Lehrstuhl für Genetik der Technischen Universität München

The Pantothenic Acid and Coenzyme A Pathway in Plants and Archaea: Analysis of Metabolite Levels in *Arabidopsis thaliana* and Characterization of Archaeal Enzymes for the Synthesis of 4´-Phosphopantetheine

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Zusammenfassung

CoenzymA ist ein grundlegender Vorläufer für verschiedene synthetische und abbauende Reaktionen im Intermediär- und Energiemetabolismus. Es wird in fünf Schritten aus Pantothenat (PA), das auch als Vitamin B_5 bekannt ist, synthetisiert. Pflanzen, Pilze und Bakterien synthetisieren PA aus β-Alanin und Pantoat in einer Reaktion, die vom Enzym Pantothenat-Synthetase (PS) katalysiert wird, während Menschen PA mit der Nahrung aufnehmen müssen. Kürzlich wurden alle biosynthetischen Gene für die CoenzymA (CoA)-Synthese aus Pflanzen, Pilzen und Menschen identifiziert und kloniert. Man nimmt an, dass die Phosphorylierung von PA durch das Enzym Pantothenat-Kinase (PanK) der entscheidende regulierende Schritt in der CoA-Biosynthese ist. Trotz der Kenntnisse über die CoA-Synthese sind verschiedene Aspekte ihres Metabolismus noch unklar. Das Ziel dieser Arbeit war es die Regulation von PA in Pflanzen und die Synthese von PA in Archäen zu erforschen.

Mit dem Ziel eine Korrelation zwischen PA und CoA zu finden wurden beide Metabolite in sich entwickelnden *Arabidopsis thaliana*-Samen zwischen 4 und 21 Tagen nach dem Blühen (Days after flowering, DAF) quantifiziert. Die wichtigste Beobachtung war, dass die PA-Mengen während der gesamten Samenentwicklung die CoA-Mengen übertrafen. PA-Konzentrationen variierten zwischen 25 und 250 nmol /g Trockenmasse (dry weight, DW) während die Gesamtmenge aus CoA und AcetylCoA immer weniger als 25 nmol/g DW betrug. Daher ist PA nicht der limitierende Faktor der CoA-Biosynthese. Die Akkumulierung von PA stimmt damit überein, dass dessen Phosphorylierung den geschwindigkeitsbestimmenden Schritt im CoA-Metabolismus darstellt, mit PanK als wichtigstem Regulator des intrazellulären CoA-Gehalts. Das Verhältnis zwischen PA und CoA veränderte sich im Laufe der Samenentwicklung um mehr als das zehnfache. Das zeigt, dass die Synthese von PA nicht direkt vom CoA-Gehalt kontrolliert wird. Zu Beginn der Samenentwicklung war der PA-Gehalt sehr hoch mit einem Maximum am 5. DAF. Danach verringerte sich der PA-Gehalt und erreichte bei 16 DAF sein Minimum. Im Gegensatz dazu war der

Gesamtgehalt an CoA und Acetyl-CoA in jungen Samen sehr niedrig und erhöhte sich bis zu einem Maximum am 12. DAF. In der weiteren Entwicklung war der CoA-Gehalt stets niedrig oder nicht detektierbar. Dies steht im Einklang mit PA als Vorstufe von CoA und kann durch die Annahme, dass ein erheblicher Anteil des neu synthetisierten CoA für die Herstellung von Acylcarrier-Protein und langkettigen Acyl-CoA-Derivaten in der Fettsäurebiosynthese verwendet wird, erklärt werden.

Im zweiten Teil dieser Arbeit wurde die PA- und CoA-Biosynthese in Archäen erforscht. Die konservierten CoA biosynthetischen Gene wurden zuerst in bereits komplett sequenzierten Genomen analysiert. Dies zeigte, dass viele Archäen-Genome keine Homologen zu bakteriellen oder eukaryotischen Genen für die Synthese von 4'-Phosphopantethein enthalten. Via phylogenetischer Analyse und „Chromosomal Proximity" wurden Kandidaten für PS-und PanK-Enzyme identifiziert. Putative PS und PanK lagen im Cluster orthologer Gene (COG) 1701 bzw. 1829. Für die klonierten Gene des COG 1701 aus *M. mazei* und *M. jannaschii* konnte gezeigt werden, dass sie in der Lage sind einen auxotrophen *E. coli*-Stamm mit fehlender PA-Synthese zu komplementieren. Die COG 1701 Gene aus *M. mazei* wurden in *E.coli* überexprimiert und die putative *M. mazei*-PS (MmPS) als His-tag-Protein aufgereinigt. Dieses Enzym war in der Lage PA aus β-Alanin, Pantoat und ATP zu synthetisieren. PA wurde mittels Dünnschichtchromatographie und Umwandlung in 4'-Phospho-PA durch PanK aus *E. coli* identifiziert. Die HPLC Analyse von Adenosinnucleotiden zeigte, dass durch die MmPS-Reaktion ATP hydrolysiert und ADP gebildet wurde. Die MmPS katalysierte Reaktion unterscheidet sich also von der Reaktion der *E. coli* PS, welche eine AMP-bildende Synthetase ist. MmPS ist in allen Archäen konserviert, unterscheidet sich aber grundlegend von der bakteriellen PS.

In dieser Arbeit wird zum erstenmal eine PS aus Archäen beschrieben und eine frühere Hypothese bestätigt, die basierend auf vergleichender Genomik von einer unabhängigen Evolution der PA-Biosynthese in Archäen und Bakterien ausgeht.

Summary

Coenzyme A is a fundamental cofactor for many synthetic and degradative reactions in intermediary and energy metabolism. It is synthesized in five steps from pantothenate (PA) also known as vitamin B_5. Plants, fungi, and bacteria synthesize PA from β-alanine and pantoate in a reaction catalyzed by the enzyme panothenate synthetase (PS), while humans have to assume it with the diet. Recently, coenzyme A (CoA) biosynthetic genes in bacteria, plants and humans have all been identified and cloned. Phosphorylation of PA, catalyzed by the enzyme pantothenate kinase (PanK), is thought to be the key regulatory step in CoA biosynthesis. Despite our knowledge on CoA synthesis, some aspects of its metabolism are as yet ill understood. The aim of this work was to investigate the regulation of PA in plants and the synthesis of PA in archaea.

In an attempt to find a correlation between PA and CoA, both metabolites were quantified in developing seeds of *Arabidopsis thaliana* between 4 and 21 days after flowering (DAF). The most important observation was that PA levels exceeded CoA levels throughout seed development. The PA content varied between 25 and 250 nmol/g dry weight (DW) while the sum of CoA and acetyl-CoA was always below 25 nmol/g DW. Thus, PA is not rate-limiting for CoA biosynthesis. Accumulation of PA is in agreement with its phosphorylation as the rate-limiting step in the CoA pathway, *i.e.* with PanK as the major regulator of intracellular CoA levels. The ratio between PA and CoA levels varies more than 10-fold during seed development, indicating that the synthesis of PA is not directly controlled by CoA levels. PA levels were very high at the beginning of seed development with the maximum observed at 5 DAF. Thereafter, PA levels decreased and reached a minimum at 16 DAF. In contrast, the combined CoA and acetyl-CoA levels were very low for young seeds and then reached the maximum at 12 DAF. Towards maturation, CoA levels were very low or undetectable. This is consistent with PA as precursor of CoA and can be plausibly explained by assuming that a substantial amount of newly synthesized CoA is used in fatty acid

biosynthesis for the production of acyl carrier protein and long chain acyl-CoA species.

In the second part of this work, the archaeal biosynthesis of PA and CoA was investigated. Conserved CoA biosynthetic genes were previously analyzed in ompletely sequenced genomes. This showed that many archaeal genomes contain no homologues to bacterial or eukaryotic genes for the synthesis of 4'-phospho-PA. Using phylogenetic profiling and chromosomal proximity, candidate genes for archaeal PS and PanK enzymes were identified in this group. Putative archaeal PS and PanK genes are contained in the Clusters of Orthologous Genes (COG) 1701 and 1829, respectively.

The cloned COG1701 genes from *M. mazei* and *M. jannaschii* were demonstrated to functionally complement an auxotrophic *E. coli* strain deficient in the synthesis of PA. The COG1701 gene from *M. mazei* was then overexpressed in *E. coli*, and the encoded putative *M. mazei* PS (MmPS) was purified as a His-tag protein. This enzyme was able to synthesize PA from β-alanine, pantoate and ATP. The identity of PA was confirmed by thin-layer chromatography (TLC) and by transformation into 4'-phospho-PA using purified PanK from *E. coli*. HPLC analysis of adenosine nucleotides showed that ATP was hydrolyzed and ADP was formed in the course of the MmPS reaction. Thus, the MmPS-catalyzed reaction differs from the one catalyzed by *E. coli* PS, which is an AMP-forming synthetase. MmPS is conserved in all archaea (COG1701) but completely unrelated to bacterial PS. This is the first report of an archaeal PS and confirms an earlier hypothesis that proposed, based on comparative genomics, independent evolution of PA biosynthesis in archaea and bacteria.

Table of contents

Abbreviations

a.a.	amino acid
AcCoA	AcetylCoA
ACP	Acyl carrier protein
ADC	Aspartate decarboxylase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	Adenosine triphosphate
bp	base pairs
c.l.	Continuous light
CMP	cytidine monophosphate
CoA	coenzymeA
CoASH	Free CoA
CS	Citrate synthase
CTP	cytidine triphosphate
CV	column volume
d/l	12h dark/ 12h light regime
DPCK	dephosphocoenzymeA kinase
dPCoA	dephosphoCoA
DTT	Dithiotreitol
DW	Dry weight
FMN	Flavomononucleotide
$FMNH_2$	Flavomononucleotide reduced form
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GTP	Guanidine triphosphate
HEPES	2-[4-(2-hidroxyethyl)-1-piperazinyl] ethan sulfonate
HPLC	High performance liquid chromatography
IPTG	isopropylthio-β-D-galactoside
IS	Internal Standard

KPHMT	ketopantoatehydroxy methyltransferase
KPR	Ketopantoate reductase
M.P.	Mobile Phase
MDH	Malate dehydrogenase
NAD	Nicotinamide adenin dinucleotide
NADH	dehydronicotinamide adenin denucleotide
NADP	nicotinamide adenin dinucleotide phosphate
NADPH	dehydronicotinamide-adenin-dinucleotide phosphate
PA	Pantothenate
PanK	Pantothenate kinase
Pi	inorganic phosphate
PPAT	phosphopantetheine adenylyltransferase
PPCDC	phosphopantothenoylcysteine decarboxylase
PPCS	Phosphopantothenoylcysteine synthetase
PPi	inorganic pyrophosphate
PS	Pantothenate synthetase
PTA	Phosphotransacetylase
THF	tetrahydrofurane
TRIS	tris(hydroxymethyl)-aminomethane
β-Ala	β-alanine

1. Introduction to CoA metabolism

1.1 Metabolism of pantothenate and Coenzyme A

Vitamins are small organic molecules that can be found in all organisms and are indispensable for their growth and functionality. Vitamins (nowadays circa 20 are known) are classified as liposoluble or hydrosoluble: the first group consists of vitamins A (retinol), E (tocopherol), D (ergosterol) and K (fitonadione), and the second group consists of vitamins C (ascorbic acid) and B. Vitamins of different groups are involved in different metabolic pathways acting as reducing agents (vitamin C), precursors of coenzymes (vitamins B), blood coagulation factors (vitamin K). The great importance of vitamins is due to the fact that animals cannot synthesize them but must include them in their diet. For this reason, studies on vitamins and their metabolism are of primary importance, and thus, much is known about their pathways.

Pantothenic acid (pantothenate, PA), also known as Vitamin B_5, is the fundamental precursor of Coenzyme A (CoA) and of acyl carrier protein (ACP). These compounds are involved in numerous metabolic pathways, like the fatty acid biosynthesis, the tricarboxylic acid cycle and other reactions of intermediary metabolism (Abiko, 1975). In the structures of both of these compounds it is possible to recognize the pantothenate molecule, like shown in figure 1-1.

Because of the great importance of CoA for all the living organisms, its biosynthesis was subject to many studies since long ago. In the year 1958, G.M. Brown clarified this pathway and indicated PA as starting molecule. This is converted in CoA in five steps (Brown, 1959).

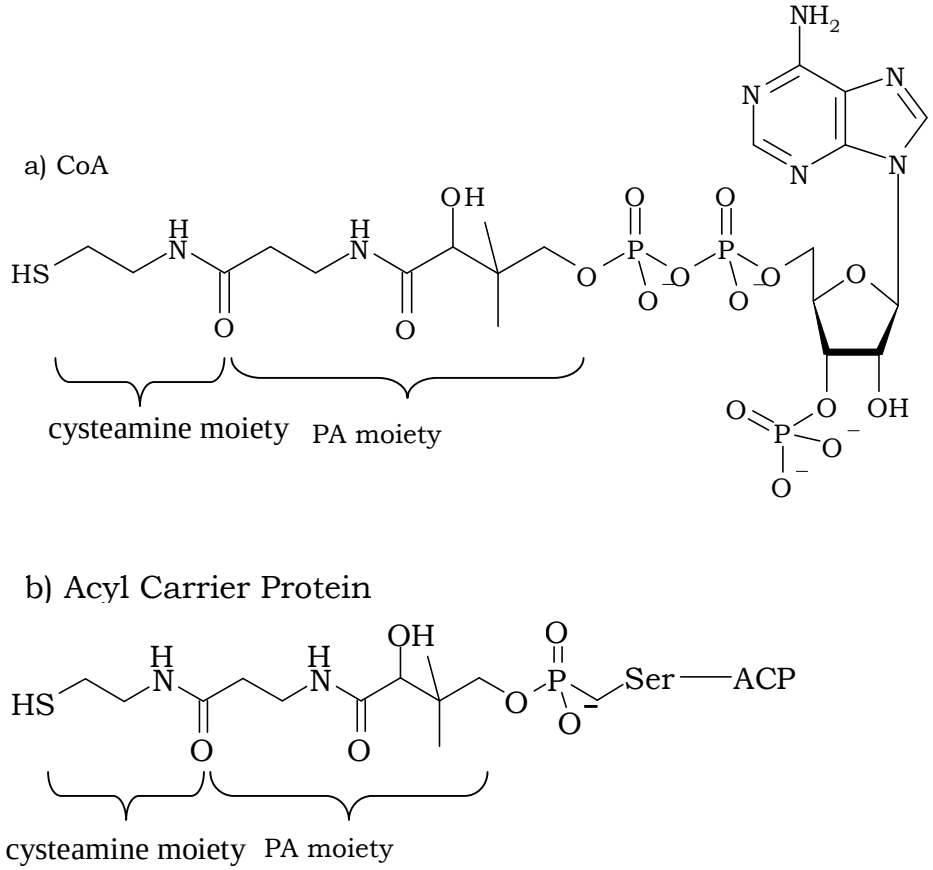

Figure 1-1: Molecular structure of CoA and ACP.

The biosynthetic pathway that leads to CoA starting from PA is present in all known organisms and for this reason is called “the universal CoA pathway”. Instead, PA is synthesized only from plants, fungi and bacteria, via a pathway that starts from α-ketoisovalerate and β-alanine (β-Ala) and, in four steps, leads to PA. These other reactions are also known as the “pantothenate pathway”.

Nowadays the CoA synthesis in bacteria is completely clarified and all the enzymes involved have been isolated and characterized (see Chapter 1.2). The scheme of CoA pathway in *Escherichia coli* is shown in figure 1-2:

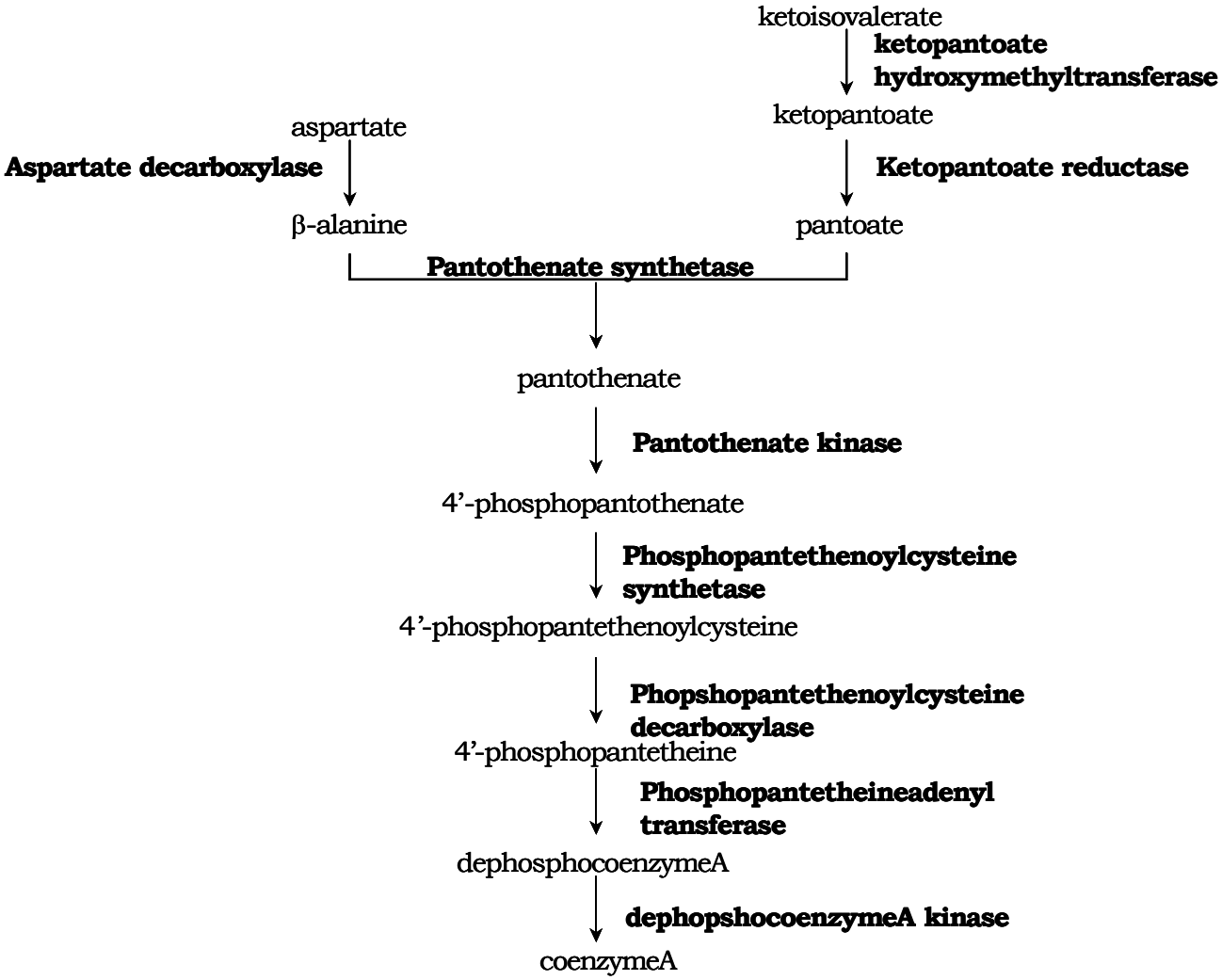

Figure 1-2: CoA and PA pathway in *E. coli*

1.2 The enzymes involved in PA and CoA biosynthesis

1.2.1 Ketopantoatehydroxy methyltransferase (KPHMT; EC 2.1.2.11)

KPHMT is the first enzyme in the pantothenate pathway. It catalyses the reaction that leads to α-ketopantoate from α-ketoisovalerate as shown in figure 1-3:

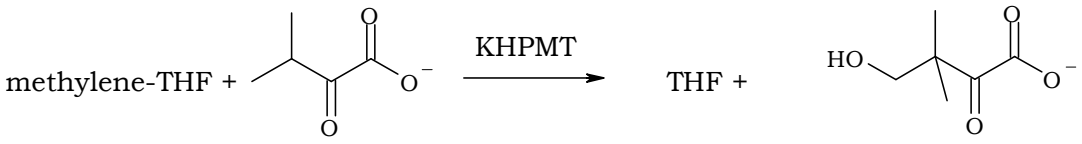

Figure 1-3: Reaction catalysed from the KPHMT

The net resulting reaction is an aldolic condensation between the ketoisovalerate and a formaldehyde-equivalent that is "carried" by the THF.

The mechanism of the reaction is not completely clear but it is known that the addition of the hydroxymethyl group proceeds with inversion of configuration at the C-3 of the ketoisovalerate (Aberhart, 1984). Furthermore it has been shown that Mg^{2+} has activating effects (Powers, 1976). A possible mechanism of the reaction is shown in figure 1-4:

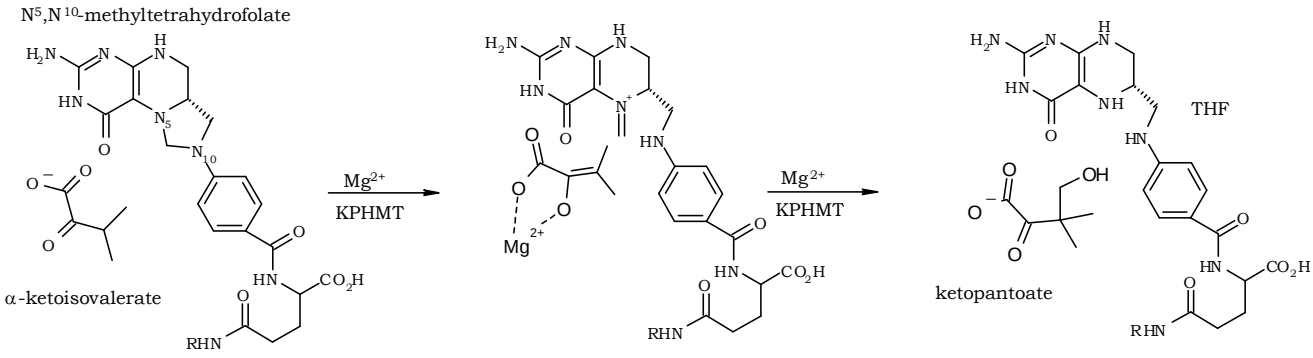

Figure 1-4: proposed mechanism for the conversion of α-ketoisovalerate and methylene-tetrahydrofolate into ketopantoate and tetrahydrofolate catalysed by KPHMT (Re-drawn from von Delft *et al.*, 2003).

This enzyme was purified from *E. coli* for the first time in 1975 by Teller et *al.* (1975) and they found a pH optimum below or equal to 8.0. By assaying the reverse reaction, they found that the apparent K_M for ketopantoate was 0.15 mM, a value that was then confirmed in a later work in which the K_M for α-ketoisovalerate and THF were also determined to be 0.18 mM and 1.1 mM respectively (Powers, 1976).

Jones and co-workers isolated the *panB* gene from *E. coli*, encoding the KPHMT, by functional complementation of a *panB* mutant strain. The gene is 792 base pairs (bp) long encoding a protein of 264 amino acids (a.a.) (Jones

et al., 1993). More recent studies on this gene showed that it is closely linked with the two genes *panC* and *panD* that encode respectively the pantothenate synthetase and the aspartate decarboxylase, the other two enzymes in this metabolic pathway.

E. coli KPHMT has a monomeric mass of 28 kDa and it appears to be a pentamer of dimers with the dimer being the functional unit (von Delft *et al*, 2003).

This enzyme, the first one in the pantothenate pathway, shows weak inhibition by the downstream products pantoate, PA and CoA. All this three effectors exhibit negative feedback, decreasing V_{max}, increasing K_M and enhancing cooperativity for ketoisovalerate (Powers *et al.*, 1976).

In regards to plants, by using BLAST with *E. coli panB* gene as query, two genes encoding putative KPHMTs were identified in the *A. thaliana* genome. The first gene, *panB1* (At2g46110), is located on chromosome II and encodes a polypeptide of 347 a.a.; the second one, *panB2* gene (At3g1530) is situated on chromosome III and codes for a polypeptide of 354 a.a. (Ottenhof *et al.*, 2004). Both of these two enzymes are targeted into the mitochondria and it seems that the transit peptides for targeting of the enzymes are in the N-terminus extension respect to the *E. coli* enzyme. The occurrence of two *panB* genes is likely to be the result of genome duplication (Coxon *et al.*, 2005). An alignment of KPHMT protein sequences from *Saccharomyces cerevisiae* (Genschel *et al.,* 1999) and *Aspergillus nidulans* (Kurtov *et al.*, 1999) shows a high degree of conservation, in particular in two regions. The first one is involved in binding Mg^{2+}, the second one is important because a deletion of the glycine, results in a PA auxotroph.

Considering the archaea, KPHMT is one of the few enzyme involved in PA biosynthesis that present homologues in eight non-methanogenic species. This gene was never cloned or studied.

1.2.2 Ketopantoate reductase (KPR; EC 1.1.1.169)

This is the second enzyme in the pantothenate pathway and catalyses the stereospecific transfer of the 4S-hydrogen of the NADPH molecule to ketopantoate leading to the formation of pantoate (Zheng *et al.*, 2000), as shown in figure 1-5:

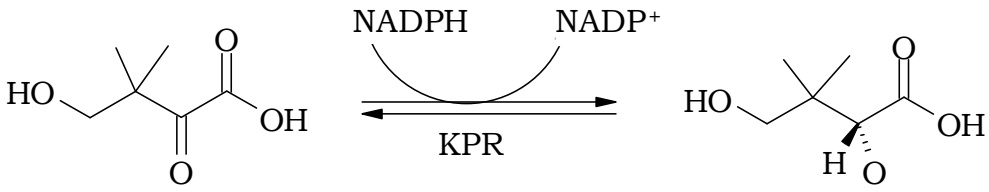

Figure 1-5: reaction catalysed from the ketopantoate reductase

The steady-state kinetic mechanism is ordered with NADPH binding first followed by ketopantoate binding and $NADP^+$ release following hydroxypantoate release. Figure 1-6 shows both the kinetic and chemical acid-base mechanism of the reaction (Zheng *et al.*, 2000).

Zheng and Blanchard cloned the *panE* gene, encoding the *E. coli* KPR and the enzyme was over expressed and purified. This procedure yielded a highly active, soluble and monomeric protein with a mass of 33 kDa. The K_M values reported for this enzyme are 0.12 mM for pantoate and 0.004 mM for NADPH (Zheng, 2000).

Because it is known that in plants the first and the final enzymes of this pathway are present, several attempts were made to identify KPR by BLAST searches of the *Arabidopsis* genome using the *E. coli panE* sequence as the query. Interestingly no matches were found. Nevertheless, reverse FUGUE identified a possible homologue of *panE* in *Arabidopsis*. Whether this protein presents KPR activity is still unsolved, but work is currently underway to test this hypothesis (Coxon *et al.*, 2005).

Concerning archaea, homologues of *E. coli* KPR are found in six genomes.

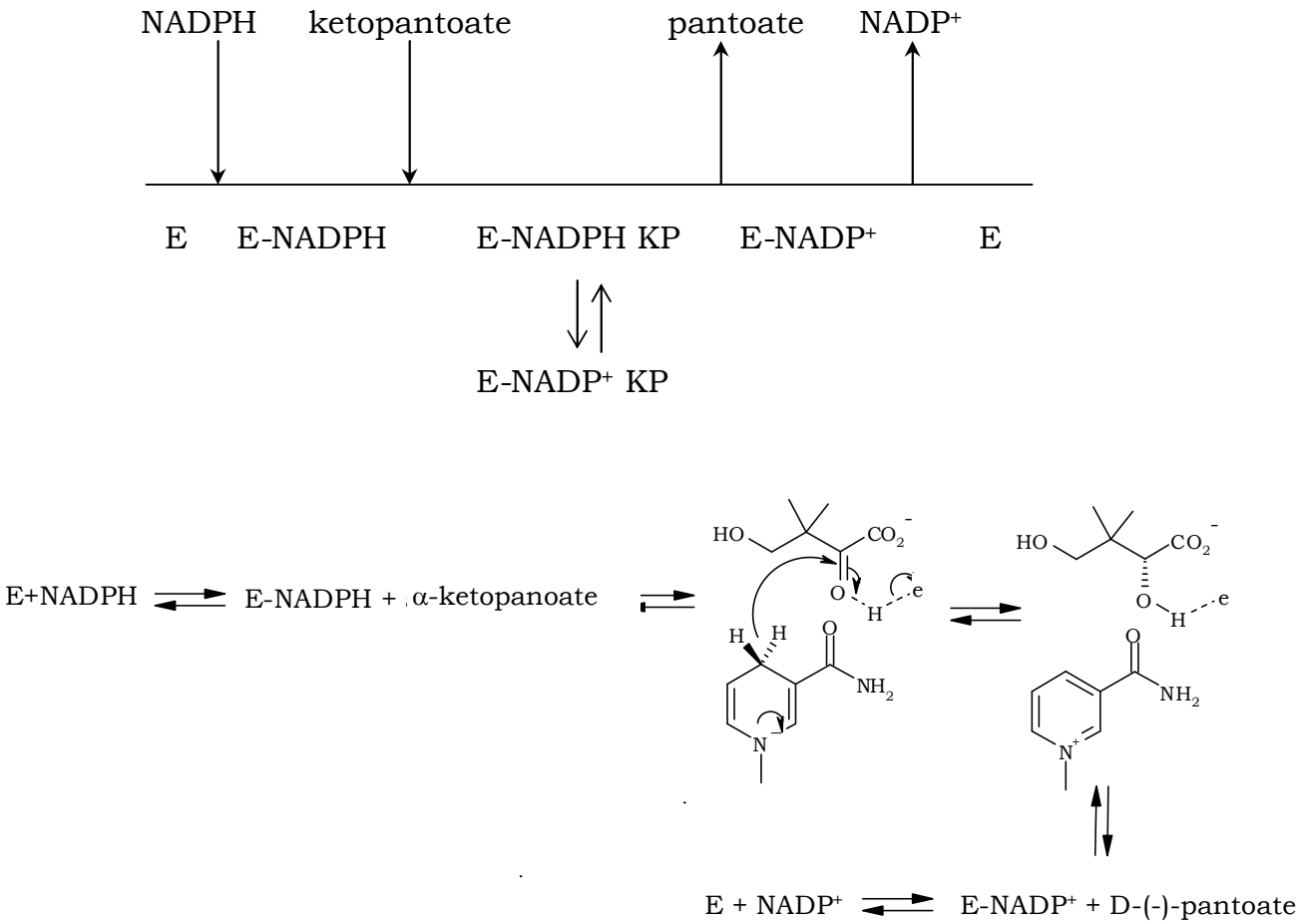

Figure 1-6: kinetic and chemical mechanism for the KPR (Re-drawn from Zheng, 2000)

1.2.3 Aspartate decarboxylase (ADC; EC 4.1.1.11).

This enzyme catalyses the decarboxylation of aspartate to β-alanine as shown in figure 1-7 (Cronan, 1980).

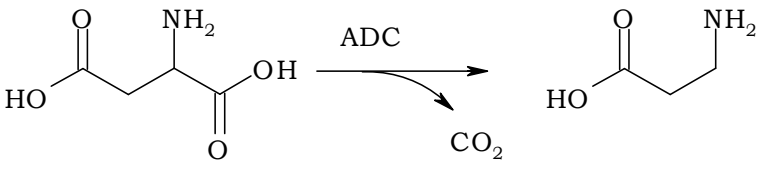

Figure 1-7: Reaction catalysed by the aspartate decarboxylate

This reaction is supposed to occur via formation of an imine between the amino group of aspartate and an integral pyruvoyl group. The proposed mechanism is shown in figure 1-8 (Saldahna, 2001).

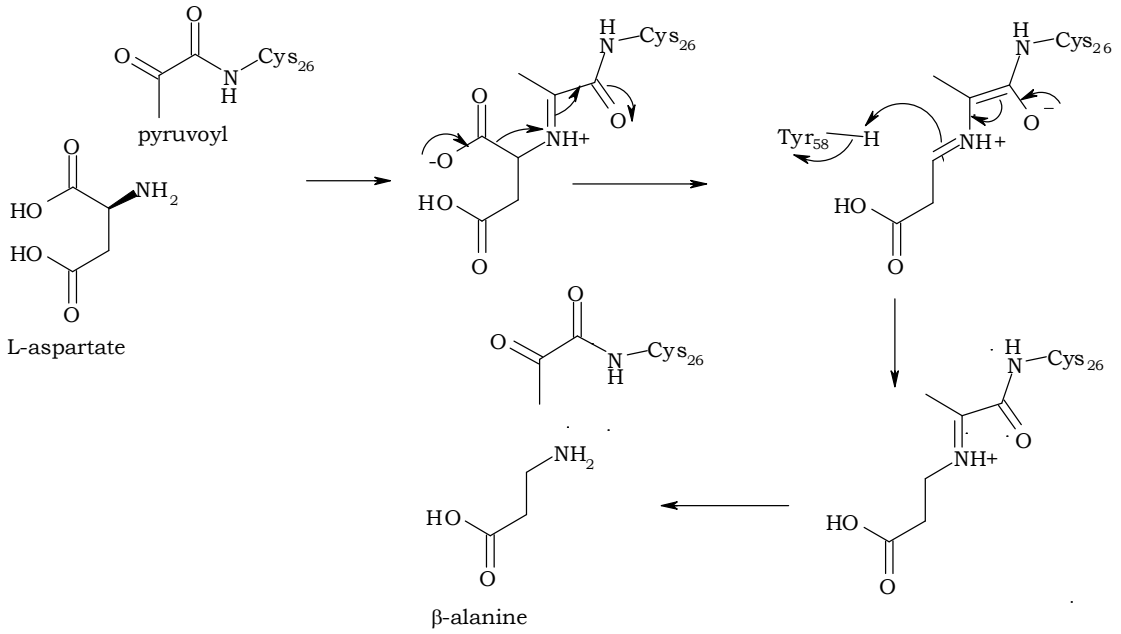

Figure 1-8: proposed mechanism for the decarboxylation of aspartate catalysed by the ADC (Re-drawn from Saldahna, 2001)

For the catalytic function of this enzyme the presence of a covalently bound pyruvoyl group is required. Further studies demonstrated that *E. coli* ADC is a pyruvoyl-dependent enzyme, and is synthesized initially as an inactive proenzyme (the π-protein). This proenzyme is proteolytically cleaved at a specific X-Ser bond to produce a β-subunit with XOH at its C-terminus and a α-subunit with a pyruvoyl group at its N-terminus, derived from the serine. The recombinant enzyme, as purified, is a tetramer, and comprises principally the unprocessed π-subunit (of 13.8 kDa), with a small proportion of the α- and β-subunits (11 kDa and 2.8 kDa respectively). (Ramjee *et al.*,1997)

The K_M value for β-Ala found for this enzyme is 0.15 mM.

Homologues of *E. coli* ADC are searched in *Arabidopsis* genome via reverse-FUGUE. Unfortunately, no matches have been found. Furthermore, no ADC homologue was identified in *Saccharomyces cerevisiae*, indicating that the gene may not have traversed the prokaryotic-eukaryotic border

(Ottenhof *et al.*, 2004). It is likely, therefore, that there is an alternative method to synthesize β-Ala for plants.

A similar situation is present in archaea, in which no matches have been found for the genomes homologue to the *E. coli* ADC.

