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ORIGINAL PAPER

**Zhongge Zhang · Jérôme N. Feige · Abraham B. Chang
Iain J. Anderson · Vadim M. Brodianski
Alexei G. Vitreschak · Mikhail S. Gelfand
Milton H. Saier Jr**

A transporter of *Escherichia coli* specific for L- and D-methionine is the prototype for a new family within the ABC superfamily

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Abstract An ABC-type transporter in *Escherichia coli* that transports both L- and D-methionine, but not other natural amino acids, was identified. This system is the first functionally characterized member of a novel family of bacterial permeases within the ABC superfamily. This family was designated the methionine uptake transporter (MUT) family (TC #3.A.1.23). The proteins that comprise the transporters of this family were analyzed phylogenetically, revealing the probable existence of several sequence-divergent primordial paralogues, no more than two of which have been transmitted to any currently sequenced organism. In addition, MetJ, the pleiotropic methionine repressor protein, was shown to negatively control expression of the operon encoding the ABC-type methionine uptake system. The identification of MetJ binding sites (in gram-negative bacteria) or S-boxes (in gram-positive bacteria) in the promoter regions of several MUT transporter-encoding operons suggests that many MUT family members transport organic sulfur compounds.
Electronic Supplementary Material Supplementary material is available for this article if you access the article at http://dx.doi.org/10.1007/s00203-003-0561-4. A link in the frame on the left on that page takes you directly to the supplementary material.

Keywords Transport · Methionine · MetD · ATP-binding cassette · *E. coli*

Z. Zhang · J. N. Feige · A. B. Chang · M. H. Saier Jr (✉)
Division of Biological Sciences,
University of California at San Diego,
La Jolla, CA 92093–0116, USA
Tel.: +1-858-5344084, Fax: +1-858-5347108,
e-mail: msaier@ucsd.edu

I. J. Anderson
Integrated Genomics, 2201 W. Campbell Park Dr.,
Chicago, IL 60612, USA

V. M. Brodianski
Integrated Genomics-Moscow,
P. O. Box 348, 117333 Moscow, Russia

A. G. Vitreschak
Institute of Information Transmission Problems,
101447 Moscow, Russia

M. S. Gelfand
State Scientific Center GosNIIGenetika, 1st Dorozhny proezd, 1,
113545 Moscow, Russia

Introduction

Methionine transport and its regulation have been extensively studied in both *Escherichia coli* and the phylogenetically related bacterium *Salmonella typhimurium*. *E. coli* has been shown to have two transport systems for L-methionine (Kadner 1974) but only one system for D-methionine (Kadner 1977). Spontaneous mutants selected for their capacity to grow on toxic methionine analogues were generated. A *metD* mutant lacks both high-affinity uptake activity for L-methionine (Kadner 1974) and lower affinity uptake activity for D-methionine (Kadner 1977; Kadner and Watson 1974). Specificity of the transport system for L- and D-methionine and related compounds has been examined (Kadner 1974, 1977), but inhibitory studies with other amino acids have not been reported. A *metD* mutant has also been isolated in *S. typhimurium* and exhibits characteristics similar to those of the corresponding mutant in *E. coli* (Ayling and Bridgeland 1972; Ayling et al. 1979; Betteridge and Ayling 1975; Poland and Ayling 1984).

Studies of energy coupling for methionine uptake in *E. coli* have suggested that transport is driven by phosphate bond energy, presumably ATP (Kadner and Winkler 1975). Moreover, the MetD transport system has been shown to be sensitive to osmotic shock and to inhibition by arsenate in both *E. coli* and *S. typhimurium* (Cottam and Ayling 1989; Kadner and Winkler 1975). It was therefore suggested that the major uptake system is an ATP-binding cassette (ABC) transporter. ABC transporters usually consist of a transmembrane protein, a cytoplasmic ATP-hydrolyzing (ABC) protein, and at least one substrate-binding receptor.

The *metD* mutation was mapped to 4.8 minutes on the *E. coli* chromosome (Berlyn 1998). Mapping of the *metD*

locus in *S. typhimurium* has revealed that several genes, when mutated, give rise to the *metD* phenotype (Grundy and Ayling 1992). In the work reported here, we identify an ABC transporter mapping very near 4.8 minutes, which proves to be the MetD transporter.

Methionine biosynthetic genes in *E. coli* are regulated by the MetJ repressor (Greene 1996; Sekowsha et al. 2000; Weissbach and Brot 1991), and a MetJ DNA binding site consensus sequence has been derived (Saint-Girons et al. 1984). Evidence is available suggesting that methionine transport is also regulated by MetJ since cells grown in medium containing methionine have lower levels of methionine transport (Kadner 1975), and methionine auxotrophs with a *metJ* mutation have higher transport activity (Kadner 1975, 1977). The recent identification of a MetJ binding site in the promoter region of the ABC transporter mentioned above (Liu et al. 2001) corroborated this hypothesis.

Recently, Gal et al. (2002) and Merlin et al. (2002) reported growth studies that led to the tentative molecular identification of this transporter (*abc-yaeE-yae-C*) which they renamed *metNIQ*. The MetJ protein, in the presence of methionine, but not in its absence, was also shown to repress expression of the operon. Although no transport studies have been reported, it has been suggested that the three-gene cluster encodes the methionine transporter characterized physiologically by Kadner and his collaborators.

In this study, we confirm and substantially extend the previously reported work. We (1) report the first transport studies with this system, revealing that MetD transports both L- and D-methionine and probably formyl methionine; (2) resolve the question as to whether both L- and D-methionine are recognized by the same receptor; (3) provide evidence that NlpA (Yamaguchi and Inouye 1988; Yu et al. 1986) may, under certain conditions, serve as a poor L- and D-methionine receptor, feeding inefficiently into MetD; (4) show that MetJ represses expression of the *metD* operon; and (5) provide detailed phylogenetic data that define a novel family within the ABC superfamily, which we have called the methionine uptake transporter (MUT) family (TC #3.A.1.23).

Materials and methods

Bacterial strains and media

E. coli strains and plasmids used in this study are listed in Table 1. All studies were conducted in the genetic background of strain BW25113. Bacteria were cultured in Luria-Bertani (LB) broth or M9 minimal medium at 37 °C (Sambrook et al. 1989). When appropriate, ampicillin (Ap) and/or kanamycin (Km) was/were added to the medium at 100 and/or 25 μg/ml, respectively. Unless otherwise stated, chemicals were purchased from Sigma-Aldrich.

