding the membrane components (Kelly & Thomas, 2001). In a non-orthologous gene displacement event, the expected ESR has been replaced by an ESR normally found associated with ABC transporters, which is homologous to the glutamine ESR family (GlnP). Experimental evidence from Flores and colleagues (Quintero *et al.*, 2001) has demonstrated that this TRAP transporter mediates glutamate transport rather than having a function in 2-oxoacid transport. This raises interesting questions about how ligand-binding specificity is imposed on the TRAP transporters and the important role of the ESR in determining this property.

The substrate for the single *E. coli* K-12 TRAP transporter (YiaMNO) has been proposed by Plantinga *et al.* (2004) to be L-xylulose. However, although these authors showed that purified YiaO apparently bound L-xylulose, their assay method was indirect, involving measurement of the residual unbound ligand. With very high (100 μ M) protein concentrations and 50 μ M L-xylulose, only 9 % of the ligand was removed from solution by their YiaO preparation. Given that the authors had purified the YiaO protein, it is notable that they did not use any biophysical methods to examine ligand binding. Using a range of such techniques, we could find no evidence that L-xylulose was able to bind, but we were able to unequivocally demonstrate that YiaO specifically binds 2,3-DKG. Significantly, Plantinga *et al.* (2004) did not use 2,3-DKG directly in any of their experiments. In addition, they performed only indirect transport assays for L-xylulose uptake in intact cells, and the difference

in rates between a *yiaMNO* deletion strain and its isogenic parent was minimal. In a separate study, Plantinga *et al.* (2005) also found that L-xylulose did not induce expression of the *yiaM* gene, and that deletion of the *yiaMNO* genes in strain MC4100 led to complex changes in growth, salt tolerance and biofilm formation that are difficult to explain but seemingly unrelated to L-xylulose transport. Hence, we conclude that 2,3-DKG and not xylulose is the physiological substrate for the YiaMNO transporter, as predicted by Yew & Gerlt (2002). Unfortunately, 2,3-DKG is relatively unstable and is unavailable commercially in a radiolabelled form, so that future transport and growth studies with the appropriate mutants will not be trivial to perform.

As ascorbate (vitamin C) is absorbed from the gut, it is likely that dehydroascorbate and hence 2,3-DKG will also be present in this environment (Bode *et al.*, 1990; Cioffi *et al.*, 2000; Deutsch, 1998a, b). Although no reliable estimates of its concentration *in vivo* are known, the use of a high-affinity TRAP system would allow scavenging of this common breakdown product of ascorbate. It is apparent from Fig. 1(a) that YiaMNO homologues are conserved in several important pathogens including *Salmonella*, *Haemophilus* and *Pasteurella* species, which may imply a significant general role for gulonate transport and metabolism *in vivo*. Expression of the *yiaK* operon in wild-type *E. coli* K-12 cells is repressed by the *yiaJ* gene product, a member of the IclR family. As yet, the inducer for this operon has not been identified (Ibanez *et al.*, 2000), and we suggest that 2,3-DKG, the identified substrate for YiaO, could be a potential inducer. Interestingly, in a separate study, strains of *E. coli* were isolated that had reduced fitness when grown on M9 glucose medium (Badarinarayana *et al.*, 2001). Some of these strains had mutations in *yiaJ*, which suggests that unregulated expression of the *yiaK* operon is detrimental to growth under these conditions.

Recently, Zhang *et al.* (2003) have described an ascorbate transporter in *E. coli* as being the products of the *sgaTBA* genes; deletion of these genes led to a complete loss of ascorbate transport in K-12 strains (Zhang *et al.*, 2003). These data are complementary to those in this study, where we have shown that ascorbate itself is not a ligand for YiaO protein. Thus *E. coli* appears to contain separate transporters for both ascorbate and its breakdown product 2,3-DKG.

Finally, the kinetic behaviour of the two proteins characterized in this study was not identical, reflecting variations in the position of the equilibrium between the closed and open forms of the proteins. A study of the effect of ionic strength and pH on the DctP CU/OU equilibrium suggested that one or more salt-bridges were involved in stabilizing the closed unliganded conformation (Walmsley *et al.*, 1992b). Relatively few proteins appear to undergo an isomerization before ligand binding, a recent example being the discovery of isomerization of antigen-binding sites in antibodies (James *et al.*, 2003). The structural basis for the observed differences in kinetic behaviour will partly be resolved by

future crystallographic studies of these and other TRAP ESR proteins.

