FEBS Letters 549 (2003) 26–30 FEBS 27478

The diverse bacterial origins of the *Arabidopsis* polyamine biosynthetic pathway

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Received 13 June 2003; revised 30 June 2003; accepted 30 June 2003

First published online 16 July 2003

Edited by Ulf-Ingo Flügge

Abstract We functionally identified the last remaining step in the plant polyamine biosynthetic pathway by expressing an *Arabidopsis thaliana* agmatine iminohydrolase cDNA in yeast. Inspection of the whole pathway suggests that the arginine decarboxylase, agmatine iminohydrolase, *N*-carbamoylputrescine amidohydrolase route to putrescine in plants was inherited from the cyanobacterial ancestor of the chloroplast. However, the rest of the pathway including ornithine decarboxylase and spermidine synthase was probably inherited from bacterial genes present in the original host cell, common ancestor of plants and animals, that acquired the cyanobacterial endosymbiont. An exception is *S*-adenosylmethionine decarboxylase, which may represent a eukaryote-specific enzyme form.

Key words: Polyamine; Agmatine iminohydrolase; Cyanobacteria; Chloroplast targeting sequence; *Arabidopsis*

1. Introduction

Polyamines are essential and ancient small polycation metabolites required for cell growth and proliferation and are found in most if not all bacterial, fungal, animal and plant cells (Fig. 1) [1]. Animal and fungal cells use ornithine decarboxylase to synthesise putrescine (1,4-diaminobutane) directly from ornithine whereas in plant and some bacterial cells there is an additional indirect route to putrescine from arginine. The plant pathway to putrescine from arginine consists of arginine decarboxylase (ADC) [2–4], agmatine iminohydrolase (AIH) [5] (also known as agmatine deiminase), and *N*-carbamoylputrescine amidohydrolase (NCPAH) [6]. In some bacteria agmatine is converted directly to putrescine by agmatine ureohydrolase [7] (also known as agmatinase). Spermidine synthase (SpdSyn) [8–10] synthesises spermidine from putrescine by the addition of an aminopropyl group acquired from decarboxylated *S*-adenosylmethionine formed by *S*-adenosylmethionine decarboxylase (AdoMetDC) [11,12]. Similarly, spermine is formed from spermidine by spermine synthase (SpmSyn) [13] through addition of another aminopropyl group. Polyamine biosynthesis in the model flowering plant *Arabidopsis thaliana* is uniquely dependent on the arginine route to putrescine due to loss of its ODC gene [14]. Mutant alleles of a SpmSyn gene in *A. thaliana* confer severe growth and cell expansion defects [13]. All *A. thaliana* genes involved in polyamine biosynthesis have been identified except for AIH (Table 1). In this paper we demonstrate by heterologous expression in the yeast *Saccharomyces cerevisiae* that an *A. thaliana* cDNA bearing sequence similarity to a *Pseudomonas aeruginosa* PAO1 AIH gene [15] is a functional plant AIH, thereby completing the molecular description of the plant polyamine biosynthetic pathway. As this paper was submitted, an independent report of the identification of the same *A. thaliana* AIH appeared [31]. The report by Janowitz et al. [31] describes the biochemical properties of recombinant AIH whereas our objective was to describe the evolution of the complete polyamine biosynthetic pathway in plants.

Oat ADC was previously demonstrated to be localised in chloroplasts [16]. Our sequence analysis of the *A. thaliana* AIH and NCPAH proteins indicates that they do not possess chloroplast targeting peptides whereas *A. thaliana* ADC1 and ADC2 do. Furthermore, the sequenced genomes from cyanobacteria thought to be most closely related to the original cyanobacterial endosymbiont that formed the chloroplast, such as *Nostoc punctiforme* [17], do not contain AIH or NCPAH homologues but do contain an agmatinase gene. However, ADC, AIH and NCPAH are found in the cyanobacterium *Thermosynechococcus elongatus* BP-1. The unicellular alga *Chlamydomonas reinhardtii* contains genomic copies of AIH and NCPAH but no corresponding expressed sequence tags (EST) and seems to have lost the gene for ADC. Plant and animal ODC and SpdSyn have homologues in eubacteria and in the case of SpdSyn in Archaebacteria but except for a possible case of gene capture by *Shewanella oneidensis*, the eukaryotic AdoMetDC may be the only eukaryotic-specific polyamine biosynthesis gene.

