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MicroCorrespondence

A regulatory system hitherto found only in Gram-positive bacteria in a Gram-negative bacterium that grows only in co-culture with a *Bacillus* strain

Sir,

Many aminoacyl-tRNA synthetase and amino acid biosynthesis genes in Gram-positive bacteria are regulated by an unusual transcription termination control system, designated the T box system. This mechanism operates by controlling the activity of an intrinsic terminator located in the leader region of the mRNA, upstream of the start of the coding sequence; interaction of the leader RNA with the cognate uncharged tRNA stabilizes the formation of an antiterminator conformation of the leader RNA, which prevents formation of the competing terminator stem–loop, thereby driving readthrough transcription (Grundy and Henkin, 1993, *Cell* **74:** 475–482; Henkin, 1994, *Mol Microbiol* **13:** 381–387). The T box system can be recognized on the basis of a complex set of primary sequence and secondary structural elements conserved in the leader regions of the target genes, the most prominent of which is the T box itself, a 14 bp highly conserved sequence. There are 19 transcriptional units with these features in the *Bacillus subtilis* genome, and many others in a variety of Gram-positive organisms. To date, no example of a T box gene has been reported in a Gram-negative organism. This raises interesting questions about the evolutionary origin of this regulatory mechanism. We have now uncovered an example of an apparent T box gene in a very unusual Gram-negative organism, *Symbiobacterium thermophilum*.

S. thermophilum was isolated as a producer of a thermostable tryptophanase (Suzuki *et al*. 1988, *J Gen Microbiol* **134:** 2353–2362). A surprising observation was that the organism could be grown only in co-culture with a specific strain of *Bacillus*, which appeared to be related to *B. stearothermophilus* and *B. coagulans*. Neither cell-free extracts nor killed cells of the *Bacillus* strain could support independent growth of *S. thermophilum*. Co-culture was optimal under low aeration at 60°C; under these conditions, the *Bacillus* strain grew first, then began to lyse in late exponential phase, concomitant with growth of *S. thermophilum*. While the *Bacillus* strain could be grown in pure culture, *S. thermophilum* appears to be an obligate symbiont, but the requirements of the symbiosis are mysterious. The two strains could be separated by differential centrifugation, permitting isolation of *S. thermophilum* DNA and cloning of the tryptophanase genes (Hirahara *et al*. 1992, *Appl Environ Microbiol* **58:** 2633–2642). Recent data deposited in GenBank (accession number AB010832) by K. Ueda and T. Beppu include additional sequence information covering the region downstream of the tryptophanase genes. This region includes a coding sequence with high similarity to leucyl-tRNA synthetases, and we found that the region upstream of the coding sequence includes the elements characteristic of members of the T box family.

The putative *S. thermophilum leuS* leader region is shown in Fig. 1. This leader region contains all of the primary sequence and secondary structural features normally conserved in T box leaders (Rollins *et al*. 1997, *Mol Microbiol* **25:** 411–421), including an appropriately placed CUC specifier sequence, corresponding to the leucine codon used as the regulatory signal in the *B. subtilis leuS* and *ilv-leu* genes. The *S. thermophilum leuS* leader also contains a putative intrinsic transcriptional terminator, with the capacity to fold into an alternative antiterminator structure, as found in other T box genes. The terminator precedes the translation initiation region, and the leader region is preceded by a standard housekeeping-type promoter (TTGACT–17 bp spacer–GATAAT). Although the function of these putative regulatory elements has not yet been demonstrated, it appears likely that *S. thermophilum leuS* is in fact a member of the T box family, the first to be uncovered in a Gram-negative organism.