1.2.4 Pantothenate synthetase (PS; EC 6.3.2.1)

The enzyme catalyses the condensation of β-Ala with pantoate to form one molecule of PA. In this reaction, illustrated in figure 1-9, one molecule of ATP is hydrolyzed (Miyatake *et al.*, 1979).

The kinetic mechanism for this reaction is a Bi Uni Uni Bi Ping Pong mechanism that suggests the formation of an enzyme-pantoyl adenylate intermediate as shown in figure 1-10 (Zheng *et al.*, 2001).

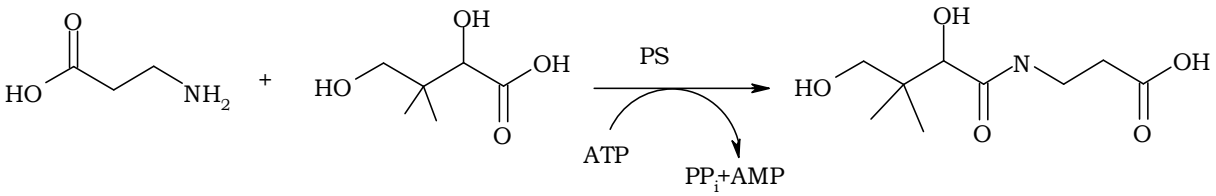

Figure 1-9: Reaction catalysed by the pantothenate synthetase

This enzyme from *E. coli* was purified to homogeneity and characterized by Miyatake *et al* (1979). It had a high pH optimum of 10, with K_M values of 150 μM for β-Ala, 63 μM for pantoate, and 100 μM for ATP.

The *panC* gene, encoding the E. *coli* PS, was then isolated and the gene mapped close to *panB* and *panD* genes, which encode KPHMT and ADC respectively. The three genes are closely linked in the clockwise order *panB panD panC*. (Cronan, 1980; Cronan *et al.*, 1982; Merkel *et al.*, 1996). The recombinant PS protein was over expressed and purified to yield a homodimeric protein with a native mass of 65 kDa and a monomeric mass of 30 kDa (von Delft *et al.*, 2001).

The cloning of the *panC* gene and functional characterization of the protein encoded by this gene in *Lotus japonica* and *Oryza sativa* (Genschel *et al.*, 1999) represents the first concrete evidence for this pathway in plants. The gene encoding the PS in *Arabidopsis* was identified with the gene At5g48840, it is 60% and 62% similar to those from *Lotus* and rice respectively and, in common with PS from other plants, there is no N-terminal extension compared with the *E. coli* protein (Ottenhof *et al.*, 2004).

This enzyme in not yet described in archaea.

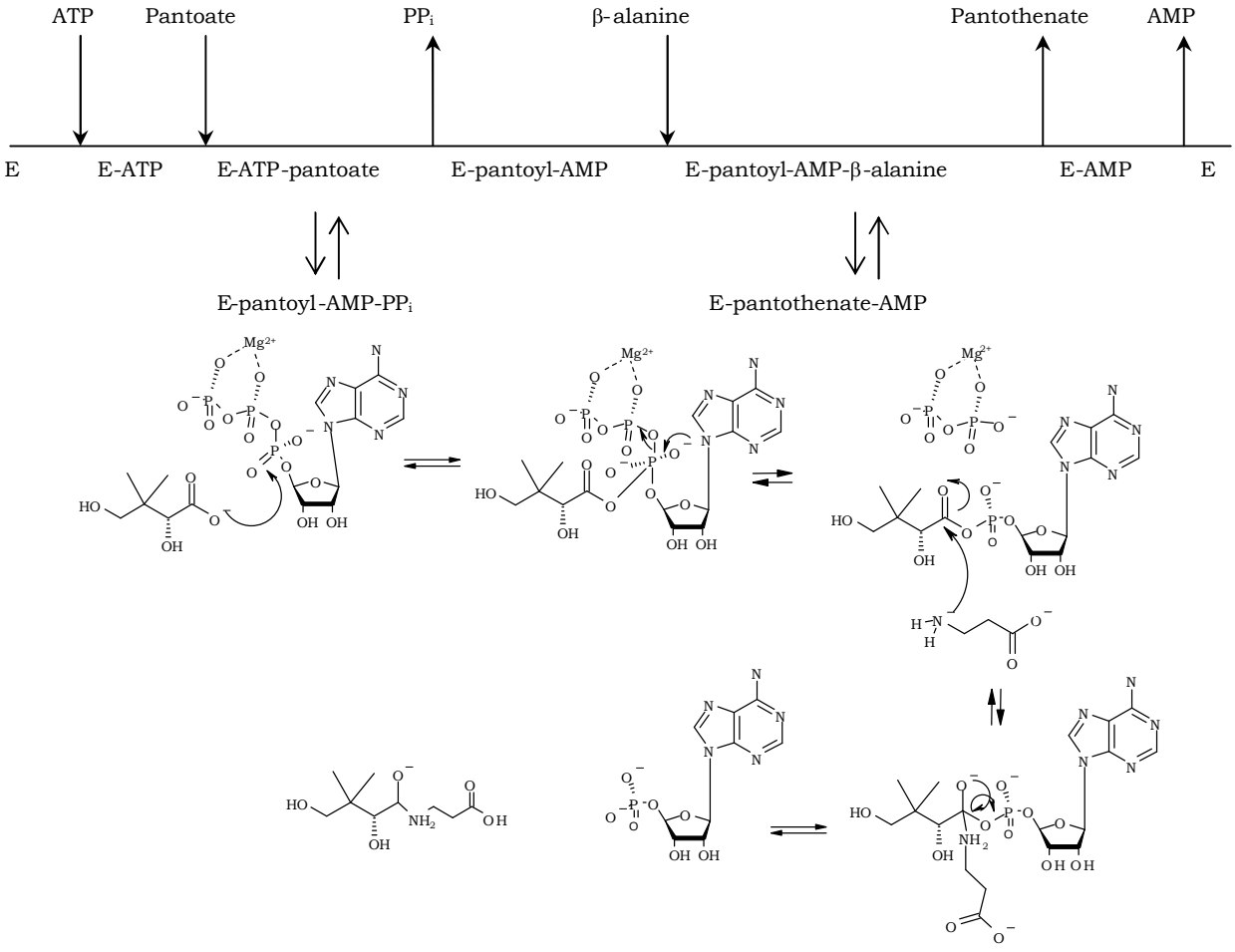

Figure 1-10: kinetic and chemical mechanism for PS (Re-drawn from Zheng *et al.*, 2001)

1.2.5 Pantothenate kinase (PanK, EC 2.7.1.33)

This enzyme, the first in the CoA pathway, is encoded by the gene *coaA* and catalyses the phosphorylation of pantothenate in the 4' position. The reaction is thought to be the rate-controlling step in the CoA biosynthetic pathway. In this reaction one molecule of ATP is hydrolyzed to give ADP, as shown in figure 1-11.

The PanK was purified to homogeneity from *E. coli* and was shown to exist as a homodimer with subunits masses of 36 kDa (Song *et al.*, 1994).

The reaction catalyzed by PanK proceeds via a compulsory ordered mechanism with ATP as the first substrate to bind. Each subunit presents a single nucleotide-binding site, and ATP binding to the homodimer is highly cooperative (Song *et al.*, 1994).

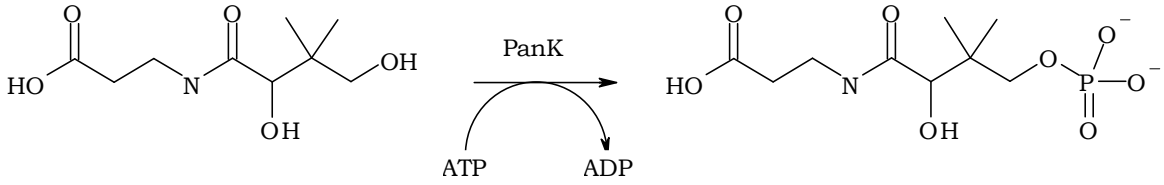

Figure 1-11: Reaction catalysed by the PanK

This enzyme catalysis the major regulated step in the CoA biosynthesis and shows a high feedback inhibition by CoASH and other CoA thioesthers (Villari *et al*, 1987). The molecular basis of this inhibition is the presence of 4 different and specific amino acid residues in the protein: Lys101 interacts with the phosphodiester of CoA and also is involved in the binding of the ATP phosphodiester. In addition, the residues Arg106, His177 and Phe247 make specific contacts with CoA but not with ATP. Mutant PanK, in which these 4 a.a. are mutated, does not show allosteric interaction or feedback regulation (Rock *et al.*, 2003).

The K_M values reported for ATP and PA are 0,225 mM and 0,009 mM respectively (Rock *et al.*, 2003).

In plants PanK was purified from spinach leaves (Falk and Guerra, 1993) and partially characterized. It is located in the chloroplasts where there is a

high demand for CoA due to the de novo fatty acid synthesis. The *A. thaliana CoaA* sequence was identified by sequence homology with *A. nidulans* PanK. It is located on chromosome 1 and it shows strong homology to yeast PanK and human PanK1b, whereas only poor homology is observed between AtCoaA and bacterial CoaA (Leonardi *et al.*, 2005).

No homologue genes are identified in archaeal genomes.

1.2.6 Phosphopantothenoylcysteine synthetase (PPCS; EC6.3.25) and Phosphopantothenoylcysteine decarboxylase (PPCDC; EC 4.1.1.36)

The *E. coli* gene *coaBC* was found to encode a flavinmononucleotide (FMN)-containing bifunctional enzyme responsible for both the 4'-phosphopantethenoylcysteine synthetase and 4'-phosphopantethenoyl-cysteine decarboxylase activities (Kupke e al., 2000; Strauss *et al*, 2001). This enzyme catalyses the reactions that lead to the formation of 4'-phosphopantetheine from 4'-phosphopantothenate like is shown in figure 1-12.

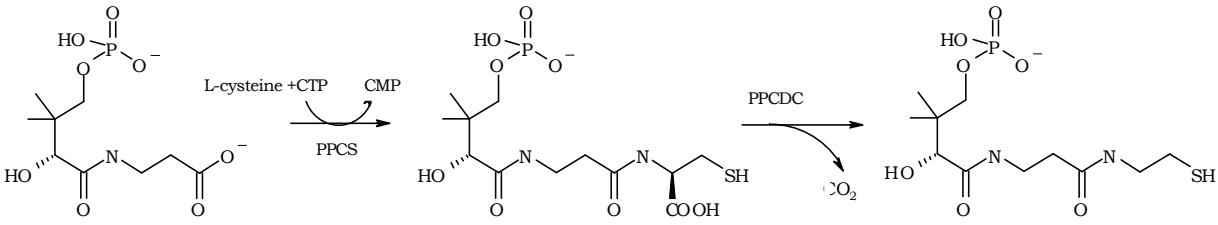

Figure 1-12: Reaction catalysed by the PPCS/PPCDC

The *E. coli* PPCS/PPCDC protein is a homodecamer of 48,3 kDa subunits. It shows a UV spectrum characteristic of flavoenzymes and the flavin mononucleotide FMN was isolated after heat-denaturation of the enzyme (Strauss *et al.*, 2001).

The two individual activity domains have been expressed and purified (Kupke, 2001; Kupke 2002). The mechanism of this reaction was clarified by

Kupke (2002) who suggested a two step process: in the first step the 4'-phosphopantothenate is activated by reaction with CTP to form a 4'-phosphopantothenoyl-cytydilate intermediate that, in the second step, is attacked by cysteine, and 4'-phosphopantothenoylcysteine is formed. This reaction occurs at the C-terminal (CoaB domain) of the enzyme. The second reaction, the FMN dependent oxidative decarboxylation of the intermediate, leads to 4'-phosphopantothenoylaminoethenthiol, which is then reduced to 4'-phosphopantetheine. This occurs at the N-terminal (CoaC) domains, as shown in figure 1-13.

4'-Phosphopantothenate shows substrate inhibition at high concentrations. The 2.7-fold higher value of K_M for phosphopantethenoyl-cysteine, compared with K_M for 4'-phosphoantothenate, suggests that this intermediate is not the physiological substrate (Strauss *et al.*, 2001).

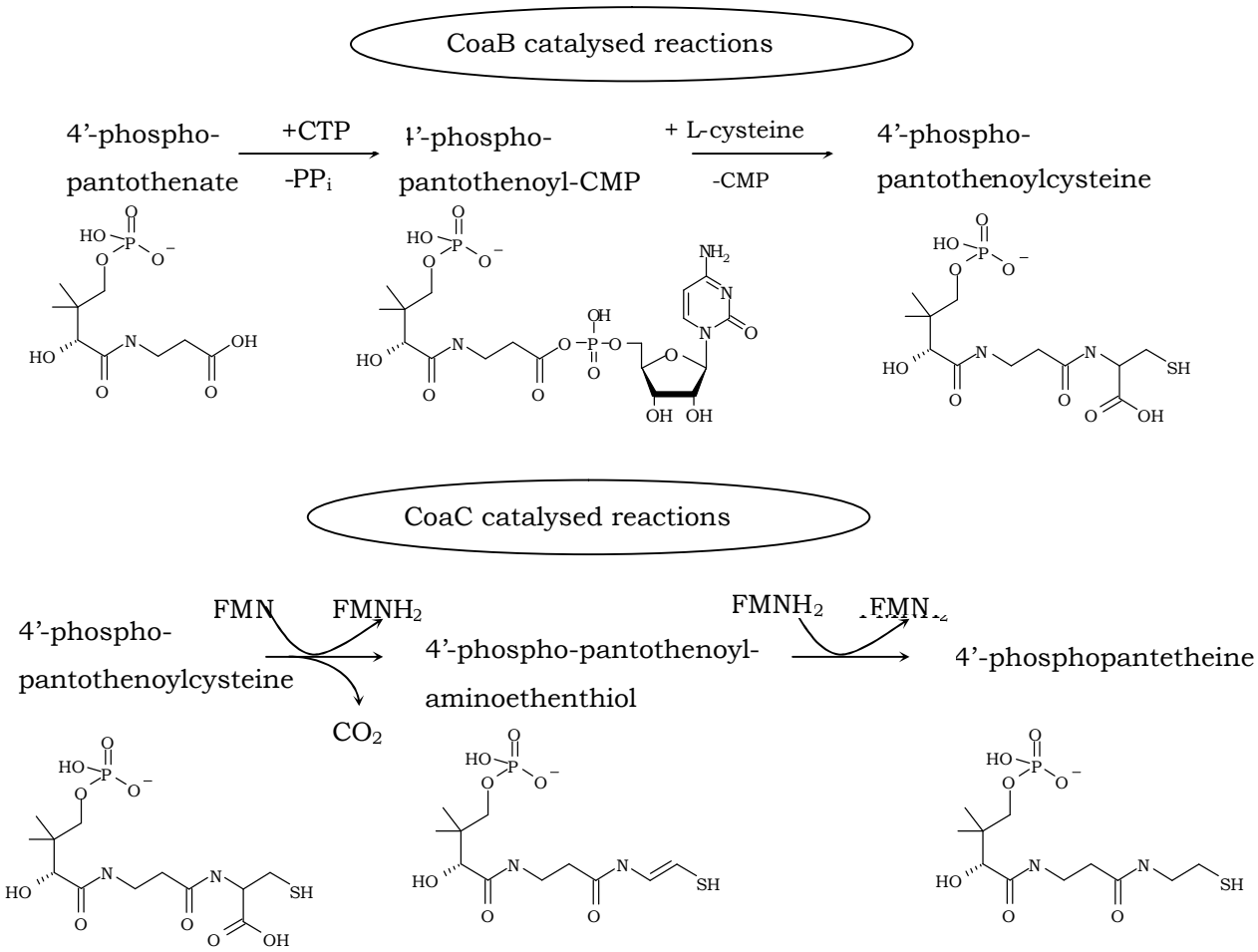

Figure 1-13:The role of CoaB and CoaC domains in 4'-phosphopantothenoylcysteine and 4'-phosphopantetheine biosynthesis (Re-drawn from Kupke, 2004)

In plants, like in all the eukaryotes, the two activities belong to two distinct enzymes: the PPCS and the PPCDC. In *A. thaliana* two proteins AtCoaB and AtcoaB* were identified as homologues of the monofunctional human PPCS (Kupke *et al.*, 2003). Both of these proteins sequences show high homology with lower and higher eukaryotic orthologs but not with the CoaB domain of the *E. coli*'s protein. However, residues important for the activity and structure of the *E. coli* enzyme are conserved in plant protein. The important difference between the plant enzyme and the *E. coli* one is that in the first case, the nucleotide used is ATP instead of CTP (Kupke *et al.*, 2003; Daugherty *et al.*, 2002).

The flavoprotein AtHAL3a (AtCoaC1, 209 a.a.) shares the phospho-pantothenoylcysteine decarboxylase signature sequence with bacterial enzymes (Kupke *et al.*, 2003). AtCoaC1 catalyses the decarboxylation of 4'-phosphopantothenoylcysteine via an oxidatively decarboxylated intermediate containing an aminoethenethiol group (Hernandez-Acosta *et al.*, 2002).

The *E. coli CoaBC gene* is the only one in the CoA pathway that presents homologous genes in all the known archaeal genomes.

Both domains of the bifunctional PPCS/PPCDC protein from *Methanococcus jannaschii* were purified as His-tag proteins and their enzymatic activities were identified and characterized (Kupke *et al.*, 2006).

Several active site residues of bacterial and eukaryotic PPCS/PPCDC enzyme are not conserved in the *M. jannaschii* enzyme, but this has apparently no influence on the decarboxylase activity. Furthermore, is demonstrated that *M. jannaschii* is a CTP-dependent enzyme, although its nucleotide-binding motif contains a Lys residue, which is characteristic for the ATP-dependent phosphopantethenoylcysteine synthetase (Kupke *et al.*, 2006).

1.2.7 Phosphopantetheine adenylyl transferase (PPAT, E.C. 2.7.7.3)

PPAT is the second rate-limiting enzyme in the CoA pathway and, together with PanK, regulates the CoA cellular content (Jackowski *et al.*, 1984). It catalyses the reversible reaction that leads to the formation of dephosphoCoA (dPCoA) starting from phosphopantetheine as in the reaction shown in figure 1-14.

PPAT is a member of the nucleotydyltransferase α/β phosphodiesterase superfamily, that catalyses the transfer of a monophosphate nucleotide moiety to different molecules by transition state stabilization by the enzyme, without involving chemistry of the functional groups (Izard, 2002).

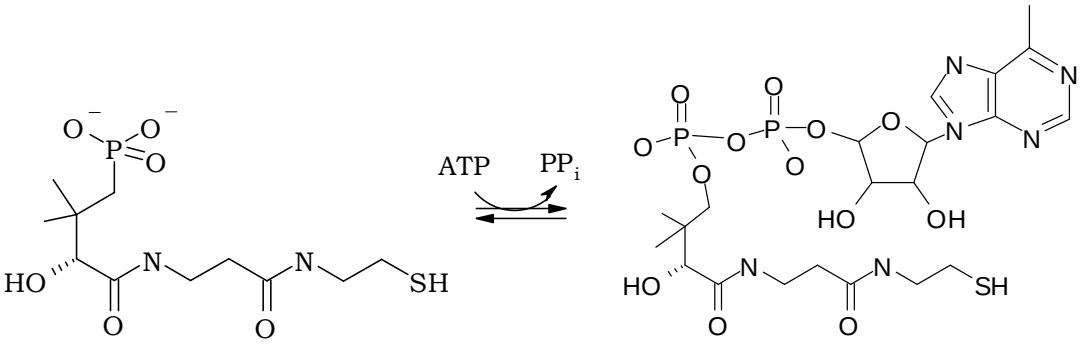

Figure 1-14: Reaction catalysed by the PPAT

The *E. coli* enzyme (encoded by the gene *coaD*) in solution shows a molecular mass of 108 kDa, which suggests a homohexameric structure with monomeric mass of 17.8 kDa (Geerlof *et al.*, 1999). The crystal structures of CoaD in binary complexes with the substrates, products and the putative inhibitor CoA, shows that CoaD is an allosteric hexameric enzyme characterized by half-of-sites reactivity. In fact, only one trimer within the hexamer actually binds dephospho-CoA and 4'-phosphopantetheine (Izard *et al.*, 1999; Izard, 2002; Izard, 2003). CoA is suggested to allosterically regulate PPAT through feedback inhibition (Izard, 2003; Rock *et al.*, 2003; Vallari *et al.*, 1988).

Reported K_M values for this enzyme are 0.007 mM for dPCoA and 0.22 mM for PP_i (pyrophosphate). No substrate inhibition is observed for dPCoA concentration up to 200μM (Geerlof *et al.*, 1999).

The *Arabidopsis AtCoaD* sequence was identified by its strong homology to the PPAT of the human bifunctional CoA synthase. The similarity of the plant enzyme to the *E. coli* monofunctional PPAT is low. Unlike AtCoaD, AtCoaE shares a high degree of homology with bacterial CoaE and the least homology is observed with the DPCK domain of the human bifunctional CoA synthase. The activity of both AtCoaD and AtCoaE has been confirmed *in vitro* (Kupke *et al.*, 2003)

1.2.8 DephosphoCoenzymeA kinase (DPCK, EC 2.7.1.24)

This enzyme catalyses the final step in the CoA biosynthesis, the phosphorylation of the 3'-hydroxy group on the sugar moiety of dPCoA, in the reaction showed in figure 1-15.

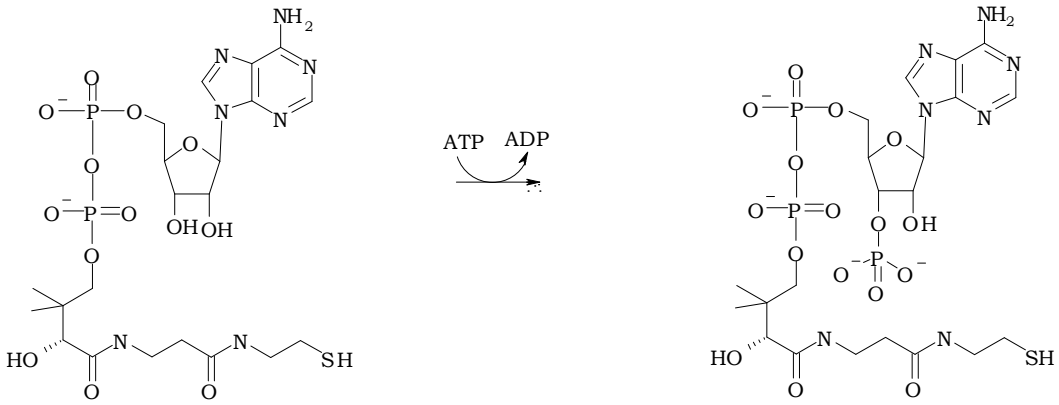

Figure 1-15: Reaction catalysed by the DPCK.

The native enzyme shows a molecular mass of 22 kDa, suggesting that it exists as a monomer in solution (Mishra *et al.*, 2001). However, the presence of sulphate ions induces the formation of trimers: for this reason DPCK in solution results in an equilibrium monomer-trimer (O'Toole *et al.*, 2003). The sulphate ion plays a decisive role in the trimer formation and it is thought that it could be replaced by the phosphate ion *in vivo*.

K_M values are 0.74 mM for dPCoA and 1.14 mM for ATP.

Recombinant DPCK shows high activity over a broad pH range, with a maximum at pH 8,5 (Mishra *et al.*, 2001).

1.3 CoenzymeA regulation in *Escherichia coli*

CoA plays a fundamental role in the metabolism of cells and its intracellular pool can vary in size and composition depending on the growth condition and the fitness of the cells. It is mainly made up of acetyl-CoA (AcCoA), followed by nonesterified CoA, succinylCoA and malonyl-CoA while long-chain acyl-CoA are not abundant (Leonardi *et al*, 2005).

For living organisms it is very important to regulate the total amount of CoA and for that reason, two enzymes in its pathway are regulated very stringently. The first and most important regulation point is at the level of the enzyme PanK that shows a very high feedback inhibition by CoA and, to a lesser extent, by its thioesters that competitively interfere with ATP binding (Yun *et al.*, 2000). The mechanism of this feedback inhibition was understood by studying the behavior of three different PanK mutants, each of them with a single amino acid mutation. These studies showed that the residues Arg 106, His 177 and Phe 247 do not play a major role in catalysis, nor do they significantly participate in the binding of ATP or PA, but they are all required for feedback regulation of the enzyme by CoA. In fact the mutants do not show inhibition by CoA or its thioesters because all the three mutant enzymes lose the capacity to bind CoA (Rock *et al.*, 2003).

The second regulatory point in the CoA pathway is at the level of the PPAT even though with a minor extent. This is evident from the release of 4'-phosphopantetheine from the bacteria to the outside medium (Jackowski *et al.*, 1984). The PPAT becomes more important when the regulation at the PanK site is disrupted (Rock *et al.*, 2003) or when the PanK protein is overexpressed (Song *et al.*, 1992).

Other important factor that regulates the upper threshold for intracellular CoA is the expression level of the *coaA* gene. The *coaA* promoter has poor homology with the consensus *E. coli coaA* promoter sequences and

the *coaA* coding sequence is populated largely by low usage codons. As a result, the bacterial CoaA protein is found at low abundance relative to the average *E. coli* protein (Leonardi *et al.*, 2005).

1.4 Bacterial degradation of pantothenate

Early studies on *Pseudomonas fluorescens* P-2 show that these bacteria are able to grow in an environment in which the only source for carbon and nitrogen was pantothenate. This means that these organisms should be able to degrade the pantothenate. A scheme proposed for the degradative pathway of pantothenate is shown in figure 1-16 (Airas, 1979).

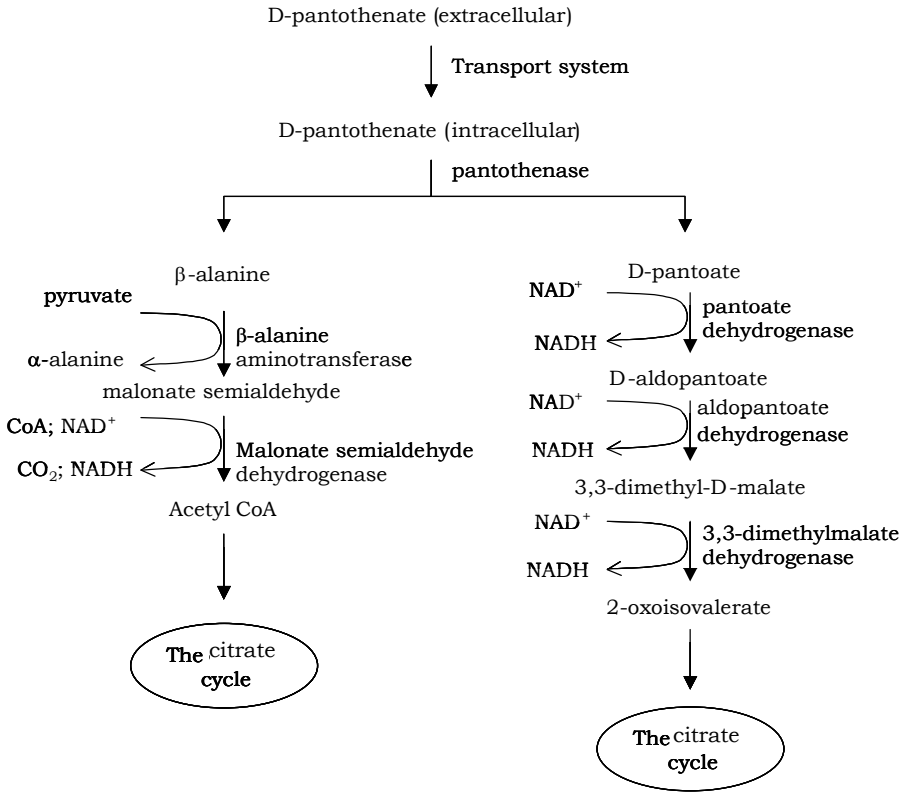

Figure 1-16: Pantothenate degradative pathway (Re-drawn from Airas, 1979)

A bacterial amidase capable of specific decomposition of pantothenate into pantoate and β-Ala, called pantothenase (EC. 3.5.1.22), was isolated from *Pseudomonas fluorescens*. This enzyme seems to be a dimer with a molecular weight of 100 kDa and monomeric mass of 50 kDa. The K_M value for pantothenate is 15 mM (Airas, 1979).

Originally, the product of the pantothenase-catalyzed reaction was suggested to be pantoic acid (Nurmikko *et al.*, 1966). In the bacterial strain used in the pantothenase-based assay of pantothenate (Airas, 1983) the product proved to be pantoyl lactone.

Later works (Airas, 1988) showed that, in effect, in the two different *Pseudomanas fluorescens* strains used, UK-1 and PS-21, two different pantothenases were present, producing, respectively, pantoyl lactone and pantoic acid.

2. Introduction to the archaea domain

2.1 Classification of living organisms

In the 4th century B. C. Aristotle created a classification scheme for all living things in which the organisms were divided in two Kingdoms: plants and animals. This classification was based on the similarity of the exterior appearance of the living organisms.

In the year 1735 Carolus Linnaeus proposed a more scientific classification, in which the same Kingdoms were further divided in classes, orders, families, genres and species. In this classification the plants were defined as organisms that were fixed to the soil and that were able to make photosynthesis (including unicellular organisms autotrophs like algae, fungi and bacteria), while animals were defined as eterotrophic organisms that could move (including the unicellular eterotrophs or protists).

In the 20th century, because this system was not really able to accommodate fungi (not moving eterotrophic organisms), protists and bacteria, a system of five kingdoms started to be accepted. In this new model, the organisms are divided in five Kingdoms: plants, animals, fungi, protists and bacteria (Whittaker, 1959; Whittaker, 1987).

At the same time another classification was accepted, in which the living organisms were divided into two groups: Eukaryotes and Prokaryotes (Chatton, 1938). The major differences between eukarya and prokarya are that the prokaryotic organisms usually possess a circular molecule of DNA (chromosome) to which different proteins are associated. The chromosome is present in a particular portion of the cell, which is called nucleolus but is not separated from the rest of the cell by a membrane. In the eukaryotic organisms (from the Greek words ευ=true and καριον=nucleus) the DNA is linear and often more than one chromosome is present. These are stored in the nucleus, separated from the cytosol by a membrane.

The characteristics of the five kingdoms are summarized in table 2-1.

Kingdom	**Cellular organisation**	**nutrition**	**reproduction**	**mouvement**	
Bacteria	Unicellular or colonial	autotrophic (photo-chemiosynthesis); eterotrophic (absorption)	Asexual	Mobile (flagella) or not mobile	Prokarya
Protists	Unicellular or colonial	Autotrophic (photosynthesis); Eterotrophic (absorption or ingestion)	Asexual and sexual	Mobile (cilia or flagella) or not mobile	Eukarya
Plants	Eukaryotic with walls (cellulose); pluricellular with tissues	Autotrophic (photosynthesis)	Sexual and asexual	Not mobile	
Fungi	Eukaryotic with walls (chitine); sinciziale (without defined border between cells)	Eterotrophic (absorption)	Sexual and asexual	Not mobile	
Animals	Eukaryotic without walls; pluricellular with tissues	eterotrophic (ingestion)	Sexual	Mobile (contractile fibres)	

Table 2-1: The five kingdoms of the living organisms

In 1977, a new classification method based on comparisons of the 16S rRNA was developed. This new method was based on the analysis of a molecule, the rRNA, which is present in all organisms with the same function in the biosynthesis of proteins. Together with this universality, the rRNA is important because it is highly conserved: this means that the 16SrRNA can be used to build the phylogenetic tree.

In the late 1970s, Carl Woese, at the University of Illinois, studying relationships among the prokaryotes using DNA sequencing and rRNA comparisons, found that those bacteria that live at high temperatures or produce methane should be clustered together but in a group that is different from both the eukarya and the prokarya. Because of these genetic differences, Woese proposed a new classification of living organisms, that was made up of three domains: Eukaryota, Eubacteria and Archaea (Woese, 1977; Woese, 1990). Archaea include inhabitants of some of the most extreme environments on the planet like the deep sea at very high pressure, over 100 degrees Centigrade, extremely saline water, extreme acidic or alkaline pH.

2.2 Morphology of Archaea

Archaea do not live only in the extreme environment from which they were first identified, but they can live everywhere: seas, rivers, soil, in ice-cold seawater and in the cow digestive system. Archaea are ubiquitous around the world, and it is estimated that circa 20% of the picoplancton is made up by archaea. (Karner *et al.*, 2001)

Archaea are very tiny, usually less than one micron long, and they present, like bacteria, a variety of different shapes and forms. Some of them are spherical (coccus), some are rod-shaped (bacillus), and they can present either single or multiple flagella. Like bacteria, archaea do not have internal membranes and their DNA exists as a single loop, the plasmid.

The major distinctions between Archaea and Bacteria are based mainly on the different types of transcription factors and on the chemical structure of the membranes. Like the cells of all the living organisms, archaeal cells present an outer membrane and, in nearly all the known archaea, outside the membrane there is a cell wall. A notable characteristic of the archaea is the chemical composition of their membranes. In fact the main constituent of the bacterial and eukaryotic membranes is D-glycerol, while the archaeal

membranes contain only L-glycerol. In figure 2-1 the major chemical constituents of archaeal and bacterial membranes are shown.

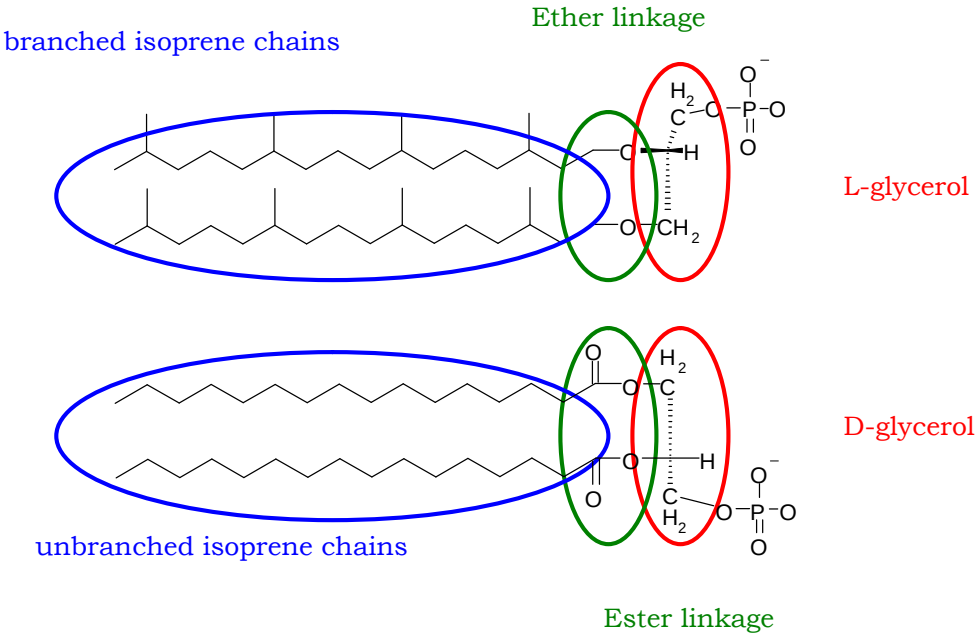

Figure 2-1: components of the cell membranes for archaea (above) and bacteria (below)

Furthermore, when the glycerol is substituted, it is possible to form linkages between different side chains: in bacteria and eukarya, the side chains are linked via ester bonds, while in archaea the side chains are bounded by ether linkages (Kates, 1993). This means that the resulting phospholipids present different chemical properties due to the lack of the additional double bond C=O.