Generation of deletion mutants

Deletion mutants were generated using the methods described by Datsenko and Wanner (2000). To prepare competent cells for transformation, BW25113 containing pKD46 was cultured at 30 °C in SOB broth (Sambrook et al. 1989) containing ampicillin and 2 mM L-arabinose. When the OD_{600} reached 0.5, the culture was centrifuged at 2,000×*g* for 5 min, and the cells were washed three times with cold 10% glycerol before being resuspended in a minimal volume of 10% glycerol (1% of the original culture). The competent cells were stored at –80 °C prior to use. PCR methods were used to clone the kanamycin gene from pKD4 using the primers described in Table 2. The PCR products were purified using a Qiagen kit, treated with *Dpn*I, and repurified by electrophoresis. BW25113 competent cells were transformed with the kanamycin gene by electroporation (Gene Pulser, pulse controller at 200 Ω, capacitance at 250 μFD, and voltage at 2.5 kV). After electroporation, the cells were grown with shaking in 1 ml of SOC (Sambrook et al. 1989) at 37 °C for 1 h, and the cultures were plated onto LB agar medium containing kanamycin. The kanamycin-resistant transformants were purified on new kanamycin-LB plates. The mutants in which the target genes were replaced by the kanamycin gene were verified by three PCRs using bacterial DNA as the template. The first PCR used forward primer k_2(5′-C G G T G C C C T G A A T G A A C T G C-3′) and reverse primer k_t (5′-C G G C C A C A G T C G A T G A A T C C-3′) (Datsenko and Wanner 2000), both of

Table 1 Strains and plasmids used in this study

Strain/plasmid	Genotype	Reference
Strain		
LJ 3001 (BW25113)	*lacI*^{q}*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner 2000
LJ 3015	BW25113 Δ*abc-yaeE* (Δ*metNI*)	This study
LJ 3016	BW25113 Δ*yaeC* (Δ*metQ*)	This study
LJ 3017	BW25113 Δ*nlpA*	This study
LJ 3018	BW25113 Δ*yaeC* Δ*nlpA*	This study
LJ 3019	BW25113 Δ*yaeE-abc* Δ*ykfD* (Δ*metNI* Δ*mmuP*)	This study
LJ3020	BW25113 Δ*ykfD*	This study
LJ 3021	BW25113 Δ*metJ*	This study
Plasmid		
pKD46	*oriR101 repA101*(ts) *araBp-gam-bet-exo* Ap^r	Datsenko and Wanner 2000
pKD4	*oriR*$_γ$ Ap^r Km^r	Datsenko and Wanner 2000
pCP20	λ*cI857*(ts) ts-rep	Datsenko and Wanner 2000
pBAD24	Expression vector, Ap^r	Guzman et al. 1995
pBAD24-metD	pBAD24 carrying *metD* operon	This study
pBAD24-metJ	pBAD24 carrying *metJ*	This study

Table 2 Primers used for generation and verification of BW25113 mutants. For all gene mutations, pKD4 was used as the template for cloning the kanamycin gene

Gene	Primers (5′→3′)
abc-yaeE (*metNI*)	Generation GATGCGGTCGCCTGCGAACTGAATTAAATAAACCAGAATGACCAGGTGTAGGCTGGAGCTGCTTC (forward) CTTAATGACGATATAAATAATCAATGATAAAACTTTCGAATATCCATATGAATATCCTCCTTAG (reverse) Verification abc-yaeE 3: CGTTACTTGCGAGTGACAGC abc-yaeE 4: GCATGTGACGCTAGTATCGC
yaeC (*metQ*)	Generation TTACAAATTGTGGAAACAGCCTAAAAATTACCAGCCTTTAACAGCGTGTAGGCTGGAGCTGCTTC (forward) AAGGAATAAGGTATGGCGTTCAAATTCAAAACCTTTGCGGCAGTGCATATGAATATCCTCCTTAG (reverse) Verification yaeC 3: ACAGCCGCTTAGCATGAGTG yaeC 4: AATTCAGTTCGCAGGCGACC
nlpA	Generation ACCGCAGCGACCTTACCGCTATAGTCAGGTAATCATTAATAAAAGGTGTAGGCTGGAGCTGCTTC (forward) TGAGAATTACCAGCCAGGCACCGCGCCACCGTTAAAAATGGTTTCCATATGAATATCCTCCTTAG (reverse) Verification nlpA 3: CGTGGTCAGTAAGAAGTGCC nlpA 4: GCTGCTGATTCTGTCATCGG
ykfD (*mmuP*)	Generation GGTTGACTTTGCATTCTGTTAACAAACGCGGTATAACAAACCGTGTAGGCTGGAGCTGCTTC (forward) GGTTGAGTAAGGAAATAAGCACCATAGCACAACGCAACAAACCATATGAATATCCTCCTTAG (reverse) Verification ykfD 3: GACTTGTTCGCACCTTCC ykfD 4: GGCTGTCGGCTAAGTTAC
metJ	Generation TGGTCTGGTCTCAATTTATTGACGAAGAGGATTAAGTATCTCATGGTGTAGGCTGGAGCTGCTTC (forward) TAGCGCATCAGGCGATTCCACTCCGCGCCGCTCTTTTTTGCTTTACATATGAATATCCTCC AATG (reverse) Verification metJ 3: CAACTGTGTGGTCTGGTCTC metJ 4: TGCGATGAGCGAGAGATCTG

which are designed from the internal kanamycin gene. The second PCR used primer k_2 and primer 4 (reverse, Table 2). The third PCR used primers 3 and 4 (Table 2). To delete the kanamycin gene from the chromosome, pKD46 was removed from the cells by growing the bacteria at 37 °C, and then pCP20 was introduced by transformation. The transformants containing pCP20 were grown overnight with shaking at 42 °C, and the cultures were plated on LB agar without antibiotics. Colonies were tested for sensitivity to kanamycin and ampicillin. To verify the loss of the kanamycin gene, the last PCR using primers 3 and 4 (see above, Table 2) was repeated. Growth experiments were carried out in M9 minimal medium, with $MgCl_2$ replacing $MgSO_4$ and with 20 µM L-methionine or 100 µM D-methionine serving as the sole sulfur source.

DNA manipulations and gene cloning

Standard methods were used for chromosomal DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (Sambrook et al. 1989). Plasmids were isolated using spin miniprep kits (Qiagen, Chatsworth, Calif., USA), and PCR products were purified using Qiaquick purification kits (Qiagen). For gene cloning, the *metD* operon (*abc-yaeE-yaeC*) and *metJ* gene were amplified from wild-type *E. coli* BW25113 chromosomal DNA by PCR. The following primers were used for gene amplification (restriction sites *Kpn*I, *Xba*I and *Hin*dIII are underlined) – for *metD*, 5′-C G C G G T A C C G A T A A A A C T T T C G A A T A T C A C C-3′ and 5′-C G C C T C T A G A T T A C A A A T T G T G G A A A C A G C C-3′, and for *metJ*, 5′-G A A T C T A G A C A T G G C T G A A T G G A G C G G C G-3′ and 5′-C G C C A A G C T T A G T A T T C C C A C G T C T C C G-3′. The PCR products were purified, treated with *Kpn*I and *Xba*I (for *metD*) or with *Xba*I and *Hin*dIII (for *metJ*), and then cloned into pBAD24 (Guzman et al. 1995).

Transport assays

Cells grown in M9 minimal medium were harvested in the exponential growth phase, washed once in Tris-maleate (TM) buffer, pH 7.0, and resuspended in the same buffer containing 0.5% D,L-lactate plus 100 µg chloramphenicol/ml. Uptake studies were conducted at 37 °C over a 10-min time interval with the cell density at an OD_{600} of 0.10 and 0.40 and the methionine concentration at 0.5 µM (50,000 cpm/ml; 55 µCi/µmol) and 5.0 µM (50,000 cpm/ml; 5.5 µCi/µmol), respectively, for the L- and D-isomers. 1-[^{14}C]L- and D-methionine were purchased from American Radiolabeled Chemicals. Aliquots (100 µl) were periodically removed from the 1-ml cell suspensions. Cells were transferred to 10 ml of ice-cold TM buffer, filtered (0.45-µm Millipore filters) and washed twice with the same buffer. After drying the filters, radioactivity was measured by scintillation counting using 10 ml of Biosafe NA scintillation fluid (Research Products International, Mt. Prospect, Ill., USA). For comparison of the uptake of L-methionine between

the *abc-yaeE* and the *abc-yaeE mmuP* mutants, L-methionine concentrations of 13 and 103 µM (12 µCi/µmol) were used since the low-affinity L-methionine transporter was analyzed. Initial uptake rates were inhibited over a 5-min time interval for both L- and D-methionine. Unless otherwise stated, the concentration of the non-radioactive inhibitory amino acid was ten times the methionine concentration. The uptake activity attributed to MetD alone was obtained by subtracting the activity remaining in the absence of MetD function from the wild-type activity (e.g., see Table 3), assuming that loss of MetD does not activate some other transporter.