As *S. thermophilum* lives in such close proximity to a *Bacillus* strain and its lifestyle includes immersion in *Bacillus* cell lysate, it is possible that the *leuS* gene may have been acquired by horizontal gene transfer. The *leuS* coding region, although incomplete, exhibits highest similarity to *leuS* from *Synechocystis* (64% amino acid identity), followed by *Aquifex* (57%), *Haemophilus* (56%), *Escherichia coli* (54%), *Rickettsia* (53%), *Borrelia* (51%), human mitochondria (50%), *Chlamydia* (51%) and *B. subtilis* (47%). The relatively low similarity to the *Bacillus* gene suggests that the *leuS* coding region was unlikely to have been acquired recently from the *Bacillus* strain with which *S. thermophilum* lives. The G + C content of the *leuS* coding region is 65%, which matches that of *S. thermophilum*, while the G + C content of the *Bacillus* strain is 53% (Suzuki *et al*. 1988, *J Gen Microbiol* **134:** 2353–2362). It remains possible that the regulatory region alone was, in fact, passed from the *Bacillus* strain to the *S. thermophilum* genome. Alternatively, another T box gene located elsewhere in the genome may have been transferred, with the

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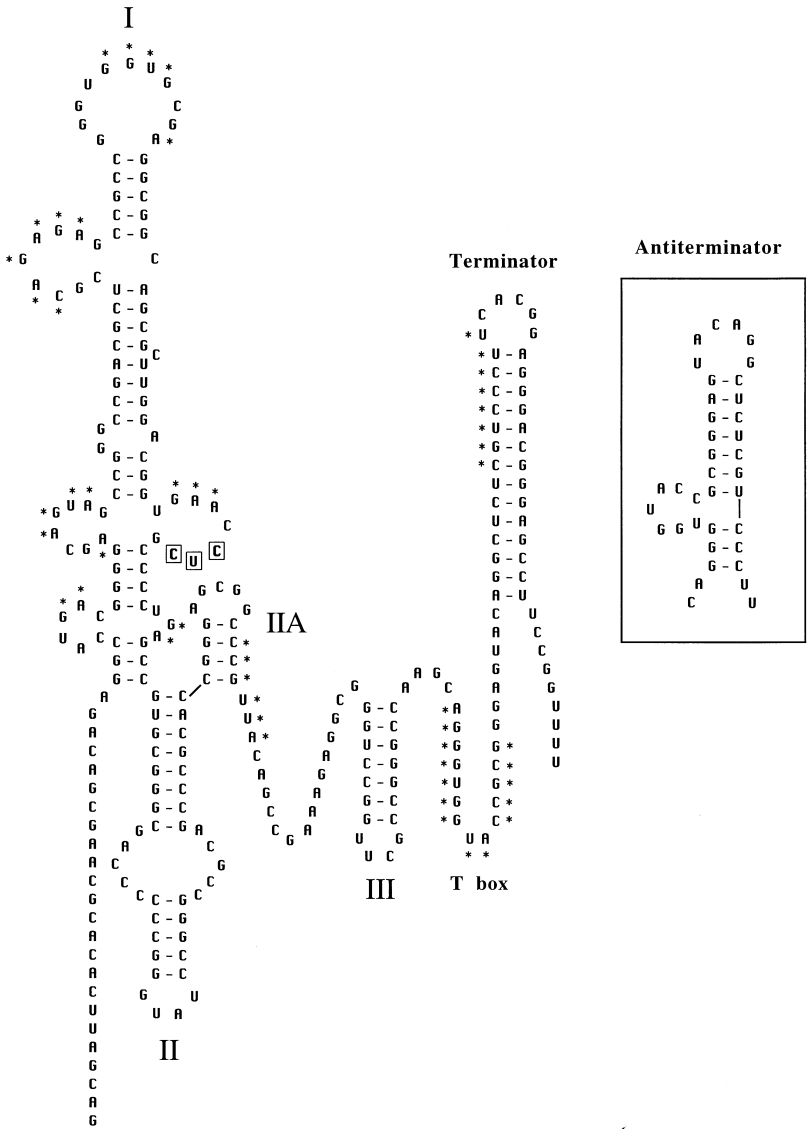

Fig 1. Structural model of the *S. thermophilum leuS* leader region. Stem–loop regions I, II, IIA and III and the T box precede the putative terminator. A portion of the T box can form an alternative pairing with a conserved sequence on the 5′ side of the terminator to form the antiterminator (shown on the right). The CUC specifier sequence, which corresponds to the CUC leucine codon, is boxed. Asterisks indicate primary sequence elements conserved in T box family members (Rollins *et al*. 1997, *Mol Microbiol* 25: 411–421).

regulatory region copied later to the *leuS* locus. A third possibility is that *S. thermophilum* is more closely related to a common ancestor of the Gram-positive lineage and retained the T box system when it was lost from other lineages. Further characterization of this unusual organism and its place on the evolutionary tree is likely to provide insight into the history of the T box system and the processes by which it arose.