ACKNOWLEDGEMENTS

We thank Mark Gibson for provision of plasmid pGIB802, Anthony Keeble for help with stopped-flow fluorescence experiments and Rosie Young for technical assistance. We would also like to thank Wen Shan Yew and John Gerlt for providing 2,3-diketo-L-gulonate. We thank Arthur Moir for N-terminal sequencing. This work was partly funded by a grant to D.J.K. from the UK Biotechnology and Biological Sciences Research Council, and the Mexican Government is acknowledged for financial support for a studentship for M. R. L.-K.

REFERENCES

- Allen, S., Zaleski, A., Johnson, J. W., Gibson, B. W. & Apicella, M. A. (2005). Novel sialic acid transporter of *Haemophilus influenzae*. *Infect Immun* **73**, 5291–5300.
- Badarinarayana, V., Estep, P. W., III, Shendure, J., Edwards, J., Tavazoie, S., Lam, F. & Church, G. M. (2001). Selection analyses of insertional mutants using subgenomic-resolution arrays. *Nat Biotechnol* **19**, 1060–1065.
- Badia, J., Ibanez, E., Sabate, M., Baldoma, L. & Aguilar, J. (1998). A rare 920-kilobase chromosomal inversion mediated by IS1 transposition causes constitutive expression of the *yiaK*-S operon for carbohydrate utilization in *Escherichia coli*. *J Biol Chem* **273**, 8376–8381.
- Bendtsen, J. D., Nielsen, H., von Heijne, G. & Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**, 783–795.
- Berriman, M. & Rutherford, K. (2003). Viewing and annotating sequence data with Artemis. *Brief Bioinform* **4**, 124–132.
- Bode, A. M., Cunningham, L. & Rose, R. C. (1990). Spontaneous decay of oxidized ascorbic acid (dehydro-L-ascorbic acid) evaluated by high-pressure liquid chromatography. *Clin Chem* **36**, 1807–1809.
- Bruggemann, C., Denger, K., Cook, A. M. & Ruff, J. (2004). Enzymes and genes of taurine and isethionate dissimilation in *Paracoccus denitrificans*. *Microbiology* **150**, 805–816.
- Chae, J. C., Kim, Y., Kim, Y. C., Zylstra, G. J. & Kim, C. K. (2000). Genetic structure and functional implication of the *fcg* gene cluster for hydrolytic dechlorination of 4-chlorobenzoate from *Pseudomonas* sp. DJ-12. *Gene* **258**, 109–116.
- Choudhary, M., MacKenzie, C., Mouncey, N. J. & Kaplan, S. (1999). RsGDB, the *Rhodobacter sphaeroides* Genome Database. *Nucleic Acids Res* **27**, 61–62.
- Cioffi, N., Losito, I., Terzano, R. & Zamboni, C. G. (2000). An electrospray ionization ion trap mass spectrometric (ESI-MS-MSn) study of dehydroascorbic acid hydrolysis at neutral pH. *Analyst* **125**, 2244–2248.
- Contzen, M., Burger, S. & Stolz, A. (2001). Cloning of the genes for a 4-sulphocatechol-oxidizing protocatechuate 3,4-dioxygenase from *Hydrogenophaga intermedia* S1 and identification of the amino acid residues responsible for the ability to convert 4-sulphocatechol. *Mol Microbiol* **41**, 199–205.
- Dailey, F. E. & Cronan, J. E., Jr (1986). Acetohydroxy acid synthase I, a required enzyme for isoleucine and valine biosynthesis in *Escherichia coli* K-12 during growth on acetate as the sole carbon source. *J Bacteriol* **165**, 453–460.
- Deutsch, J. C. (1997). Gas chromatographic/mass spectrometric measurement of ascorbic acid and analysis of ascorbic acid degradation in solution. *Methods Enzymol* **279**, 13–24.