Computer methods

Sequences of the proteins that comprise the three constituents of MUT family permeases were obtained by initial BLAST searches (Altschul et al. 1997) using the sequences of the three *E. coli* MetD permease constituents as query. The resulting hits were filtered through a program manipulating the BLAST program to eliminate the sequences more related to other families in the ABC superfamily (C. Tran and M.H. Saier, Jr., unpublished program).

Multiple sequence alignments were constructed using the Clustal X program (Thompson et al. 1997). The gap penalty and gap extension values used with the Clustal X program were 10 and 0.1, respectively, although other combinations were tried. The HMMTOP (Tusnady and Simon 1998, 2001) and TMHMM (Krogh et al. 2001; Sonnhammer et al. 1998) programs were used to determine the predicted numbers of transmembrane segments. Phylogenetic trees were derived from alignments generated with the Clustal X program using the BLOSUM 62 scoring matrix. The trees were drawn using the TreeView program (Page 1996). Complementary trees were constructed using the Phylo_win program (Galtier et al. 1996) with the neighbor-joining method and PAM distances as the model of evolution. This study was conducted independently for the three protein constituents of the ABC transporters that comprise the MUT family, and the sequences obtained were checked manually to see whether all three ABC elements had been identified for each transporter.

G+C content was analyzed with the GeeCee program (Rice et al. 2000), and codon usage was analyzed with the Countcodon program from the Codon Usage Database website (http://www.kazusa.or.jp/codon/countcodon.html). The lipoproteic structure of the receptors was predicted with the Lipop section of the PSORT program (http://psort.nibb.ac.jp). S-boxes were predicted with the RNApattern program (Vitreschak et al., unpublished program).

Results

Growth studies

Figure 1 shows the growth of the isogenic strains described above with 100 µM D-methionine as the sole source of sulfur. The wild-type strain, the *nlpA* mutant, and the *ykfD* (Thanbichler et al. 1999) mutant grew equally well, but the *abc-yaeE* mutant and the *yaeC nlpA* double mutant as well as the *abc-yaeE-ykfD* triple mutant grew very poorly. Both the growth rates and growth yields were substantially depressed. These effects were completely reversed by inclusion of a plasmid (pBAD24) bearing the *metD* (*abc-yaeE-yaeC*) operon (data not shown, see supplementary Fig. S1 in the electronic supplementary material). The *yaeC* single mutant grew substantially better than the two double mutants but much less well than the wild-type strain. When 20 µM L-methionine served as the sole source of sulfur, the difference between the wild-type and the double mutants was less pronounced than when D-methionine

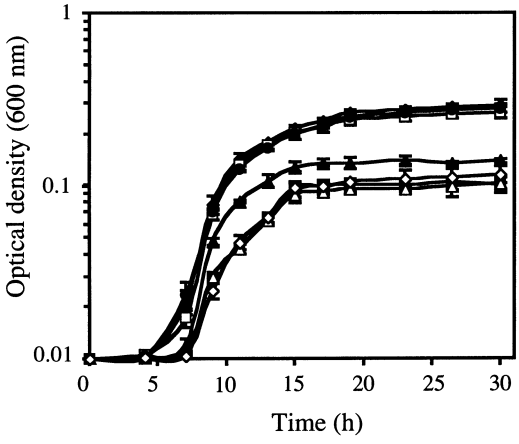


Fig. 1 Growth of *Escherichia coli* as a function of time in M9 minimal medium in which $MgSO_4$ was replaced by 1 mM $MgCl_2$ and D-methionine was added at a concentration of 100 µM as the sole sulfur source present. The growth experiments were conducted three times, and the results were averaged. The following strains were examined: wild-type (BW25113) (◆), Δ*abc-yaeE* (■), Δ*yaeC* (▲), Δ*nlpA* (●), Δ*yaeC*-Δ*nlpA* (◇), Δ*ykfD* (□) and Δ*abc-yaeE* Δ*ykfD* (Δ). *Error bars* indicate standard deviations

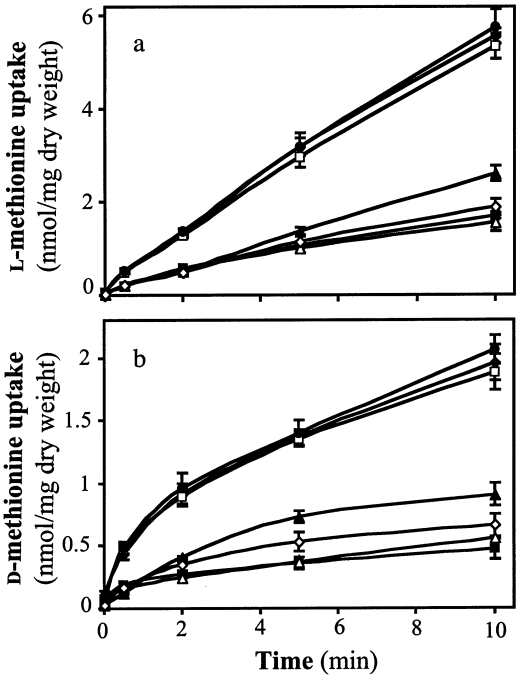


Fig. 2 Uptake of 1-[^{14}C]L-methionine (**a**) and 1-[^{14}C]D-methionine (**b**) by wild-type (BW25113) (◆), Δ*abc-yaeE* (■), Δ*yaeC* (▲), Δ*nlpA* (●), Δ*yaeC*-Δ*nlpA* (◇), Δ*ykfD* (□) and Δ*abc-yaeE* Δ*ykfD* (Δ) cells. Cells grown in M9 minimal medium were prepared as described in Materials and methods. Uptake assays were carried out at 37 °C over a 10-min time interval with the optical densities (600 nm) at 0.1 (**a**) or 0.4 (**b**) and the methionine concentrations at 0.5 µM (55 µCi/µmol) or 5 µM (5.5 µCi/µmol) (both at 50,000 cpm/ml), respectively, for the L- or D-isomers. Values are expressed in pmol L- or D-methionine retained per mg bacterial dry weight. The experiment was conducted three times, and the results reported represent an average of the values obtained. *Error bars* indicate standard deviations

was used (Fig. 1; data not shown). However, depressed growth suggested that the transporter could accept both D- and L-methionine as substrates.