Acknowledgements

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Note added in proof

Recent analysis of *S. thermophilum* 16S rRNA sequence indicates that by this criterion this organism should be placed at the outermost branch of Gram-positive bacterium, at the border of Gram-positive and Gram-negative organisms (T. Beppu and K. Ueda, personal communication). It therefore appears that despite its cell wall characteristics, *S. thermophilum* is more closely related to the Gram-positive group, and that the T box system is indeed widely distributed in the Gram-positive lineage.

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Possible origin of the *Legionella pneumophila* virulence genes and their relation to *Coxiella burnetii*

Sir,

Legionella pneumophila, the causative agent of legionnaires' disease, is a facultative intracellular pathogen that multiplies within and kills human macrophages, as well as free living amoebae. *L. pneumophila* evades the microbicidal defences of the phagocytes by maintaining the phagosome pH near neutrality and by preventing phagosome–lysosome fusion. Once inside the specialized phagosome, the bacteria multiply exponentially until the cell eventually lyses, releasing bacteria that can start new rounds of infection.

Two regions of genes required for human macrophage killing and intracellular multiplication have been discovered in *L. pneumophila* (Segal and Shuman, 1998, *Trends Microbiol* **6:** 253–255; Vogel and Isberg, 1999, *Curr Opinion Microbiol* **2:** 30–34). Region I contains seven genes (*icmV, W,* and *X*, and *dotA, B, C* and *D*), and region II contains 17 genes (*icmT, S, R, Q, P, O, N, M, L, K, E, G, C, D, J, B* and *F*). Most of these genes are also known to be required for intracellular growth in the protozoan host *Acanthamoeba castellanii* (Segal and Shuman, 1999, *Infect Immun* **67:** 2117–2124).

Five proteins encoded by the *icm/dot* genes (IcmP, IcmO, Icml, IcmE and DotB) have homology to conjugation-related proteins encoded from four different plasmids. *L. pneumophila* can conjugate RSF1010-related plasmids to bacteria in an *icm/dot*-dependent manner (Segal *et al*., 1998, *Proc Natl Acad Sci USA* **95:** 1669–1674; Vogel *et al*., 1998, *Science* **279:** 873–876), and *L. pneumophila* intracellular growth and human macrophage killing were shown to be inhibited by an active RSF1010 conjugation system (Segal and Shuman, 1998, *Mol Microbiol* **30:** 197–208).

New information that might indicate the origin of several *icm/dot* genes comes from the complete sequence of the *Shigella sonnei* colIb-P9 IncI plasmid (Sampei and Mizobuchi, 1999, accession no. AB021078). This 93 kb plasmid contains a large transfer region, which consists of 29 *tra* and *trb* genes. Fourteen of the *icm/dot* genes were found to be homologous to *tra/trb* genes (Fig. 1; Table 1). The *tra/trb* genes are located in one contiguous region on the colIb-P9 plasmid that contains homologues to *icm/dot* genes from both region I and region II. In addition, in four cases, the gene organization in both the *icm/dot* regions (*icmP–icmO*, *icmL–icmK*, *icmG–icmC–icmD–icmJ–icmB* and *dotD–dotC–dotB*) and the *tra/trb* region (*trbA–trbB–trbC*, *traM–traN*, *traP–traQ–traR–traS–traT–traU* and *traH–traI–traJ*) are similar (except *trbB* and *traS,* which do not have homologues in the *icm/dot* system). Of particular interest is DotA, which contains eight transmembrane (TM) segments, three of them before a large periplasmic domain and five after it (Roy and Isberg, 1997, *Infect Immun* **65:** 571–578). Its homologue TraY lacks the last two TM segments, the remaining TM segments being those regions most similar to DotA; the two large periplasmic domains share only a little similarity. Homologous proteins in the two systems are predicted to have the same bacterial cell location, and the extents of similarity and identity between all the Icm/Dot and Tra/Trb proteins were found to be similar (Table 1). These similarities indicate that *icm/dot* genes from both regions have the same origin and probably perform their function together.