2. Materials and methods

2.1. cDNA sequencing and plasmid construction

An *A. thaliana* EST encoding the putative AIH was obtained from the Arabidopsis Biological Resource Centre (http://www.arabidopsis.org/home.html). The cDNA insert was contained in the λZiplox plasmid with a 5′ *Sal*I site and a 3′ *Not*I site. Both DNA strands of the cDNA insert were sequenced and the open reading frame (ORF) of the AIH was polymerase chain reaction (PCR) amplified from the λZiplox plasmid with *Pfu* polymerase (Stratagene) using the 5′ primer (5′-CCACGCGGCCGCAAACATCG-3′) and the 3′ primer

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0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00756-7

(5′-TCAGCGGCCGCGATTCATAAATCC-3′). The PCR product contained *Not*I sites at each end of the AIH ORF. After digestion with *Not*I the PCR product was inserted into the *Not*I site of the pFL61 multicopy yeast expression vector [18] for constitutive expression.

2.2. Expression in yeast

The pFL61 plasmid containing the AIH ORF was transformed into the wild-type *S. cerevisiae* strain 2602 (MATα, *ura3-52*, *his6*, *leu2*) using the method of Elble [19]. For polyamine analysis, transformed yeast cells containing pFL61:AIH were grown in SD minimal medium minus uracil and with or without 100 μM agmatine (Sigma), at 30°C to an OD_{600} of 0.5.

2.3. High performance liquid chromatography (HPLC) analysis of N-carbamoylputrescine and polyamines

N-Carbamoylputrescine was synthesised according to the method of Sri Venugopal and Adiga [20]. Crystals were washed with ether and the products were checked using ninhydrin after separation by thin layer chromatography on Whatman PE SIL G/UV plates in a solvent of *n*-butanol/acetic acid/water (60/15/25, v/v/v). RFs were 0.60, 0.31 and 0.14 (dicarbamoylputrescine, *N*-carbamoylputrescine and putrescine respectively). Dicarbamoylputrescine, saved from earlier stages of purification and which does not contain a primary amine, was detected with 4-dimethylaminocinnamaldehyde.

For analysis of polyamines in yeast, cells were pelleted by centrifugation and washed with sterile, distilled water and repelleted. The washed cell pellet was resuspended in 5% trichloroacetic acid (TCA) and vortexed five times with an equal volume of glass beads (425–600 μm, Sigma). Disrupted cells were incubated on ice for 2 h and cell debris was removed by centrifugation for 2 min at 13 000×*g* and the supernatant stored at −20°C. Polyamines were derivatised overnight with dansyl chloride and analysed by HPLC as described by Hanfrey et al. [14], using a Luna 5μ C18 (2) 150×4.6 mm column (Phenomenex, Macclesfield, UK). Post-column derivatisation of the TCA-extracted polyamines with *o*-phthaldehyde was performed using the method of Seiler and Knödgen [21] with the modifications described by Wallace et al. [22], using the same column. The authenticity of our synthesised *N*-carbamoylputrescine was validated by HPLC comparison of an *N*-carbamoylputrescine standard kindly supplied by Dr. Markus Piotrowski, Ruhr Universität Bochum, Germany [6]. Other polyamine standards were obtained from Sigma.

2.4. Comparative sequence analysis

PSI-BLAST [23] was used to search the archaebacterial and eubacterial genome protein databases using the NCBI BLAST server (http://ncbi.nlm.nih.gov/blast/). The completed genome of *C. reinhardtii* was searched using TBLASTN at the *Chlamydomonas* Resource Center (http://www.biology.duke.edu/chlamy_genome). Presence or absence of chloroplast targeting sequences were assessed using the ChloroP 1.1. program [24] at the Technical University of Denmark (http://www.cbs.dtu.dk/services/ChloroP-1.1/). Alignment of the *A. thaliana* AIH with the *P. aeruginosa* PAO1 agmatine deiminase and alignment of the *A. thaliana* AdoMetDC with the human and *S. oneidensis* proteins was performed using the multiple sequence alignment program of DNAMAN sequence analysis software.