Transport studies

Figure 2 shows the uptake of [^{14}C]L-methionine (Fig. 2a, 0.5 µM) and [^{14}C]D-methionine (Fig. 2b, 5 µM), respectively. Relative rates of uptake of the two substrates by the wild-type and mutant strains were essentially the same. Thus, the wild-type, and the mutant strains *ykfD* and *nlpA* took up the amino acids at nearly the same rate; the two double mutants (*abc-yaeE* and *yaeC nlpA*) took up both substrates poorly, and the *yaeC* single mutant took up both substrates poorly but slightly better than the double mutants. These depressed uptake rates were largely reversed by inclusion of the plasmid-encoded *metD* operon in these strains (data not shown; see Fig. S2 in the electronic supplementary material). It was therefore concluded that: (1) both L- and D-methionine are substrates of the Abc-YaeE-YaeC transporter, and (2) the binding receptor YaeC, and possibly NlpA, can activate both D- and L-methionine uptake. If NlpA acts as a receptor for the Abc-YaeE system, it is a much less effective receptor than YaeC. For both isomers, residual uptake was observed in the *abc-yaeE* mutant, suggesting that a second transporter exists both for L- and for D-methionine. We tested the transport of L- and D-methionine at concentrations of 10 and 100 µM in the *mmuP abc-yaeE* triple mutant, but no significant difference was observed relative to the *abc-yaeE* mutant (data not shown). This result is consistent with the indistinguishable growth rates between the *abc-yaeE-yaeC* and the *abc-yaeE-yaeC mmuP* mutants observed by Gal et al. (2002) in a methionine auxotrophic strain grown in minimal medium supplemented with L-methionine. It can therefore be assumed that the S-methylmethionine permease MmuP is not responsible for the residual transport of L- or D-methionine observed in the *abc-yaeE* mutant.

Inhibition studies

Table 3 summarizes the inhibitory effects of several non-radioactive amino acids present at ten-fold the concentration of the radioactive amino acid on uptake of both L- and D-methionine. Uptake of L-methionine by the wild-type bacteria was strongly inhibited by L-methionine and weakly

Table 3 Inhibition of L-methionine (top) and D-methionine (bottom) uptake by the L- and D-isomers of several aimno acids. Assays were done as described in Materials and methods with the non-radioactive inhibitory amino acids at 10× the concentrations of the radioactive substrate. Rate of uptake is expressed in pmol/(min×mg dry weight). Wild type–Δ*abc-yaeE* is the uptake activity attributed to MetD alone

Inhibitor	Wild-type		Δ*abc-yaeE*		Wild-type–Δ*abc-yaeE*[c]	
	Rate	%	Rate	%	Rate	%
–	688.8±88	100	230.4±16	100	458.5±72	100
L-Methionine	47.5±5	7	41.8±8	18	5.7±3	1
D-Methionine	635.3±45	92	298.6±16	129	336.8±30	73
N-Formyl-L-methionine	268.3±18	39	258.9±13	112	9.3±5	2
L-Alanine	627.4±56	91	176.9±20	77	451.1±35	98
D-Alanine	649.5±40	94	281.6±22	122	367.9±18	80
L-Leucine	581.8±40	84	179.7±16	78	402.1±24	88
D-Leucine	677.5±56	98	245.6±36	107	431.9±20	94
L-Valine	528.1±50	77	159.1±18	69	369.0±31	80
L-Serine	833.2±105	121	271.7±17	118	561.5±88	122
D-Serine	590.3±57	86	304.8±25	132	285.5±34	62
L-Threonine	612.7±76	89	151.7±25	66	461.0±51	101
D-Threonine	649.8±72	94	245.6±34	107	404.1±38	88
Inhibitor	Wild-type		Δ*abc-yaeE*		Wild-type–Δ*abc-yaeE*	
	Rate	%	Rate	%	Rate	%
–	287.8±31	100	68.4±11	100	219.3±20	100
D-Methionine	34.2±4	12	28.8±5	42	5.4±1	2
L-Methionine	66.7±12	23	36.5±5	53	31.1±6	14
N-Formyl-L-methionine	71.6±11	25	45.8±8	67	25.73±3	12
L-Alanine	273.0±25	95	77.8±9	114	195.2±16	89
D-Alanine	306.5±29	106	73.1±7	107	233.33±22	106
L-leucine	269.1±24	94	75.4±14	110	193.7±10	88
D-Leucine	258.2±26	90	84.0±4	123	174.2±22	79
L-Valine	232.6±21	81	65.3±9	95	167.2±12	76
L-Serine	327.5±36	114	70.8±10	103	256.7±26	1L7
D-Serine	338.3±23	118	85.6±7	125	252.8±17	115
L-Threonine	345.3±33	120	84.0±11	123	261.3±22	119
D-Threonine	329.8±33	115	87.9±6	128	24L9±27	110

inhibited by *N*-formyl L-methionine. However, no other amino acid inhibited strongly. Uptake by the *abc-yaeE* mutant was most strongly inhibited by L-methionine and to a lesser extent by L-threonine, L-valine, L-alanine, and L-leucine. When uptake of D-methionine was studied in wild-type *E. coli*, L-methionine was most inhibitory followed by *N*-formyl L-methionine and D-methionine in that order, but no other amino acid inhibited. In the *abc-yaeE* mutant, only L-methionine inhibited strongly. The mild increase of transport rate observed in the presence of some amino acids is unexplained. The results are consistent with the conclusion that the MetD transporter is specific for L- and D-methionine as well as *N*-formyl-L-methionine. The relative inhibitory effects of L- and D-methionine on uptake of these two radioactive substrates are in line with the relative affinities reported by Kadner (1974, 1977) (K_m values of 75 nM for L-methionine (Kadner 1974) and 1.2 µM for D-methionine (Kadner 1977)). Inhibition studies of D-methionine uptake were also conducted with 100- and 1,000 fold excess of both L- and D-methionine. The results were consistent with the existence of a second low-affinity transporter for methionine (data not shown). Surprisingly, the uncharacterized methionine transporter present in the mutant is also fairly specific for methionine.

The effects of energy poisons were also examined (data not shown; see Fig. S3 in the electronic supplementary material). The conditions used were essentially the same as those reported in Zhang et al. (2003). Five mM sodium arsenate virtually abolished uptake of both L- and D-methionine under the same conditions used in the experiment reported in Fig. 2. FCCP (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone) at a concentration of 2 µM was substantially less inhibitory (see Fig. S3 in the electronic supplementary material). The results clearly suggest that uptake is energy dependent and are consistent with the expectation that ATP is the energy source.