Another important difference between archaea and other organisms is that, in Archaea, the side chains of the phospholipids are built from isoprene, the simplest member of the terpene class, while both bacteria and higher organisms build the membranes with unbranched fatty acids. The presence of terpenes in the side chains gives the archaeal membranes some interesting properties. For instance, the isoprene side chains can be joined together. This means that the two side chains of a single phospholipid can join together, or they can be joined to side chains of another phospholipid on the other side of the membrane. No other group of organisms can form such

transmembrane phospholipids. Another interesting property of the side branches is their ability to form carbon rings, which are thought to provide structural stability to the membranes, since they seem to be more common among species that live at high temperatures.

From the genome point of view, the majority of archaeal proteins, particularly the metabolic enzymes and proteins involved in cell division and cell wall biogenesis, are most similar to their bacterial counterparts. On the contrary, a minority, primarily proteins involved in genome replication and expression, most closely resemble their eukaryotic orthologs (Makarova, 1999). Furthermore, considering the transcription factors in archaea, it was possible to find tRNAi introns, that before the discovery of archaea were thought to be a characteristic only of the eukarya (Kaine *et al.*, 1983).

2.3 Classification of archaea

On the basis of the 16S rRNA analysis, archaea consist of three phylogenetically distinct groups: Chrenarchaeota, Euryarchaeota and Korarchaeota. For this last group only the nucleic acids have been detected, and no organisms have been isolated or cultured. Figure 2-2 shows the phylogenetic tree of archaea, bacteria and eukaryotes.

Physiology of Archaea allows a classification in three types: methanogenes (that produce methane), extreme halophiles (that live at very high concentration of salts) and extreme (hyper) thermophiles (that live at very high temperatures).

The Chrenarcheota consists mainly of hypothermophilic sulphur-dependent prokaryotes and the Euryarchaeota contains the methanogens and the extreme halophiles. 16SrRNAs of the Korarchaeota have been obtained from hypothermophilic environments similar to those inhabited by Chrenarchaeota.

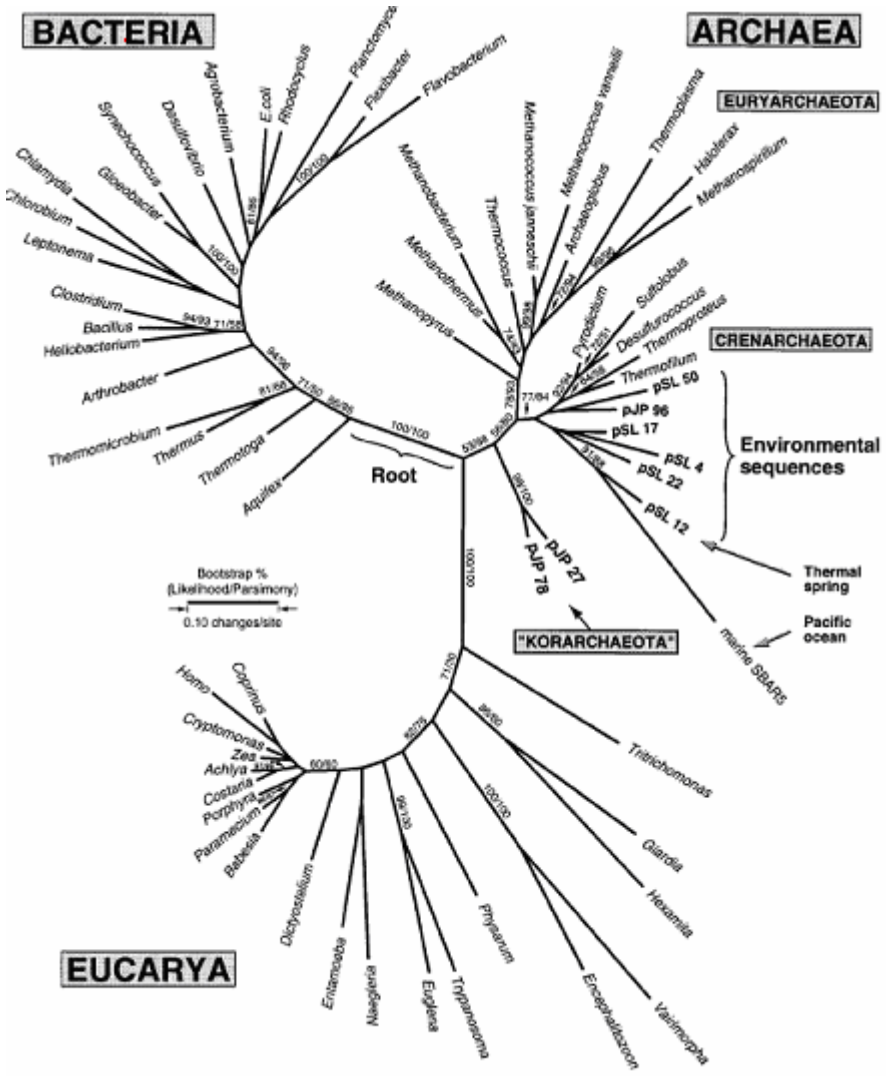

Figure 2-2: Phylogenetic tree of Archaea (from Barn *et al*. Proc. Natl. Acad. Sci. USA 93, 1996)

2.4 Methanogens

Methanogens are obligate anaerobes that do not tolerate even brief exposure to oxygen. Their metabolism uses H_2 as an energy source and CO_2 as an electron acceptor. In the metabolic processes, these organisms produce CH_4, which accumulate in their environment. Methanogenic organisms produce most of the natural reserves of natural gas that are nowadays used as an energy source for domestic or industrial uses.

2.4.1 Extreme halophiles

These organisms live in extreme environment such as the Dead Sea, the Great Salt Lake or evaporating ponds of seawater in which the concentration of NaCl is very high (5M). They require salt to grow and are not able to live at low salt concentration because of their cell walls, ribosomes and enzymes that are stabilized by Na^+.

Because the high concentration of NaCl reduces the availability of O_2, these organisms are able to supplement their ATP-producing capacity by converting light-energy into ATP using bacteriorhodopsin, which reacts with light forming a proton gradient on the membrane. It is this gradient that allows the formation of ATP.

2.4.2 Extreme thermophiles

These archaea live in hot, sulphur-rich environment usually associated with volcanism. Their main characteristic is that they require very high temperatures (80°C to 105°C) to grow. In addition, most of them require elemental sulphur. They can be anaerobic and use sulphur as an electron acceptor for respiration instead of oxygen or aerobic and in this case they can oxidize sulphur as an energy source. Also, because of oxidizing S^0 produces sulphuric acid (SO_4^{2-}), they acidify their growing environment reaching very low values of pH (less than 2).

2.5 Archaeal strains used in this work

In this work, genomic DNA of three different archaea is used: *Methanococcus jannaschii*, *Methanosarcina mazei* and *Sulfolobus solfataricus*.

2.5.1 Methanococcus jannaschii

It is a single-celled microbe, which grows at pressures of up to 200 atm and at an optimum temperature of 85°C. It is an autotroph that gets its energy from hydrogen and carbon dioxide producing methane and it is capable of nitrogen fixation.

These organisms live in the harshest environment on earth, boiling water holes in Italy, the ice of Antarctic seas, and hydrothermal vents at the bottom of the ocean.

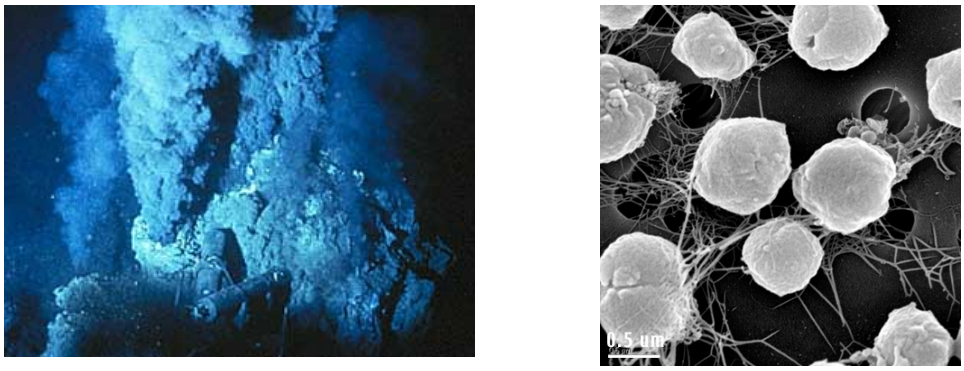

Figure 2-3: On the left: Methanococcus jannaschii isolated near a "smoker," a hydrothermal vent 2,600 meters deep in the Pacific Ocean (© Woods Hole Oceanographic Institution). On the right: Methanococcus jannaschii grown at 78°C and 30 psi. Cultivated by Chan B. Park, Dept. Chem. Eng., UC Berkeley (© Berkeley Microscope Lab).

The genome sequence of *Methanococcus jannaschii* was the first complete genome sequence as a representative of the Archaea. It was named after Holger Jannasch of the Woods Hole Oceanographic Institute in Massachusetts, the microbiologist who led the research expedition that identified the organism.

2.5.2 Methanosarcina mazei

This organism and related species are of great ecological importance as they are the only organisms fermenting acetate, methylamines and methanol to methane, carbon dioxide and ammonia (in case of methylamines).

Figure 2-4: Colony of the archaebacterium *Methanosarcina mazei* (© Ralph Robinson).

Methanococcus mazei was first described in mixed culture by Maze in 1903. In the year 1980 Mah isolated and described a pure culture of *Methanococcus mazei* (Mah, 1980) and has now placed this species within the genus *Methanosarcina*.

Cells are irregular and grouped into multicellular sarcinal colonies, which may disaggregate in older cultures. The protoplast is bounded by a typical trilaminar plasma membrane, outside of which is a matrix of loose fibrils. Polymers extruded by *M. mazei* are likely quite adhesive in nature, which accounts for its strong adherence to surfaces and hardiness as compared to many other methanogens (Robinson, 1985).

2.5.3 Sulfolobus solfataricus

It is a single-celled organism, grows optimally at 80°C and can be found in sulphur-rich, acidic environment with pH values from 2-4. It can live either litoautotrophically by oxidizing sulphur, or chemoheterotrophically on reduced carbon compounds. The sequenced P2 strain was isolated from a sulphur hot spring near Naples, Italy.

Figure 2-5: On the left: the red of these rocks is produced by Sulfolobus solfataricus, near Naples, Italy. (Tony Phillips, Science@NASA). On the right: a cell of Sulfolobus solfataricus (D.Janckovik/W.Zillig).

Sulfolobus solfataricus is a model species for investigating DNA replication, the cell cycle and RNA processing in archaea.

3. Aims of this work

The high number of studies on CoA pathway reflects its importance in all the living organisms. In fact it acts as important intermediates in the synthesis and breakdown of lipids and fatty acids. In addition, acyl CoA esters have been shown to be involved in regulating metabolism and cell signaling in bacteria, yeast and mammalian cells (Graham *et al.*, 2002). As described in the introduction, our comprehension of this metabolic pathway in bacteria (*Escherichia coli*) is more or less complete. All the enzymes involved in this metabolism are identified, the genes are cloned and the protein fully characterized. Same thing, unfortunately, is not true for plants. In this case, in fact, not all the enzymes involved in PA biosynthesis are identified and characterized even though the presence of KPHMT and PS allow to hypothesis that this pathway is present.

Other aspect that is not understood in plants is the regulation of PA synthesis. In bacteria, an excess of PA is excreted in the media and in this way the intracellular concentration of PA is maintained below certain limits. Furthermore, an enzyme that is able to degrade PA into β-Ala and pantoyl lacton or pantoic acid is described in *Pseudomonas fluorescens*.

The goal of the first part of this work is to find out if in plants, and particularly in *Arabidopsis thaliana*, a mechanism to degrade pantothenate is present. If a pantothenase-like activity is present, the following step would be to identify the enzyme that presents this activity.

In the second part of this work, the CoA pathway in Archaea is considered. As described in the introduction, only few enzymes of the PA and CoA pathways (KPHMT, CoaBC) have homologues in archaea. The presence of these two enzymes suggests that these pathways must be present in archaea too. The fact that not obvious homologues are present can mean that they are encoded from not related genes.

Via chromosomal proximity analyses, nearly all the putative genes encoding for the enzymes involved in CoA biosynthesis are identified in archaeal genomes. Target of this part of the work is to verify if these putative

genes present the supposed activity. For this reason, three of these genes were cloned from different organisms, the proteins overexpressed (when possible) and the resulting recombinant enzyme characterized.

4. Materials and methods

4.1 Reagents and materials

All the chemicals used in this work are obtained commercially (analytical grade) from the companies: BioRad (USA), Fluka (Switzerland), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg), Sigma-Aldrich (USA).
The oligonucleotides used as primers in the cloning reactions were from Biomers (Germany).

The enzymes for the DNA restriction analysis are obtained from the companies Gibco BRL (USA), New England Biolabs (USA), Qiagen (Hilden), Roche (Switzerland), Serva (Heidelberg), Sigma-Aldrich (USA) und Stratagene (USA).

The radioactive reagents are obtained from the companies ARC (American Radiolabeled Chemicals) ([1-^{14}C]-ß-Ala 55mCi/mmol; 0,1 mCi/ml; [1-^{14}C]-PA 50 mCi/mmol; 100 µCi/ml), Sigma-Aldrich ([3-^{14}C]-β-Ala 1,0 mCi/ml; 49 mCi/mmol), Amersham Bioscience ([γ-^{32}P]-ATP 10 mCi/ml; ~3000Ci/mmol).

The purification of the DNA band from agarose gel was carried out with the GFX™ DNA and Gel Band Purification Kit from Amersham Pharmacia Biotech (Freiburg).

Purification of the recombinant proteins expressed in *E. Coli* is obtained with Ni-NTA Agarose (Hilden).

DNA sequencing was carried out with the BigDye® Terminator v1.1 Cycle Sequencing Kit from Applied Biosystems (USA).

TLC analyses were carried on using TLC Aluminum sheets (Silica gel 60 F_{254}) from Merck using mobile phases as specified below.

4.2 Instruments

- *Sonicator*
  - Branson Sonifier W-250D
- *Centrifuges*
  - Beckman Allegra™ X-22R Rotor Sx4250
  - Beckman GS-15R Rotor F2402H
  - Beckman J2-HC Rotor JA20
  - ALC microCENTRIFUGETTE 4214
- *HPLC system*
  - PDA-100 Photodiode Array Detector
  - ASI-100 Automated sampler
  - P-680 HPLC Pump
- *Electrophoresis supplier*
  - Biotech-Fischer PHERO-stab 500
- *Phosphor screen*
  - Molecular Dyamics STORM 860
- *P.C.R*
  - Biometra Thermoblock UNO
- *Sequencer*
  - Applied Biosystems capillar sequencer ABI PRISM® 310 Genetic Analyzer.
- *Cool-dryer*
  - CHRIST® ALPHA 1-4
- *Äkta*
  - Amersham Pharmacia Biotech - Äkta explorer

4.3 Solutions and media

(* Sterilized by autoclave) (° Sterilized by filtration)

TE buffer*	Tris/HCl	pH 8,0	10 mM
	EDTA	pH 8,0	1 mM
TAE 50x buffer	Tris/Acetate	pH 8,0	2 M
	EDTA	pH 8,0	50 mM
Solution I* *(plasmid prep)*	Tris/HCl	pH 8,0	50 mM
	Glucose		50 mM
	EDTA	pH 8,0	15 mM
Solution II *(plasmid prep)*	NaOH		0,2 N
	SDS		1%
Solution III *(plasmid prep)*	K Acetate/acetic acid		$[K^+]$ = 3M
			[Acetate] = 5M
3x Lämmli buffer *(SDS-PAGE)*	Tris/HCl	pH 6,8	150 mM
	DTT		300 mM
	SDS		6% (w/v)
	Bromophenol blue		0,3% (w/v)
	Glycerol		30% (v/v)
Lysis buffer *(protein purification)*	NaH_2PO_4		50 mM
	NaCl		300 mM
	Imidazole		10 mM
	Adjust pH to 8,0 with NaOH		
Washing buffer *(protein purification)*	NaH_2PO_4		50 mM
	NaCl		300 mM
	Imidazole		20 mM
	Adjust the pH to 8,0 with NaOH		
Elution buffer *(protein purification)*	NaH_2PO_4		50 mM
	NaCl		300 mM
	Imidazole		250 mM
	Adjust the pH to 8,0 with NaOH		
Coomassie staining solution *(SDS-PAGE)*	Coomassie Brilliant Blue R-250		0,25 g
	$^{i}PrOH:H_2O$ (1:1)		90 ml
	Acetic acid glacial		10 ml

Lauria-Bertani medium (LB)*	peptone	10 g/l
	yeast extract	5 g/l
	NaCl	10 g/l
	(Agar-agar	14 g/l)
	NaOH	~0,2 ml (to bring pH to 7,0)
Terrific broth medium (TB)*	peptone	12 g/l
	yeast extract	24 g/l
	glycerol	4 ml/l
	(Agar-agar	14 g/l)
	After autoclaved add	
	0,17M KH_2PO_4, 0,72M K_2HPO_4*	100 ml/l
SOC Medium*	peptone	20 g/l
	yeast extract	5 g/l
	NaCl	0,5 g/l
	(Agar-agar	14 g/l)
	KCl 250 mM	100 ml/l
	NaOH	~0,2 ml (to bring pH to 7,0)
	After autoclave add	
	glucose 1M	200 ml/l
	Before use add	
	$MgCl_2$ 2 M	5 ml/l
10xGB1 buffer*	KH_2PO_4	136 g/l
	$(NH_4)_2SO_4$	20 g/l
	Adjust pH to 7,0	
Amino acid solution*:	L-Arginine	8,5 g/l
	L-Histidine	1,1 g/l
	L-Proline	15,3 g/l
Adenine solution° (in HCl 0,1N):	Adenine	13,6 g/l
Nutrition solution°	D-Glucose	200 g/l
	$MgSO_4$·7H2O	12,5 g/l
	$FeSO_4$·7H2O	12,5 mg/l
	Thiamine	250 mg/l
1xGB1 Minimal Media	10xGB1	100 ml/l
	Nutrient solution	20 ml/l
	Adenine solution	5 ml/l
	amino acid solution	15 ml/l
	(Agarose*	1%)

4.4 Biological systems

4.4.1 Plant material

Arabidopsis thaliana plants (ecotype Col 0) are grown on autoclaved soil and kept 3 days at 4°C in the darkness before transferring in a green chamber at 18°C under continuous light (protein extraction) or with a 12h/12h light/dark regime (metabolites quantification).

4.4.2 *E. coli* strains

The *E. coli* strains used during this work are shown in table 4-1.

Strain	Genotype	Usage
E. coli XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac- lac [F' proAB+ lacIq lacZΔM15 Tn10(Tetr)]*; (Bullock *et al.*, 1987).	Standard cloning procedure
E. coli BL21(DE3)	F- dcm ompT hsdSB (rB- mB-) gal (DE3); (Studier and Mofat, 1986).	Expression of protein
E. coli At1371	*panC4, Δ(gpt-proA)62, lacY1, tsx-29, glnV44(AS), galK2, LAM-, rac-0, hisG4(Oc), rfbD1, xylA5, mtl-1, argE3(Oc), thi-1* (Cronan *et al.*, 1982)	Complementation experiments

Table 4-1: *E. coli* strains used.

4.4.3 Oligonucleotides

The oligonucleotides used as primers in PCR reaction for cloning procedures are shown in table 4-2. The blue letters represent silent changes, for adaptation to *E. coli* codon bias. In some cases, the primers were designed with the addition, at the 5'-end, of the ribosomal binding site. This was necessary for the usage of the specific plasmid in complementation experiments.

The name of the restriction enzyme used to cut the gene is shown in the table.

Name	Sequence 5'→ 3'
MJ PS-pBKS-FOR	XhoI cgcgg ctcgag aa gga gat ata cat atg ttg tcg gtg atg aga atg cag
MJ PS-pBKS-REV	BamHI gcgc ggatcc ta taa ttc atc taa gct taa att
MJ PK-pET-FOR (N-terminal His-tag)	NdeI gcgcg catatg ttt gct cca ggg cac ata aca gga t
MJ PK-pET-REV (N-terminal His-tag)	HindIII gcgcg aagctt tca gta ata aat att aca aac tat tgg a
MM PS-pET-FOR (N-terminal His-tag)	NdeI gcgcg catatg acc gat att ccg cac gat cac ccg cgc tac gaa tcc
MM PS-pET-REV (N-terminal His-tag)	XhoI gcgcg ctcgag tta gta gcc ggt ttc cgc ggc cat ggt
MM PSc-pGEM-FOR (complementation)	gcgcg agaaggaga tat acc atg acc gat att ccg cac gat cac ccg cgc
MM PSc-pGEM-REV (complementation)	gcgcg ctc gag tta gta gcc ggt ttc cgc ggc cat ggt
MM PK1-pET-FOR (C-terminal His-tag)	NcoI gcgcg ccatgg tg tat aca tac gag agt gaa ggg gca ga
MM PK1-pET-REV (C-terminal His-tag)	HindIII gcgcg aag ctt gtc ata gag taa tct ggg tat gca ggt g
MM PK2-pET-FOR (N-terminal His-tag)	NdeI gcgcg catatg tat acc tac gag agc gaa ggc gcg gat ttt ttc gca aaa gcg tat gct cca ggg cat
MM PK2-pET-REV (N-terminal His-tag)	HindIII gcgcg aagctt a gtc ata cag taa gcg ggg tat gca ggt gct
MM CoaBC-pET-FOR (N-terminal His-tag)	NdeI gcgcg catatg aaa gag ccg caa agt aag aag gat
MM CoaBC-pET-REV (N-terminal His-tag)	EcoRI gcgcg gaattc tta cag aac ctg gcg tga att taa aat

SS PS-pET-FOR	NcoI gcgc ccatgg at aaa gcg cag gat agc aaa ccg tgg agc att cgt gat ctg atc ccg gaa aac cat ccg cgc cgt gaa tcc ctg ctg atc cgc gaa aag ttg
SS PS –pET-REV	XhoI gcgc ctcgag tta cag gct cag aga cag ctg ggt cag gcg gtt agc gat aaa ttt cag tga ttc agc
SS PK1-pET-FOR	NcoI gcg cccatg gcg cgt ctg aaa agc cag ctg ctg ctg gtg aaa att att aat aac agc ttt att gtg ctg ggc gtg gag atc aaa gta cca att
SS PK2-pET-FOR	NcoI gcg cccatg gtg ctg ggc gtg gaa atc aaa gtg cca att tct att
SS PK1-pET-REV SS PK2-pET-REV	EcoRI XbaI gcgc gaattc tctaga tta aat cac gta tgc gcc acg gct tgc gat ctt gtg ctt tat

Table 4-2: Primers used to clone the archaeal genes. In the boxes: restriction sites: in green: ribosomal binding sites; in red: start and end codon; in blue: silent changes.

Oligonucleotides used as primers for sequencing reactions are shown in table 4-3.

Name	**Sequence 5'→3'**	**description**
T4	aat taa ccc tca cta aag gg	pBKS sequencing
T7	gta ata cga ctc act ata ggg c	pBKS, pGEM, pET sequencing
SP6	att tag gtag aca cta tag aa	pGEM sequencing
T3 univ primer	gga tcg aga tct cga tcc cgc	pET sequencing
M13	gta aaa cga cgg cca gt	pET sequencing
mm2283-int-upper	gaa cgg ggc aga agt cca	internal primer for mm2283 sequencing
mm2283-int-lower	cca gtt ccg aaa gca cag tc	internal primer for mm2283 sequencing
mm2281- int-upper (284 → 303)	ccg gag cca gga ttg aag ta	internal primer for mm2281 sequencing
mm2281-int-lower (457 → 474)	ggg gga cga cga cga cat c	internal primer for mm2281 sequencing

Table 4-3: oligonucleotides used for sequencing

4.4.4 Plasmids

The plasmids used during this work are shown in table 4-4.

Vector	Source	Selection	Organisms	Usage
pBluescript KS	Stratagene	Ampicillin	*E. coli*	standard cloning
pET 28a (+)	Stratagene	Kanamycin	*E. coli*	protein expression
pRIL	Stratagene	Chloramphenicol	*E. coli*	helper plasmid
pGEM-T easy vector	Promega	Ampicillin	*E. coli*	standard cloning

Table 4-4: Plasmids used.

4.5 Molecular biological methods

4.5.1 Isolation of plasmidic DNA

Plasmidic DNA is isolated from bacteria using the method of the alkaline lysis in combination with the detergent SDS (Birnboim and Doly, 1979).

1,5 ml of an overnight culture is centrifuged (5 min, 4000 rpm, 4°C), the supernatant removed and the pellet dissolved in 100 µl of solution I to which RNAase is added to a final concentration of 0,2 mg/ml. After complete dissolution of the pellet, 300 µl of solution II are added, the tube is inverted 6-8 times (the solution become transparent) and 200 µl of solution III are added. The tube is kept on ice 3-5 min then centrifuged (14000 rpm, 4°C, 10 min) and the supernatant moved in another clean tube in which 0,6-0,7 volumes of isopropanol are added. The tube is then again centrifuged (14000 x rpm, 4°C, 10 min), the pellet is washed with 100 µl EtOH 100% and then, after removal of EtOH and dried, the precipitated DNA is dissolved in 50 µl of TE-buffer and stored at –20°C.

4.5.2 Isolation of genomic DNA

1,5 ml of a bacterial overnight culture is centrifuged (5 min, 4000 x rpm) and the medium is completely removed. The pellet is resuspended in TE buffer (467 µl) and then 30 µl of SDS 10% and 3 µl of proteinase K 20 mg/ml are added. After 1 hour incubation at room temperature, an equal volume of phenol/chloroform (50:50) is added and mixed (2 times). The upper aqueous phases are collected and 1/10 volume of Na acetate and 0,6 volumes isopropanol are added. The DNA is then collected by centrifugation; the pellet is washed with EtOH 100% and resuspended in TE buffer.

4.5.3 PCR

The standard PCR reactions (Sambrook and Russell, 2001) are carried on in a total volume of 50 µl reactions containing 50 mM Tris-HCl pH 8,3; 10 mM KCl; 2 mM $MgCl_2$; 3 % DMSO; 1 µM each primer; 0,5 mM dNTPs and 1 unit *Taq* DNA Polymerase (Roche) or *Pfunds* polymerase. The PCR is carried on, with the standard procedure, like hot-start-PCR.
The PCR program is:

1.	95°C	5 min	
2.	94°C	1 min	←
3.	specific T	1min	40 times
4.	72°C	1min 10 sec	
5.	72°C	5 min	
6.	4°C	15’	

4.5.4 Agarose gel and DNA purification from a gel band

DNA electrophoresis is carried on using agarose gels (from 0,6 to 1,2%) to which ethidium bromide is added. Gel electrophoresis are performed using

TAE buffer as running buffer and applying a voltage of 8 V/cm. Gels are analyzed under UV light and the band of the right size is excised from the gel and purified using the GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences) according with the manifacture's protocol. The DNA is then stored at –20°C.

4.5.5 Sequencing

Sequencing procedure is based on the didesoxy method described by Sanger *et al.* (1977). The sequencing reactions are carried on using the Big-Dye® Terminator v1.1 Cycle Sequencing Kit from Applied Biosystems.

Sequencing reaction mixture:		
	Ready Reaction Premix 2,5x	2,7 µl
	Big Dye® Sequencing buffer 5x	2,6 µl
	Primer	3,2 pmol
	Plasmidic DNA	100 ng
	Water	to 20 µl.

Sequencing PCR program:

1.	96°C	1 min (denaturation)
2.	96°C	10 sec (denaturation) ←
3.	50°C	5 sec (annealing)
4.	60°C	4 min (elongation) ┘
5.	4°C	10 min.

4.5.6 Cloning

The genes of interest are cloned from the genomic DNA of the different species using specific primers (see table 4-2).

4.5.6.1 Enzymes from Methanococcus jannaschii

The genes encoding for both the MjPS (*mj0209)* and the MjPanK (*mj0969*) were cloned from genomic DNA by PCR reaction using *Pfu*-polymerase. The

primers are designed in such a way that the ribosomal binding site is added at the 5'-end of the gene. The PCR product was then ligated into the pBKS vector, and amplified. The orientation of the gene was then checked via restriction analysis. The construct with the right orientation was used for expression and, in case of the MjPS; complementation experiments.

Cloning procedure, purification protocol for the recombinant protein and complementation experiments were carried out by Uli Binder during his Bachelor's work.

4.5.6.2 Enzymes from Methanosarcina mazei

The three genes encoding for the MmPS (*mm2281)*, the MmPanK (*mm2282*) and for the MmPPCS/PPCDC (*mm2283*) are cloned from the genomic DNA by a PCR reaction using *Pfu*-polymerase. The PCR product were then ligated into the pBKS vector, and amplified. The genes were then cut from the pBKS vector with specific pairs of restriction enzymes and ligated in pET 28(a) vector. These constructs were then used for expression experiments

Cloning procedure and preliminary attempts for protein expression of the MmPPCS/PPCDC were developed by Annika Fischer during her Bachelor's thesis work.

For complementation experiments, the gene *mm2281* was cloned from the pET-mmPS construct and, using a completely new pair of primers, the ribosomal binding site was added at the 5'-end of the gene. The amplified fragment was then ligated in pGEM T-easy vector, the orientation of the gene checked via restriction analysis and the construct used to transform the *E. coli* strain At1371 for complementation experiments.

4.5.6.3 Enzymes from Sulfolobus solfataricus

The two genes encoding for the SsPS (*ss2399)*, the SsPanK (*ss2397*) are cloned from the genomic DNA by a PCR reaction using *Taq*-polymerase. In this case, in fact, because of the high diversity between its genome and the *E. coli*'s, a high number of divergences were introduced in the primers. In this condition, if the PCR reaction was carried on using *Pfu*-polymerase,

when the products were sequenced, they showed a very high number of mistakes due to deletion, addition and exchange of single or multiple bases in the primers part of the cloned gene. This was probably due to the high frequency of silent changes introduced, which the *Pfu*-polymerase was not able to introduce in the genes in the right way. The PCR product was then ligated into the pGEM vector, and amplified. The genes were then cut from the pGEM vector with specific pairs of restriction enzymes and ligated in pET 28(a) vector. These constructs were then used for expression experiments.
All the enzymes cloned present no His-tag.

4.6 Analytical techniques

4.6.1 TLC

TLC analyses were carried on using TLC Aluminum sheets (Silica gel 60 F_{254}) from Merck.
Reactions were stopped by addition of 2V of acetone (isotope exchange experiments, enzymatic assays on mazei enzymes) or by heating at 100°C to denaturate the enzymes. The reaction mixtures were then centrifuged 15 min at 14000 x rpm at 4 °C. 5 µl of the supernatant were then spotted on the TLC sheet.
TLC sheets were then put into a TLC chamber containing 50 ml of the right mobile phase and allowed to run till the solvent front arrived circa to 1 cm from the top of the sheet. The radioactive TLC were then developed via exposure to a phosphor screen for circa 4 hours.
The mobile phase used were the mixture Ethanol:NH_3=4:3 for a good β-Ala/PA separation and dioxane:NH_3:H_2O=9:1:4 for a good P-PA/β-Ala/P-Pantetheine separation.

4.6.2 HPLC

HPLC analysis was used to determine the products of the reaction catalyzed by MmPS. The reactions were stopped via addition of 2 volumes of acetone and kept at –20°C for at least 4 hours. The reaction mixtures were then centrifuged (14000 x rpm; 15 min; 4°C) and the supernatant collected and further centrifuged on a Vivaspin filter for proteins. The filtrates were then collected and 100 µl each injected into the HPLC.

The column used for the analysis was the RP-18e 5 mm from Lichrospher® 100 250-4. The analysis is carried out with a flow of 1 ml/min with a gradient that started with 0% B and kept it for the next 3 minutes. The percentage of B is then brought to 10% in the next 7 minutes and to 100% in the following 5 minutes. The gradient remains at 100% B for 1 minute and, in 12 seconds it came back to 0% B and remains in this condition for the next minute. After 17 minutes the run is stopped. In this gradient A=ammonium acetate 15 mM; B= methanol

4.6.3 Affinity chromatography

The affinity chromatography was carried out to purify the *M. mazei* enzymes once they were expressed from recombinant *E. coli* cells. The purification was carried out using a Äkta instrument via injection into a monoQ column CV (column volume)=1 ml. The eluent used for the run were A= 50 mM TRIS-Cl pH 8,8 and B=50 mM TRIS-Cl pH 8,8; 1 M KCl.

The gradient used was the following: for 5 CV the percentage of solution B was maintained at 0% then, in 20 CV it was brought from 0 to 100% and maintained at 100% for further 5 CV.

4.7 Plant extracts and metabolites assays

4.7.1 Plant extracts

Plant extracts were prepared by grinding specific tissues under liquid nitrogen. The extract were then prepared by addition of the appropriate buffer (showed in table 4-5) with a ratio of 100 µl extraction buffer for every 100 mg plant material and centrifuged at 14000 x rpm for 30 min. The supernatant was then collected and stored at –70°C.

The plant tissues considered were leaves (8 days old; 3 weeks old), roots (8 days old), siliques (from the very young to the very old one, from the central stem) and seeds (mixed from all the developmental stages).

All the buffers were used with and without addition of 1x complete as proteases inhibitor and the extract was or not dialyzed against the same extraction buffer.

Extraction buffer	**Constituent**
HEPES	50 mM HEPES-KOH pH 7,6 ; 1 mM DTT
TRIS	50 mM TRIS-HCl pH 8,0 ; 1 mM DTT
MES	50 mM MES pH 6,3 ; 1mM DTT

Table 4-5: extraction buffers used for pantothenase activity tests in plant extracts.

4.7.2 Seed extracts

Plants were harvested 22 days after they started to flower. Siliques, marked with threads of different colors, were collected so that every sample was

formed by siliques of the same age. Siliques were then completely dehydrated and then opened to collect the seeds.

To 20 mg of seeds are then add 200 µl of TCA 16% and. The seeds are then extracted using a micropestel and the centrifuged (14000 x rpm, 4°C, 20') to remove the debris. The extract is spotted on a small piece of parafilm to remove the oil, and collected.

4.7.2.1 Extracts for CoA quantification

To half of the seed extract collected, as described in 4.7.2, an equal volume of 1M TRIS solution (which pH was not adjusted) was added to bring the pH to neutrality, the extract aliquoted in 30 µl aliquots and stored at –70°C.

4.7.2.2 Extracts for PA quantification

To half of the seed extract collected, as described in 4.7.2, an equal volume of NaAc solution (1 ml NaAc 4M; 1,3 ml NaOH 5M; 7,7 ml H_2O) was added to bring the pH to circa 6, together with 25 µl of a solution containing 5,22 µg/ml (21,9 µM) of an isotopomer of PA. Extracts were then aliquoted in 2x100 µl aliquots and stored at –70°C.

4.7.3 CoA quantification

CoA quantification is carried on with a cyclic assay like described by Allred *et al.* (1969) with some modifications.