Only one *icm/dot* protein (IcmE), which was shown to share sequence homology with a plasmid-encoded protein (TrbI from the IncP plasmid RK2), does not have a homologue in colIb-P9. This result indicates that whereas 14 of the *icm/dot* genes originated from the *tra* region of an IncI plasmid other *icm/dot* genes may have originated from other sources, such as IncP plasmids.

Additional, very important, sequence homologies were found with random sequences generated from the chromosome of the intracellular pathogen *Coxiella burnetii*,

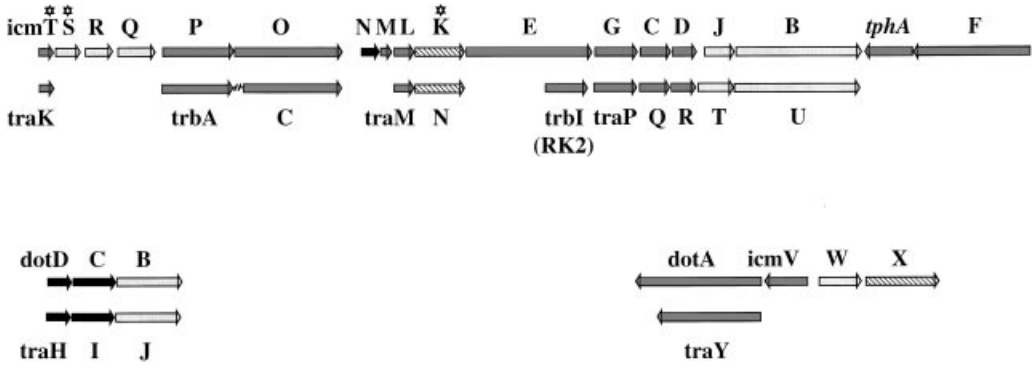

Fig. 1. Linkage map of the two *icm/dot* regions. Region I contains *icmVWX–dotABCD*, region II contains *icmTSRQPONMLKEGCDJB–tphA–icmF*. Coding regions are indicated by bold arrows. The homologues from the IncI plasmid colIb-P9 are indicated under the corresponding *icm/dot* gene, the *trbI* homologue is from the IncP plasmid RK2. Shading indicates the predicted location of the protein in the bacterial cell: black, lipoprotein;light grey, cytoplasm; dark grey, inner-membrane; hatched, periplasm. The star indicates *icm* genes that were found to have homologues in *C. burnetii*.

Table 1. Proteins homologous to the *Legionella pneumophila* Icm/Dot proteins.

Icm/Dot	ColIb-P9 (IncI)		RK2 (IncP)		*Coxiella burnetii*	
Protein/length	Protein/length	Identity/similarity	Protein/length	Identity/similarity	Protein/length	Identity/similarity
IcmT/86	TraK/96	24.5/32.3			Orf85/85	47.1/60.9
IcmS/114					Orf/67[a]	50.7/61.2
IcmP/376	TrbA/402	20.0/33.3				
IcmO/783	TrbC/763	23.8/35.5				
IcmI/212	TraM/230	26.0/40.1				
IcmK/360	TraN/327	26.9/38.9			Orf159/145[a]	68.3/76.5
IcmE/1048			TrbI/462	22.5/34.4[b]		
IcmG/269	TraP/234	20.4/33.9				
IcmC/194	TraQ/175	19.7/31.2				
IcmD/132	TraR/134	19.0/31.1				
IcmJ/208	TraT/266	18.7/29.8[b]				
IcmB/1009	TraU/1014	26.2/39.6				
DotA/1048	TraY/745	22.2/36.0[c]				
DotB/377	TraJ/382	30.3/44.2				
DotC/303	TraI/272	25.2/35.7				
DotD/163	TraH/152	25.1/36.5				

a. Sequence is available only for part of the Orf.
b. Similarity was calculated only for the C-terminal part of the proteins.
c. Similarity between DotA and TraY was found only in the three TM segments before and three after the big periplasmic domain, see text.