Regulation of *metD* operon expression by MetJ

A *metJ* knockout mutant was constructed, and *metJ* was cloned into plasmid pBAD24 for complementation studies. Using [^{14}C]D-methionine as the uptake substrate, the effects of *metJ* expression plus and minus L- and D-methionine were studied (Fig. 3). In Fig. 3a, it can be seen that the presence of either L- or D-methionine substantially reduced uptake of D-methionine into wild-type cells. In Fig. 3b, the same experiment conducted with the *metJ* mutant revealed that (1) the loss of *metJ* enhanced uptake above that observed for the wild-type strain grown without methionine and (2) methionine present during growth did not exert a repressive effect. In Fig. 3c, it can be seen that the plasmid bearing *metJ* depressed methionine uptake activity in the wild-type background and depressed the much greater activity of the *metJ* mutant even more. As expected, the wild-type strain expressing *metJ* on the plasmid exhibited lower D-methionine uptake than observed for the *metJ* mutant bearing the same plasmid. These

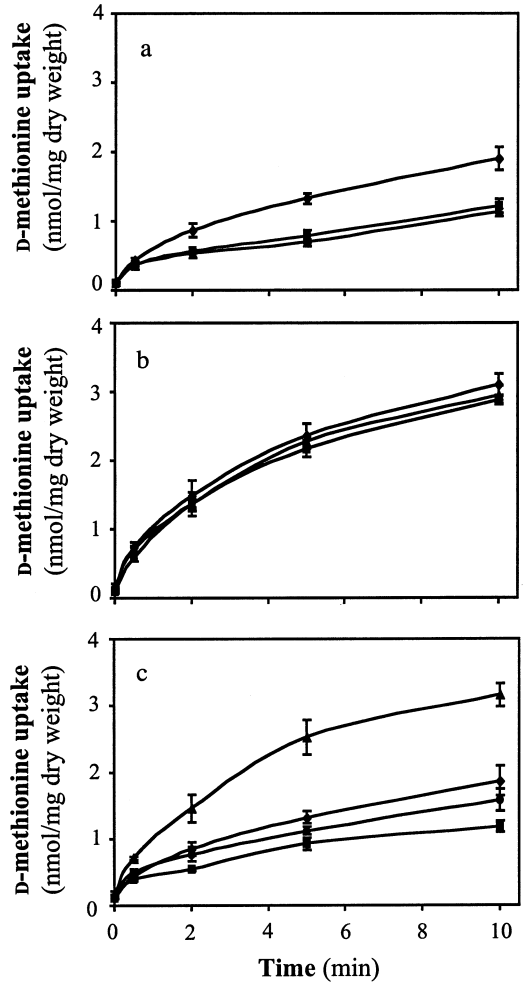


Fig. 3a–c Uptake of [^{14}C]D-methionine by cells grown in M9 medium with or without L- or D-methionine. Uptake experiments were conducted as described in the legend to Fig. 2. **a** Wild-type cells grown without methionine (◆), with 25 µM L-methionine (■), or with 25 µM D-methionine (▲). **b** The same conditions and symbols were used for the *metJ* mutant. **c** Complementation studies with the *metJ* bearing pBAD24 plasmid. Wild-type cells bearing pBAD24 (◆), wild-type cells bearing pBAD24-*metJ* (■), *metJ* mutant with pBAD24 (▲), *metJ* mutant with pBAD24-*metJ* (●). All cells in (**c**) were grown in medium M9 containing 100 µg ampicillin ml^{-1} and 2 mM L-arabinose

results are in accord with the expected *metJ* gene dosages. Measurements of L-methionine uptake were similar, but differences were substantially less pronounced as expected (data not shown).

Phylogenetic studies

Our preliminary results suggested that the *E. coli* ABC methionine transporter identified by Gal et al. (2002) and Merlin et al. (2002) and in the work described here belongs to a novel family within the ABC superfamily. Surprisingly, this family is more closely related to the polar amino acid uptake transporter (PAAT) family (TC# 3.A.1.3) than to the hydrophobic amino acid uptake transporter (HAAT)

Table 4 Members of the methionine uptake transporter (MUT) family. All full-length homologues of the membrane protein were predicted to exhibit 5 TMSs using the TMHMM or HMMTOP programs. * indicates an organism for which the complete genome sequence is not available in GenBank. GenBank accession numbers are given. Lipoprotein: *Y*, yes, the receptor is predicted to be a lipoprotein; *N*, no, it is not predicted to be a lipoprotein; *?*, the prediction is uncertain

Phylogenetic cluster	Organism	Source	Protein abbreviation	ABC proteins		Membrane proteins		Substrate-binding receptors		
				Accession number	Size (aa)	Accession number	Size (aa)	Accession number	Size (aa)	Lipoprotein ?
1	*Salmonella typhimurium*	γ-Proteobacteria	Styl	AAL19210	343	AAL19209	217	AALI9208	271	Y
	Escherichia coli	γ-Proteobacteria	Eco	AAC73310	343	AAC73309	217	AAC73308	271	Y
				–		–		AAC76685	272	Y
	Yersinia pestis	γ-Proteobacteria	Ype1	CAC89916	343	CAC89915	217	CAC89914	271	Y
	Vibrio cholerae	γ-Proteobacteria	Vch	AAF94069	344	AAF94068	225	AAP94067	275	Y
	Pasteurella multocida	γ-Proteobacteria	Pmu	AAK03812	344	AAK03813	229	AAK03814	276	Y
	Haemophilus influenzae	γ-Proteobacteria	Hin	AAC22280	345	P46492	198	AAC22279	273	Y
2	*Lactococcus lactis*	Firmicutes; Bacillales	Lla	CAB59828	368	AAK04421	231	CAB59827	286	N
	Streptococcus pneumoniae	Firmicutes; Bacillales	Spn	AAK98953	353	AAK98954	230	AAK98951	284	Y
	Streptococcus pyogenes	Firmicutes; Bacillales	Spy	AAM7884I	354	AAM78842	230	AAM78840	281	Y
	Streptococcus mutans	Firmicutes; Bacillales	Smu	AAL04079	354	AAL04080	229	AAL04077	280	Y
3	*Fusobacterium nucleatum*	Fusobacteria	Fnu	AAL94856	335	AAL94855	233	AAL94854	261	N
	Helicobacter pylori	ε-Proteobacteria	Hpy	AAD086I6	327	AAD08617	215	AAD08604	271	N
4	*Salmonella typhimurium*	γ-Proteobacteria	Sty2	AAL19465	338	AAL19466	219	AAL19464	276	N
5	*Streptomyces coelicolor*	Firmicutes; Actinobacteria	Sco	CAB76078	368	CAB76077	240	CAB76076	275	Y
6	*Yersinia pestis*	γ-Proteobacteria	Ype2	CAC90148	328	CAC90149	223	CAC90147	274	N
7	*Sinorhizobium meliloti*	α-Proteobacteria	Sme	CAC47469	358	:CAC47468	221	CAC47467	258	N
	Agrobacterium tumefaciens	α-Proteobacteria	Atu	AAL4528I	346	AAK88954	222	AAK88953	259	N
8	*Deinococcus radiodurans*	Thermus-Deinococcus group	Dra	AAF10928	325	AAF10929	218	AAP10931	256	N
				–		–		AAF10930	256	N
9	*Treponema pallidum*	Spirochaetales	Tpa	AAC65110	269	AAC65109	219	AAC65789	268	Y
10	*Pseudomonas aeruginosa*	γ-Proteobacteria	Pae1	AAG05738	369	AAG05739	217	AAG07318	259	N
11	*Brucella melitensis*	α-Proteobactena	Bme	AAL53579	369	AAL53578	230	AAL53580	278	N
				–		–		AAL53135	268	N
	Mesorhizobium loti	α-Proteobacteria	Mio	NP_105583	365	NP_105582	218	NP_105584	284	N
12	*Neisseria meningitidis*	β-Proteobacteria	Nme	CAB83797	245	CAB83798	228	CAB83799	287	Y
13	*Ralstonia solanacearum*	β-Proteobacteria	Rso	CAD14622	350	CAD14623	217	CAD14624	266	N