<u>*Materials:*</u>

Spectrometer

1 ml plastic cuvette

Reaction mixture A (500 µl) (4°C):	Tris-HCl	pH 7,4	24 mM
	Malate		100 mM
	Acetylphosphate		4,5 mM
	NAD		1,4 mM

Reaction mixture B (300 µl) (4°C):	PTA	10 units/300 µl
	CS	8 units/300 µl
	MDH	20 units/300 µl

Plant extract

Procedure

To 500 µl reaction mixture A add 25 µl plant extract and 175 µl H_2O. Equilibrate at RT for 5 min. Add 300 µl reaction mixture B. Wait 5 min at RT and follow the increasing in absorbance at 340 nm for 20 min.

4.8 Complementation experiments

The *E. coli* mutant At1371 was transformed with the recombinant plasmid carrying the pantothenate synthetase genes from *M. jannaschii* (pBKS) and *M. mazei* (pGEM).

4.8.1 Complementation experiments on solidified media

A 5 ml culture of transformed bacteria was grown overnight in LB media + Amp100.

The day after the culture was harvested and bacteria were washed twice with 1xGB1 buffer. For complementation with MjPS, the cells suspensions were diluted to OD_{600} values of 0,2; 0,02; 0,002 and 1 µl each suspension was plated on one DYT plate (control), one GB1 plate and GB1 plates added with PA (1 mM), pantoate (10 mM), pantoyl lactone (PL; 10mM) or β-Ala (10 mM). After 24h incubation at 37°C, there were on the DYT plate clear colonies and the plate was photographed. On the GB1 plates no colonies were visible, so they were kept another 24h at 37°C.

For complementation with the MmPS, the OD_{600} was measured and the appropriate volume of 1xGB1 buffer was added to bring the OD_{600}=0,3. Bacteria were then added to 10% glycerol, aliquoted (500 µl each) and stored

at –70°C. Known amount of bacteria were then spotted on different GB1 minimal media plates, containing several addition substances (see table 4-6). In both cases the plates were photographed after 24h and 48h.

	Construct transformed	**Growth conditions**
liquid culture	pBKS-EcPS pBKS empty pGEM-MmPS	GB1 minimal plate GB1 minimal plate + 1 mM PA GB1 minimal plate + 1 mM pantoate GB1 minimal plate + 5 mM pantoate GB1 minimal plate + 10 mM pantoate; GB1 minimal plate + 20 mM pantoate; GB1 minimal plate + 50 mM pantoate
solid culture	pBKS-EcPS pBKS empty pBKS-MjPS	DYT plate GB1 minimal plate GB1 minimal plate + 10 mM β-ala GB1 minimal plate +10 mM pantoyl lactone GB1 minimal plate + 10 mM pantoate GB1 minimal plate + 1 mM PA
solid culture	pBKS-EcPS pBKS empty pBKS-MmPS	LB plate GB1 minimal plate GB1 minimal plate + 1 mM PA GB1 minimal plate +10 mM pantoyl lactone GB1 minimal plate + 1 mM pantoate GB1 minimal plate + 10 mM pantoate GB1 minimal plate + 100 mM pantoate

Table 4-6: complementation scheme with constructs and additional substances

4.8.2 Complementation experiments in liquid culture

A 5 ml culture of transformed bacteria was grown overnight in LB media + Amp100.

The day after the culture was harvested, and bacteria were washed twice with 1 volume 1xGB1 buffer each. The OD_{600} was measured and the appropriate volume of 1xGB1 buffer was added to bring the OD_{600}=0,3.

Bacteria were then added to 10% glycerol, aliquoted (500 μl each) and stored at –70°C.

Different liquid cultures were then prepared inoculating 20 ml of GB1 minimal media with 100 ml IPTG and a certain amount of transformed bacteria so that every flask had the same amount of bacteria (based on OD_{600}). The flasks were kept at 37°C and shacked at 120 rpm and 1 ml aliquots were taken every hour to follow the growth of bacteria measuring the OD_{600}.

4.9 Protein techniques

4.9.1 Protein expression

50 ml liquid culture of *E. coli* cells transformed with the vector carrying the gene of interest was growth at 37°C until the culture reaches a OD_{600}=0,3-0,6. The overexpression of the protein is then induced by adding IPTG to a final concentration of 1 mM. The culture is then allowed to grow other 16-48h and harvested.

gene	protein	plasmid	antibiotics	media	temperature
mm2281	MmPS	pET	KM50	LB	37°C
mm2282	MmPK	pET	KM50	TB	20°C
mm2283	MmCoaBC	pET	KM50	LB	37°C
ss2399	SsPS	pET	KM50	SOC	20°C
ss2397	SsPK1	pET	KM50	SOC	20°C
ss2397	SsPK2	pET	KM50	SOC	20°C
mj0209	MjPS	pBKS	Amp100	LB	37°C
mj0969	MjPK	pBKS	Amp100	LB	20°C

Table 4-7: *E. coli* strains, plasmid and conditions used for the expression of the proteins.

4.9.2 Protein purification

4.9.2.1 Protein carrying one His-tag terminal

Bacteria are harvested by centrifugation (5 min, 4°C, 4000 rpm) and the cell pellet is dissolved in 5 ml lysis buffer and sonicate (6 x 10 sec bursts, 10 sec pause, 30% amplitude). After sonication the cellular debris are removed by centrifugation (20 min, 14000 rpm, 4°C) and the supernatant is loaded on Ni-NTA Agarose column (Qiagen). Supernatant is washed with 2 x 4 ml washing buffer and then eluted with 4 x 0,5 ml elution buffer. The eluted fraction are collected, dialyzed against 2 x 100 ml dialysis buffer at 4°C and stored at –70°C.

For purification of MmCoaBC, the elution steps were changed. It consisted of 2 x 0,5 ml elution buffer containing 50 mM imidazole; 2 x 0,5ml elution buffer with 100 mM imidazole; 2 x 0,5 ml elution buffer with 150 mM imidazole and 3 x 0,5ml elution buffer with 500 mM imidazole.

4.9.2.2 Protein not carrying one His-tag terminal

Bacteria are harvested by centrifugation (5 min, 4°C, 4000 rpm), the cell pellet is dissolved in lysis buffer and sonicate (6 x 10 sec bursts, 10 sec pause, 30% amplitude). After sonication the cellular debris are removed by centrifugation (20 min, 14000 rpm, 4°C) and the supernatant is heated at 80°C for 15 min, allowed to slowly cool to RT, centrifuged (14000 rpm, 20 min, 4°C) and the supernatant is collected and stored at –70°C.

4.9.3 Polyacrylamide-SDS gel electrophoresis

The separation of the proteins based on their molecular weight is carried on with a TRIS-glycine-SDS-polyacrylamide gel (Lämmli, 1970): the separation gel is 12% or 15%-SDS gel, and the stacking gel is 5%.
The gels were stained with the Coomassie staining solution.

4.10 Enzymes assays

The activity of the enzymes is tested with the addition of radioactive substrates or by injection of the reaction mixture in the HPLC. In the first case, the reaction is stopped by addition of two volumes of acetone for the complete denaturation of the enzymes. After centrifugation (14000 rpm, 4°C, 10 min) the reaction mixture is spotted on a TLC sheet and, after running, the radioactive spots are revealed by a phosphor screen analysis.

In the case of the HPLC analysis, the reactions were stopped by boiling for 5 min. The reaction mixture was then centrifuged and the supernatant centrifuged through a membrane filter (Vivaspin 6, 10 kDa, vivascience) to remove the enzymes. The clear reaction mixture is then injected in the HPLC system.

In every set of experiments is included a control reaction in which the same reaction mixture is treated exactly in the same way except that no enzymes were added.

The control enzymes EcPs and EcPanK were expressed and purified from constructs in pET28a. The EcPS was cloned by Rafał Jończyk (Dr Genschel's lab) and the EcPanK was cloned from Dr. G. D. Wright from the Antimicrobial Research Center, Department Biochemistry, Mc Master University, Ontario, Canada.

4.10.1 Pantothenase activity assay

The plant extracts were incubated at 24°C in presence of 0.1 mM [1-^{14}C]-PA. The reaction time was of 10, 30, 60, 90, 120 or 180 min. The concentration of PA varied from 0,1,2,5,30 mM. The concentration of ATP, $MgCl_2$, $CaCl_2$ was 0 or 5 mM. The constituents of the standard reaction are listed in table 4-8.

Reagents	Final concentrations
[1-^{14}C]-PA	0.5 nCi/µl
PA	5 mM
ATP	5 mM
$MgCl_2$	5 mM
$CaCl_2$	5 mM

Table 4-8: Components of the assay for pantothenase in plant extracts.

4.10.2 Assay for MmPS and MmPK activity

MmPS activity was tested in presence of EcPanK enzyme. To test different conditions, a big mixture of all the components the reaction common to all the experiments (including radioactivity) was prepared each time and splitted into different eppendorf tubes. The varying components were then added to the single tubes and the reaction started by addition of the enzymes. The reactions were carried out at 37°C, for a maximum of 3 hours.

The reaction was carried out in presence of different concentration of ATP (1; 5; 10; 20 mM), β-Ala (0.5; 1 mM), and pantoate (1; 5; 10; 20; 50 mM) and with the addition of different cations (Ca^{2+}; Ni^{2+}; Fe^{2+}).

To find the best conditions, different buffers (MES, HEPES, TRIS) were tested at different values of pH (5.5; 6.5; 7; 7.5; 8; 9).

The best conditions found for the activity tests are listed in table 4-9.

Reagents	Final concentration (TLC)	Final concentration (HPLC)
EcPanK	0. µg/µl	0.1 µg/µl
MmPS	0.1 µg/µl	0.1 µg/µl
[3-^{14}C]-β-Ala	0.004 µCi /µl	0 mM
Tris-SO_4 pH 8,0	50 mM	50 mM
$MgSO_4$	5 mM	5 mM
K_2SO_4	7.5 mM ($[K^+]$=15 mM)	7.5 mM ($[K^+]$=15 mM)
β-Ala	0.5 mM	1 mM
Pantoate	20 mM	20 mM
DTT	5 mM	5 mM
ATP	5 mM	3 mM

Table 4-9: Best condition to test MmPS activity

4.10.3 Isotope exchange experiments

Each set of experiments included a reaction in which all the components, including all the products, were presents. In all the other experiment of the set, one or the other of the components were omitted. The constituents of the standard experiment are listed in table 4-10.

Reagents	**Final concentration (TLC)**
MmPS	0,9 µg/µl
Tris-SO_4 pH 8,0	50 mM
[3-^{14}C]-β-Ala	0,004 µCi /µl
β-Ala	0,5 mM
K_2SO_4	7,5 mM ([K^+]=15 mM)
$MgSO_4$	5 mM
DTT	5 mM
Pantoate	*20 mM*
ATP	*5 mM*
ADP	*5 mM*
P_i	*5 mM*

Table 4-10: Components of the reaction mixture for isotope exchange experiments. The components in italic were omitted or replaced from AMP and PP_i in some cases

4.10.4 Assay for MmPPCS/PPCDC activity

MmPPCS/PPCDC activity was tested at 37°C in condition listed in table 4-11. The reactions were carried on in presence of [1-^{14}C]-P-PA and [^{32}P]-P-PA.

Reagents	**Final Concentration**
MmPPCS/PPCDC	1 µg/µl
TRIS-SO_4 pH 8,0	50 mM
KCl	5 mM
MgSO4	5 mM
DTT	10 mM
P-PA	1 mM
*P-PA	0,004 µCi/µl
ATP	5 mM
CTP	5 mM

Table 4-11: Components of the reaction mixture for MmCoaBC activity tests.

Part 1:

Analysis of Pantothenate and Coenzyme A levels in *Arabidopsis thaliana*

5. Aims of this project

As discussed in the introduction, PA is the fundamental precursor of CoA, a cofactor that is involved in a high number of metabolic pathways (i.e. fatty acid biosynthesis). Plants, fungi and bacteria can afford a *de novo* PA synthesis while animals introduce it with the diet.

Regulation of CoA synthesis was extensively studied in bacteria, and it was found that the most regulated enzyme in the pathway is PanK, the enzyme that catalyses the phosphorylation of PA (Leonardi *et al.*, 2005).

For what concerns the regulation of PA synthesis, earlier studies on microbes suggested that either PS or KPHMT catalyses the rate limiting steps in PA synthesis (Cronan *et al.*, 1982; Jackowski *et al.*, 1981). Other studies show that a mutation in the promoter of the *panB* gene (encoding KPHMT) results in over-expression of KPHMT and increased levels of PA and CoA (Rubio *et al.*, 2002). Furthermore it is known that bacteria can produce 40-fold more PA than is necessary for their metabolism. The excess of PA is then excreted in the culture media from which, in case of necessity, can be absorbed again. All these experimental evidences suggest that PS is not a highly regulated enzyme in bacteria and it seems that PA can be synthesized to a very high concentration. The bacterial way to keep its concentration under certain levels consists of its excretion in the culture media.

In plants, the regulation of this pathway is not clear. It is known that PS is subject to substrate inhibition (Genschel *et al.*, 1999), suggesting that this step may be regulated *in vivo.*

An alternative to a strict regulation of PS could be the presence of a new pathway to degrade exceeding amounts of PA. Such degradative pathway is known in *Pseudomonas sp.*, in which PA is degraded to β-Ala and pantoate in a reaction catalyzed by the enzyme pantothenase (Nurmikko *et al.* 1966). This enzyme was purified and characterized from *Pseudomonas fluorescens* (Airas, 1988) and *Lactobacillus plantarum* (Solberg et. al, 1979). Unfortunately, pantothenase was never sequenced, so it is not possible to find homologues in other organisms.

Earlier work (Wieland *et al.*, 1963), on the other side, described an interesting behavior of *E. coli* PS. When this enzyme is incubated with a synthetic homologue of adenoyl-pantoate in presence of PP_i, a considerable amount of ATP is detectable in the reaction mixture. Figure 5-1 shows the reaction observed.

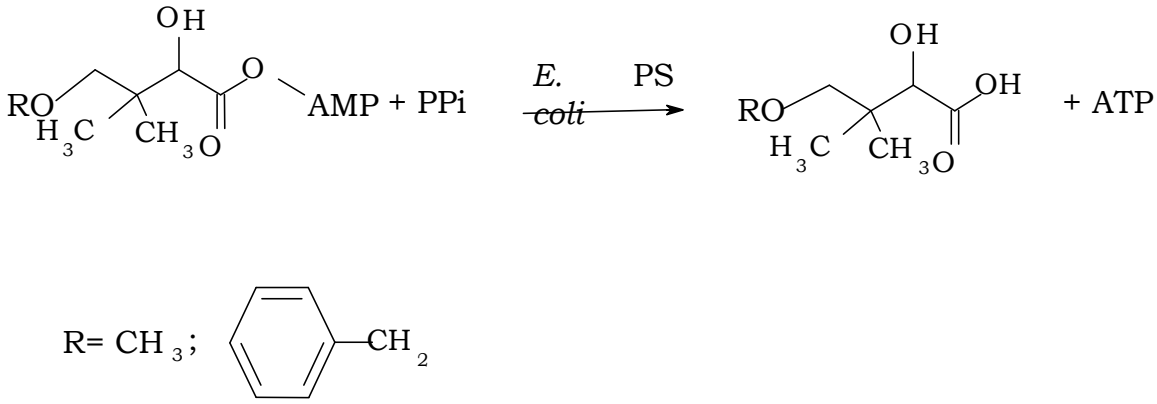

Figure 5-1: Reaction catalysed by the E. coli reaction as described by Wieland *et al.* (1963).

Because it was not possible to synthesize the pantoate adenylate, two homologues were synthesized, carrying a methylic or a benzylic group that stabilize the molecule. This experiment shows that the reaction that leads to the formation of PA and catalyzed by the *E. coli* PS is reversible, at least for what concern the first half.

The aim of this project is to find out if the PA synthesis is regulated in *A. thaliana* and, more precisely, if there is a degradative pathway for PA. The possibility of this kind of regulation was investigated in this project via two different approaches. The first one, a direct approach, consisted in incubating extracts of different plant tissues with ^{14}C-PA. Using a simple TLC system it is possible to separate PA from both β-Ala and pantoate. The appearance of spots for these two metabolites is then proof that degradation of PA is occurring.

The second approach consisted in the quantification of PA and CoA. The profiles of PA and CoA levels were obtained during seed development in order to provide hints to the degradation of PA and to test the dependency of CoA on PA.

6. Enzyme assays for pantothenase activity in *Arabidopsis thaliana*

One of the simplest possible experiments to test the presence of pantothenase activity in *A. thaliana* involves the use of radioactivity. It should suffice, in fact, to incubate the plant extracts in presence of a radioactive substrate and to follow the destiny of radioactivity in order to detect degradation of PA.

An advantage of using radiolabelled PA ([1-^{14}C]-PA; *PA) is in the possibility to run the reaction directly using the crude tissue extracts without further purification steps. In fact the only detectable substances at the end of the reaction would be the radioactive ones without visible interferences from all the other substances present in the extract.

*PA used in this experiment has the radioactive carbon on the β-Ala part of the molecule. This means that if a pantothenase activity is present some radioactive *β-Ala should be produced.

Figure 6-1 and 6-2 show two typical TLC obtained after these reactions. In both of them it is visible, in the middle, the line with the *PA reference. The identity of the spots in the TLC can be determined by comparison the *PA standard, in which a small amount of *β-Ala is present. This is due to the hydrolysis of PA that occurs even though *PA is stored at –20°C.

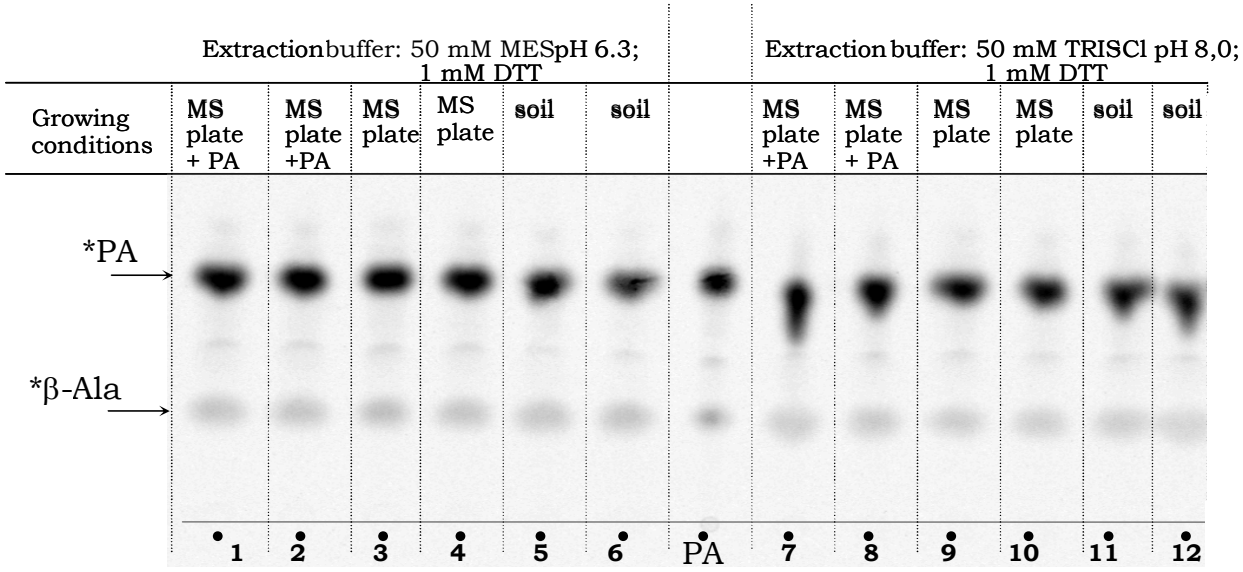

Figure 6-1: TLC of PA degradation in leaves (M.P.: $^{n}BuOH:H_2O:C_2H_5COOH=10:7:5$). Plants grew (18°C, c.l.) on MS plates were 8 days old, on soil 4 weeks old. All the extracts were incubated with 0,1 mM [1-^{14}C]-PA. In lanes 2-4-6-8-10-12 additional 5 mM PA is present. T=RT; t=3 hours.

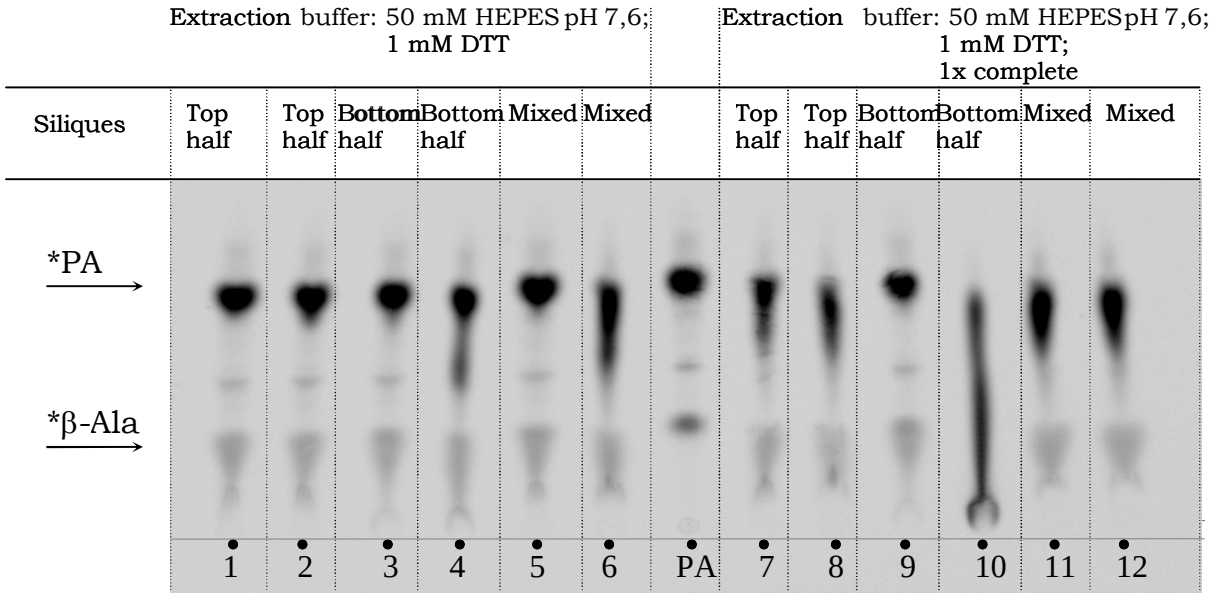

Figure 6-2: TLC of the assay for degradation of ^{14}C-PA in seeds (M.P.: $^{n}BuOH:H_2O:C_2H_5COOH=10:7:5$). T=RT; t=1h 30 min. All the extracts were incubated with 0,1 mM [1-^{14}C]-PA. In lanes 2-4-6-8-10-12 additional 5 mM PA are present. T=RT; t=1 hour 30 min.

What is clear from these TLCs is that there is no net degradation of PA, at least with the condition used for these experiments. New extracts were then prepared, using different extraction buffers (TRIS; MES; HEPES), pH (from 6,3 to 8,5), presence or absence of protease inhibitors, cations and substrates but all the attempts to find a net PA degradation were unsuccessful.

The only case in which the TLC shows the appearance of new spots is when the assay was carried out with seed extracts, like shown in figure 6-2. Unfortunately, all attempts to improve the quality of this assay failed and no clear answer came from the analysis of these TLC.

7. Quantification of CoA and PA

As showed above, the assays to test the presence of pantothenase activity in plants give no clear answer to the question if a degradation of PA is occurring.

A second approach to this problem did not involve a direct enzymatic assay but the analysis of PA and CoA levels in plant tissues. With this other approach it could be possible not only understand if a net degradation of PA is occurring, but also if there is a correlation between PA and CoA levels.

This kind of approach has been used in several other cases of metabolic studies. In particular, analysis of metabolites was very useful to understand lysine metabolism and catabolism (Galili *et al.*, 2005; Stepansky *et al.*, 2005). In these works was found, for instance, that various hormonal, metabolic and stress signals can trigger the degradation of cellular proteins, resulting in significant accumulation of free lysine.

Considering that CoA is a fundamental cofactor in fatty acid biosynthesis and that seeds have a very active lipids metabolism, developing seeds were chosen for PA and CoA quantification in this work.

7.1 Stages of seed development

The phenomenon of plant flowering and the development of seeds are very well understood in *A. thaliana*. According to Ferrándiz *et al.*, (1999) and Smyth *et al.*, (1990), 20 stages are recognized in flower development (see table 7-1). These stages start from the first appearance of the flower until the falling of the seeds from the open siliques. In this classification, stage 14 is defined as 0 hours after flowering and represents the beginning of silique and seed development.

Stage	Morphological maker	Development event
1	Flower buttress arises	
2	Flower primordium forms and separates from the inflorescence meristem	
3	Sepal primordia arise	
4	Sepals overlie flower meristem	
5	Petal and stamen primordia arise	
6	Sepals enclose bud	Gynoecial primordium arises
7	Long stamen primordia stalked at base	Gynoecial primordium elongates and develops into an open-ended cylinder
8	Locules appear in long staens	Placentae appear. Main vascular bundles differentiate
9	Petal primordia stalked at base	Ovule primordia appear. Carpel wall differentiates into exocarp, mesocarp, and endocarp. Lateral vascular bundes appear
10	Petals level with short stamens	Gynoecium closes at its upper end. Septum forms
11	Stigmatic papillae appear	Style epidermis distinct from ovary. Endocarp differentiates into two layers. Main vascular bundles branch at the tip of the gynoecium. Each ovule develops a funiculus
12	Petals level with long stamens	General growth of the structures. Transmitting tract differentiates
13	Anthesis	Pollination takes place
14	Long anthers extend above stigma	Fertilization takes place. Sutures at both sides of the replum visible
15	Stigma extend above long anthers	Xylem lignifies
16	Petals and sepals withering	General expansion
17A	All organs fall from siliques	Separation layer forms. Exocarp develops outer cuticule; enb cells develop into schlerenchyma
17B	Silique reaches its final length	Lignification of enb and separation layer cells begin
18	Siliques turn yellow	Ena disintegrates, and enb completely lignifies. Mesocarp drying out
19	Valves separates from dry siliques	
20	Seed falls	

Table 7-1: Stages of flower development in *A. thaliana*, with the morphological markers that define the beginning of each stage and the carpel development events associated. (Re-drawn from Ferrándiz *et al.*, 1999.)

Figure 7-1 shows siliques of different developmental stages. The numbers refer to the stage.

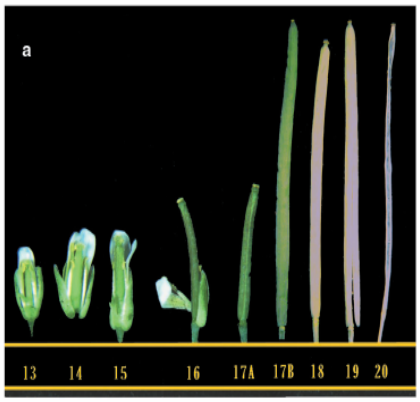

Figure 7-1: Post fertilisation stages of gynoecium development (from Ferrándiz *et al.*, 1999).

7.2 Plant materials

Two batches of *Arabidopsis thaliana* (ecotype Colombia 0) plants, of 47 and 36 plants respectively, were growth in single pots in a green chamber at 18°C under a regime 12h/12h light/darkness.

After circa 8 weeks, plants started to flower and, circa three days from the appearance of the flower, the pollination took place. From this point, the plants were checked every day and new forming siliques were marked with little colored cotton wires, as shown in figure 7-2. Different wires colors represent different days.

Siliques were then harvested and collected in different samples, each of them representing one day after flowering, frozen in liquid nitrogen and kept one week in the cool dryer, to remove completely the water. Figure 7-3 shows representative for each sample, in the developmental series.

Siliques are then opened, the seeds collected and stored at –70°C until used for quantification.

Figure 7-2: *Arabidopsis thaliana* plant on the day of harvesting.

Small aliquots of seeds, of circa 2 mg each and containing a maximum of 193 seeds and a minimum of 85, were brought slowly to room temperature and counted with the help of a binocular. These aliquots were then weighed on a precision balance. The weighing procedure was repeated at least 3 times for each sample.

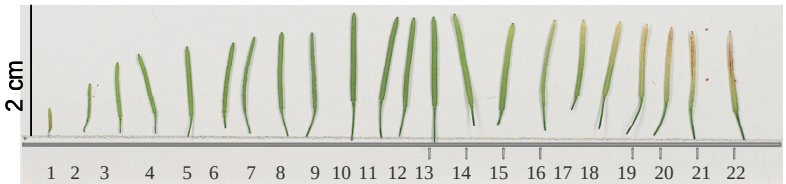

Figure 7-3: Appearance of siliques after harvesting and lyophilization. The numbers indicate the DAF.

The weight so determined was then divided by the number of seeds in the sample and the average weight per seed was obtained.

Figure 7-4 shows the increasing seed weight during the development of the seeds. The minimum error calculated on this measurement is about 0,03% and the maximum of 1,05%. The very small error bars, not really visible in the graph, show the high reproducibility of this procedure.

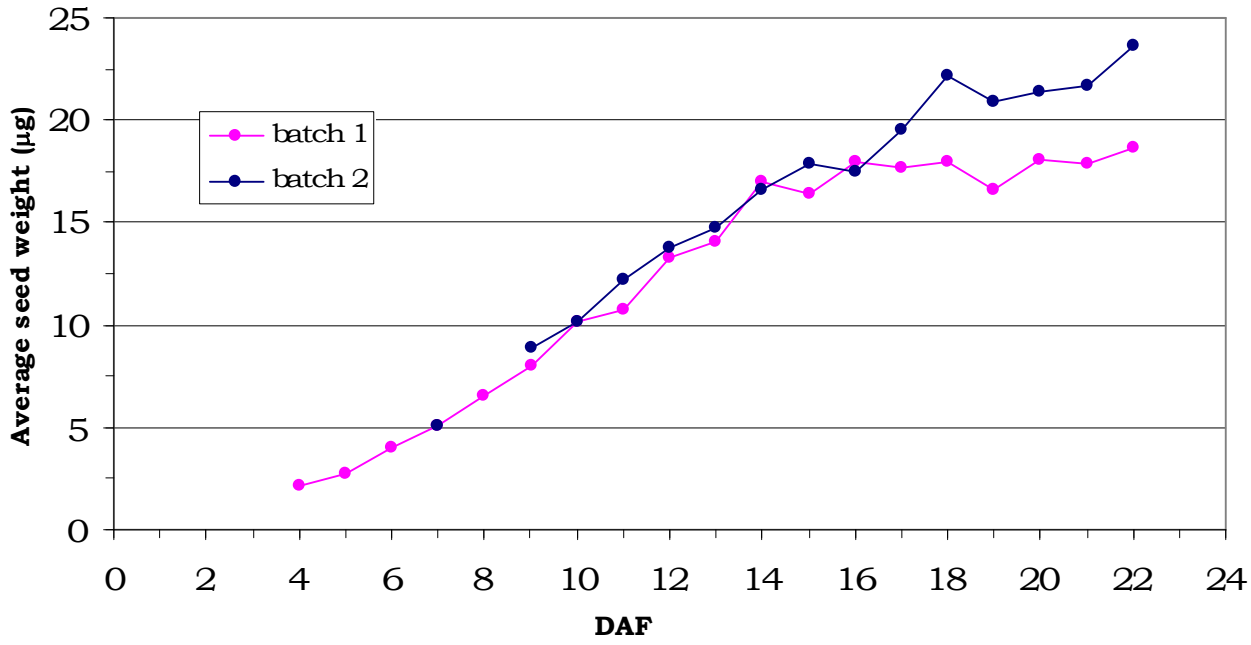

Figure 7-4: Average weight per seed determined on an average of 3 different weights each sample. The errors were between 0,0050 and 0,1458 µg/seed. The resulting error bars are smaller than the symbols in the graph.

7.3 Assays for determination of metabolites levels

The determination of PA and CoA levels was carried out with two specific assays, one for each metabolite.

The metabolites extraction was carried out with TCA 32% as described in Materials and methods (see 4.7.2). The extracts were then divided in two equivalent parts and the pH was adjusted to values close to neutrality by addition of TRIS 1M (CoA assay) or NaAc 4M (PA assay). In case of addition of TRIS, the pH reached a neutral value, in case of NaAc, the value reached was about 5,5. The necessity to bring the pH to values close to neutrality is due to the fact that at very low pH and for extended time hydrolysis of PA

occurs. In case of PA analysis NaAc was the most indicated buffer because TRIS could interfere with the LC-MS analysis.

The extracts were then aliquoted and stored at –70°C until the analyses were carried out. For each analysis, a new aliquot of extract was used.

7.3.1 Determination of PA levels

The most common method used for PA analysis was measuring turbidimetrically the growth of *Lactobacillus plantarum* in a PA deficient medium (Tanner *et al.*, 1993). This procedure presented the problem of having a very high incubation time (up to 24 h) and it was necessary to maintain sterile working conditions for a long time.

Chromatographic methods have not been found to be suitable for different reasons: HPLC suffers from the weak absorption peak of PA above 230 nm, and GC requires derivatization of PA.

The method used in this work to quantify PA in *A. thaliana* seeds is an innovative method based on a stable isotope dilution assay. This assay was established in the laboratory of Dr. Rychlik at the Institut für Lebensmittelchemie der TUM in Garching and the analysis of the seeds extracts were carried by his group.

The assay is based on the addition of an isotopomer of PA to the extract that was synthesized in Rychlik's laboratory. This PA isotopomer is exactly like the natural PA, but all the carbon atoms present on the β-Ala part of the molecule are substituted with the heavier isotope C-13 and the nitrogen atom with the N-15. The synthetic scheme is shown in figure 7-5.

The analysis consists of a LC-MS carried out with an extract including a known amount of the PA isotopomer. Analyte and Internal Standard (IS) were extracted into ethyl acetate and analyzed by LC-MS.

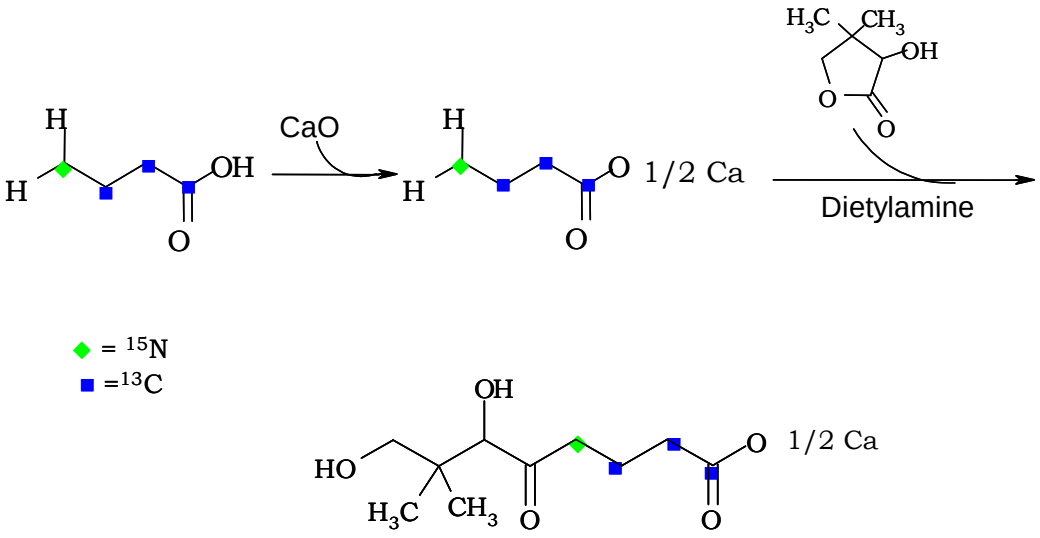

Figure 7-5: Synthetic route used in the preparation of [^{15}N,$^{13}C_3$]-PA (Re-drawn from Rychlik, 2000)

The ratio between the two peaks (*m/z* 291 and 295), specific for the natural PA and the IS respectively, is constant for the single analysis and, because the amount of the internal standard in the sample is known, it allows to determine the absolute amount of PA in the sample. This analysis exhibits excellent sensitivity and reliability and, by using the isotopomers of the analytes like internal standard, losses during clean up and extraction can be exactly corrected (Rychlik, 2000).