Table 1
The *A. thaliana* polyamine biosynthetic pathway

Gene	Locus	Protein size (a.a.)
ADC1 (SPE1)	At2g16500	702
ADC2 (SPE2)	Atg34710	711
ODC absent	–	–
AIH	At5g08170	383
NCPAH	At2g27450	299
AdoMetDC1	At3g02470	366
AdoMetDC2	At5g15950	362
SpdSyn1	At1g23820	334
SpdSyn2	At1g70310	340
SpdSyn3/SpmSyn	At5g53120	359
SpmSyn (ACL5)	At5g19530	339

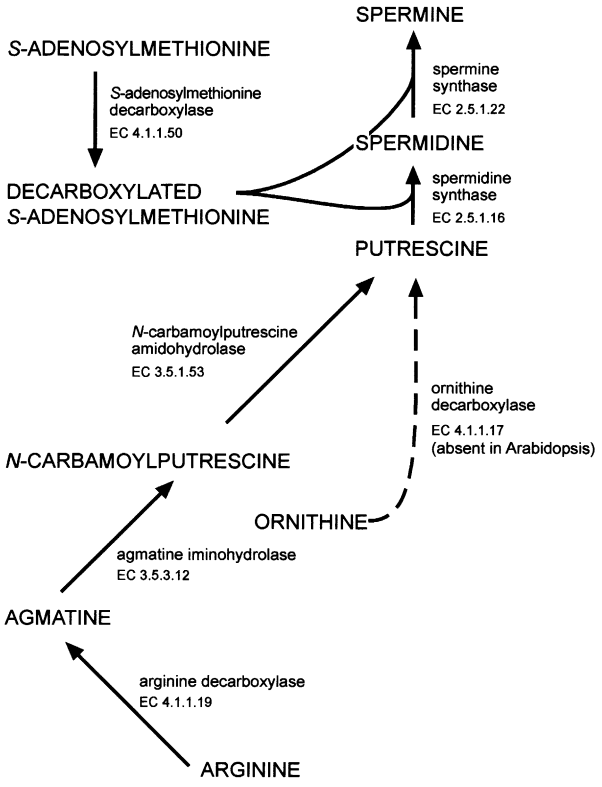

Fig. 1. The plant polyamine biosynthetic pathway. From arginine to putrescine, the pathway is specific to plants and some bacteria whereas ornithine to spermine is common to all eukaryotes.

3. Results and discussion

3.1. Functional identification of the Arabidopsis AIH

Using the amino acid sequence of the recently discovered agmatine deiminase gene from *P. aeruginosa* PAO1 [15], we detected by TBLASTN an *A. thaliana* EST with amino acid sequence similarity to the N-terminus of the bacterial protein. The *A. thaliana* EST (clone 106O4T7) was fully sequenced (1471 bp) and found to be identical to a full length cDNA of unknown function (acc. no. AK118589 corresponding to At5g08170) although the cDNA we sequenced was 21 bp shorter at the 5′ end. The sequenced EST possessed an ORF of 383 amino acids and there was an in-frame UGA stop codon three bases upstream of the initiating AUG. Amino acid sequence alignment of the *P. aeruginosa* PAO1 protein and the putative *A. thaliana* AIH is shown in Fig. 2. PSI-BLAST searches indicated highest sequence identity between *A. thaliana* AIH and the *P. aeruginosa* PAO1 enzyme (56%) followed by a *Listeria monocytogenes* ORF (54%, acc. no. CAC98253) and an ORF from *Pseudomonas syringae* pv. syringae B728a (acc. no. ZP_00128043).

To assess whether the *A. thaliana* cDNA did indeed encode a protein with AIH activity, we expressed the cDNA in *S. cerevisiae* strain 2602. Yeast does not possess AIH activity and so does not accumulate *N*-carbamoylputrescine. The *A. thaliana* cDNA was introduced into yeast and constitutively expressed in the multicopy vector pFL61. Fig. 3 shows the *o*-phthaldehyde-derivatised, post-column HPLC detection of *N*-carbamoylputrescine accumulation in yeast cells expressing the *A. thaliana* cDNA, dependent on exogenous supply of 100 μM agmatine. *N*-carbamoylputrescine accumulated to a