14	*Caulobacter crescentus*	α-Proteobacteria	Ccr	AAK24636	332	AAK24635	224	AAK24631	268	Y
	Pseudomonas aeruginosa	γ-Proteobacteria	Pae2	AAGOB888	335	AAG08889	225	AAG08890	260	N
	Xylella fastidiosa	γ-Proteobacteria	Xfa	AAP83685	334	AAF83684	235	AAF83683	261	N
	Xanthomonas axonopodis	γ-Proteobacteria	Xax	AAM38512	335	AAM38511	231	AAM38510	269	N
	Xanthomonas campestris	γ-Proteobacteria	Xca	AAM42900	335	AAM42899	232	AAM42898	266	N
15	*Bacillus anthracis*	Firmicutes; Bacillales	Ban1	NP_654117	346	NP_654116	222	NP654118	270	Y
	Listeria innocua	Firmicutes; Bacillales	Lin2	CAC95545	338	CAC95544	220	CAC95546	273	Y
	Listeria monocytogenes	Firmicutes; Bacillales	Lmo2	CAD008H	338	CAD00810	220	CAD00812	273	Y
16	*Staphylococcus aureus*	Firmicutes; Bacillales	Sau1	BAB56999	341	BAB57000	231	BAB57001	273	Y
	Bacillus anthracis	Firmicutes; Bacillales	Ban2	NP_653454	341	NP_653453	221	NP_653451	268	Y
	Bacillus anthracis	Firmicutes; Bacillales	Ban2	–		–		NP_JS53452	270	Y
	Bacillus halodurans	Firmicutes; Bacillales	Bha	BAB07200	338	BAB07I99	218	BAB07198	246	Y
	Bacillus subtilis	Firmicutes; Bacillales	Bsu	CAB 15264	341	CAB 15263	222	CAB 15262	274:	Y
				–		–.		CAB 12739	263	Y
	Listeria innocua	Firmicutes; Bacillales	Lin1	CAC97741	340	CAC97740	224	CAC97739	276	Y
	Listeria monocytogenes	Firmicutes; Bacillales	Lmol	CAD00497	340	CAD00496	224	CAD00495	276	Y
17	*Staphylococcus aureus*	Firmicutes; Bacillales	Sau2	BAB56624	341	BAB56625	219	BAB56626	280	Y
18	*Corynebacterium glutamicum*	Firmicutes; Actinobacteria	Cg1	NP599870	360	NP599869	225	NP599871	299	Y
19	*Chlamydia pneumoniae*	Chlamydiales	Cpn	AAD 18429	341	AADI8428	221	AAD 18427	272	.N
20	*Clostridium acetobutylicum*	Firmicutes; Bacillus-Clostridium group	Cac	AAK78960	320	AAK7896I	218	AAK78962	272	Y
	Campylobacter jejuni	ε-proteobacteria	Cje	CAB73039	336	CAB73038	303	CAB73037	257	N
				–		–		CAB73036	256	N
				–		–		CAB73035	258	N
	*Providencia stuartii**	γ-Proteobacteria	Pst	–		AAF71397	146	–		
	*Mannheimia haemolytica**	γ-Proteobacteria	Mha1	–		–		AAA25538	277	Y
			Mha2	–		–		AAA25547	277	Y
			Mha3	–		–		AAA25540	263	Y
	*Legionella pneumophila**	γ-Proteobacteria	Lpn	–		–		CAA06664	259	Y
	*Neisseria gonorrhoeae**	β-Proteobacteria	Ngo	–		–		AAF44768	288	Y

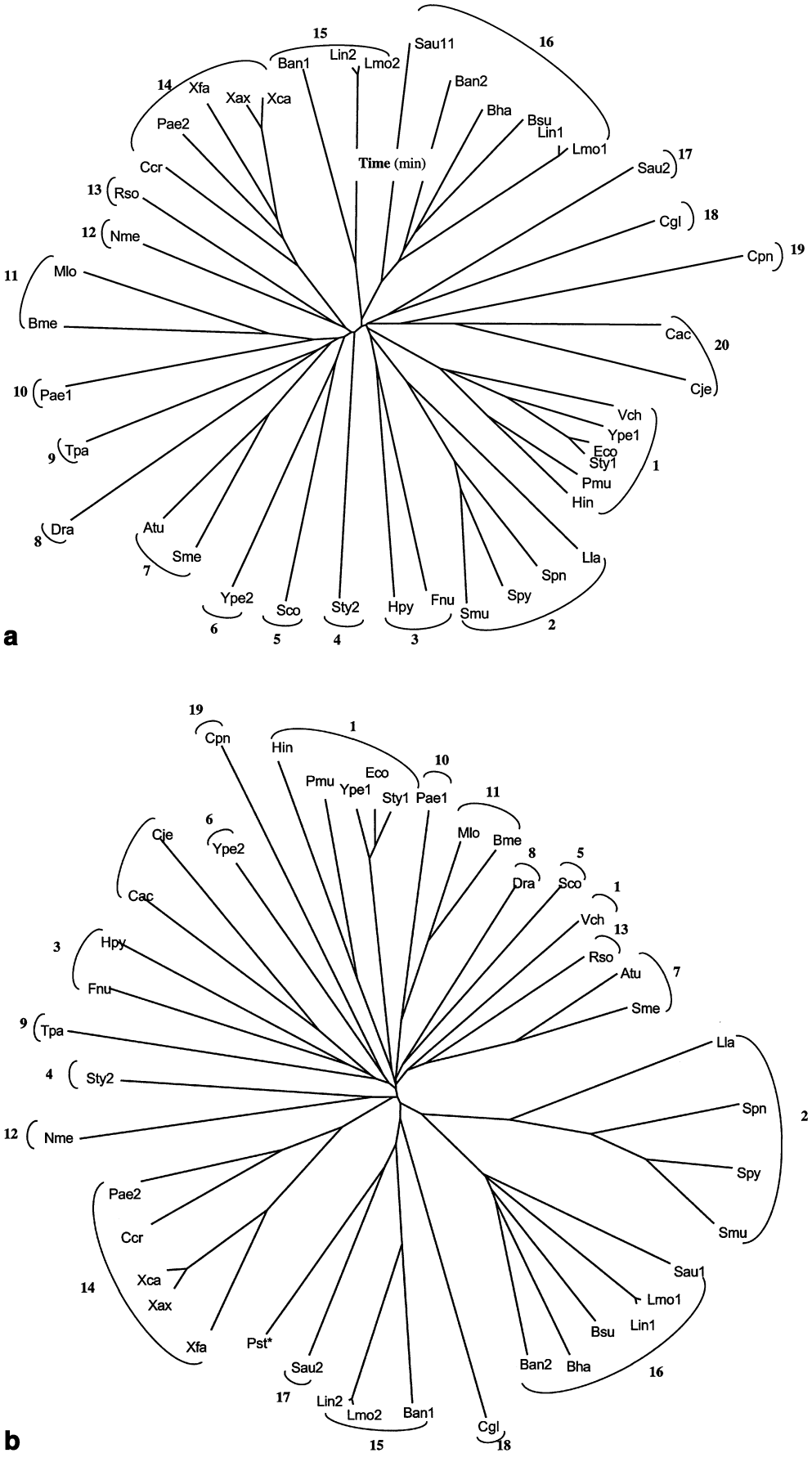
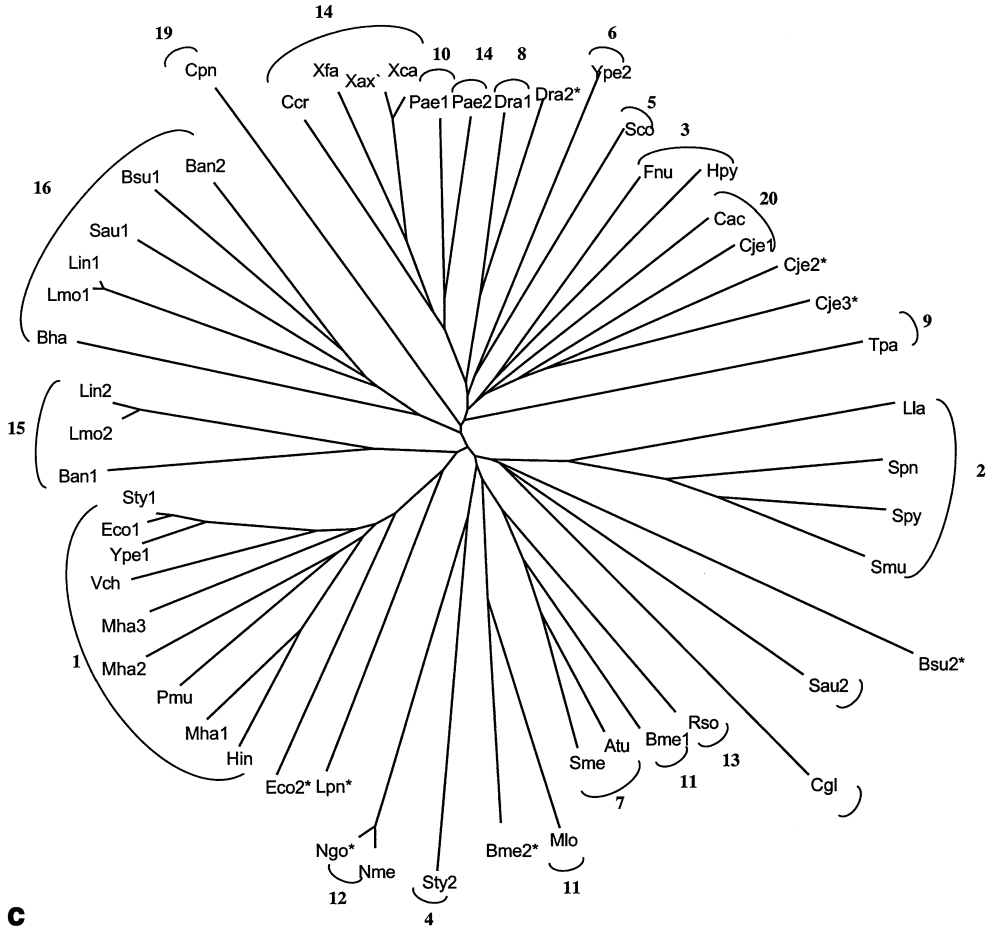