The determination of PA in seed extracts was repeated on developing seeds from two different batches of plants, and each extract was analyzed at least twice. Figure 7-6 shows the measured PA concentrations in nmoles of PA per gram of dry weight (A) and in pmoles of PA per seed (B).

These graphs show that the values obtained are quite reproducible: in both batches there is a decrease in PA level (nmoles/g DW) of about 20 fold from the maximum to the minimum. After 10-11 DAF, the minimum level is reached and then it remains constant during the further seed development.

Considering the pmol of PA per seed, there is not a clear trend: it seems that the content per seed remains quite constant, between 0,1-0,6 pg/seed during seed development.

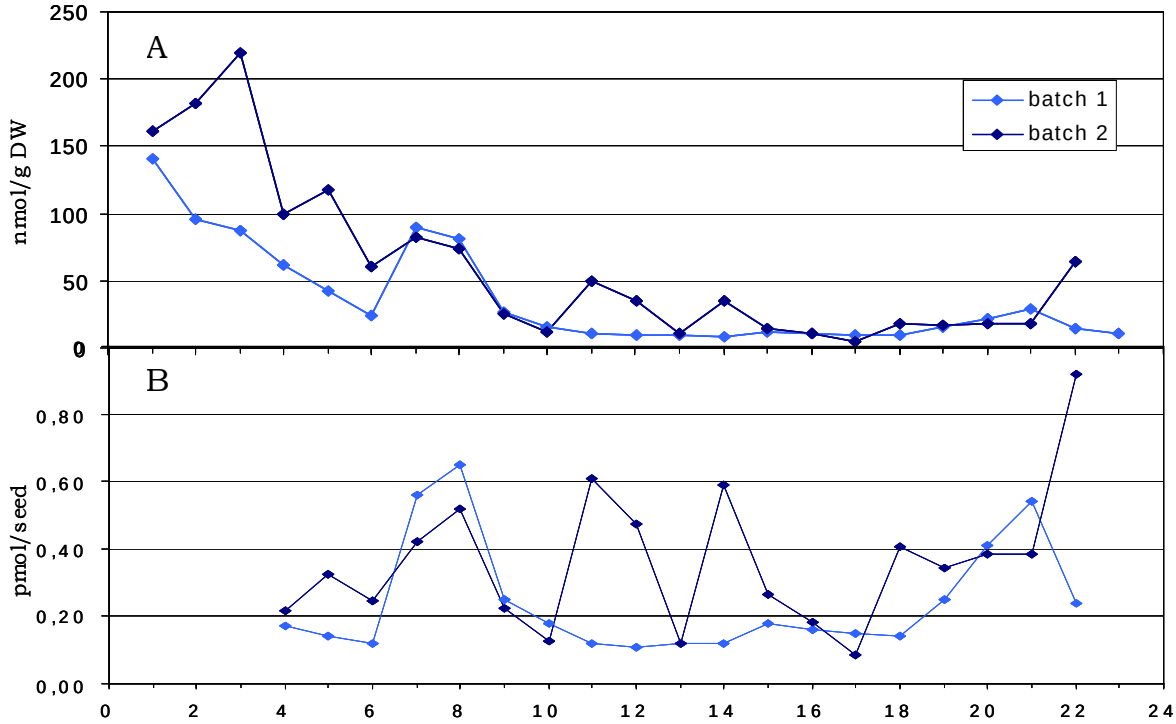

Figure 7-6: Determination of PA: amount of PA in developing seed per gram of dry weight (A) and per seed (B).

7.3.2 Determination of CoA

The method used to quantify the CoA in seed extracts (Allred *et al.*, 1969, with some modification) is based on a sequence of reactions catalyzed by coupling enzyme, as shown in figure 7-7.

The first reaction of this cycle is the acetylation of CoA, catalyzed by the enzyme phosphotransacetylase (PTA). AcetylCoA is then, in presence of oxalacetate, converted to citrate and CoA by citrate synthase (CS). The cycle will then start again. Oxalacetate is produced from malate in a reaction catalyzed by malatedehydrogenase (MDH). During this reaction a molecule of NADH is produced. The rate of production of NADH, measured by following the increase of absorbance at 340 nm, is proportional to the amount of CoA and AcCoA (acetylCoA) present in the solution. Clearly, with this method, the sum of CoA and AcCoA concentrations is determined.

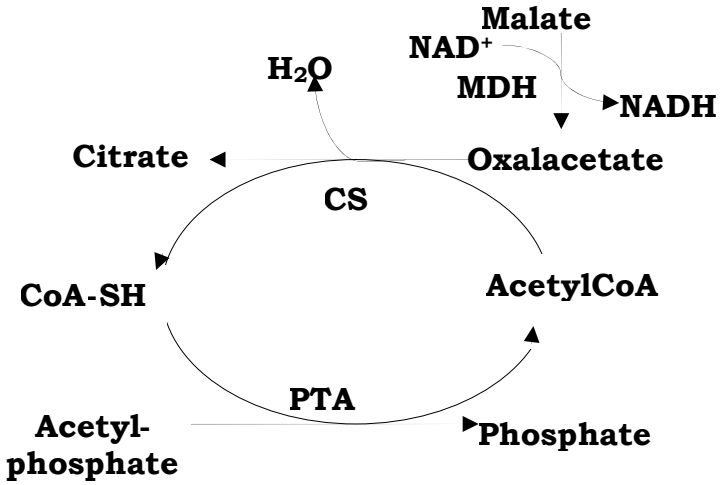

Figure 7-7: Scheme of the coupling enzyme reactions for CoA quantification.

The concentration of CoAs in the sample is obtained from a calibration curve. This is determined using known amounts of CoA and plotting the dA/min against the pmol of CoA.

An example of calibration curve is shown in Figure 7-8.

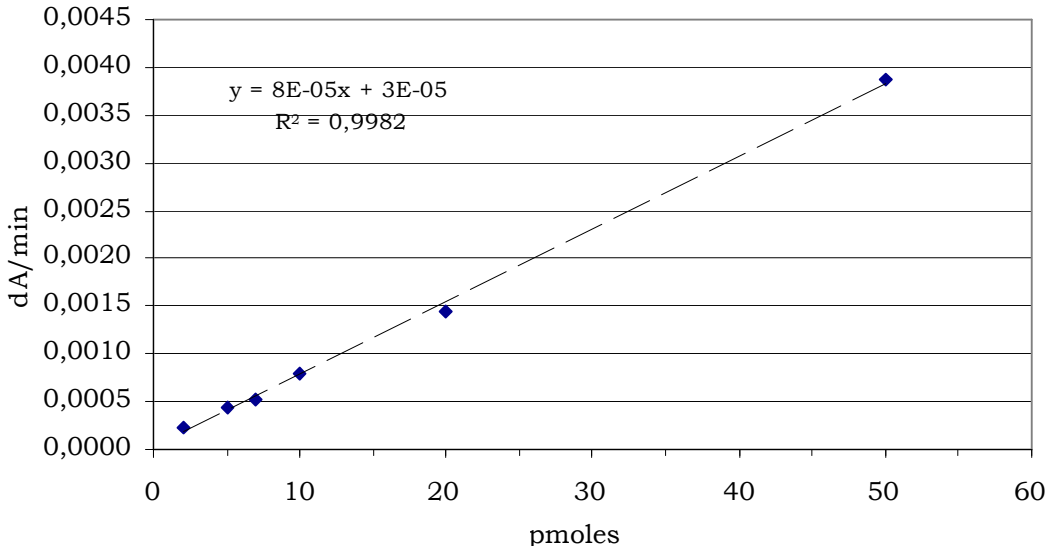

Figure 7-8: Calibration curve for CoA analysis.

The analyses used for CoA determination is based on coupling enzyme. As shown in figure 7-7 the change in absorbance is due to the production of NADH with a rate that is proportional to the amount of CoA+AcCoA in the

cuvette. It is clear that a small variation in the ratio of the three enzymes involved (CS, PTA and MDH) means variation in the calibration curve and in the sensibility of the assay. For this reason, every series of determination were carried out using a mixture of enzyme freshly prepared and kept on ice during the assay, for a maximum of three hours. A new calibration curve was determined for each enzyme mixture used for the CoA assay.

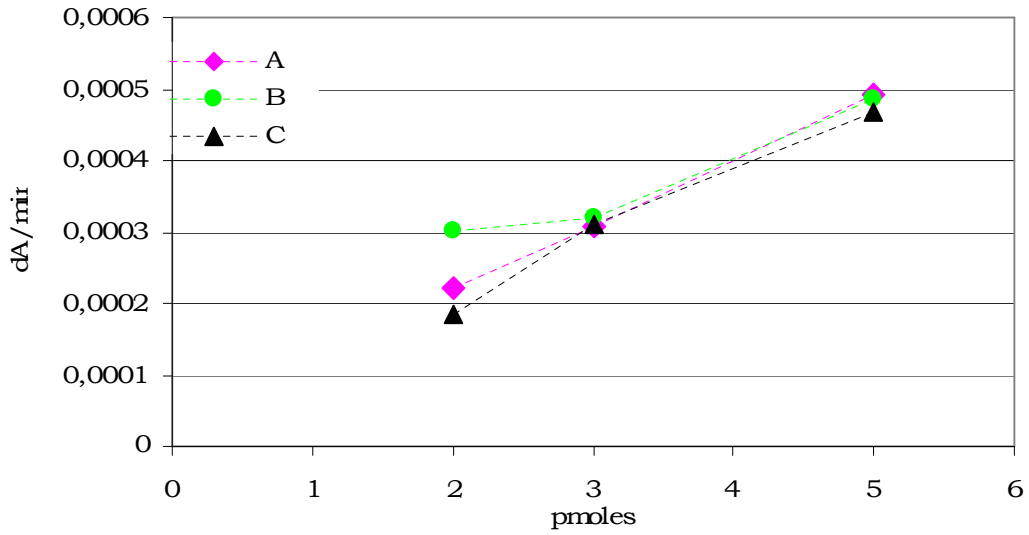

Figure 7-9: Particular of three different calibration curve obtained with different enzyme mixture. In case of curve B, 2 pmoles was taken as lower limit of detection, in case of curves A and C 2 pmoles was taken as minimum value.

As shown in figure 7-9, in which the lower values of three calibration curves are compared, calibration slopes were different for the different reaction mixtures. Furthermore, the lower limits of detectibility are different. In fact, in case of curves A and C, the linearity of the curve is maintained for very small values and 2 pmoles of CoA is taken as lower limit of detectibility. In case of curve B, the point corresponding to 2 pmoles is clearly out of the linear range of the calibration curve. In this case, 3 pmoles were taken as lower detection limit.

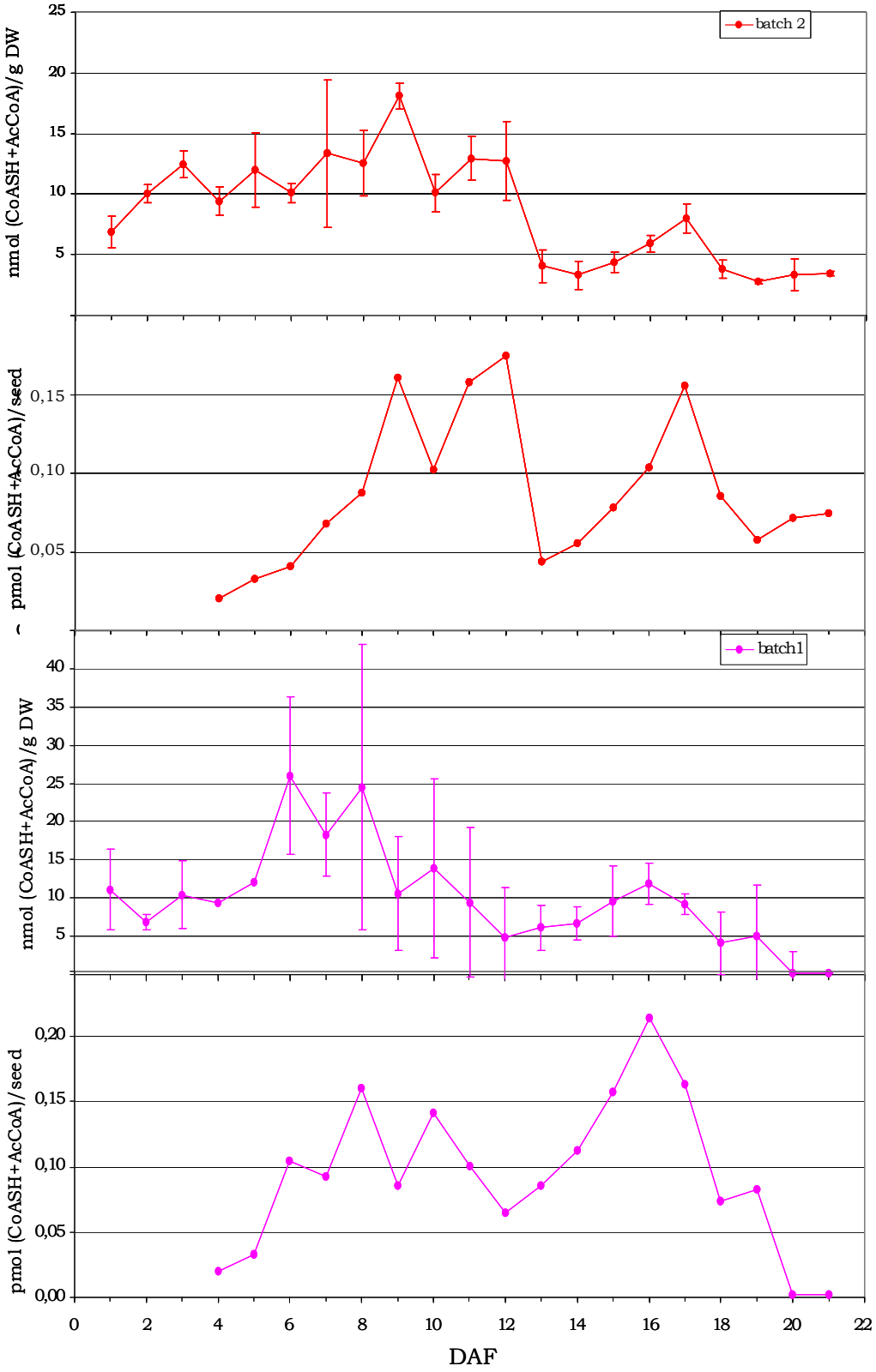

Figure 7-10: Determination of CoAs: amount of CoASH and AcCoA in developing seed per gram of dry weight (up) and per seed (low).

The quantification of CoA and AcCoA in seeds was afforded on two different batches of plants. Figure 7-10, reports the data from both batches. These graphs show that the data are in good agreement and the profiles of the curves look very similar.

Each value reported is an average of at least three determinations. The standard errors, as shown by the error bars, are large for the first batch and typically below 10% for the second batch.

The higher values of the errors for the first batch were probably due to the experimental conditions used at that time. In fact each time different amount of extracts (20,40 and 60 μl) were analyzed. After the finding that different amount of TRIS buffer in the cuvette change the absolute value of absorbance read from the spectrometer, the results of the first batch were taken as preliminary data, as hint to determine the right amount of extract to be analyzed each time (25 μl) and to establish the right procedure of analysis.

From figure 7-10 it is clear that, at the beginning of seed development, the levels of AcCoA+CoASH increase to reach a maximum of circa 25 nmol/ g DW. Around the 8^{th}-9^{th} DAF, the levels of AcCoA+CoASH decrease to reach very low values of about 3 nmol/g DW at the end of the development. Similar trend can be found when the content of CoA per seed is considered. In this second case, the CoA content reaches a maximum of about 0,15 pmol/seed in the middle of the development. After a short decreasing phase, in which a minimum of 0,05 pmol/seed is reached, CoA levels increase again and then remain relatively stable around 0,07 pmol/seed at the end of the development.

The content of CoASH+AcCoA found in developing seeds is in good accordance with what reported in literature: Gibon *et al.* (2002) report a value of AcetylCoA in *Arabidopsis* seeds of maximum 30 nmol/g DW. Furthermore, Rubio *et al.* (2006) report a content of circa 0,25 pmol/seed of acetyl CoA in *Arabidopsis* seedlings.

7.3.3 Comparison between CoA and PA levels

The data collected for PA and CoA were then put together, to find a relation between these two metabolites. In figure 7-11 the relative behavior of the two metabolites are shown.

It is clear that, first of all, the PA levels remain nearly always higher than the AcCoA+CoASH levels. This could mean that the regulation of PA levels is independent from the CoA levels. This is shown in part A of the graph, which shows the concentration of the two metabolites per gram of DW.

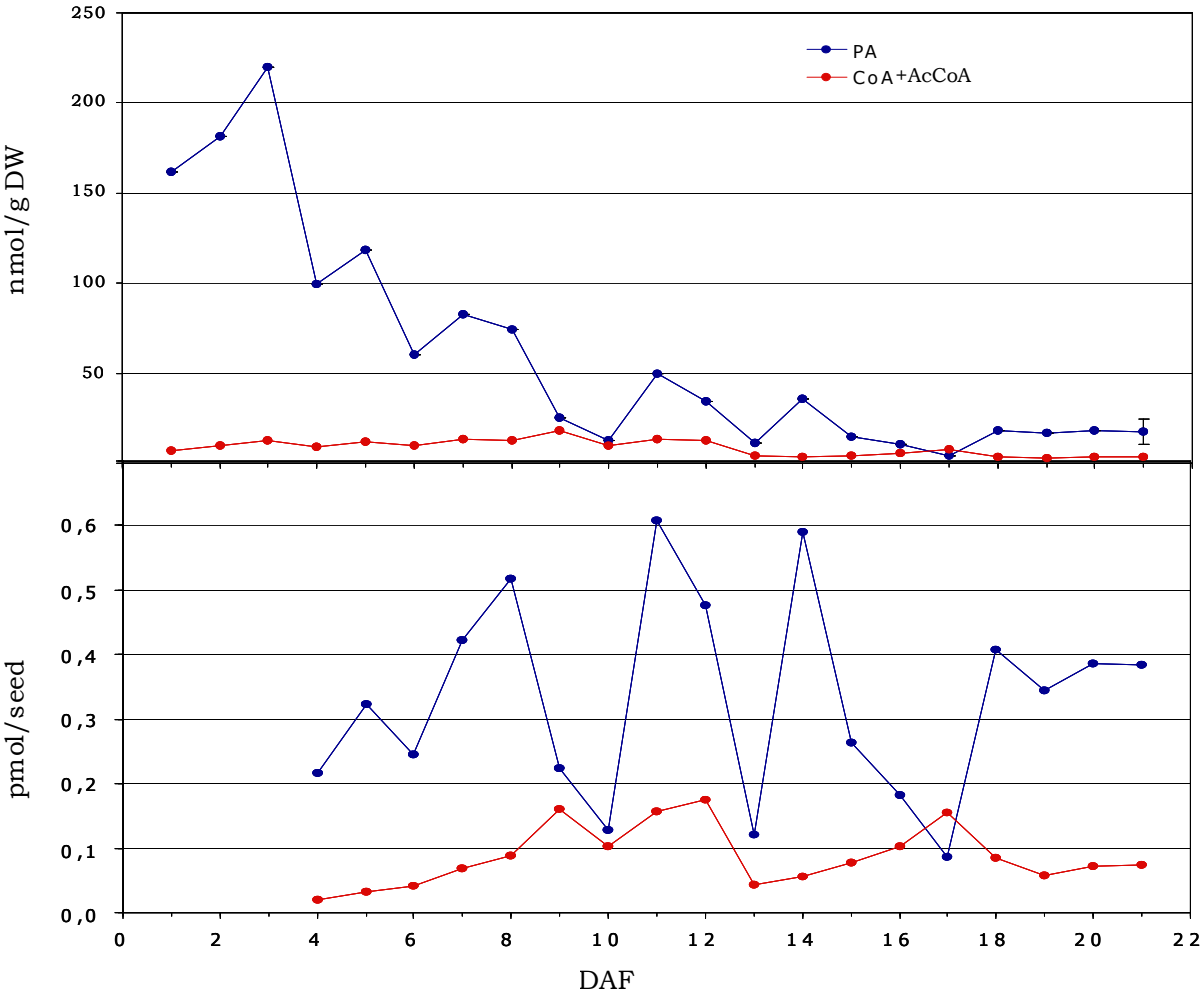

Figure 7-11: Comparison between CoA and PA levels in developing seeds expressed in nmol/ g DW (A) and pmoles/seed (B).

Another representation of the data in figure 7-11 is shown in figure 7-12, where the ratio between the two concentrations during the seed development

is plotted. From this figure, it is clear that the ratio continues to change throughout the entire development without a clear trend, and also that it is always higher than 1. This means that PA is, in every case, in excess.

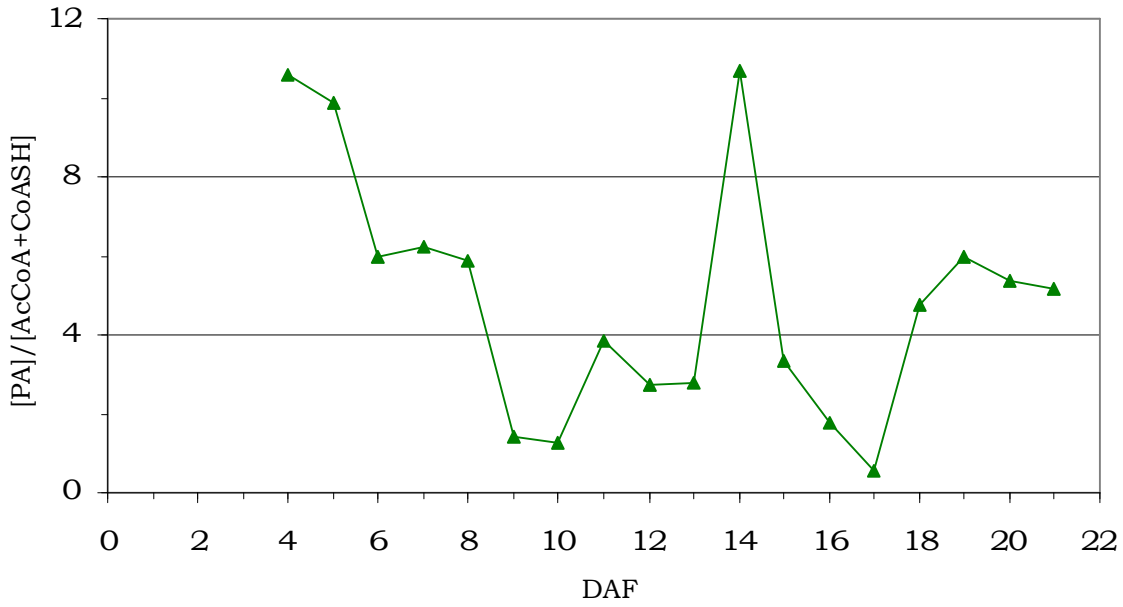

Figure 7-12: Ratio [PA]/[CoA] during the seed development.

The finding that PA accumulates to higher levels respect to CoA is consistent with PanK as the major regulatory enzyme of CoA biosynthesis. PanK catalyzes the phosphorylation of PA to P-PA that represents the key intermediate in the synthesis of CoA. An accumulation of PA suggests that its phosphorylation is the low-rate step in the pathway.

Another important comment that can be made on the figure 7-11 is that the profile of the two metabolite concentrations provides no direct evidences for the occurrence of PA degradation. In fact, looking at the PA content per seed, it seems that the amount of PA per seed increase, even in the early stages. Thereafter, PA levels drastically decreased (from 0,5 to 0,1 pmol/seed) while, on the other side, CoA levels increased to reach the maximum circa at 9 DAF. However, the increasing in CoA levels is not enough to explain the drastic decreasing of PA. This could suggest that, at least at the beginning of the development, a net synthesis of PA is occurring. In the middle and later developmental stages the amount of PA per g DW is then decreasing to the minimum level (~ 5 nmol/g DW). This decreasing of

PA levels could be explained or by net degradation or by conversion of PA in the other intermediate of the pathway (as phosphopantetheine or dephosphoCoA). In fact, the decreasing of PA levels at early stages can also be explained by CoA synthesis, increasing of biomass or by its degradation.

8. Discussion

As described at the beginning of this section, two different strategies were used to detect if some mechanism for degradation of pantothenate is present in plants. The direct strategy, that involves enzymatic tests carried out on specific plants extract, did not produce evidence of a net PA degradation.

The second method used in this work was based on the quantification of metabolites. In this way, from the analysis of PA and CoA levels, informations on PA regulation could be achieved.

Analyses for PA, CoA and AcylCoA are known from literature and it was described, at least for bacteria, that the sum of the CoA and AcetylCoA was close to 90% of the total amount of Acyl-CoA (Jackowski, 1986). For this reason, the decision to quantify the content in CoA and AcetylCoA, without considering the longer-chain CoAs, was taken. More recent papers (Rubio *et al.*, 2006; Germain *et al.*, 2001) show a profile of total acylCoA in *A. thaliana* that confirm that AcetylCoA is the most abundant, but the long-chain acylCoAs are not always negligible. As shown in figure 8-1 in fact, the AcetylCoA is the most abundant of all the different CoA species present in *A. thaliana* wild type but other long-chain Acyl-CoAs are present in amount that are, in some case, confrontable with AcCoA. This suggests that if it is true that CoASH+AcCoA represents the major component of the CoAs pool, the sum of all the long-chain AcylCoAs is not always negligible respect to the CoASH+AcCoA.

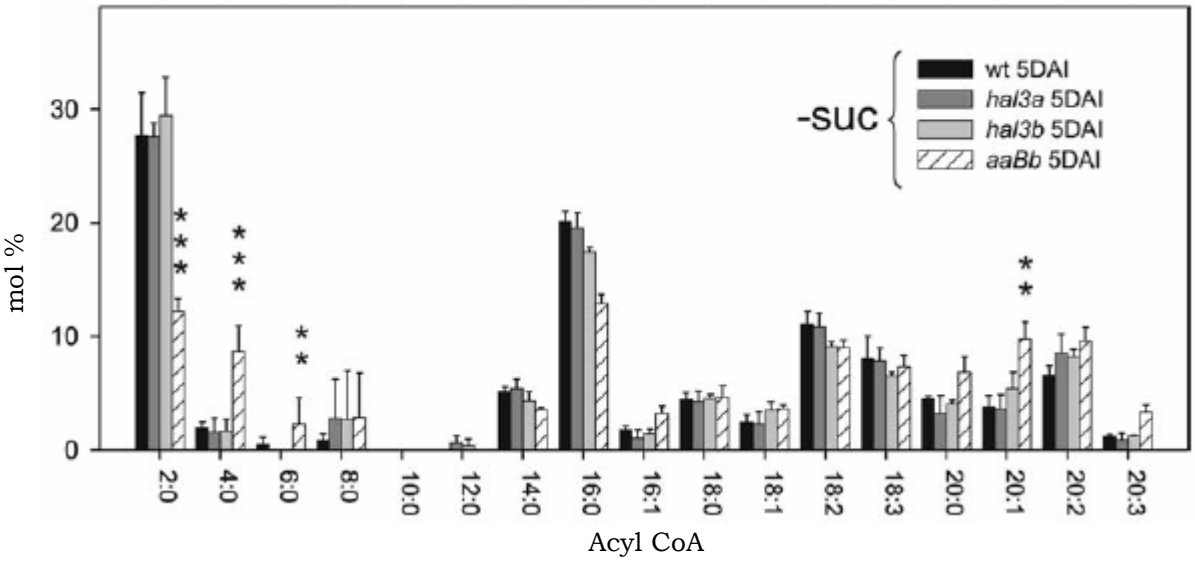

Figure 8-1: profile of AcCoA as reported by Rubio *et al.*, (2006).

The results of the investigation on the concentration of PA and CoA in developing seeds, showed in figure 7-11, suggest that there is not evident correlation between them. From a further analysis, is possible to find some trend, but only for the very young and the very old seed (till the 8th-9th DAF and again from the 17th-18th DAF). Considering the data expressed in nmoles/g DW, it is clear that for early developmental stages the amount of PA goes down while the amount of CoA+AcCoA goes up. At the 11th DAF the two levels are very similar. Unfortunately, the absolute decrease of PA is not explainable just with its conversion in CoA, which levels remain at very low concentration in every case (about 20 nmoles/ gDW). For old seeds, from the 18th DAF till maturity, the shape of the curve is the opposite: the level of PA goes up while the level of CoA approaches 0 (in some case they are below the lower limit of detectibility). In the middle of the development of the seeds, the levels of the two metabolites oscillate and apparently there is no relation between them.

These results could be explained assuming that there is not a net degradation of PA but, on the contrary, that PA is synthesized to adjust its level to the requirement of the plant tissue for the behavior of the curves at the latest developmental stages. On the other side, the founding that PA levels are varying from 0,6 to 0,1 pmol/seed while CoASH+AcCoA are always comprise between 0,18 and 0,02 could suggest the presence of a mechanism

for PA degradation. A further explanation for this founding could be that this "disappearing" PA is converted in CoA and then acylated and used, for instance, to build ACP for fatty acid biosynthesis.

A partial confirmation of this hypothesis comes from the analysis of the expression levels of the enzymes involved in CoA biosynthesis, like listed by the Genevestigator®; database (Zimmermann *et al.*, 2004)

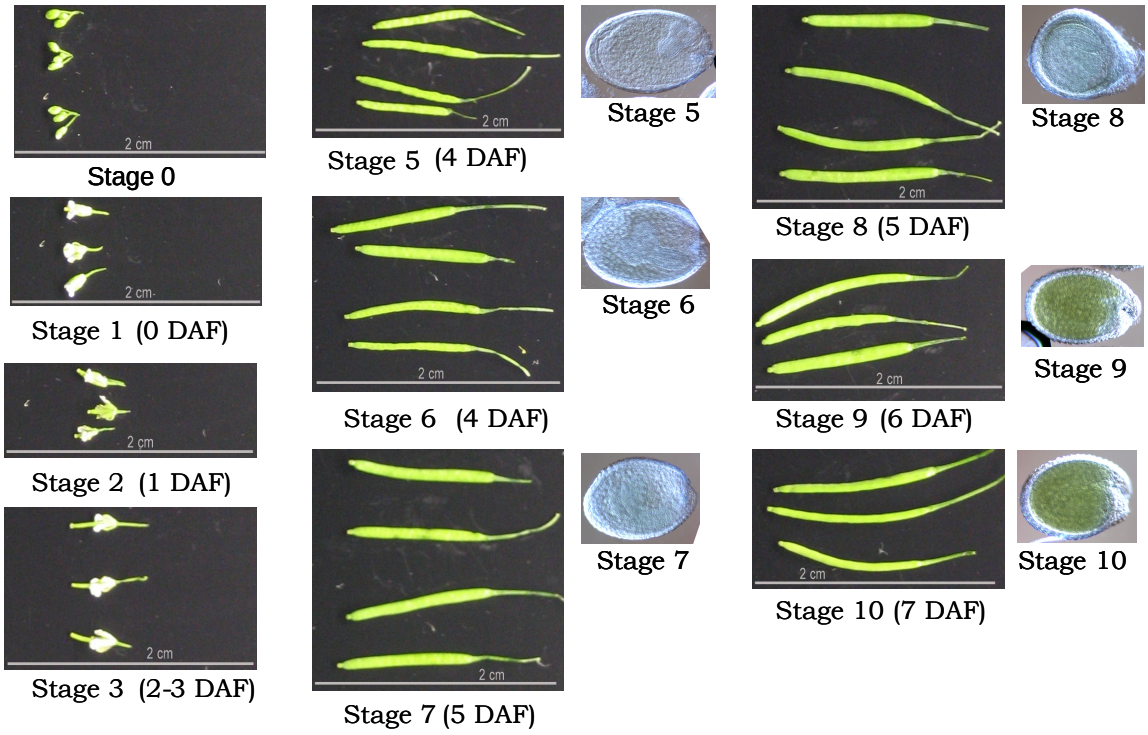

Figure 8-2: Appearance of seeds and siliques analysed for gene expression levels. The stage indicated refers to the GeneVestigator databases. In brackets, the corresponding DAF as used in this work.

In this database, it is possible to find the expression values of all the genes involved in CoA biosynthesis in specific tissues. Of major interest for this work were the values from developing seeds. Unfortunately, in the database, another classification for the developing seeds stages is used but, as reported in figure 8-2, they represent what I defined 0-7 DAF.

Figure 8-3 shows the expression profile for the genes encoding the enzymes involved in CoA synthesis. (To compare the stages with the DAF, refer to figure 8-2).

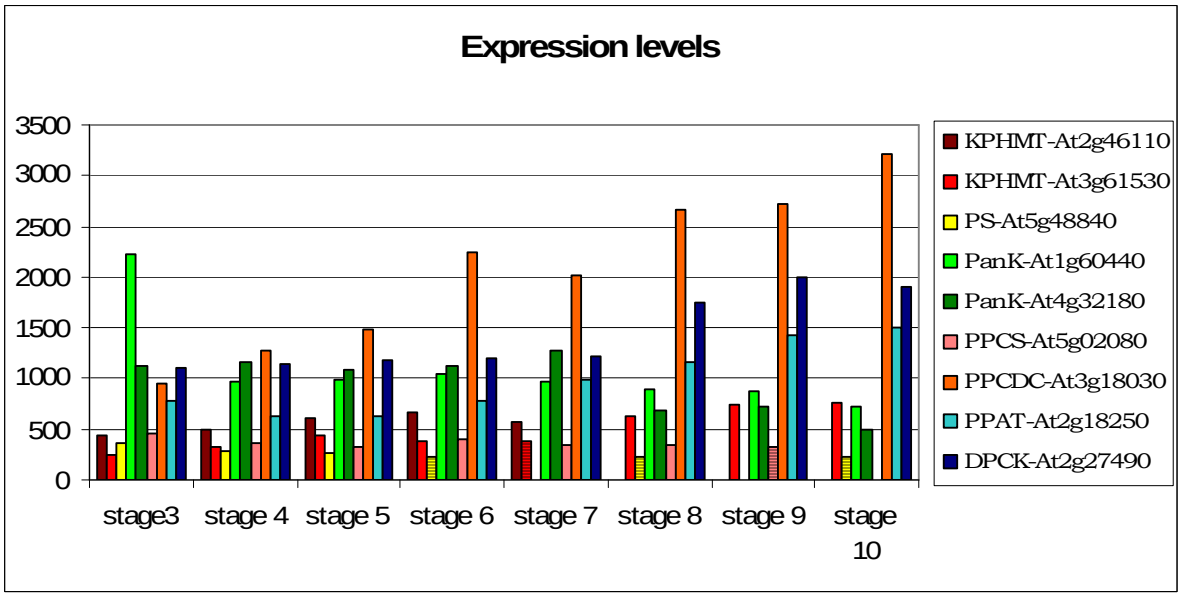

Figure 8-3: Expression level of enzymes involved in PA and CoA biosynthesis. Values obtained from an average of three values, one of which designed as not significant, are reported as striped rectangles. The background was subtracted to all the values.