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At ..WDSHAQTWIGWPERQDNWRHNALPAQRVFADVAKAISK 38
Pa .............PERpDNWRnggkPAQaaFAaVAKAIar 27
                  *** ****     ***   ** *****
At FEPVTVCASPAQWENARKQLP.EDIRVVEMSMNDSWFRDS 77
Pa FEPVTVCASagQyENARarLddgnIRVVEiSsdDaWvRDt 67
   *********  * ****  *    ***** *  * * **
At GPTFIVRKRPVKLSSLNRNIAGIDWNFNAWGGANDGCYND 117
Pa GPTFviddk........gdvrGvDWgFNAWGGfegGlYfp 99
   ****                  * ** ******   * *
At WSHDLLVSRKILALERIPRFQHS.MILEGGSIHVDGEGTC 156
Pa WqrDdqVaRKILeiERraRyrtddfvLEGGSIHVDGEGTl 139
   *  *  * ****  **  *        ************
At LVTEECLLNKNRNPHMSKEQIEEELKKYLGVQSFIWLPRG 196
Pa itTEECLLNhNRNPHlSqaeIErtLrdYLaVeSiIWLPnG 179
     ******* ***** *   **  *  ** * * **** *
At LYGDEDTNGHIDNMCCFARPGVVLLSWTDDETDPQYERSV 236
Pa LYnD.eTdGHvDNfCCyARPGeVLLaWTDDqdDPnYlRcq 218
   ** *  * ** ** ** **** *** **** ** * *
At EALSVLSNSIDARGRKIQVIKLYIPEPLYMTEEESSGITQ 276
Pa aALrVLeeSrDAkGRKlvVhKmpIPgPLYaTqEEcdGvdi 258
    ** **  * ** ***  * *  ** *** * **  *
At DGEAIPRLAGTRLAASYVNFYIANGGIIAPQFGDPIRDKE 316
Pa vegsqPRdpsiRLAgSYVNFlIvNGGIIAPsFdDPk.DaE 297
       **    *** ***** * ******* * **  * *
At AIRVLSDTFPHHSVVGIENAREIVLAGGNIHCITQQQPAE 356
Pa AraiLqrvFPeHeVVmvp.gREIlLgGGNIHCITQQQPAp 336
   *   *   ** * **     *** * *************
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Fig. 2. Alignment of the amino acid sequences encoded by the *A. thaliana* (At) putative AIH cDNA and the *P. aeruginosa* PAO1 (Pa) agmatine deiminase gene (acc. no. AGG03681). Identical residues are indicated by asterisks.

level of 54 pmol/10^6 cells ± 10.9 pmol/10^6 cells (experiment performed on triplicate cultures) but was not detectable in yeast transformed with the empty vector nor in yeast transformed with the *A. thaliana* cDNA but without exogenous agmatine. Accumulation of *N*-carbamoylputrescine due to

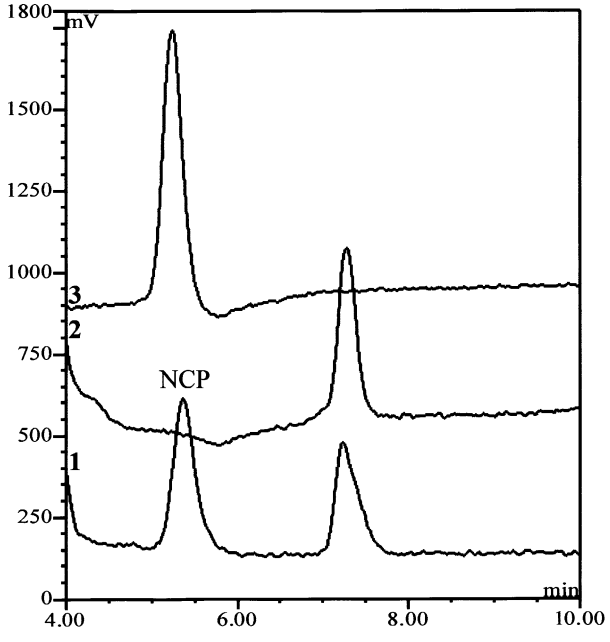

Fig. 3. Post-column HPLC detection of *N*-carbamoylputrescine in yeast cells expressing the *A. thaliana* AIH cDNA. Yeast cells expressing the AIH cDNA in the (1) presence; (2) absence of 100 μM exogenously supplied agmatine; (3) *N*-carbamoylputrescine standard. *N*-Carbamoylputrescine was detected by derivatisation with *o*-phthaldehyde.