Fig. 4a–c Phylogenetic trees of the three constituents of the MUT family of ABC transporters: **a** ATP-binding cassette (ABC) constituents, **b** membrane constituents, **c** solute binding receptors. The multiple alignments were generated with the Clustal X program (Thompson et al. 1997) using the BLOSUM 62 scoring matrix. The trees are based on the neighbor-joining method and were drawn with the TreeView program (Page 1996). **c** * refers to a receptor that is not encoded with a gene for an ABC protein or a membrane protein

Fig. 4c



family (TC# 3.A.1.4). We have termed this family the methionine uptake transporter (MUT) family (TC# 3.A.1.23).

We identified the three constituent proteins that comprise MUT family permeases (Table 4). Most organisms having MUT family representation have only one homologue within this family, but a few have two. Organisms with two paralogues include *Salmonella typhimurium*, *Yersinia pestis*, and *Pseudomonas aeruginosa* (all γ-Proteobacteria) as well as *Bacillus anthracis*, *Staphylococcus aureus*, and two species of *Listeria* (all low G+C gram-positive bacteria). No organism has three or more paralogues within the MUT family. The database entry for the Hin membrane protein-encoding gene in *Haemophilus influenzae* was found to be truncated at both the N- and C-termini due to (1) an incorrect initiation codon assignment and (2) a frameshift mutation in the structural gene. This frameshift mutation has been shown to be authentic and not due to a sequencing error. Hin may therefore be encoded by a pseudogene. The reconstructed protein was 198 residues long. The database entries for the Vch and Bha receptors were also found to be erroneous due to incorrect initiation codon assignments (Table 4).

The phylogenetic trees shown in Fig. 4a, b, c for the ABC, membrane and receptor protein constituents of the transporters, respectively, were analyzed according to phylogenetic cluster. Cluster 1 includes the *E. coli* methionine uptake transporter, and only γ-proteobacterial proteins are represented. All of the members of cluster 1 have lipoproteins as receptors. The phylogenies of the proteins follow those of the organisms thereby suggesting orthology. One system, Vch, has its ABC protein and its receptor in cluster 1, but its membrane protein is loosely clustered with the α-proteobacterial proteins of clusters 7 and 13. This may possibly represent an unusual case of shuffling of constituents between systems (Kuan et al. 1995). Only the membrane protein behaved anomalously. However, using PAM distances with the Phylo_win program (Galtier et al. 1996), the membrane constituent Vch clustered together with other cluster 1 proteins. For all complete cluster 1 members, predicted MetJ boxes were found in the promoter regions of the operons as shown in Fig. 5.

Cluster 2 consists of proteins from streptococci and a closely related lactic acid bacterium. These proteins are also probably orthologous to each other.

Cluster 3 proteins from *Helicobacter pylori* and *Fusobacterium nucleatum* always cluster together in spite of the great phylogenetic distance between these two organisms. It seems possible that horizontal transfer had occurred. Therefore, the G+C contents and codon usages of the genes encoding the ABC transporters of these two organisms were compared, but no significant differences were observed relative to those of the genomes. This negative result does not eliminate the possibility of horizontal transfer.

a

Eco	AGACGTCT	GGATGCCT	TAACATCC	-67
Sty1	AGACGTCT	GGATGCCT	TAACATCC	-68
Ype1	AGCCGTCT	AGACGCCT	TAACATCC	-64
Vch	AGACGTCT	AGACGTAA	AAATATCT	-92
Hin	AGCAATCT	AGACATCT	-39	
Pmu	GGAAATCT	AGACGTCT	-40	

b

Eco	8/8	5/8	4/8
Sty1	8/8	5/8	4/8
Ype1	7/8	7/8	4/8
Vch	8/8	6/8	5/8
Hin	5/8	7/8	
Pmu	5/8	8/8	

Fig. 5a, b Conserved MetJ binding sites (Met boxes) found between *abc* and *yaeD* from enterobacteria. **a** An alignment of proposed Met boxes from *Escherichia coli* (Eco), *Salmonella typhimurium* (Sty1), *Yersinia pestis* (Ype1), *Vibrio cholerae* (Vch), *Haemophilus influenzae* (Hin), and *Pasteurella multocida* (Pmu). Protein abbreviations are as indicated in Table 4. The numbers at the *right* indicate the distance from the start codon of *abc*. **b** The number of matches to the consensus Met box sequence (AGACGTCT) is shown for each predicted Met box

Clusters 4–13 consist of only one or two proteins per cluster. In clusters 7 and 11, the two proteins in each cluster are from α-Proteobacteria, suggesting orthology. However, some of the distantly related proteins belong to closely related organisms (e.g., Sty2 and Ype2; Nme and Rso). This clearly suggests that sequence-divergent primordial proteins resulted from early gene duplication events and that these early paralogues were not transmitted to most of the organisms. Moreover, this conclusion is confirmed by the presence of several lipoproteic receptors from gram-negative organisms (Ccr, Nme, Tpa) although most are soluble (Table 4).