the causative agent of Q-fever (Willems *et al*., 1998, *J Bacteriol* **180:** 3816–3822). *C. burnetii* and *L. pneumophila* are evolutionarily closely related (Weisburg *et al*., 1989, *J Bacteriol* **171:** 4202–4206), but phagosomes containing these bacteria have opposite intracellular fates. The hallmark of *L. pneumophila* is its ability to prevent phagosome–lysosome fusion (Horwitz, 1983, *J Exp Med* **158:** 2108–2126; Horwitz and Maxfield, 1984, *J Cell Biol* **99:** 1936–1943), whereas *C. burnetii* resides in phagosomes that rapidly fuse with lysosomes (Hackstadt and Williams, 1981, *Proc Natl Acad Sci USA* **78:** 3240–3244; Heinzen *et al*., 1999, *Trends Microbiol* **7:** 149–154). Homologues of three *icm* genes (*icmT*, *icmS* and *icmK*) were found in *C. burnetii*, and the *icmT* and *icmS* homologues are located next to each other in both bacteria. The degree of similarity between the *C. burnetii* homologues to *icm/dot* genes is much higher than that between the *icm/dot* and *tra/trb* genes (Table 1); thus, the *icm/dot* genes of *L. pneumophila* and *C. burnetii* are more closely related to one another then to the *tra* system of colIb-P9.

We propose that *L. pneumophila* and *C. burnetii* both incorporated an IncI–plasmid conjugation system to export effectors into host cells. Because the intracellular fates of the two organisms are so different, it is likely that the molecules that are not shared among them and the IncI plasmids correspond to the effectors that determine this fundamental difference in the intracellular fate.

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Is FatP a long-chain fatty acid transporter?

Sir,

The mechanisms of fatty acid transport across cell membranes have long been a controversial topic of investigation (Kleinfeld *et al*., 1998, *Biochemistry* **37:** 8011–8019). In a recent publication, Hirsch, Stahl and Lodish (1998, *Proc Natl Acad Sci USA* **95:** 8625–8629) reported the identification of a family of fatty acid transporters conserved from mycobacteria to man. Previously, Faergeman *et al*. (1997, *J Biol Chem* **272:** 8531–8538) had disrupted a gene (*FATP* or *FAT1*) in *Saccharomyces cerevisiae* that appeared to facilitate long-chain fatty acid uptake in this unicellular eukaryote. The yeast FATP protein proved to be homologous to the mammalian proteins studied by Hirsch *et al*. (1998, *Proc Natl Acad Sci USA* **95:** 8625–8629). In the paper by Hirsch *et al*., representatives of these putative plasma membrane proteins were shown to increase long-chain fatty acid uptake when expressed in cultured mammalian cells, and a homologue from *Mycobacterium tuberculosis* was shown to increase the rate of long-chain fatty acid uptake when expressed in *Escherichia coli*. The identified FATP homologues were claimed to show significant homology to other proteins only in a

small stretch of amino acids, previously identified as an AMP-binding motif found in a multitude of other proteins such as acyl CoA ligases (synthases) and gramicidin S synthetase component II. The relevance of this motif to fatty acid transport was not clear to the authors. Several FATP isoforms were identified in mammals such as mice and humans.

One of the FATPs identified, mouse FATP2, proved to be the murine homologue of a rat gene previously identified by Uchiyama *et al*. (1996, *J Biol Chem* **271:** 30360–30365) as a very long-chain acyl CoA synthase (VLACS). Hirsch *et al*. (1998 *Proc Natl Acad Sci USA* **95:** 8625–8629) claimed that this protein 'shares no sequence homology with other cloned acyl-CoA synthases.' They explained the result of Uchiyama *et al*. (1996, *J Biol Chem* **271:** 30360–30365) by suggesting that the rat homologue of mouse FATP2 increased acyl CoA synthase activity when expressed in CHO cells by assuming that it increased uptake of fatty acids from the medium, and that this in turn stimulated transcription of the VLACS gene (Suzuki *et al*., 1990, *J Biol Chem* **265:** 8681–8685). Recently, Watkins *et al*. (1998, *J Biol Chem* **273:** 18210–18219) studied expression of the FATP gene of *Saccharomyces cerevisiae* and provided evidence that it codes for a peroxisomal VLACS.