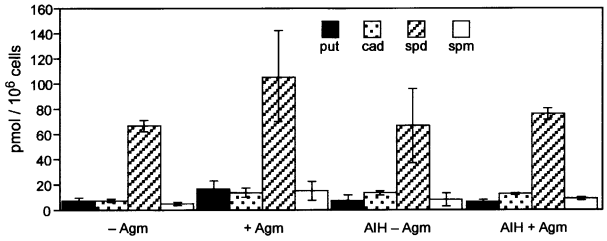

Fig. 4. Pre-column HPLC detection of polyamines in yeast cells expressing the *A. thaliana* AIH cDNA. Cells were grown in the presence (+) or absence (−) of 100 μM exogenously supplied agmatine and with or without the AIH cDNA in the pFL61 constitutive, multcopy yeast expression plasmid. put, putrescine; cad, cadaverine; spd, spermidine; spm, spermine.

expression of the *A. thaliana* cDNA in cells supplied with agmatine was approximately half the level of spermidine accumulation in those cells (Fig. 4). The presence of *N*-carbamoylputrescine did not affect the accumulation of the normal yeast polyamines. As yeast cells do not contain the arginine route to putrescine and do not normally accumulate *N*-carbamoylputrescine, we conclude that the *A. thaliana* cDNA encodes a functional AIH.

3.2. Bacterial origins of the plant polyamine biosynthetic pathway

The three enzymes required for putrescine biosynthesis from arginine in plants: ADC, AIH and NCPAH, are clearly of bacterial origin. The closest match to the *A. thaliana* ADC1 protein is with the cyanobacterium *N. punctiforme* (acc. no. ZP_00111883), which exhibits 40% identity. The other closest matches are all with cyanobacteria: *Nostoc* sp. PCC 7120, *T. elongatus* BP-1, *Synechococcus* sp. WH8102, *Trichodesium erythaeum* IMS101, *Prochlorococcus marinus* and *Synechocystis* sp. PCC 6803. Amongst sequenced cyanobacterial genomes, the overall complement of genes of *N. punctiforme* is thought to be most similar to the endosymbiont ancestor of plant chloroplasts [17]. In contrast to ADC, both AIH and NCPAH are absent from all the above cyanobacterial genomes except for *T. elongatus* BP-1, which contains ADC, AIH and NCPAH, as determined by TBLASTN searches. In all the cyanobacterial genomes that lack AIH and NCPAH genes, BLASTP searches detect an agmatinase sequence. The highest PSI-BLAST amino acid sequence similarities to the *A. thaliana* NCPAH are found in the plant pathogenic bacterium *P. syringae* (64% identity) and *P. aeruginosa* PAO1 (62% identity), indicating a high degree of conservation of the NCPAH primary structure. Several other photosynthetic bacteria possess the ADC/AIH/NCPAH enzymes including the green sulphur bacterium *Chlorobium tepidum* TLS. It was previously noted by Nakada and Itoh [25] that part of the peptidylarginine deiminase of *Porphyromonas gingivalis* exhibits some homology to AIH. Although *P. ginivalis* is associated with human gum infections, its genome is most closely related to the photosynthetic *C. tepidum* TLS [26] and it is therefore reasonable to assume that the peptidylarginine deiminase evolved from a pre-existing AIH.

One intriguing aspect of the *A. thaliana* ADC/AIH/NCPAH pathway is the subcellular location of the enzymes. It was previously shown that the oat ADC is localised in chloroplasts [16] and indeed the chloroplast targeting sequence prediction program ChloroP indicates a potential targeting sequence of