From this data some interesting observation can be done. First of all, it seems that the enzymes involved in PA synthesis (KPHMT and PS) are expressed at very low level if compared with all the others. Furthermore, their expression levels seems to decrease during seed development: the gene At2g46110 encoding one of the two KPHMT is not expressed anymore after the 7th stage and the values collected for PS become less significant with the proceeding of the stage. Considering the expression levels of the *PanK* gene it appears to be the most expressed gene of this metabolism at the very early stage. With the development of the seed, its expression level decreases but in every case remains detectable. This could mean that at the beginning of the seeds development, the phosphorylation of PA occurs very fast and great amount of P-PA are produced. This one is then converted into CoA by the other enzymes in the pathway.

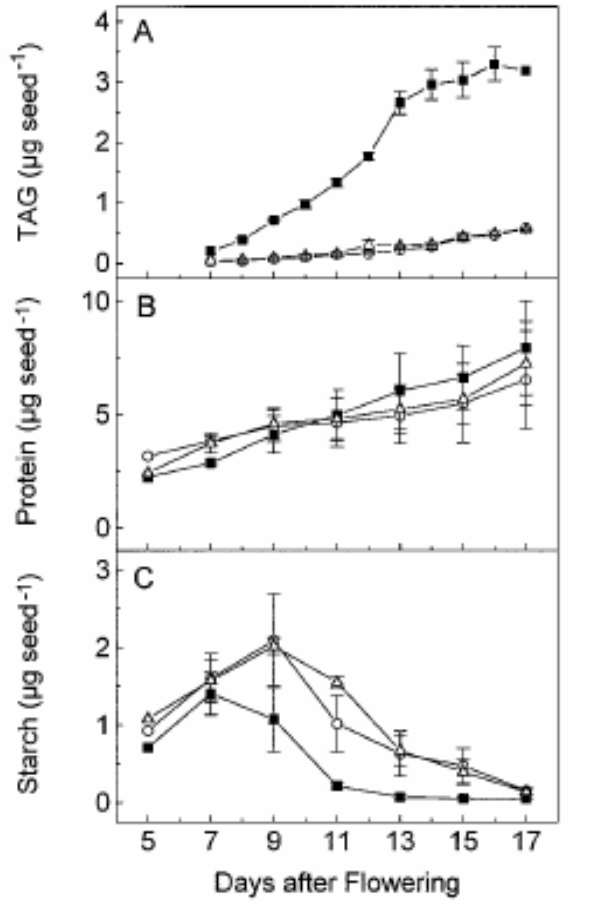

Figure 8-4: Time courses of triacylglycerol (TAG), protein, and carbohydrate accumulation in developing seeds of the wild type (■), wri1–1 (○), and wri1–2 (∆) mutant. A, Triacylglycerols; B, total protein; C, starch (from Focks, 1998)

These observations are in agreement with the hypothesis that CoA is not accumulating but used for the synthesis of ACP involved in lipid metabolism. As shown in figure 8-4 (A) the fatty acid biosynthesis starts at around the 8th DAF (Focks, 1998) and is very active until the 16th DAF. During this time, probably new CoA is synthesized and immediately converted in acylCoA. When the fatty acid biosynthesis finishes, a degradation of CoA can occur, to keep the CoA levels under certain limits. This degradation could bring to an increase in PA level, which could be enough to explain the increasing in PA concentration observed for very old seeds.

The conversion of CoA into acylCoA and ACP seems to agree with the hypothesis that PA is synthesized at the beginning of the development of seeds to high levels. While the growth of the seed continues, PA is then used to support CoA production.

Unfortunately, it was not possible to find expression levels data for older developing seeds, so nothing can be said to further demonstrate the hypothesis of a net synthesis of PA to maintain its concentration in accordance with tissue and CoA metabolism requirements.

Part 2.

Characterization of Archaeal enzymes for the synthesis of 4’-phosphopantetheine

9. Theoretical basis of this project

As discussed before, the pathway for the *de novo* synthesis of CoA is present in all organisms. This pathway is well known and all the enzymes are identified and characterized in bacteria (Leonardi *et al.*, 2005).

Genes encoding for the enzymes involved in this pathway were identified in most bacteria and eukarya. In figure 9-1 the phylogenetic profile for the 9 bacterial and eukaryotic enzymes of the pathway is shown.

As is clear from the picture, the enzymes are quite conserved across the 20 bacterial genomes considered. Furthermore, the bacterial genomes contain homologues to PPCDC and to the DPCK domain of human CoA synthetase. There are no highly conserved bacterial homologues to human PanK, PPCS and PPAT.

When homologues of these genes are searched in the archaeal genomes no obvious homologues can be found (with the exception of homologues to the *E. coli* KPHMT and to the two domains PPCS and PPCDC of the *E. coli* bifunctional enzyme PPCS/PPCDC). The fusion of PPCS and PPCDC in a biunctional enzyme is conserved in most bacteria and all archaea. Most archaea also contain obvious homologues to the human PPAT domain but not to the *E.coli* PPAT. However, conserved genes encoding ADC, PS, PanK and DPCK are generally lacking in archaea. Moreover, methanogenic archaea lack KPHMT and KPR (Genschel, 2004).

The presence of homologues to PPCDC, PPCS and PPAT in all the archaeal genomes analyzed and the presence of homologues to KPHMT in some of them is a hint that at least a subset of archaeal species should be able to convert pantoate into P-PA. The fact that it is not possible to determine homologues of all the genes involved in this pathway could simply mean that unrelated genes encode the enzymes required for the complete synthesis of CoA.

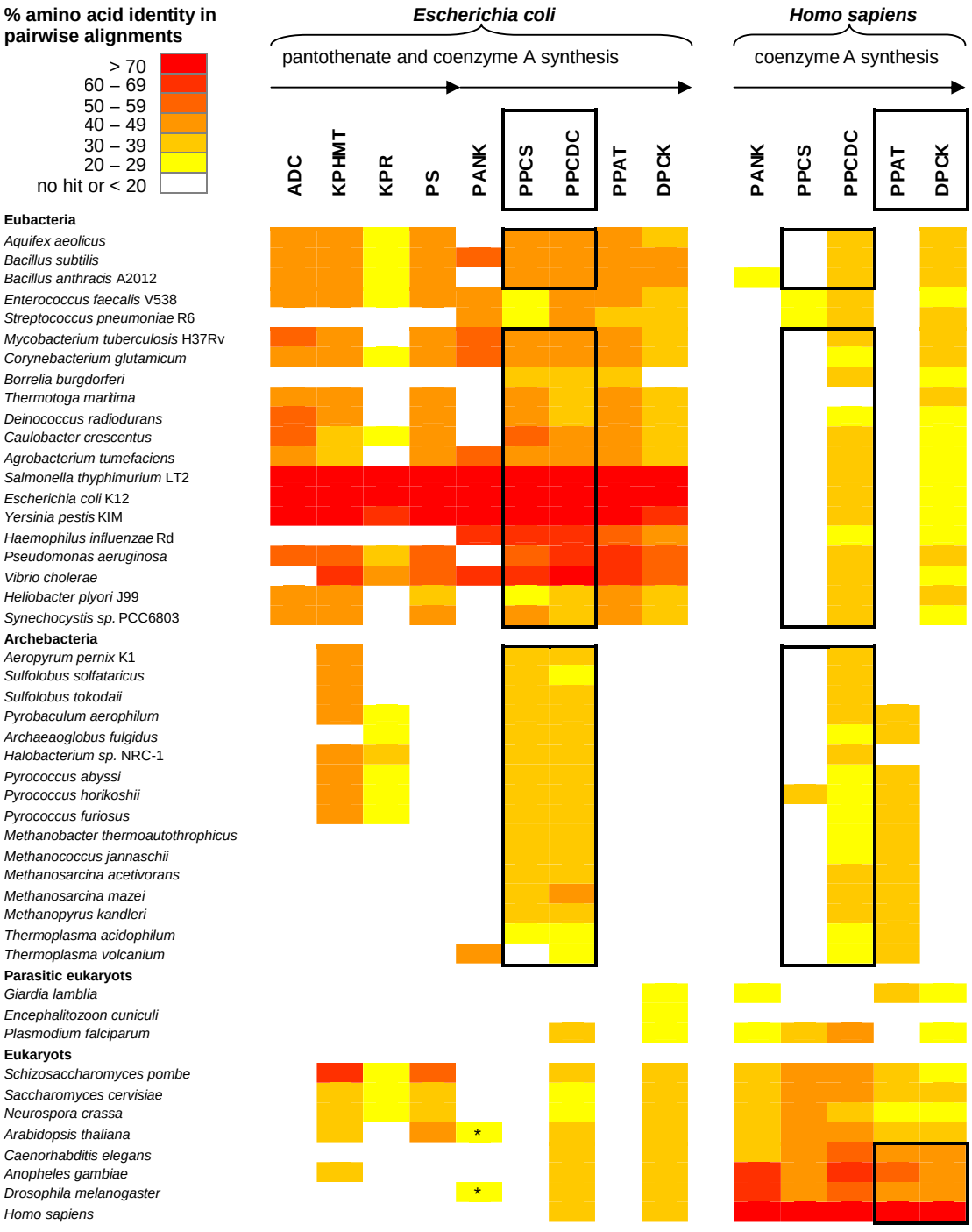

Figure 9-1: Phylogenetic profile of E. coli and human CoA biosynthetic genes across 47 completely sequenced genomes. (From Genschel, 2004)

The problem of the identification of putative genes encoding enzymes in the PA and CoA pathways is solved using a chromosomal proximity method and inferring functional links to archaeal genes for KPHMT, KPR and PPCS/PPCDC. (Genschel, 2004).

This method is particularly useful in Archaea which genomes are very often organized in Operons. This means that very often genes encoding for enzymes involved in the same pathway are close to each other in the genome (Achenbach-Richter *et al.,* 1988). Because of the presence of these operons, it is very likely that enzymes involved in PA and CoA biosynthesis are located very closely in the archaeal genomes.

In several archaeal genomes homologues of bacterial KPHMT, KPR and PPCS/PPCDC and of human PPCDC and PPAT are present. By inferring functional links to archaeal genes for KPHMT; KPR and PPCS/PPCDC using the chromosomal proximity method, it was found that archaeal genes for PPCS/PPCDC (COG0452) were chromosomally linked to members of COG0388 (amidohydrolases), COG1701 (uncharacterized protein conserved in archaea) and COG1829 (predicted archaeal kinase). In addition, archaeal KPHMT genes (COG0413) are linked to COG1701 and COG1829 by physical proximity. This is a very reliable hint that COG1701 and COG1829 are very likely to represent archaeal forms of PS and PanK respectively (Genschel, 2004)

9.1 Aim of this project.

As described above, the COG1701 and COG1829 are very good candidates for representing the archaeal PS and PanK.
This work wants to prove that the genes grouped together in these two COGs are really encoding for ArPS and ArPanK.
For this reason, putative PS and PanK from 3 different archaeal genomes were cloned. The putative PS from *M. mazei* and *M. jannaschii* were shown to functionally complement an *E. coli* strain deficient in the synthesis of PA. Furthermore, the putative PS from M. mazei was overexpressed in E. coli, purified and used in simple experiments to confirm its activity as pantothenate synthetase.
The putative ArPPCS/PPCDC from *M. mazei* was also cloned, to confirm the ability of archaea to synthesize phospho-pantetheine from pantothenate.

10. Experimental results

10.1 Cloning strategies

The genes of interest, which are the putative ArPS (pantothenate synthetase; COG1701), ArPanK (pantothenate kinase; COG1829), and ArCoaBC (bifunctional enzyme phosphopantothenoylcysteine synthetase and phosphopantothenoyl-cysteine decarboxylase; COG0452), were cloned from the genomic DNA using specific primers (see Materials and methods).

Different strategies are used for the cloning procedure, depending on the method chosen for expression and purification of the recombinant protein. All the constructs were sequenced and the obtained sequences compared with the genomic DNA sequences. The constructs generated and used in this work are shown in figure 10-1.

In the cases of *M. mazei* and *S. solfataricus* genes, the primers used for the cloning procedure contain several silent point mutations in order to adjust the codons to the codon usage in *E. coli* (for the complete primer sequences see Materials and methods).

All the genes were cloned in pET and expressed in E. coli BL21. Every plasmid carried only one recombinant gene.

Organism	Enzyme	Construct	Comments	Use
M. jannaschii	MjPS	pBKS-mj0209	The rbs (ribosomal binding site) is added to the primers	Expression and Complementation
	MjPanK	pBKS-mj0969	The rbs is added to the primers	Expression
		pET-mj0969	N-terminal His-tag	Expression
M.mazei	MmPS	pBKS-mm2281		Amplification of the gene
		pET-mm2281	N-terminal His-tag	Expression
		pGEM-mm2281 (MMPS)c	The rbs is added to the primers	Complementation
	MmPanK	pBKS-mm2282		amplification
		pET-mm2282-A (MMPK1)	C-terminal His-tag	Expression
		pET-mm2282-B (MMPK2)	N-terminal His-tag	Expression
	MmCoaBC	pGEM-mm2283		Amplification
		pET-mm2283	N-terminal His-tag	Expression
S. solfataricus	SsPS	pGEM-ss2399		Amplification
		pET-ss2399	No His-tag	Expression
	SsPanK	pGEM-ss2397		Amplification
		pET-ss2397-A (SSPK1)	No His-tag. The gene is entirely cloned	Expression
		pET-ss2397-B (SSPK2)	No His-tag. 54 bp shorter than the complete gene	Expression
	Double construct	pET-ss2399-ss2397 (SSPS/SSPK1)	No His-tag	Expression
		pET-ss2399-ss2397 (SSPS/SSPK2)	No His-tag	Expression

Figure10-1: Construct used for this project

```
M. mazei                    ----------MYTYESEGADFFAKAYAPGHITGFFQIHEHN--DPHRKGS
M. thermautotrophicus       ------------MFREVFKMKPRVFVPAHITGFFEVVMQR--DPLRSGS
P. horikoschii              --------------------MLIRAFVPAHITAFFVPIIRE--DPQNSGS
P. abissii                  -----------MRASSGVPKMLVRAFVPAHITAFFVPIIRD--DPQLSGS
Halobacterium sp. NRC-1     ----------------MSDGESAVAFAPGHVTGFFSVDTGATEAAVASGS
A. fulgidus                 ----------------------MIFAPASITCIFSPHVDS--NPKNSGS
M. jannaschii               ----------------------MFAPGHITGFFVICKSS--NKLKTGS
A. pernix                   -MGLRLELTGSIQPSARGLRVARCLSTPHHITALFRPVPGP--GPGYSGS
S. solfataricus             MMRLKSQLLLVKIINNSFIVLGVEIKVPISISGIWYPVIEK-ENLIKSGS
                                                    .*  :: ::           .**

M. mazei                    TGCGIVLNGGVTTEVKVGKSVE-NTEIFLNG---KKVEGKTTRTVVEMMT
M. thermautotrophicus       RGAGVVLDRGVKTGVKLRGSDD-TTKVRVNG---RRDDDGVSIRAVEILR
P. horikoschii              LGAGINLSKGTNVFISFEESLEKHVHVAFNGEPVKKEDARITYHVIEKIL
P. abissii                  LGAGVNLSRGTNVFISFEESLERHVHVALNGEPVKREDAKVTFHVIDKML
Halobacterium sp. NRC-1     RGAGVALSAGVTVTVTRAENTG----VVLNG---------TPTEIASVRG
A. fulgidus                 IGVGFTIERGLQASPSRKTTIN-----------------GEEWNFPTLD
M. jannaschii               IGAGITIDRGVNVELKEGNGSIFYNNKKVN---------ICAVEKVIEHY
A. pernix                   IGVGLAVEPRARFCPGLDGWEP-------------------GYDLPQFNE
S. solfataricus             IGLTLTLEPYITAEIRNGSGIEFN----------------GIEIQLPNYE
                             *  . :.

M. mazei                    DEP-----VRVKSWAEIPVGCGFGASGAGALGAAYALNRALSLNRTVNGL
M. thermautotrophicus       ELTGFSEGVSIHHEVEVPIGCGFGTSAACALGSVLAIASELELPITFNMA
P. horikoschii              PEDYIG-EVEVWQYFDYPTGHGYGNSAGGALGTALGLSYKFGG--TLLSA
P. abissii                  PQEYVG-EVEVWQYFDYPTGHGFGNSGGGALGTALCLAYKFRR--TLLSA
Halobacterium sp. NRC-1     VLDALGVTARVTAETPLPLGAGFGVSGATALAAALAANEVFGCGCSENEL
A. fulgidus                 DVLEKVGLEGVEIKASLPFGCGFGMSGAAALATAVLGN------LGYIEA
M. jannaschii               KKFGYNDDYDIIFSSDFPLGSGLGMSGGCALILAKKLNEMLNLN---ENY
A. pernix                   AAELLGSRPGGRLVYPLPPGRGYAVSAASAILGALAAAMERGAG--VFRA
S. solfataricus             ILKKKLGEYKLSVYSEVPLGYGYGLSGAISLAYALGVKELTPIS--EEDA
                                        * * * . *.. ::  .

M. mazei                    TEYSHVAEVVNRSGLGDVAAQSS-GGVVIRLHPGGPQFGSVDRIPAPEAR
M. thermautotrophicus       GEVAHRAEVELGTGLGDLIAEVT-GGMVIRTREGPPGYGRVDRIIQDGLH
P. horikoschii              SRIAHEAEVIHKGGLGDIVAQLA-GGIEIRIKEGGPGVAVVDNILVEGFK
P. abissii                  AKVAHEAEVLNRGGLGDIVAQLA-GGVEVRIKEGGPGVAIVDNLIVEGFK
Halobacterium sp. NRC-1     VQVAHVADAEAGTGLGDVVAQAR-GGLPVRVEPGAPPHGSLDGVPASG-R
A. fulgidus                 ADIAHVAEVENFTGLGDVVTQTF-GGVVVRKSAACPSRAELERFFWNA-E
M. jannaschii               AEIAHISEVECGTGLGDVIAQYV-KGFVIRKTPGFP-INVEKIVVDDDYY
A. pernix                   YRAAHEVEVRRSTGLGDVASIACGVGLVLRYSPGPPGEALVDCIPTPPGL
S. solfataricus             VNTAHLSDVLSGNGLGDVIAQYYGGGLVYRKKAGGLGYGEVEVINMDWSY
                               :*  :.    ****: :     *.  *   .       . .
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```
M. mazei                    VFCIVLGEISTDSVLADETAAGKINAAGKAAMLELLKTPTLENFMHQAKN
M. thermautotrophicus       VITRTLGELDTASVIHDDAHVRAINRMGGGMIGRLLKRPAPARFMELSRE
P. horikoschii              VLTISIGKLETR-KVLDSEVVEKIKIAGKQALENLLRDPRPETLMKEARS
P. abissii                  VLTISLGRLSTR-EVLDSDVVEKIKVEGQKSLENLLANPTLETLMREARR
Halobacterium sp. NRC-1     IEYVSFGGLSTSDVIGG--ETDALSAAGADALAALRADPTVAEFVAASRR
A. fulgidus                 FDFVVMGEISTKDVIGDELKRRRIGEAGKRWTKEFLKKPTLENLFHCSNG
M. jannaschii               IIIEIFGKKETKEIITNDIWIKKINEYGERCLNELLKNPTLENFVNLSYE
A. pernix                   YIIAGEAGSMDTSRLIEMYSSQRFVDEVSRSLSRIFREPSLDRFFEEAER
S. solfataricus             YPIFSQPISQLP-----TKSIIKRSEIALKLIDDFLKNPSPLKFIEVAQQ
                                                          :   *    :.  :

M. mazei                    FASSTGLMSSTAQDVIEAAYANG-GLASQAMLGDTVFAIAPYSQEFPLYE
M. thermautotrophicus       FAEGAGLIPRELREPLEELSEGT-LGASMAMLGNTVFALSEDPDAG----
P. horikoschii              FAVETGLMSEDLIEIANEIDREISLPSSMIMLGKGIFALVREEEVEKVKE
P. abissii                  FAEVTGLMDDELKEIAREVDKVISLPSSMIMLGKGIFAMVRNEEVERVKE
Halobacterium sp. NRC-1     FARAAGLVDAAVADAIAAVQAAG-GEAAMAMLGRTVFALGTG--------
A. fulgidus                 FARETGLLELVGDAVEAVESAGG--KAAMVMLGKTVFAVNGFEALK----
M. jannaschii               FAVNTGLINEKILSICEDLKFTV--GASQSMLGNTLFCISKKE-------
A. pernix                   VTRLLSLDSRLVGVEAHRILSGVGDVYGYYVKKRVLVVAAGEGSVGEIVG
S. solfataricus             FTSSLGFSSEYPYSYRKKGIIVKIFEPEYGVWIKHKIASRGAYVI-----
                            .:    .:                        :      .

M. mazei                    ALQEFGQVLEYGISTCIPRLLYD-----
M. thermautotrophicus       ----GAGTSTYGIDQLGARFI-------
P. horikoschii              IVKDLDLQYDISYIYWGKPVVGRWLESE
P. abissii                  LVKDLGVPYDIAEIYWGKPTVGRWFGES
Halobacterium sp. NRC-1     LSDAGYDAASCAIHHGGASLR-------
A. fulgidus                 ---EFGEPFKARLDCCGVRRL-------
M. jannaschii               ---TLEDALSILKNPIVCNIYY------
A. pernix                   RMAGAGLDVRLLKPSTGPPRLE------
S. solfataricus             ----------------------------
```

Figure 10-2: CLUSTAL W (1.83) multiple sequence alignment of 9 genes grouped in the COG1829 encoding for the putative ArPanK. (Matrix: BLOSUM 62). The red star indicate the presence of the same amino acid, the blue points the exchange of the amino acid of the same type, the green point the replace of one amino acid with another one.

Concerning the cloning of the gene *ss2397*, that encodes the putative SsPank, a different strategy was planned. In fact from the alignment of the sequences, it is clear that this gene presents a "head" of circa 90 amino acids. This could mean that SsPanK is synthesized as a proenzyme and subsequently, some cleavage mechanism can cut this "head" to form the active enzyme.

To verify this hypothesis, the cloning procedure was designed so that not only the complete gene but also the gene without the first 90 amino acids was cloned.

Furthermore, because of the expression of all the archaeal PanK cloned (MmPanK and MjPanK) presents big problems of solubility and degradation, this time one goal of the cloning procedure was to obtain a double construct in which the two genes (SsPS+SsPanK) were together in the same plasmid and in the same order as in the genome. This new strategy was planned hoping that the presence of SsPS could help to express and keep soluble the SsPanK.

10.2 Complementation experiments

The *E. coli* strain AT1371 is an auxotrophic mutant lacking the gene for pantothenate synthetase. Because of this mutation, this strain is not able to grow in the absence of PA. This made it suitable for complementation experiments with the cloned putative archaeal PS.

E. coli AT1371 was transformed with both constructs pBKS-MjPS and pGEM-MmPS (for experimental procedure see Materials and methods) and its ability to grow in presence and absence of PA was tested.

10.2.1 Complementation with MjPS

In this experiment the *E. coli* strain AT1371 is transformed with the empty plasmid (negative control), the pBKS-EcPS construct (positive control) and

with the pBKS-MjPS construct (for experimental conditions see Material and methods). The results of this complementation experiment are summarized in figure 10-3.

All the three constructs grew on the DYT plate and on the GB1 plate plus PA (1mM). Bacteria transformed with the pBKS-EcPS construct can grow in all the plates and the dimension of the colonies look very similar, indicating that these bacteria are not affected by the addition of nutrients. Bacteria transformed with the empty plasmid can only grow on the DYT plate and on the GB1+PA plate. This is expected because these bacteria lack PS.

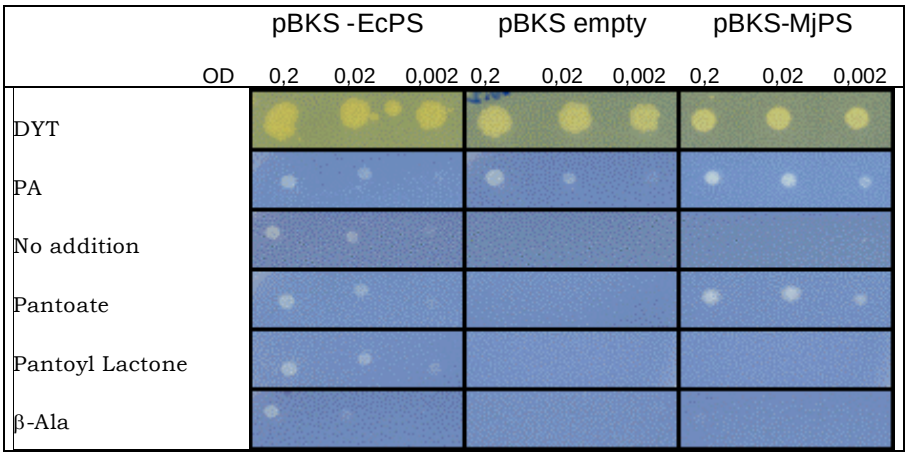


Figure 10-3: Colonies of *E. coli* mutant AT1371 carrying the pBKS-EcPS, pBKS empty plasmid or pBKS-MjPS constructs after 48h incubation at 37°C.

Bacteria transformed with MjPS can grow in presence of PA and, in addition, they grow in presence of pantoate. This means that the enzyme that was suggested be the archaeal PS is really involved in the synthesis of PA.

10.2.2 Complementation with MmPS

Complementation experiments with putative PS cloned from *M. mazei* were carried in both liquid culture and on solid media. The experimental procedures are explained in Materials and Methods.
Like in the case of the MjPS, the experiment was planned with a negative control (bacteria transformed with the empty pBKS), a positive control (bacteria transformed with pBKS-EcPS) and with the archaeal enzyme (pGEM-MmPS) to compare the behavior of the three differently transformed bacteria.

10.2.2.1 Solid culture

In this case, the different aliquots of the same cells suspensions described above and transformed with the three different constructs were spotted on GB1 minimal media plates, in presence of different nutrient.

The results of this complementation experiment are summarized in figure 10-4. In this case, like in the liquid culture, the bacteria transformed with EcPS can grow on all media. The negative control, as expected, can grow in presence of PA; however, it showed limited growth also in presence of 10 mM or 100 mM pantoate. Bacteria transformed with MmPS can grow in presence of PA, but also in absence of any addition and in presence of PL and pantoate. What is different is the shape of the colonies, in that bacteria transformed with pGEM-MmPS that do not grow equally well in all the conditions. From figure 10-4 it is clear that the colonies that grew on 1 mM pantoate or 1 mM PL are very small, especially if compared with the positive control. By increasing the amount of pantoate present in the plates, the colonies become bigger, reflecting the increased growth of bacteria.

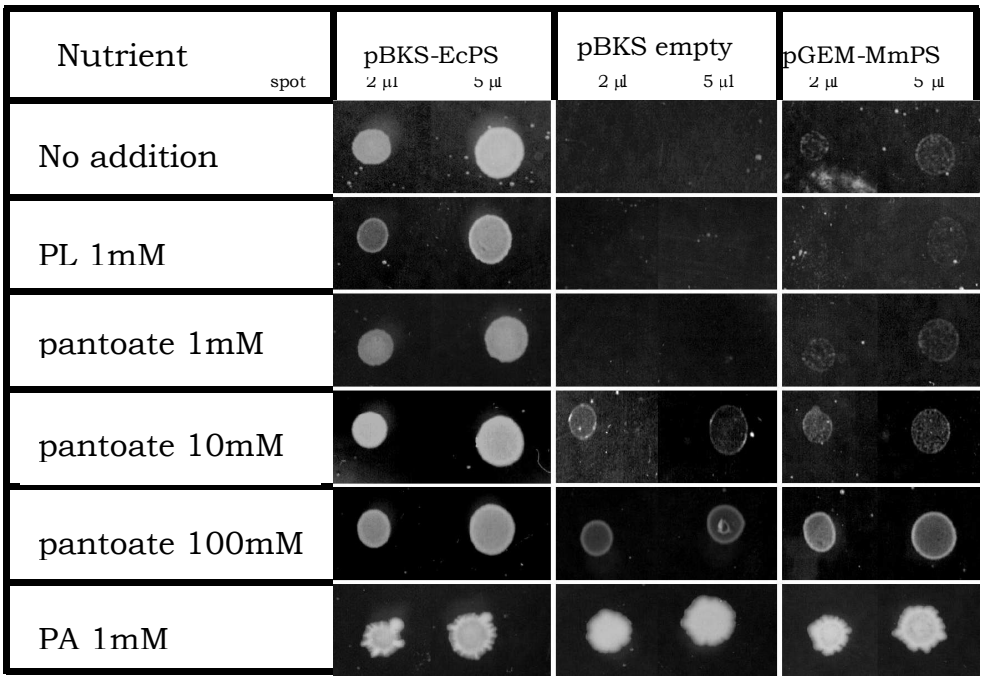


Figure 10-4: Complementation of he *E. coli* strain At1371 with the empty plasmid, the EcPS and the MmPS

10.2.2.2 Liquid culture

The experiment started with the inoculation of 20 ml aliquots of GB1 minimal media with 200 µl of each bacterial prep (OD_{600}=0,3; prepared as described in Materials and Methods and stored at –70°C). The flasks were then put into an incubator at 37°C. One ml aliquots were taken every hour from the flasks to follow the bacterial growth via OD_{600} measurements. Two cultures for each transformant were started, one early in the morning and the second late in the evening so to minimize the hours in which no aliquot could be taken.

Figure 10-5 shows the growth of the three different transformed bacteria in the presence of different nutrient added. As is clear from this figure, all constructs enable growth of *E. coli* At1371 in presence of 1 mM PA, without a big difference between the different cultures. In case of the negative control, as expected, PA is the only nutrient that allows bacterial growth.

When the *E. coli* strain AT1371 is transformed with the plasmid carrying EcPS, the bacteria can grow in all the conditions used but it seems that high concentrations of pantoate (10, 20, 50 mM) have an inhibitory effect on the growth, which becomes higher as the concentration increases.

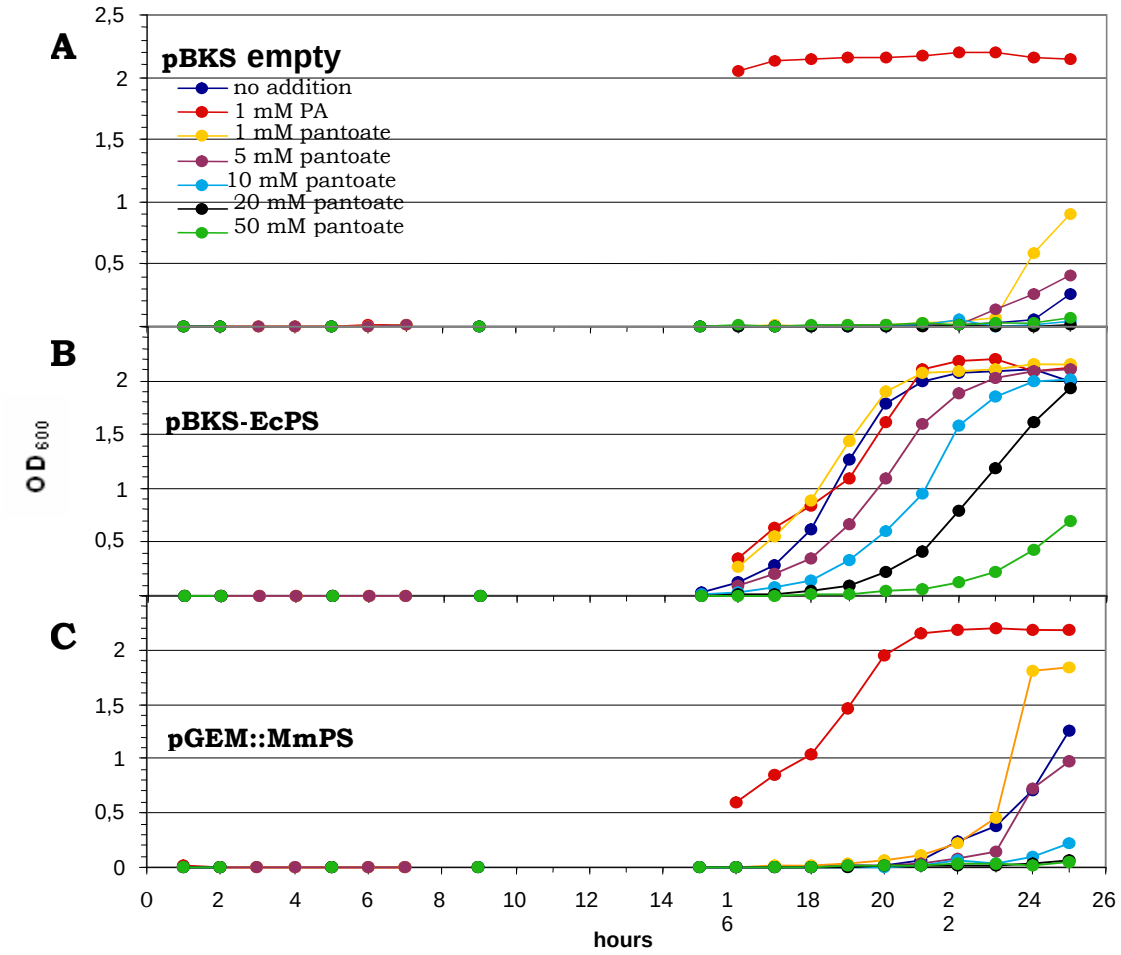


Figure 10-5: Bacterial growth with the addition of the different nutrient substances.

Bacteria transformed with the plasmid carrying MmPS can grow in presence of PA as well as in the presence of 1 or 5 mM pantoate and also when no nutrients are added to the minimal media, showing that this enzyme is able to produce PA.

For a better comparison of the effects of the nutrient added on the growth of bacteria transformed with the specific constructs, figure 10-6 shows the growth curves of the three transformed *E. coli* AT1371 in absence of nutrient and when single addition were present. From this figure, is clear that the cells transformed with MmPS are able to grow in presence of 1 mM pantoate, reaching a maximal OD_{600} similar to the one reached when

bacteria are transformed with EcPS. When 5 mM pantoate is present, the growth is slow but bacteria grow better than the transformed with the empty vector.