Finally, Hirsch *et al*. (1998 *Proc Natl Acad Sci USA* **95:** 8625–8629) claimed that the *E. coli* genome does not encode a FATP homologue, that another *E. coli* protein, FadL, mediates uptake of fatty acids from the medium as noted for example by Black (1991, *J Bacteriol* **173:** 435–442), and that the mycobacterial FATP clustered on the phylogenetic tree with the mouse and human FATP5 isoforms. The tree published by Hirsch *et al*. (1998 *Proc Natl Acad Sci USA* **95:** 8625–8629) was based on a 48 amino acyl residue segment of the aligned proteins.

While conducting sequence analyses of the FATP protein family members identified by Hirsch *et al*., we made the following observations.

1 The putative FATP transporters are in fact homologous to dozens of long-chain fatty acyl CoA synthases (ligases) found ubiquitously in all living organisms including *E. coli* (see Table 1 for representative examples). We refer to this superfamily as the acyl CoA synthase (ACS) superfamily (Fulda *et al*., 1994, *Mol Gen Genet* **242:** 241–249).

2 FatPs are also homologous to other acyl CoA ligases including carnitine/crotonobetaine CoA synthase of *E. coli* and 4-coumarate CoA synthases of plants. A few of these functionally distinct, but mechanistically similar, enzymes of the ACS superfamily are included in Table 1.

3 The FatP proteins recognized by Hirsch *et al*. (1998 *Proc Natl Acad Sci USA* **95:** 8625–8629) comprise one of several coherent phylogenetic families within the ACS superfamily. These proteins thus exhibit distinctive motifs and characteristics that are not shared by all members of the ACS superfamily.

4 FadL is an outer membrane porin-like protein in *E. coli*, not a cytoplasmic membrane transporter. Therefore, although it stimulates uptake of fatty acids into the cell, it cannot account for transport of fatty acids across the cytoplasmic membrane.

5 Topological analyses of the various FatP proteins identified by Hirsch *et al*. (1998 *Proc Natl Acad Sci USA* **95:** 8625–8629) using the TOP PRED II program (von Heijne, 1992, *J Mol Biol* **225:** 487–494) revealed that these proteins exhibit between zero and three hydrophobic regions that might correspond to transmembrane α-helical spanners (TMSs). A topology of 1–3 TMSs per polypeptide chain is a characteristic of some channel-like proteins, but not of secondary carriers (see our website: http://www-biology.ucsd.edu/~msaier/transport/titlepage.html).

A partial multiple alignment of the sequences of representative members of the putative long-chain fatty acid

Table 1. Representative proteins of the acyl-coenzyme A synthase (ACS) superfamily[a]

Abbreviation	Name or description	Organism	Comparison Score (SD)[b]	Accession no.[c]
FATP1 *Mmu*	Fatty acid transport protein 1	*Mus musculus*	–	Q60714
VLCS *Rno*	Very long-chain acyl-CoA synthetase (ligase)	*Rattus norvegicus*	56	P97524
FAT1 *Sce*	Probable long-chain fatty acid transport protein	*Saccharomyces cerevisiae*	30	P38225
CAIC *Eco*	Probable crotonobetaine/carnitine-CoA ligase	*Escherichia coli*	19	P31552
4CL2 *Stu*	4-Coumarate-CoA ligase 2	*Solanum tuberosum*	11	P31685
LCFA *Eco*	Long-chain fatty acid-CoA synthase (ligase)	*Escherichia coli*	10	P29212
4CL1 *Pcr*	4-Coumarate-CoA ligase 1	*Petroselinum crispum*	10	P14912

a. Proteins included are members of the putative FATP family (FATP1 *Mmu*; FAT1 *Sce*), an established fatty acyl CoA ligase (LCFA *Eco*), a postulated fatty acyl CoA ligase (VLCS *Rno*) or established acyl CoA ligases specific for other substrates (CAIC *Eco*, 4 CL2 *Stu*, and 4 CL1 *Pcr*).
b. The comparison scores presented were obtained by comparing FATP1 *Mmu* with the full-length sequence of the indicated protein using the GAP program (Devereux *et al*., 1984, *Nucleic Acids Res* **12:** 387–395) with 500 random shuffles and a gap penalty of 5. A value of nine standard deviations (SD) (corresponding to a probability that the degree of sequence similarity observed occurred by chance is less than 10^{-19}) is considered sufficient to establish homology for a protein domain of 60 residues or more in length (Dayhoff *et al*., 1983, *Methods Enzymol* **91:** 524–545; Saier, 1994, *Microbiol Rev* **58:** 71–93).
c. Accession numbers in the SWISSPROT database are provided.