Cluster 14 consists of γ-proteobacterial proteins except for Ccr, which is from an α-proteobacterial species. The clustering patterns in Fig. 4a, c are consistent with orthology, but the clustering of Ccr in Fig. 4b is anomalous, and the same topology was obtained using PAM distances as a model of evolution.

Clusters 15 and 16 clearly represent two sequence-divergent groups of paralogues, both represented in *Bacillus anthracis* and two *Listeria* species. *Staphylococcus aureus* as well as *Bacillus subtilis* and *Bacillus halodurans* encode within their genomes only the second of these homologues (cluster 16). The clustering of Bha in Fig. 4c is anomalous, and this was also observed for the tree drawn using PAM distances. S-boxes have been predicted upstream of the operons encoding all transporters in clusters 15 and 16 with the exception of Bha (data not shown).

Cluster 19 includes a single chlamydial protein although three chlamydial species have been sequenced. In spite of their close phylogenetic relationship, the other two close relatives of *Chlamydophilia pneumoniae* must have lost the corresponding orthologues.

Finally, in cluster 20, Cac and Cje are always together (Fig. 4a, b, c). Because of the great distance between *Clostridium acetobutylicum* (a gram-positive bacterium) and *Campylobacter jejuni* (a gram-negative ε-proteobacterium), we suggest that horizontal transfer has occurred. However, this assumption can not be confirmed as both organisms have similar overall G+C content, and the G+C content and codon usage patterns of the MUT genes did not prove to be different from those of the complete genomes. An S-box was also predicted in the region of the operon encoding the Cac system.

With the exception of Lla, all receptors from gram-positive organisms were predicted to be lipoproteins. For Lla, a signal peptide with high similarity to those of Spn, Spy, and Smu was found. However, the conserved cysteine residue essential for lipid anchorage was replaced by a glycine residue. This might be explained by a sequencing error or by a single-point mutation since the codons for these two residues differ by only one nucleotide.

Discussion

The results presented here confirm and extend the molecular characterization of the MetD transporter identified earlier in the laboratories of Kadner and Ayling. *abc* and *yaeE* encode, respectively, the ATP-binding cassette (ABC) and membrane proteins of the MetD transporter. YaeC, the receptor encoded with the other components of the transporter, is the major binding protein for both L- and D-methionine. Inhibition studies revealed that the transporter is specific for both methionine isomers and their analogues including *N*-formyl methionine (Table 3; Kadner 1974, 1977). However, the YaeC-related paralogue NlpA (lipoprotein 28) may also exhibit the slight capacity to bind the two isomers of methionine. The *abc-yaeE-yaeC* genes were renamed *metNIQ* by Gal et al. (2002) and Merlin et al. (2002).

We confirmed Kadner's observation that L-methionine effectively competes for D-methionine transport while D-methionine does not strongly compete for L-methionine transport. This led Kadner to suggest that the *metD* locus encodes a component of at least two transport systems but may not encode the initial methionine-binding site (Kadner 1977), a suggestion reiterated by Merlin et al. (2002). However, the data presented here show that MetD is a single transport system with a single major methionine-binding receptor. The difference in inhibition observed for the two isomers is a consequence of the low K_m for L-methionine (75 nM) (Kadner 1974), which is 15-fold lower than that for D-methionine (1.2 μM) (Kadner 1977). We further demonstrate that MetJ is an effective repressor of *metD* expression.

Phylogenetic analyses led to the conclusion that MetD belongs to a new ABC family, which we named the methionine uptake transporter (MUT) family (TC #3.A.1.23). The MUT family is widely represented among bacterial subdivisions. All homologues of each of the three components are of a similar size, and all membrane proteins exhibit five putative transmembrane α-helical segments (TMSs). The overall topology of the trees presented does not fol-

low the phylogenies of the organisms, suggesting that several genetic duplications encoding these systems had occurred early during the evolutionary history of the family. The existence of several sequence-divergent primordial paralogues is likely to explain the topology of the phylogenetic trees. However, no more than two of these paralogues have been transmitted to any currently sequenced organism.

It is interesting to note that *Yersinia pestis* and *Salmonella typhimurium*, two close relatives of *E. coli*, possess two paralogous systems within the MUT family whereas only the receptor is present twice in *E. coli*. The possibility that the common ancestor of these three organisms had two paralogous systems, and that *E. coli* specifically lost the membrane and ABC proteins of one of them is highly unlikely. Ype2 and Sty2 do not cluster with Ype1 and Sty1 although the two receptors, Eco2 and Eco1, are found together in cluster 1. We therefore propose that *nlpA* (encoding Eco2) arose by a much more recent duplication of the *yaeC* gene precursor (encoding Eco1). This conclusion is corroborated by the observation that both Sty2 and Ype2 are not lipoproteins although Eco1 and Eco2 are.

Cluster 1 includes a group of γ-proteobacterial proteins conserved in all three trees. For this cluster, the phylogenies of the proteins agree with those of the organisms. Moreover, we identified MetJ binding sites in the promoter regions of all members of this cluster, and all cluster 1 receptors are predicted to be lipoproteins. These observations strongly suggest that all cluster 1 systems are orthologous methionine transporters, a suggestion confirmed by the presence of the functionally similar MetD transporter in *S. typhimurium* (Ayling and Bridgeland 1972; Ayling et al. 1979; Betteridge and Ayling 1975; Poland and Ayling 1984).

Several members of the MUT family from gram-positive bacteria are likely to transport sulfur compounds. Thus, almost all cluster 15 and 16 constituents (Figs. 4a-c) are encoded in operons regulated by S-boxes (Grundy and Henkin 1998). S-boxes are gram-positive bacterial consensus sequences for the transcriptional control of sulfur metabolism (Grundy and Henkin 1998). The presence of transporters likely to be involved in sulfur acquisition in positions throughout the phylogenetic tree suggests that this entire family may be involved in the transport of organic sulfur compounds. Further experimentation will be required to determine the substrate ranges of the transporters in this family.

The MUT family is of pharmaceutical interest since several members are required for bacterial pathogenicity. *sfbA* of *S. typhimurium* is found in a pathogenicity islet and is essential for infection in a mouse model (Pattery et al. 1999), although its specific contribution to pathogenicity is unknown. *sfbA* was predicted to encode the binding protein of an ABC transporter for iron because its expression was increased under iron-limiting conditions. Despite its regulation by iron, a Fur regulatory binding site was not found close to this operon (Panina et al. 2001). Based on the analyses presented here, we suggest that the Sfb transporter is involved in the uptake of an amino acid or sulfur compound during infection. The *H. influenzae hlpA* gene is not essential for infection, but a mutation in this gene results in reduced invasion in rats (Chanyangam et al. 1991). *Helicobacter pylori* also contains a MUT family transporter (AbcBCD) of unknown function that is required for maximal production of urease, essential for colonization (Hendricks and Mobley 1997). Since many *Yersinia pestis* strains require exogenous methionine (Brubaker 1972), methionine transporters may be drug targets for these organisms.

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