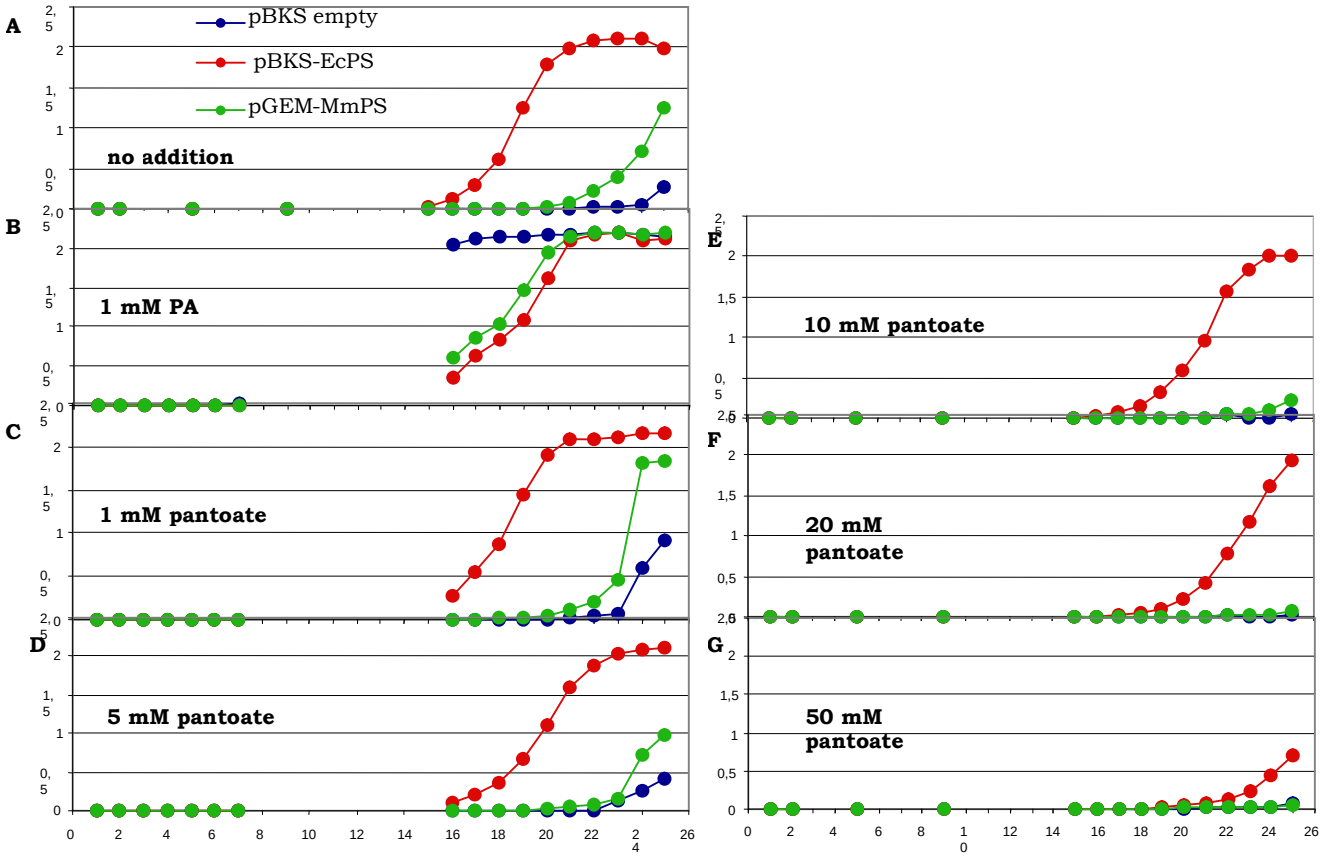


Figure 10-6: Growth curves of bacteria transformed with pBKS empty, pBKS-EcPs and pGEM-MmPS in presence of different nutrients

The very long lag phase can be due to the fact that the cultures are inoculated directly with bacteria stored at –70°C that were not allowed to grow on a rich media to regenerate before starting the experiment.

10.3 Expression and purification of the proteins

The expression of all the enzymes was carried out using standard methods as described in Materials and Methods, with some specific modification.

10.3.1 Archaeal pantothenate synthetases

10.3.1.1 MjPS

MjPS was expressed in *E. coli* (strain BL21) under the control of the *lac* promoter. The overexpression of the protein started with the addition of IPTG (to a final concentration of 0,5 mM). The bacteria were then kept at 37°C for 16 or 24 hours before harvesting.

The purification procedure was based on the consideration that, if *M. jannaschii* grows at an optimal temperature of 85°C, its proteins must be stable too at this high temperature. Because this is not true for *E. coli* proteins, which are temperature labile, heating the protein mixture to 80°C was expected to be a good method to purify MjPS from the *E. coli* cell preparation.

After lysis of the cells by sonication, the presence of the overexpressed protein was checked by SDS-PAGE gel. As shown in figure 10-7, the MjPS is nicely expressed in *E. coli* and, after heat treatment a relatively pure preparation of the enzyme was obtained. The presence of some residual *E. coli* proteins was not considered a problem because they enzymes should be completely inactivated from the heat treatment. For this reason, the enzyme was not purified further. Several preps obtained in this way, were tested for activity.

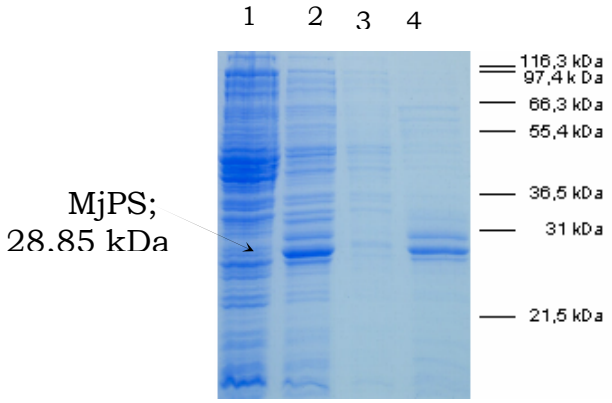


Figure 10-7: SDS-PAGE of the purified MjPS. Lane 1-crude extract before adding of IPTG; Lane 2- soluble extract after addition of IPTG; Lane 3- Insoluble fraction after heat treatment; Lane 4- Soluble fraction after heat treatment (80°C; 15 min.).

10.3.1.2 MmPS

MmPS was expressed in *E. coli* strain BL21 under the control of the *T7* promoter. After their growth, bacteria were harvested and the protein was purified under standard condition using a Ni-column procedure (as described in Materials and methods).

The degree of purification of the enzyme was checked by SDS-PAGE gel (see figure 10-8) and, because the purity was not optimal, the enzyme was further purified by anion exchange purification.

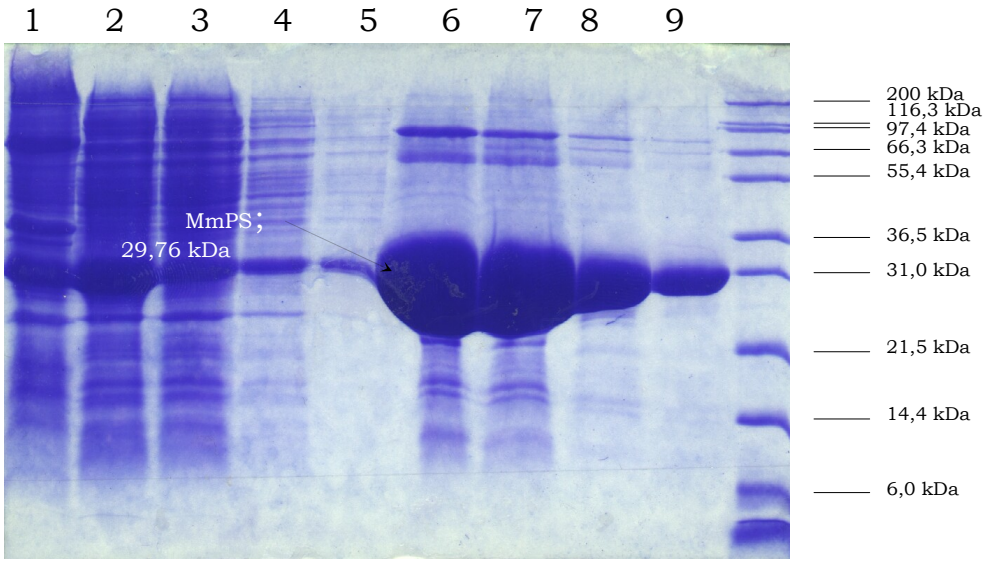


Figure 10-8: SDS-PAGE gel 15% of the purified MmPS. Lane 1- soluble fraction after induction; Lane 2- insoluble fraction after induction; Lane 3- flow through the Ni-NTA column; Lane4- washing step 1; Lane 5- washing step 2; Lane6- elution step 1; Lane 7- elution step 2; Lane 8 elution step 3; Lane 9 elution step 4.

Figure 10-9 shows the protein prep after the anion exchange purification.

10.3.1.3 SsPS

SsPS was expressed in *E. coli* under the control of the *T7* promoter as described in Materials and methods.

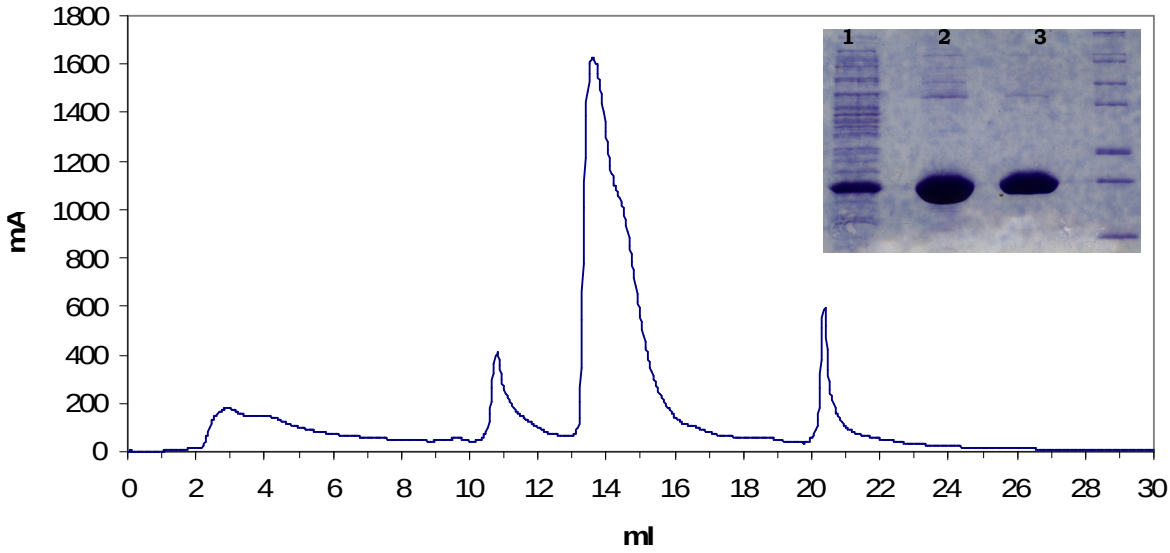


Figure 10-9: Chromatogram of the Äkta purification. In the small box, is the aspect of the SDS-PAGE gel (12%), after purification: Lane1-extract soluble part; Lane2-soluble part after Ni-agarose column; Lane3-Soluble part after gel filtration purification.

After breakage of the cells by sonication, the presence of the over-expressed protein was checked by SDS-PAGE gel. As shown in figure 10-10, the SsPS is not nicely expressed in *E. coli* but by increasing the volume of the bacterial culture, relatively high amounts of the protein were obtained. As in the case of MjPS, this protein is expressed without His-tag and the purification procedure is based on heat treatment. Unfortunately, perhaps due to the low expression level, in this case the purification procedure is not as good as in the case of the MjPS.

Considering that the *S. solfataricus* live in very acidic environments (pH ca 2-4), the breakage of the cells was carried out not only at pH 8,0 (using TRIS buffer) but also at pH 6,5 (using MES buffer). As shown in figure 10-10, the pH seems to not influence the solubility of the protein, which is instead influenced by the temperature of expression.

Expression and purification were checked by SDS-PAGE gel.

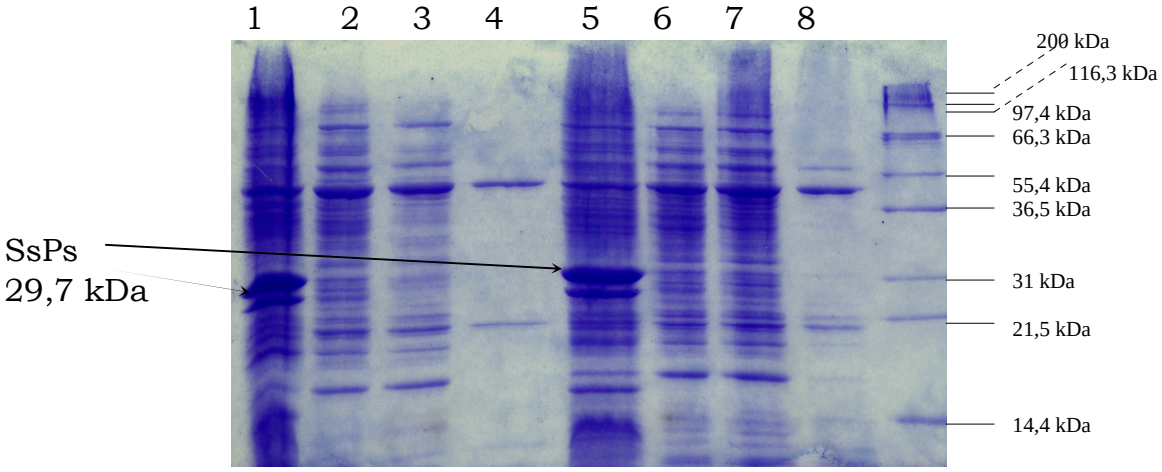


Figure 10-10: SDS-PAGE gel 12% of the SsPS expression in SOC medium at 37°C. Lysis buffer: lanes 1 to 4 – MES pH 6,1; lanes 5 to 8 – TRIS-Cl pH 8,0. Lanes 1- and 5 Insoluble fraction; lanes 2 and 6 – soluble fraction; lanes 3 and 7 - soluble after 15' at 50°C; lanes 4 and 8 – soluble after 15' at 80°C.

10.3.2 Pantothenate kinases

10.3.2.1 MjPanK

Several attempts to express this protein were carried on using different growing temperature (20°C and 37°C), different media (LB, TB, SOC in presence or absence of 1% glucose), different *E. coli* strains (XL1Blue and BL21), different constructs (in pBKS and in pET) and the addition of the helper plasmid pRIL that encodes for rare codons in *E. coli* which are frequently used in *M. jannaschii* gene. However, MjPanK was never expressed.

Figure 10-11 shows a typical SDS-GEL of the expression of the MjPanK, both in *E. coli* XL1Blue and BL21.

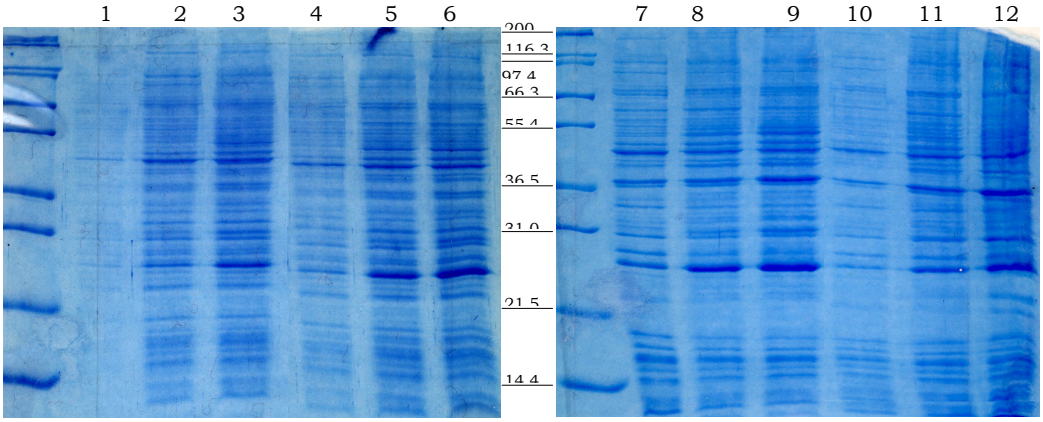


Figure 10-11: SDS-PAGE gel 15% of MjPanK expression in XL1Blue (lines 1-6) and BL21 (lines 7-12). Lanes 1 and 7: pBKS empty plasmid before IPTG; Lanes 2 and 8- pBKS empty plasmid 2 h after IPTG; Lanes 3 and 9- pBKS empty plasmid 4 h after IPTG; Lanes 4 and 10- pBKS-MjPK before IPTG; Lanes 5 and 11- pBKS-MjPK 2 h after IPTG; Lanes 6 and 12- pBKS-MjPK 4 h after IPTG.

10.3.2.2 MmPanK

The MmPanK protein is expressed as a His-tag protein in *E. coli* strain BL21 under the control of the *T7* promoter.

The first attempt to obtain a soluble MmPanK was carried out with the protein called MmPK1, which carries the His-tag at the C-terminal. The expression of the protein was induced by addition of IPTG (to a final concentration of 1 mM) to a liquid culture of *E. coli* strain BL21 transformed with the plasmid pET-mm2282 and grew till an OD_{600} of circa 0,3. After addition of IPTG the cells were allowed to grow further for 16 hours and then the expression of the protein was checked via SDS-PAGE gel.

As shown in figure 10-12, this protein is nicely expressed but unfortunately, it appeared to be insoluble. A number of different attempts to increase the solubility of the protein were carried out, changing the expression media (LB, TB, SOC, with or without the addition of 1% glucose), the expression temperature (20°C or 37°C), the OD_{600} induction values (0,2; 0,5; 1,0), and the expression time (4 hours, 24 hours, 48 hours). None of these attempts increased the solubility of the protein.

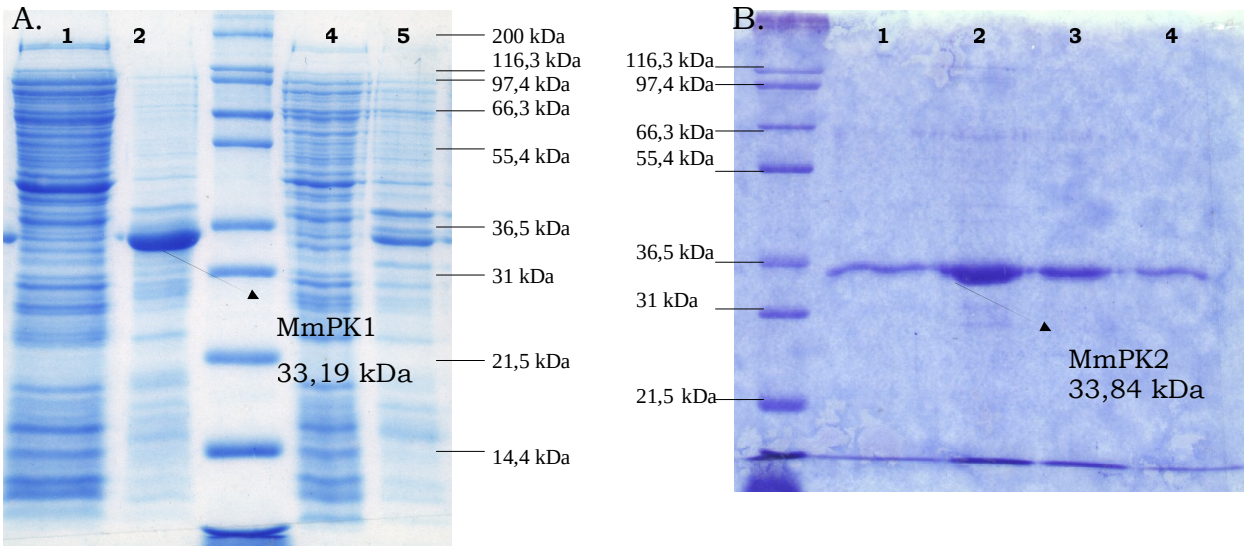


Figure 10-12: SDS-PAGE gels of MmPanK. A. (15% gel): MmPanK1- Lanes 1-2: expression at 37°C; Lanes 4-5: expression at 20°C. Lane 1- soluble fraction; Lane 2- insoluble fraction; Lane 4- soluble fraction; Lane 5: insoluble fraction. B. (12% gel): MmPanK2- Lanes 1-4: fractions collected after Ni-agarose column.

The next step was to clone the gene again, but this time the construct presented the His –tag at the N-terminal. This second protein was expressed in *E. coli* at 20°C in SOC medium, as the previous one. This time the protein was expressed, in little amount, in a soluble form too. Unfortunately, MmPanK2 was subjected to degradation during the purifying procedure. This was checked by SDS-PAGE gel, in which the protein appeared smaller after each purification step. Figure 10-12 shows he MmPanK2 protein immediately after the purification on the Ni column.

10.3.2.3 SsPanK

Several attempts to express this protein were carried on using different growing temperature (20°C and 37°C), different media (LB, TB, SOC in presence or absence of 1% glucose), different *E. coli* strains (XL1Blue and BL21), and the addition of the helper plasmid pRIL that encodes for the rare codons in *E. coli* that are encoded by the *S. solfolobus* gene. Unfortunately, neither the SsPanK1 either the SsPanK2 were expressed in a soluble fraction as shown in figure 10-13.

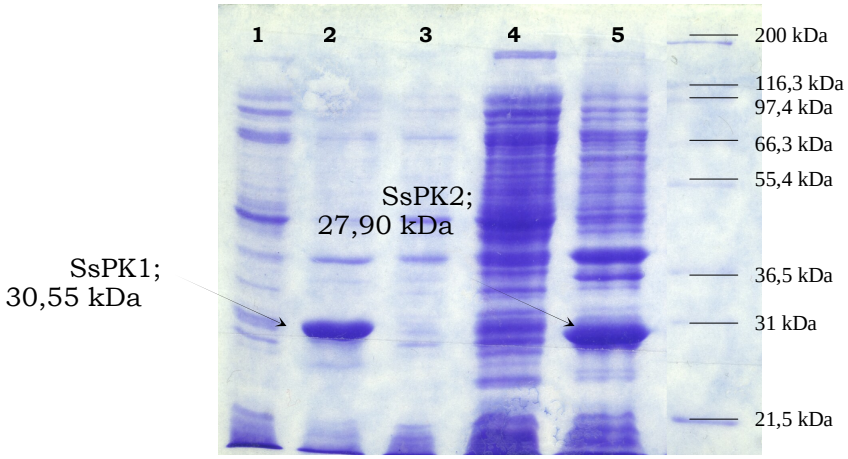


Figure 10-13: SDS-PAGE gel 12% of SsPanKs. Lane1- SsPK1 soluble fraction; Lane 2- SsPK1 insoluble fraction; Lane 3- SsPK2 before induction with IPTG; Lane 4- SSPK2 soluble fraction; Lane 5- SsPK2 insoluble fraction.

10.3.3 PPCS/PPCDC

MmPPCS/PPCDC protein is expressed as His-tag protein in *E. coli* strain BL21 under the control of the *T7* promoter.

The expression of the protein presented no problem and it is easily expressed in soluble form. Purification of the protein proceeded as described in Materials and Methods.

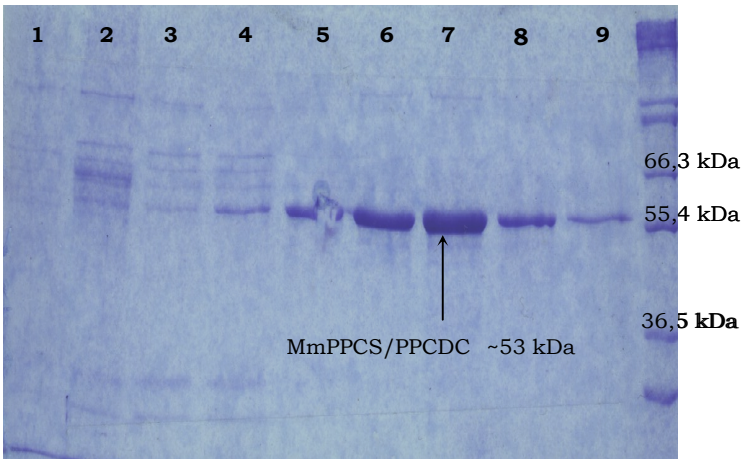


Figure 10-14: SDS-PAGE gel 12% of the MmCoaBC purification. Lane 1- elution 1 (50 mM imidazole); lane 2 – elution 2 (50 mM imidazole); lane 3 – elution 3 (100 mM imidazole); lane 4 - elution 4 (100 mM imidazole; lane 5 – elution 5 (150 mM imidazole); lane 6 – elution 6 (150 mM imidazole); lane 7 – elution 7 (500 mM imidazole); lane 8 – elution 8 (500 mM imidazole); lane 9 – elution 9 (500 mM imidazole). For the complete description of purification procedure see Materials and methods.

10.4 Pantothenate synthetase activity of MmPS

10.4.1 Coupled assay

To test the activity of the putative PS from *M. mazei*, the reaction was carried on as described for *E. coli* PS (Miyatake *et al.*, 1979) with some modification. Figure 10-15 shows the TLC of a typical assay for MmPS activity. In this assay, EcPS+EcPanK were used to identify the position of substrate and products on the TLC (for experimental details see Materials and methods).

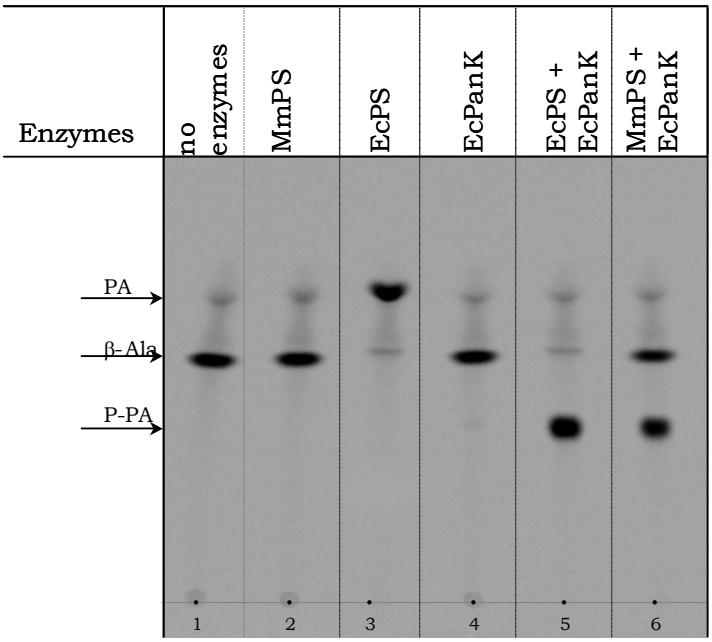


Figure 10-15: TLC of activity test of MmPS. The reaction is carried on at 37°C for 3 hours. Mobile phase: EtOH:NH_3=4:3. Lane 1- control (reaction w/o enzyme); lane 2- MmPS; lane 3- EcPS; lane 4-EcPanK; lane 5 EcPS+EcPanK; lane 6- MmPS+EcPanK;

When the reaction is carried out in presence of MmPS, after 3 hours no detectable PA is produced (lane 2), but when the same experiment was repeated in presence of EcPanK, formation of P-PA was observed (lane 6). To test if the new P-PA spot was due to EcPS contamination in the EcPanK prep, the same reaction is carried out in the presence of only EcPanK. As shown in figure 10-15, lane 4, in this case no P-PA spots appear on the TLC. This suggests that to be active MmPS requires the presence of MmPanK.

This assumption could be supported to some extent by the fact that in the major part of the archaeal genomes the putative PS and PanK are very close to each other. It was no possible to confirm this hypothesis in a direct way because, as described above, it was never possible to purify enough MmPanK to carry out an experiment.

A second plausible explanation for this apparent absence of pantothenate synthetase activity could be that the enzyme is not highly active, at least in the condition used for the experiment. If for instance the releasing of PA is slow, just few amount of free PA is produced, amount that could be under the limit of detection. The addition of a PA consuming enzyme like the EcPanK should facilitate its releasing from the archaeal PS. Alternatively increasing the concentration of the enzyme should help the detection of the product.

Conclusion of this first assay is that the putative MmPS is really catalyzing a reaction that produces PA from β-Ala and pantoate in presence of ATP. The next step of this study was to understand something more about the stoichiometry of the reaction and the kinetic of the same.

10.4.2 Is EcPanK strictly necessary?

The isotope exchange experiment, carried on in presence only of the MmPS suggested that, to catalyze the formation of PA, the presence of the PanK is not necessary. To test this hypothesis, the same standard reaction was carried on in presence and absence of the EcPanK using increasing amount of MmPS. The results of the reactions are shown in figure 10-16.

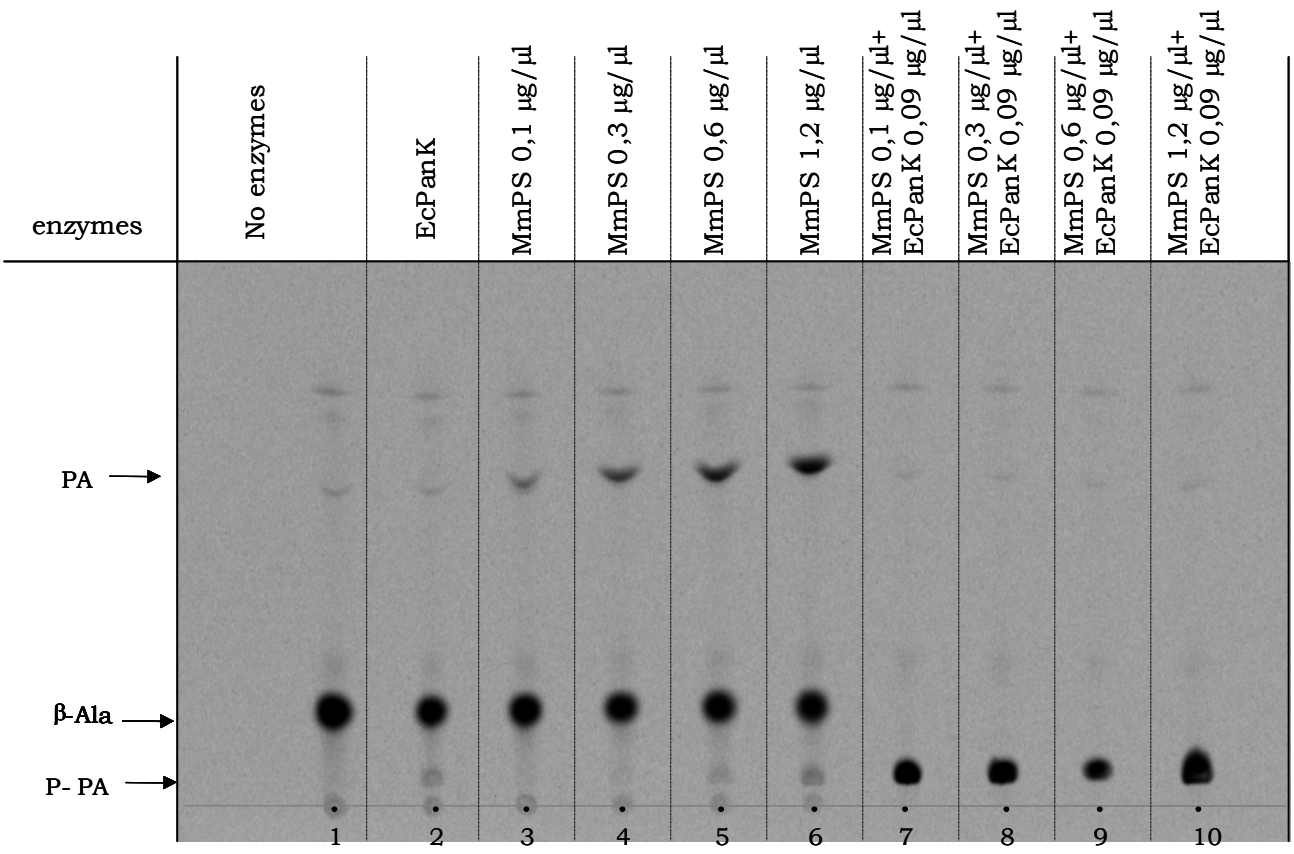


Figure 10-16: TLC of the reaction catalysed by the MmPS carried on presence of increasing amount of the enzyme. The reaction is carried out in standard condition (see Materials and methods), and aliquots were taken after 2, 6 (shown) and 21h. Mobile phase: Dioxane:NH_3:H_2O=9:1:4

This figure shows that, either in absence of EcPanK (lines 3-6) MmPS is able to convert β-Ala and pantoate into PA. In fact, after 6 h and in presence of 0,1 µg/µl of enzyme, the 1,8% of the radioactivity was found in the PA spot (line 3). Increasing the amount of enzyme, the percentage of conversion increases and, in presence of 1,2 µg/µl of MmPS, the 100% is reached (after 21h). The same reaction carried on in presence of 0,1 µg/µl of EcPanK shows 100% conversion in all cases after 2 h.

This experiment shows that, alone, MmPS is able to convert β-Ala and pantoate into PA at a rate of 0,52 $nmolmin^{-1}mg_{enzyme}^{-1}$.

When the same reaction is carried out in presence of EcPanK, after 2 hours the complete conversion of β-Ala into PA is observed. This means that the MmPS, when in presence of a PanK, can convert more than 49 $nmolmin^{-1}mg_{enzyme}^{-1}$ of substrates into PA. In other words, when MmPS is coupled with

EcPanK the rate of the reaction is more than 100 fold faster than when MmPS is alone.

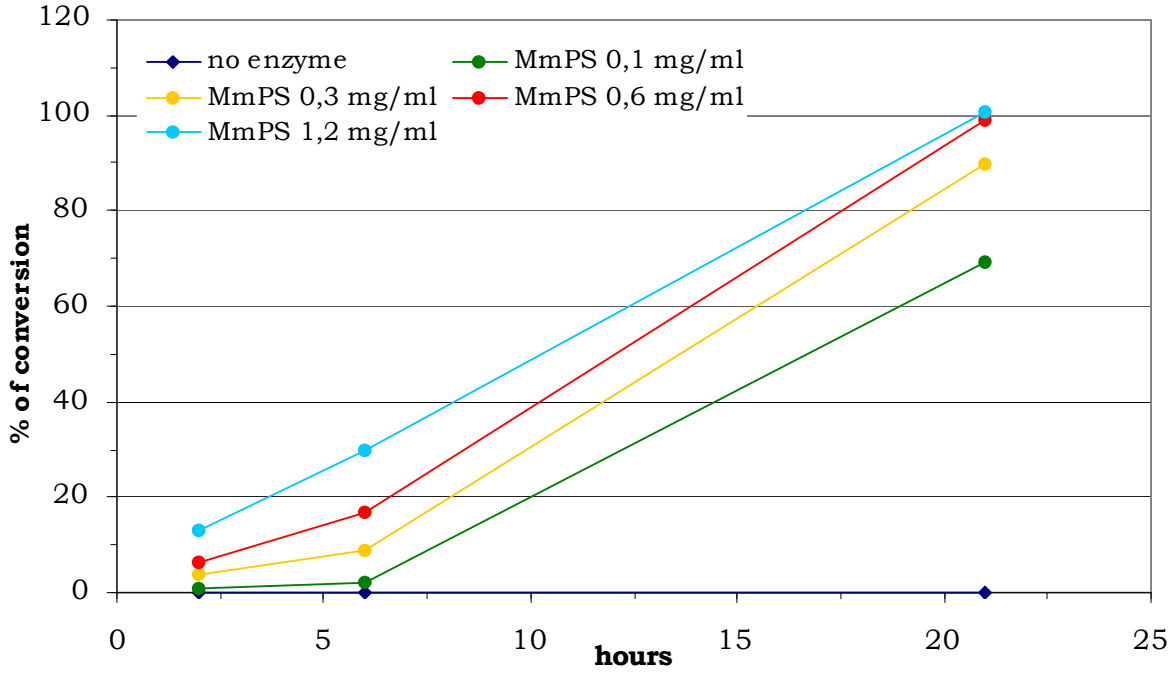


Figure 10-17: Percentage of conversion of β-Ala and pantoate into PA in presence of increasing amount of MmPS.