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At ..LGLRALTKSQLDEILTPAACTIVSSLSNDQLDSYVLSE 38
Hs ...dLRtiprSewDilLkdvqCsIiSvtktDkqeaYVLSE 37
So .............ttlvacAnaeIlSkiSNkdcDaYlLSE 27
                            * *         * ***
At SSFFVYPYKVIIKTCGTTKLLLSIPPLLKLAGELS..LSV 76
Hs SSmFVskrrfIlKTCGTTlLLkalvPLLKLArdySgfdSi 77
So SSlFVwdnKilIlTCGnstLieaacyfinslGseh....i 63
   ** **        ***   *
At KSVKYTRGSFLCPGGQPFPHRSFSEEVSVLDGHFTQLGLN 116
Hs qSffYsRknFmkPshQgyPHRnFqEEiefLnaiFp....N 113
So aalcYqRk...neyqaqlqsttFaEdiaqLrtlis..... 95
      * *               * *    *
At SVATLMGNDDETKKWHVYAASAQDSSNCNNNVYTLEMCMT 156
Hs gagycMGrmn.sdcWylYtldfpeSrvisqpdqTLEilMs 152
So gqAfrvGhlDahhhyvfcAAgvp....itqvgtrLElmMy 131
         *                          **  *
At GLDREKAAVFYKDEADKTGSMTDNSGIRKNLPKSEICDFE 196
Hs eLDpavmdqFYmkdgvtakdvTreSGIRdliPgSvIdatm 192
So hir.gelAeylnDpvqteqgivhrlkltqlfPefqldmhc 170
                                  *
At FEPCGYSMNSIEGD.AISTNHVTPEDG...FSYASFEAVG 232
Hs FnPCGYSMNgmksDgtywTiHiTPEpe...FSYvSFEtn. 228
So FkPtGfSlNaIqGs.hyfTlHiTPsaGqgehSYvSFEtn. 208
   * * * * *          * * **        ** ***
At YDFNTLDLS.QLVTRVLSCFEPKQFSVAVHSSVGANS... 268
Hs ..lsqtsyd.dLirkVvevFkPgkFvttlfvnqsskcrtv 265
So ..lNlaaypypvVaqliSlFaPsswdlvsvnqlistag.. 244
                        * *
```

Fig. 5. Alignment of the amino acid sequences encoded by the *A. thaliana* (At; acc. no. AAB17665) and human (Hs; acc. no. P17707) AdoMetDC cDNAs and the *S. oneidensis* (So; acc. no. NP_717479) AdoMetDC-like gene. Identical residues are indicated by asterisks.

52 amino acids for the *A. thaliana* ADC1. In contrast, neither AIH or NCPAH from *A. thaliana* are predicted to contain a chloroplast targeting sequence. This would seem to suggest that agmatine must be transported from the chloroplast to the cytoplasm. It has been predicted that more than half the proteins of cyanobacterial origin in *A. thaliana* are not targeted to the chloroplast [17]. One potential advantage of a cytoplasmic pool of agmatine could be the formation of *N*-hydroxycinnamoyl conjugates of agmatine, which in some species act as precursors of defense-related compounds [27]. The completed genome of the unicellular alga *C. reinhardtii* suggests that this organism once possessed the ADC/AIH/NCPAH pathway but is now dependent solely on the ODC route to putrescine. The genome sequence does not contain ADC nor are there any corresponding ESTs and although there are genomic copies of AIH and NCPAH, there are no cognate ESTs for these two genes. In contrast there are over 50 ESTs for ODC. The presence of AIH and NCPAH gene sequences in the genome of *C. reinhardtii* nevertheless confirms an ancient origin of the plant arginine polyamine pathway. Although AIH and NCPAH are not present in most of the sequenced cyanobacterial genomes, the presence of AIH and NCPAH sequences in an alga and ADC, AIH and NCPAH in *T. elongatus* BP-1 suggests a cyanobacterial origin of the plant arginine polyamine pathway. Nakada et al. [15] detected the presence of AIH and NCPAH genes in the chlorella virus PBCV-1 where there is no recognisable ADC gene but there is an ORF with homology to ODC. It is of interest therefore that the ODC of this virus behaves more like ADC [28].

Plant ODC belongs to group IV of the PLP-dependent decarboxylases, which includes eukaryotic ODC, PLP-dependent biosynthetic plant and bacterial ADC and diaminopimelate decarboxylases [29]. It was previously thought that the eukaryotic ODC was specific to eukaryotes. However, a eukaryotic-like ODC has been found recently in the strictly anaerobic Gram-negative bacterium *Selenomonas ruminantium* [30] and PSI-BLAST searches reveal that the eukaryotic-like ODC is present in several other eubacterial species such as *Thermotoga maritima* (acc. no. NP_229669) and *Brucella melitensis* (acc. no. NP_542111). It seems likely therefore that the plant, fungal and animal ODC is thus directly descended from a bacterial ODC progenitor. SpdSyn is found in archaebacteria, eubacteria and all eukaryotes and SpmSyn has undoubtedly evolved from SpdSyn. The one possible exception to the bacterial origin of the plant polyamine biosynthetic pathway is AdoMetDC. There is an ORF in the facultatively aerobic Gram-negative bacterium *S. oneidensis* (acc. no. NP-717479) that exhibits similarity at the amino acid level to the human and *A. thaliana* AdoMetDC1 (Fig. 5) but the single instance of a eukaryotic-like AdoMetDC in one bacterial species suggests that this gene was more probably captured by horizontal transfer from a eukaryotic source.

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