FATP1 *Mmu*	485	D S A Y L S **G D** V L V M **D** E L G Y M Y F R **D R** S G D T F R W R G E N **V** S T T **E** V E A V	527
VLCS *Rno*	458	D V Y F N S **G D** L L M I **D** R E N F I Y F H **D R** V G D T F R W K G E N **V** A T T **E** V A D I	500
FAT1 *Sce*	501	D A W Y R C **G D** L L K A **D** E Y G L W Y F L **D R** M G D T F R W K S E N **V** S T T **E** V E D Q	543
CAIC *Eco*	402	D G W L H T **G D** T G Y R **D** E E D F F Y F V **D R** R C N M I K R G G E N **V** S C V **E** L E N I	444
4CL2 *Stu*	416	E G W L H T **G D** I G F I **D** D D D E L F I V **D R** L K E L I K Y K G F Q **V** A P A **E** L E A L	458
LCFA *Eco*	431	N G W L H T **G D** I A V M **D** E E G F L R I V **D R** K K D M I L V S G F N **V** Y P N **E** I E D V	473
4CL1 *Pcr*	414	E G W L H T **G D** I G F I **D** D D D E L F I V **D R** L K E I I K Y K G F Q **V** A P A **E** L E A L	456
Consensus		D G W L H T **G D** – – – – **D** E – – – – Y F V **D R** – – D – I – – K G E N **V** – – – **E** – E – –	

Fig. 1. Partial multiple alignment of seven representative protein members of the acyl-coenzyme A synthase (ACS) superfamily. Proteins included and their abbreviations are shown in Table 1. Residue number is indicated at the beginning and end of each aligned sequence. Fully conserved residues are indicated in bold , and residues occurring in a majority of the sequences at any one position are presented in the consensus sequence below the alignment. The TREE program (Feng and Doolittle, 1990, *Methods Enzymol* 183: 375–387) was used to generate the multiple alignment.

transporters of mouse and yeast with those of various established members of the ACS superfamily is presented in Fig. 1. The region presented does not correspond to that which includes the conserved AMP-binding motif, but does encompass the coenzyme A binding motif. These proteins exhibit highly significant sequence similarity throughout their entire lengths except for their extreme N- and C-termini (data not shown). The region shown reveals that many residues are fully conserved among the functionally divergent proteins selected for study, and a majority of the residues in this region appear in the consensus sequence. The comparison scores presented in Table 1 (in which each protein listed is compared with the full-length mouse FatP1) establish that all of these proteins are homologous (see footnote to the table).

On the basis of these observations, we suggest that the FATP homologues identified by Hirsch *et al*. comprise a coherent cluster within the ubiquitous ACS superfamily. Whether or not any of these proteins actually catalyses transport of fatty acids across the cytoplasmic membrane remains an open question. Increased uptake rates observed by Hirsch *et al*. could have been due to cytoplasmic trapping of the fatty acid when the coenzyme A derivative is formed in the cytoplasm. This notion is in agreement with the recently published results of Watkins *et al*. (1998, *J Biol Chem* **273:** 18210–18219) showing that the yeast FatP homologue is probably a very long-chain fatty acyl-CoA synthase. If the FatP proteins are in fact transporters, we suggest that the N-terminal transmembrane moiety functions as a simple channel, and the remainder of the protein catalyses the thioacylation reaction. Fatty acid transport would then precede ligation with coenzyme A in a normal sequence of metabolic events leading to fatty acid degradation. It is interesting to note that over a quarter of a century ago, Frerman and Bennett (1973, *Arch Biochem Biophys* **159:** 434–443) suggested that long-chain fatty acids are transported by acyl CoA synthases using a group translocation mechanism [Saier, 1998, *Advances in Microbial Physiology,* Poole, R.K., (ed.), pp. 81–136. Academic Press: San Diego]. Further experimentation will be required to define the biochemical function(s) of the interesting group of FatP proteins.

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