- Deutsch, J. C. (1998a). Spontaneous hydrolysis and dehydration of dehydroascorbic acid in aqueous solution. *Anal Biochem* **260**, 223–229.
- Deutsch, J. C. (1998b). Oxygen-accepting antioxidants which arise during ascorbate oxidation. *Anal Biochem* **265**, 238–245.
- Deutsch, J. C. (2000). Dehydroascorbic acid. *J Chromatogr A* **881**, 299–307.
- Felsenstein, J. (1989). PHYLIP – Phylogeny Inference Package, version 3.2. *Cladistics* **5**, 164–166.
- Forward, J. A., Behrendt, M. C., Wyborn, N. R., Cross, R. & Kelly, D. J. (1997). TRAP transporters: a new family of periplasmic solute transport systems encoded by the *dctPQM* genes of *Rhodobacter capsulatus* and by homologs in diverse gram-negative bacteria. *J Bacteriol* **179**, 5482–5493.
- Grammann, K., Volke, A. & Kunte, H. J. (2002). New type of osmoregulated solute transporter identified in halophilic members of the bacteria domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine in *Halomonas elongata* DSM 2581^T. *J Bacteriol* **184**, 3078–3085.
- Higgins, C. F. (2001). ABC transporters: physiology, structure and mechanism – an overview. *Res Microbiol* **152**, 205–210.
- Hillmer, P. & Gest, H. (1977). H₂ metabolism in the photosynthetic bacterium *Rhodospseudomonas capsulata*: H₂ production by growing cultures. *J Bacteriol* **129**, 724–731.
- Horlacher, R., Xavier, K. B., Santos, H., Diruggiero, J., Kossman, M. & Boos, W. (1998). Archaeal binding protein-dependent ABC transporter: molecular and biochemical analysis of the trehalose/maltose transport system of the hyperthermophilic archaeon *Thermococcus litoralis*. *J Bacteriol* **180**, 680–689.
- Hosie, A. H., Allaway, D. & Poole, P. S. (2002). A monocarboxylate permease of *Rhizobium leguminosarum* is the first member of a new subfamily of transporters. *J Bacteriol* **184**, 5436–5448.
- Ibanez, E., Campos, E., Baldoma, L., Aguilar, J. & Badia, J. (2000). Regulation of expression of the *yiaKLMNOPQRS* operon for carbohydrate utilization in *Escherichia coli*: involvement of the main transcriptional factors. *J Bacteriol* **182**, 4617–4624.
- Jacobs, M. H., van der Heide, T., Driessen, A. J. & Konings, W. N. (1996). Glutamate transport in *Rhodobacter sphaeroides* is mediated by a novel binding protein-dependent secondary transport system. *Proc Natl Acad Sci U S A* **93**, 12786–12790.
- James, L. C., Roversi, P. & Tawfik, D. S. (2003). Antibody multispecificity mediated by conformational diversity. *Science* **299**, 1362–1367.
- Kapatral, V., Anderson, I., Ivanova, N. & 22 other authors (2002). Genome sequence and analysis of the oral bacterium *Fusobacterium nucleatum* strain ATCC 25586. *J Bacteriol* **184**, 2005–2018.
- Kelly, D. J. & Thomas, G. H. (2001). The tripartite ATP-independent periplasmic (TRAP) transporters of bacteria and archaea. *FEMS Microbiol Rev* **25**, 405–424.
- Kolker, E., Makarova, K. S., Shabalina, S. & 9 other authors (2004). Identification and functional analysis of ‘hypothetical’ genes expressed in *Haemophilus influenzae*. *Nucleic Acids Res* **32**, 2353–2361.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Miller, D. M., Olson, J. S. & Quioco, F. A. (1980). The mechanism of sugar binding to the periplasmic receptor for galactose chemotaxis and transport in *Escherichia coli*. *J Biol Chem* **255**, 2465–2471.
- Miller, D. M., III, Olson, J. S., Pflugrath, J. W. & Quioco, F. A. (1983). Rates of ligand binding to periplasmic proteins involved in bacterial transport and chemotaxis. *J Biol Chem* **258**, 13665–13672.
- Nielsen, H., Brunak, S. & von Heijne, G. (1999). Machine learning approaches for the prediction of signal peptides and other protein sorting signals. *Protein Eng* **12**, 3–9.
- Overbeek, R., Larsen, N., Walunas, T. & 19 other authors (2003). The ERGO genome analysis and discovery system. *Nucleic Acids Res* **31**, 164–171.
- Page, R. D. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Plantinga, T. H., van der Does, C., Badia, J., Aguilar, J., Konings, W. N. & Driessen, A. J. M. (2004). Functional characterization of the *Escherichia coli* K-12 *yiaMNO* transport protein genes. *Mol Membr Biol* **21**, 51–57.
- Plantinga, T. H., van der Does, C., Tomkiewicz, D., van Keulen, G., Konings, W. N. & Driessen, A. J. M. (2005). Deletion of the *yiaMNO* transporter genes affects the growth characteristics of *Escherichia coli* K-12. *Microbiology* **151**, 1683–1689.
- Prakash, S., Cooper, G., Singhi, S. & Saier, M. H., Jr (2003). The ion transporter superfamily. *Biochim Biophys Acta* **1618**, 79–92.
- Quintero, M. J., Montesinos, M. L., Herrero, A. & Flores, E. (2001). Identification of genes encoding amino acid permeases by inactivation of selected ORFs from the *Synechocystis* genomic sequence. *Genome Res* **11**, 2034–2040.
- Quioco, F. A. & Ledvina, P. S. (1996). Atomic structure and specificity of bacterial periplasmic receptors for active transport and chemotaxis: variation of common themes. *Mol Microbiol* **20**, 17–25.
- Rabus, R., Jack, D. L., Kelly, D. J. & Saier, M. H., Jr (1999). TRAP transporters: an ancient family of extracytoplasmic solute-receptor-dependent secondary active transporters. *Microbiology* **145**, 3431–3445.
- Rohrbach, M. R., Braun, V. & Koster, W. (1995). Ferrichrome transport in *Escherichia coli* K-12: altered substrate specificity of mutated periplasmic FhuD and interaction of FhuD with the integral membrane protein FhuB. *J Bacteriol* **177**, 7186–7193.
- Sanchez, J. C., Gimenez, R., Schneider, A., Fessner, W. D., Baldoma, L., Aguilar, J. & Badia, J. (1994). Activation of a cryptic gene encoding a kinase for L-xylulose opens a new pathway for the utilization of L-lyxose by *Escherichia coli*. *J Biol Chem* **269**, 29665–29669.
- Severi, E., Randle, G., Kivlin, P., Whitfield, K., Young, R., Moxon, R., Kelly, D., Hood, D. & Thomas, G. (2005). Sialic acid transport in *Haemophilus influenzae* is essential for lipopolysaccharide sialylation and serum resistance and is dependent on a novel tripartite ATP-independent periplasmic transporter. *Mol Microbiol* **58**, 1173–1185.
- Stein, M. A., Schafer, A. & Giffhorn, F. (1997). Cloning, nucleotide sequence, and overexpression of *smoS*, a component of a novel operon encoding an ABC transporter and polyol dehydrogenases of *Rhodobacter sphaeroides* Si4. *J Bacteriol* **179**, 6335–6340.
- Tam, R. & Saier, M. H., Jr (1993). Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol Rev* **57**, 320–346.
- Tetsch, L. & Kunte, H. J. (2002). The substrate-binding protein TeaA of the osmoregulated ectoine transporter TeaABC from *Halomonas elongata*: purification and characterization of recombinant TeaA. *FEMS Microbiol Lett* **211**, 213–218.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Ullmann, R., Gross, R., Simon, J., Unden, G. & Kroger, A. (2000). Transport of C₄-dicarboxylates in *Wolinella succinogenes*. *J Bacteriol* **182**, 5757–5764.
- Walmsley, A. R., Shaw, J. G. & Kelly, D. J. (1992a). The mechanism of ligand binding to the periplasmic C₄-dicarboxylate binding

protein (DctP) from *Rhodobacter capsulatus*. *J Biol Chem* **267**, 8064–8072.

Walmsley, A. R., Shaw, J. G. & Kelly, D. J. (1992b). Perturbation of the equilibrium between open and closed conformations of the periplasmic C₄-dicarboxylate binding protein from *Rhodobacter capsulatus*. *Biochemistry* **31**, 11175–11181.

Walmsley, A. R., Zhou, T., Borges-Walmsley, M. I. & Rosen, B. P. (2001). A kinetic model for the action of a resistance efflux pump. *J Biol Chem* **276**, 6378–6391.

Weaver, P. F., Wall, J. D. & Gest, H. (1975). Characterisation of *Rhodopseudomonas capsulata*. *Arch Microbiol* **105**, 207–216.

Winnen, B., Hvorup, R. N. & Saier, M. H., Jr (2003). The tripartite tricarboxylate transporter (TTT) family. *Res Microbiol* **154**, 457–465.

Wyborn, N. R., Alderson, J., Andrews, S. C. & Kelly, D. J. (2001). Topological analysis of DctQ, the small integral membrane protein of the C₄-dicarboxylate TRAP transporter of *Rhodobacter capsulatus*. *FEMS Microbiol Lett* **194**, 13–17.

Yew, W. S. & Gerlt, J. A. (2002). Utilization of L-ascorbate by *Escherichia coli* K-12: assignments of functions to products of the *yjf-sga* and *yia-sgb* operons. *J Bacteriol* **184**, 302–306.

Zhang, Z., Aboulwafa, M., Smith, M. H. & Saier, M. H., Jr (2003). The ascorbate transporter of *Escherichia coli*. *J Bacteriol* **185**, 2243–2250.