10.4.3 Stability of the enzyme

The stability of the enzyme was tested carrying on the same standard reaction in presence of 1,2 μg/μl of MmPS from different aliquots of the same prep stored at –70°C for 1 day, 11 days and 23 days. Results of the reaction are summarized in figure 10-18. All this reactions were carried out in absence of EcPanK.

From this graph is clear that the enzyme is not stable: after 11 days it shows that the 25% of the activity of the enzyme 1 day old.

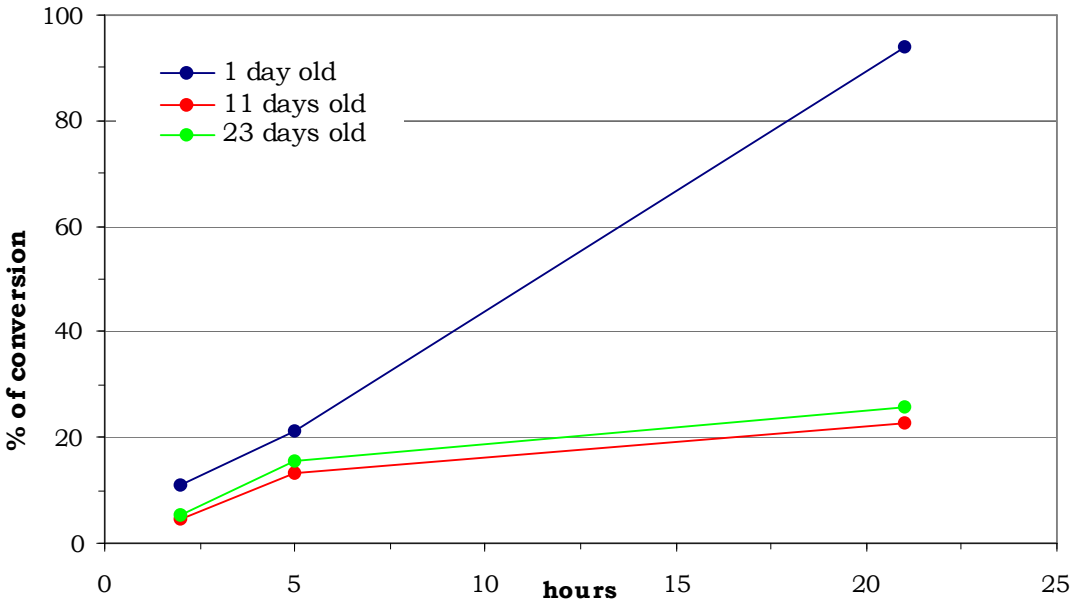


Figure 10-18: Percentage of conversion of the substrates into the product using enzyme 1,11,23 days old. The reaction is carried on in standard condition (see Materials and methods) at 37°C.

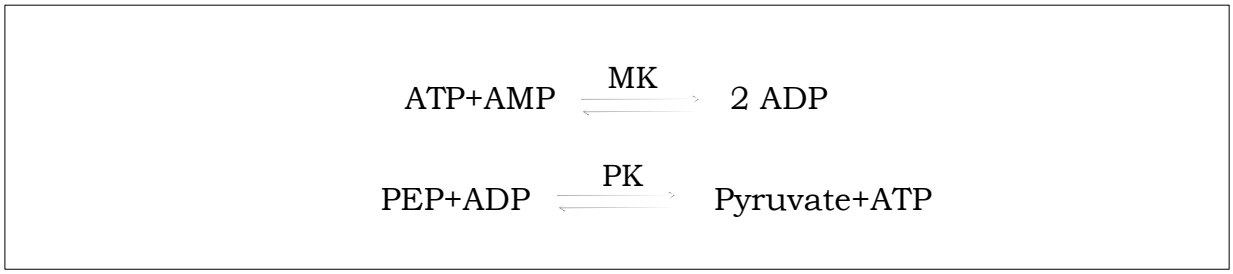
10.4.4 Identification of substrates and products

Figure 10-16 suggested that MmPS showed activity only if the assay is carried out in presence of EcPanK. This observation could be explained with the assumption that the enzyme is inhibited by the products, and in particular by PA. In this case the conversion of PA into P-PA catalyzed by EcPanK is necessary for MmPS activity. A second explanation could be that the reaction catalyzed by MmPS is close to the equilibrium. If this is more on the side of the substrates than of the products only very few PA can be synthesized, and these probably remain under the detection limit. A third possible explanation could be that MmPS requires activation by unspecific interaction with another enzyme. This hypothesis was tested via incubation of MmPS with pantoate, β-Ala and ATP in presence of BSA (data not shown). In this case no formation of PA was observed and this suggests that unspecific interaction with other proteins are not so important for the activation of MmPS.

If release of PA is rate limiting, the removal of one of the other products should have no effect on the amount of PA formed. If the reaction is only regulated by equilibrium laws, the removal of one of the other products should help to form more PA. Till now nothing is known about this enzyme, the stoichiometry of the reaction is unknown. If it proceeds as in case of EcPS the other products should be AMP and PP_i but is not possible to exclude the possibility that ADP and P_i are formed.

To distinguish between inhibitory effect and equilibrium proximity, the assay was repeated in presence of Myokinase (MK) to remove AMP or pyruvate kinase (PK) and phosphoenolpyruvate (PEP) to remove ADP. The reactions catalyzed by these two enzymes are reported in scheme 10-1.

Furthermore, the addition of these enzymes could help to understand if the second product is AMP or ADP:



Scheme 10-1: Reaction catalyzed by Mk and PK

As shown in figure 10-19 it seems that the presence of these enzymes alone does not influence the proceeding of the reaction (lanes 7-9-11). In fact it seems that the reaction happens only when PanK is present, independently from the addition of the other two enzymes.

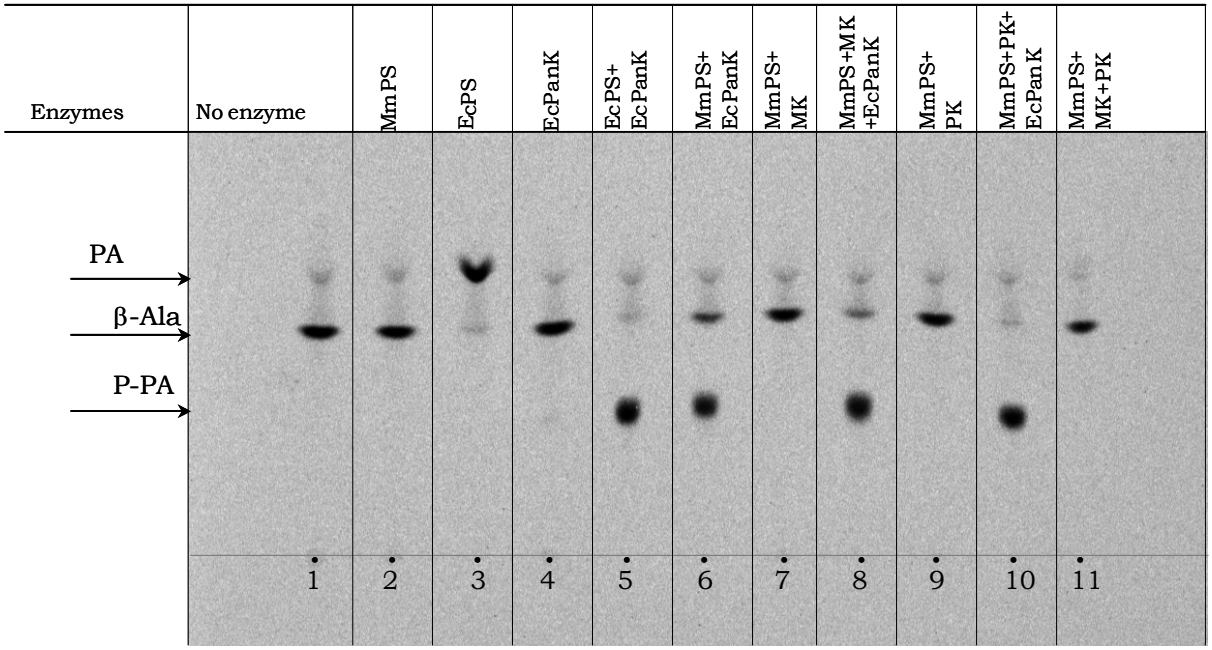


Figure 10-19: TLC of the reaction catalyzed by MmPS in presence of MK, PK+PEP and EcPanK. The reaction is carried on at 37°C for 3 hours. Mobile phase: EtOH:NH_3=4:3.

These results suggest that this reaction is not regulated by the equilibrium between substrates and products because the removal of the other product of the reaction that is not PA does not allow the proceeding of the reaction. Furthermore, because the addition of PK increases the conversion of β-Ala into PA, the other product of the reaction should be ADP and not AMP like in case of EcPS.

As shown in figure 10-20, the reaction shows a maximum of PA production when it is carried on in presence of ATP. The fact that after 21 hours, the reaction carried on with ADP shows a maximum value of conversion that is very close to the first one, means that the enzyme can use ADP, but it prefers ATP. The fact that if the reaction is carried on in presence of CTP no PA formation is observed, means that the enzyme requires ATP and can not use other nucleotides except for ADP.

After identification of all the reagents, a new assay was planned to identify exactly the products of the reaction. The standard reaction was carried on with 1,2 μg/μl of MmPS in a big volume (1 ml reaction) using ATP or ADP as nucleotides. The reaction was allowed to run for 24h (but aliquots

are taken after 2, 5 and 24h) and then analyzed by injection of the reaction mixture into the HPLC.

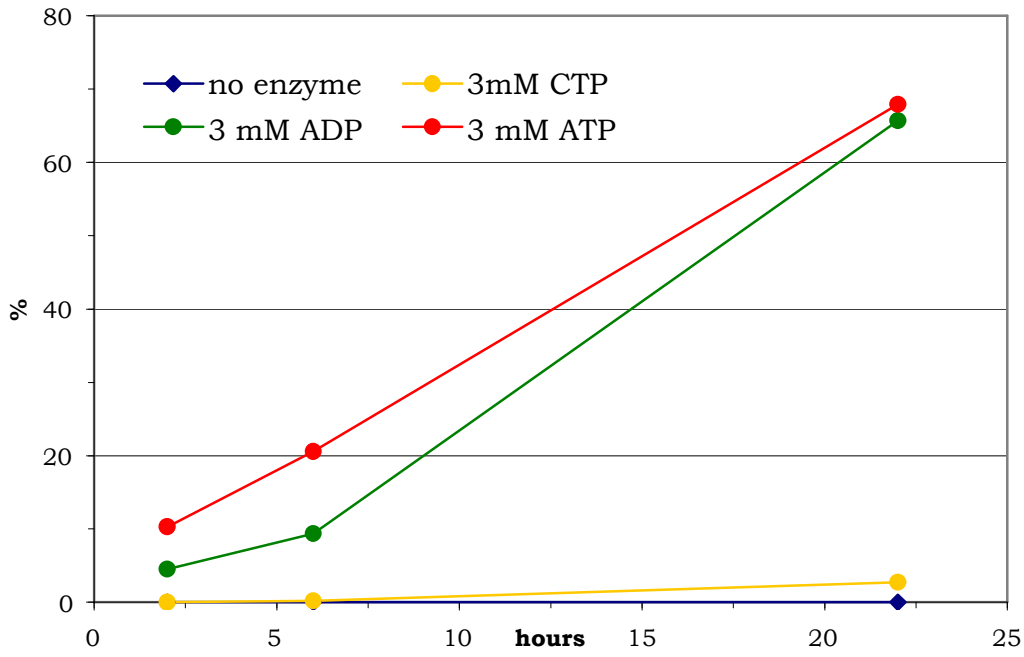


Figure 10-20: Reaction catalysed by MmPS. Aliquots of the reactions, carried on as the standard reaction described in Materials and methods, were taken after 2h, 6h and 22h. The amount of radioactivity present in the single spots was quantified via exposure on a phosphor-screen.

To check the proceeding of the reaction, 50 µl aliquots was taken at the beginning and added with *β-Ala. By developing the TLC for this reaction, it was possible to follow the appearing of *PA spot.

In figure 10-21 the results of the HPLC analyses are shown. When the reaction is carried on in presence of ADP (part B), peaks for all the three nucleotides are present; when the reaction is carried on in presence of ATP, only the peak of ATP and ADP are present. This means that the reaction catalyzed by the MmPS hydrolyzed only one of the three phosphoric bonds in the ATP molecule and that the product of the reaction is ADP. The reaction can also use ADP, and in this case the product would be AMP.

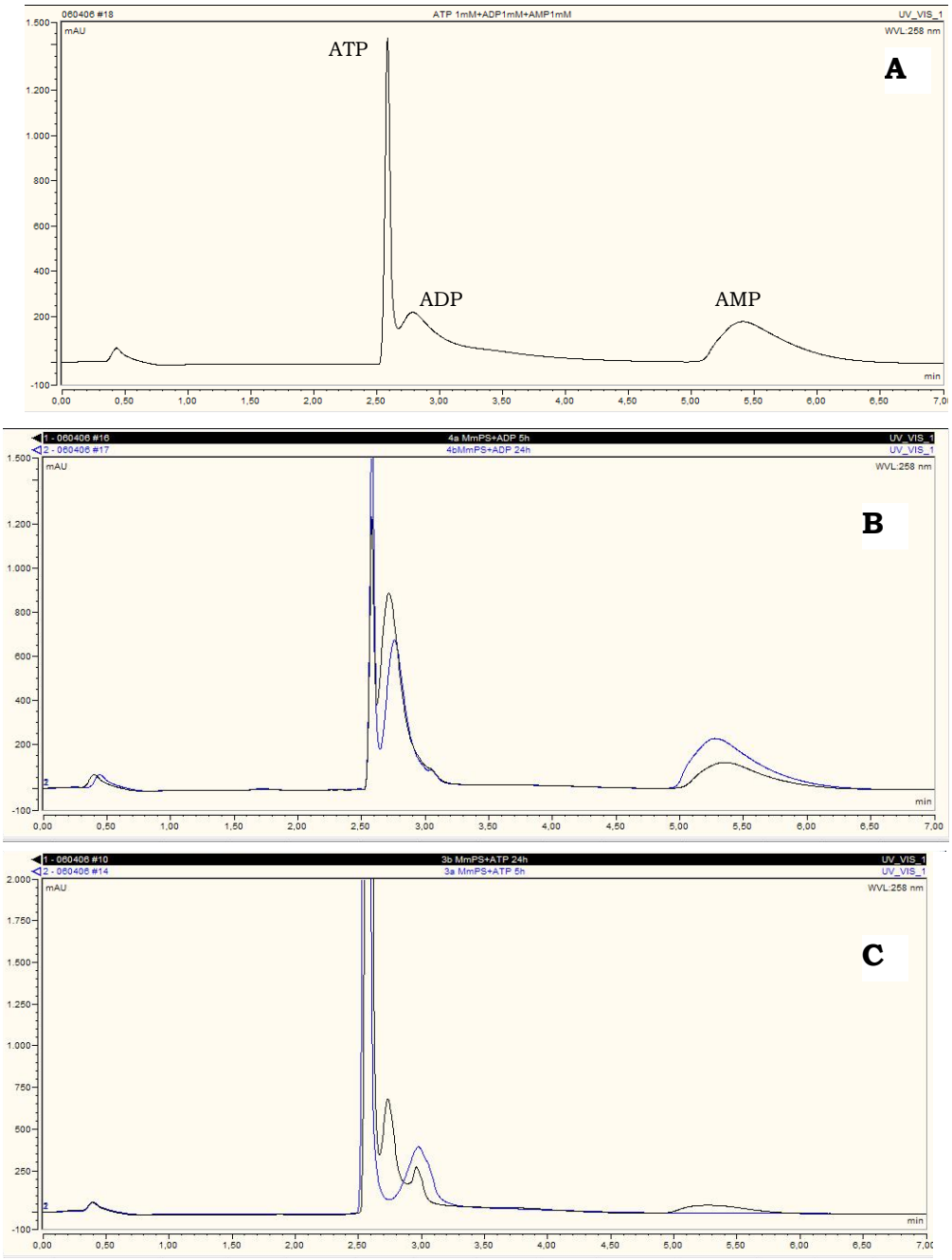


Figure 10-21: HPLC chromatogram of the reaction catalysed by MmPS carried on in absence of radioactivity. A. Mixture of ATP, ADP and AMP 1 mM each; B: reaction carried on in presence of ADP after 5 and 24h; C: reaction carried on in presence of ATP after 5 and 24h.

The appearance of the ATP peak when only ADP is added to the reaction could suggest that the reaction is close to the equilibrium and that, after a certain amount of PA is formed, this can be hydrolyzed in the inverse reaction, resulting in the formation of ATP by phosphorylation of one ADP

molecule. The presence of some AMP in the reaction mixture could mean that AMP is not a substrate for the inverse reaction either.

10.4.5 Kinetic of the reaction

To try to define more precisely which are the products of the reaction and the order with which substrates bound to the enzyme and products are released, some isotopic exchange experiments are planned. This technique is very useful to study the kinetic of the reactions. Isotope exchange experiments depend on the assumptions that isotopes of specific atoms do not influence their chemical properties and that the influences on their kinetics are small enough to be neglected. In this way, the radioactive atoms are used just like molecular markers that help identify a specific molecule in a sea of identical one in which it could be lost. The fundamental observation on which the isotope exchange technique is based is that even if a chemical reaction is at the equilibrium (when its net rate is zero by definition), the unidirectional rates through the steps can be measured by means of isotopic tracers.

Two important assumptions are normally needed for analyzing isotope exchange experiments kinetically:

1. a reaction that involves radioactive substrates follows the same mechanism as the normal reaction, with the same rate constants
2. the concentrations of all radioactive species are so low that they do not have a perceptible effect on the concentrations of the unlabelled species.

Isotope exchange experiments were carried out in this work to identify which is the minimal set of reactant that must be present to have exchange and, as consequence, which is the order with which the reagents bind to the enzyme and the product leave.

The reaction catalyzed from MmPS is:

$$\beta\text{-Ala+pantoate+ATP} \rightleftharpoons \text{PA+ ADP+P}_i \text{ (or + AMP+PP}_i\text{)}$$

As shown in figure 10-22, in the absence of pantoate or of a nucleotide, the exchange reactions do not proceed. In presence of pantoate and ATP, the maximum exchange is observed. When just ADP is added to the reaction mixture, the extent of the exchange is not so high; when P_i is added to ADP the extent of the exchange increases and, after 22h, it reaches a value close to the one reached with the addition of only ATP.

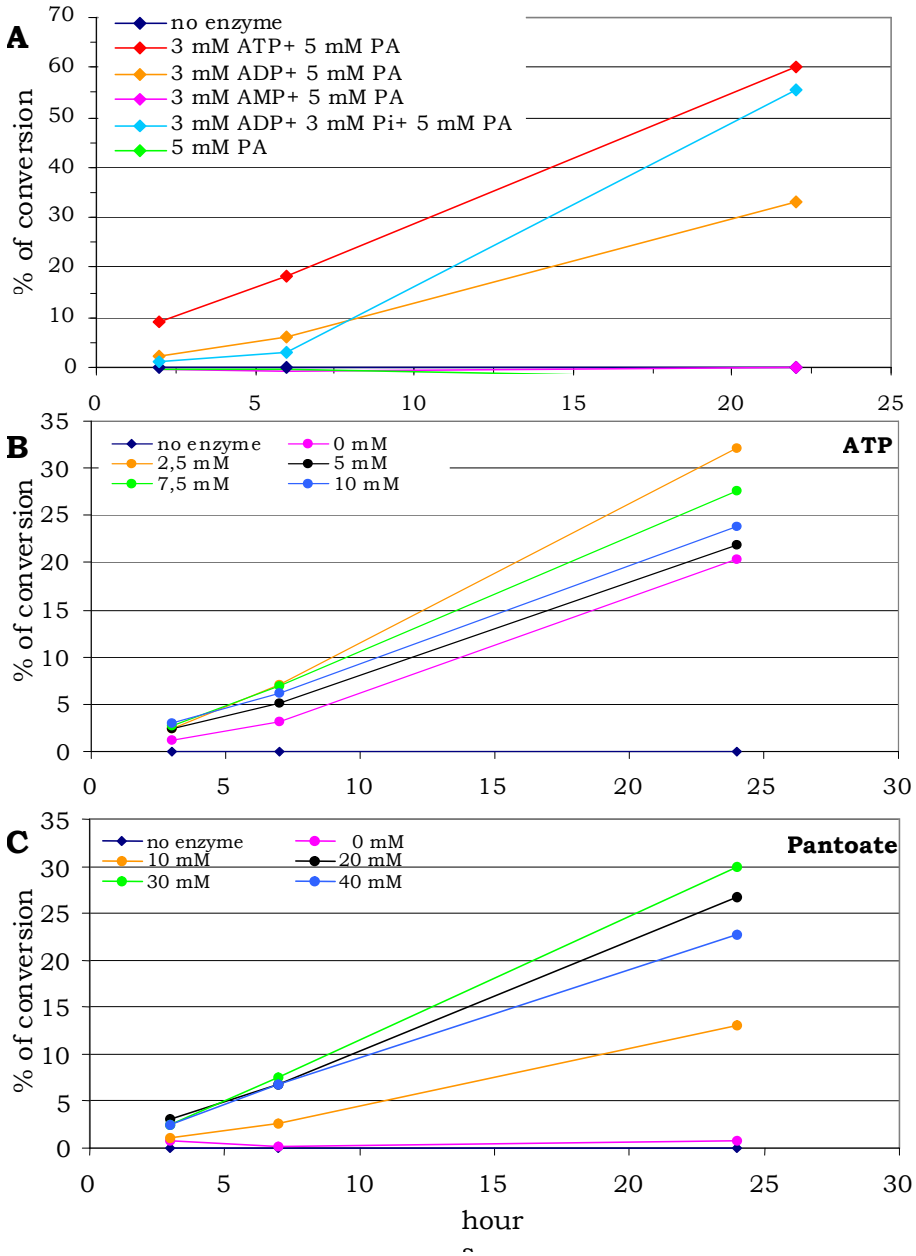
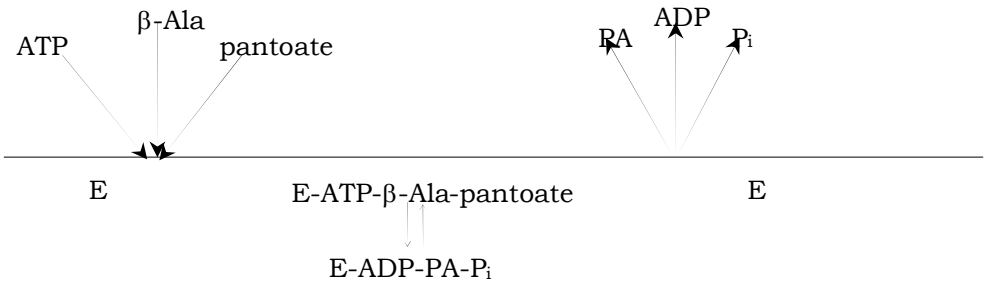


Figure 10-22: Isotope exchange reaction. Aliquots of the reactions, carried on as described in materials and methods, were taken after 2h, 6h and 22h. Graph A shows the influence of the nucleotides on the reaction; graphs B and C the influence of the concentration of ATP and Pantoate respectively.

These results suggest that pantoate binds to the enzyme after β-Ala and that the ATP is the first one to bind. Because no exchange is observed when AMP is added, this id in agreement with the HPLC analysis that showed that the product of the reaction is ADP. For what concern the order with which the products are released, nothing can be said apart that the reaction proceed with a mechanism that is different from the one described for the EcPS. In case of the coli enzyme, in fact, the mechanism described is a Bi Uni Uni Bi Ping Pong (see figure 1-10) that is not possible for the MmPS considering the results obtained. The most probable mechanism is a sequential one in which all the substrates bind to the enzyme and then all the products are released. With the data collected is not possible, at the moment, determine the order of binding and releasing of substrates and products respectively.

The suggested mechanism is shown in scheme 10-2.



Scheme 10-2: Suggested mechanism for the reaction catalysed by the MmPS

10.5 Activity of MmPPCS/PPCDC

To test the activity of the MmPPCS/PPCDC, the initial conditions were chosen as described for EcPPCS/PPCDC (Strauss, 2001). As in the case of MmPS, the reaction was carried on in presence of radioactive substrates. In this case, substrate was *P-PA, synthesized from β-Ala and pantoate in presence of EcPanK. The reaction was then spotted on a TLC, the spot of *P-PA scraped from the TLC, *P-PA extracted with ethyl acetate and then used for MmCoaBC assays.

Radioactive P-PA synthesized in this way, presented the radioactivity on the phosphorous atom or on the C atom, depending on whether the previous reaction was carried on in presence of *β-Ala or *γ-ATP. Figure 10-23 shows the results of this reaction.

The putative archaeal *CoaBC* encoding gene was identified by homologies with both the bacterial and the human homologue. The PPCS domain of this bifunctional enzyme is more similar to the bacterial one, and the PPCDC domain shows high similarity with the homologues in eukarya.

Eukaryotic PPCS uses preferably ATP as a cofactor, whereas the bacterial one use preferably CTP. The ATP binding site implies three residues in cofactor binding. Of these three, two are conserved in both bacterial and eukaryotic PPCS, the third one is conserved only in eukarya. On this basis, archaeal PPCS would be expected to use CTP as was shown for the *E. coli* enzyme (Genschel, 2004).

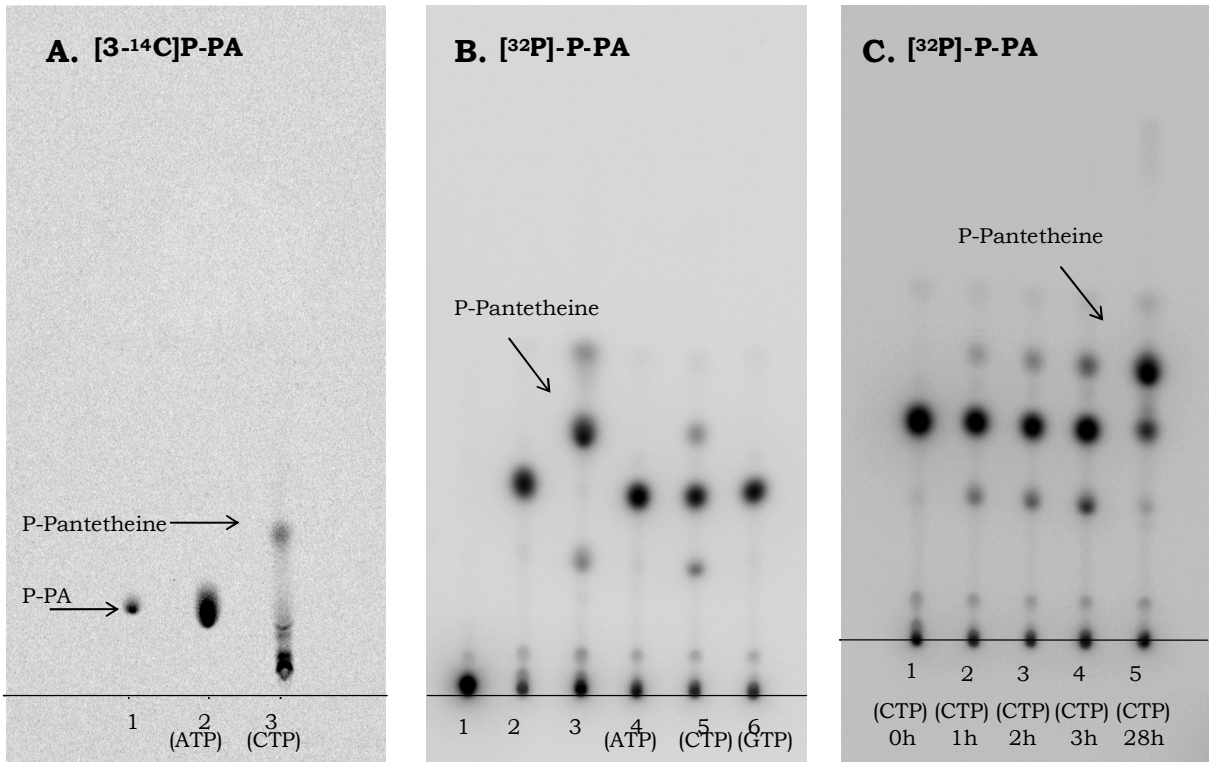


Figure 10-23:TLC of the reaction catalysed by MmPPCS/PPCDC. Part A.: Lane 1-*P-PA standard; lane 2-: MmPPCS/PPCDC+ATP; lane 3- MmPPCS/PPCDC+ CTP; Mobile phase: Dioxane: NH_3: H_2O=9:1:4. Part B. Lane 1- standard *ATP; lane 2- [^{32}P]-P-PA standard; lane 3- [^{32}P]-P-pantetheine standard; lane 4- MmPPCS/PPCDC+ATP; lane 5- MmPPCS/PPCDC+CTP; lane 6- MmPPCS/PPCDC+ GTP; Mobile phase: EtOH:NH_3=4:3. Part C.: Lane 1 – reaction at t=0h; lane 2 – reaction at t=1h; lane 3 – t=2h; lane 4: t=3h; lane 5 – t=28h. Mobile phase: EtOH:NH_3=4:3.

For this reason the assay to test the activity of the MmPPCS/PPCDC was carried out in presence of both nucleotides, ATP and CTP. In addition, GTP was tested too. As showed in figure 10-23, after the incubation of the MmPPCS/PPCDC with P-PA and cysteine in presence of CTP, a clear spot of P-pantetheine appears on the TLC. This means that the enzyme individuated and cloned from the *M.mazei* genome is really the MmPPCS/PPCDC and it behaves like the EcCoaBC. In fact, when the same reaction is carried on in presence of ATP or GTP, no P-pantetheine is produced.

The other spots present on the TLC, especially when the radioactivity is on the P atom, are probably due to oxidation products such as P-pantethine or partially phosphorilated P-pantethine. The addition of mercaptoethanol or DTT to the running buffer helps to reduce the number of spots visible but, unfortunately, affect the run of the substances and the spots appear larger and not so clear.

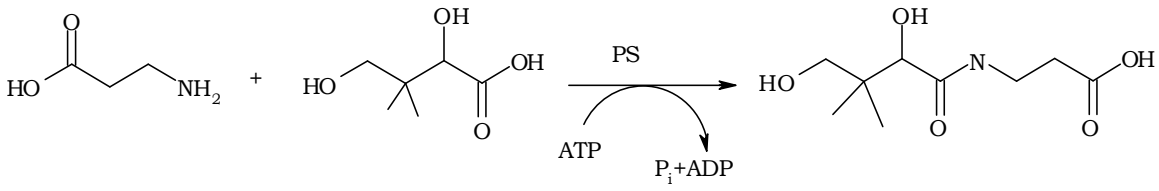
11. Discussion

All the experimental results showed up until this point are in good accordance with the original theory that the PA pathway is a universal pathway and that in archaea the enzymes involved in this metabolism are present. The genes encoding the putative ArPS, ArPanK and ArPPCS/PPCDC were cloned and, if possible, the proteins expressed in E. coli, purified and tested for the specific activity.

The complementation experiments, carried on at the beginning of this work, suggested that the enzymes that were identified were really the ArPS. In fact the complementation experiments show that the bacteria transformed with both the MjPS and the MmPS can grow in absence of PA and in presence of pantoate. The fact that bacteria transformed with these enzymes show a different growth curve in respect to the one transformed with the EcPS is not so surprising if the high differences between *Coli*'s and archaeal's proteins and genome is taken into account. Furthermore, the temperature at which the experiments are carried on is too low for the archaeal enzymes, especially in case of complementation with the MjPS. The pH of the media can also influence the activity of the enzyme: archaea can grow in extreme conditions of pH. All these factors can bring to enzymes that are not completely active.

The MmPS activity is then confirmed by enzymatic assay that prove its activity. Using radioactivity, it was possible to identify the products of the reaction and, by isotopic exchange experiments, it was possible to hypothesize a mechanism of the reaction.

The results of the experiments carried on during this work, shows that the MmPS catalyses the reaction:



Scheme 11-1: Reaction catalysed by MmPS

In this reaction, MmPS behaves differently from the EcPS. First of all, the products of the reaction catalyzed by EcPS are PA, AMP and PP_i. In case of the archaeal PS, as demonstrated by HPLC analysis of the products of the reaction, the products are PA, ADP and P_i instead. Another difference between the two enzymes is that the MmPS can use also ADP as nucleotide for the reaction, and in this case the product of the reaction are AMP, P_i and PA. HPLC analysis shows, furthermore, that when the reaction is carried on in presence of ADP, the products of the reaction are both ATP and AMP. This can mean that the reaction is reversible and, probably, close to the equilibrium. This means that when in the reaction mixture are present ADP, PA and Pi, the enzyme can catalyze the inverse reaction that bring to formation of ATP, β-Ala and pantoate. The fact that AMP is also accumulating in the reaction mixture means that it is not a substrate of the reaction.

The equilibrium of the reaction is more on the side of the reagents than on the side of the products: this is more evident by the fact that, when PA is removed from the reaction mixture via phosphorylation, the reaction is fast and arrive very soon to completion. On the other side, when PA remains in the reaction mixture, its synthesis can be afforded only in presence of high concentration of pantoate (20 mM) and the reaction is never complete.

Isotopic exchange experiments show that pantoate is the last substrate to bind to the enzyme and that ATP is the first one. Unfortunately, with the system used that places radioactivity onto the β-Ala, it is not possible to say anything regarding the order with which products leave the enzyme.

The experiments on the MmPPCS/PPCDC show that this enzyme presents the same activity as the *coli*'s and uses CTP as cofactor. This was hypothesized on the basis of the structural analysis of the enzyme.

The fact that the genes encoding these enzymes are present into the archaeal genomes confirms the universality of PA and CoA pathways.

Part 3

Conclusions and future work

12. Conclusion

This work clarified some new aspects of PA and CoA metabolism in *Arabidopsis thaliana* and demonstrated the presence of these pathways in archaea.

Regarding plants, studies on the quantification of the levels of metabolites, in particular of CoA and PA in developing seeds, were carried on to find possible correlations between them. In particular, the first aim for this part of this project was to find out if in plants there is a net degradation of PA.

From the analysis of the levels of CoA and PA, it seems that, not only this degradation is not present in *A. thaliana*, but neither a kind of correlation between this two metabolites. Their levels in developing seeds seem not related each other and it seems that a synthesis of PA is present rather than a degradation.

In the second part of this work, the attention was focalized on the archaea. Using the technique of the chromosomal proximity, candidate genes encoding for all the enzymes involved in both PA and CoA metabolisms were identified. The genes encoding for the putative ArPS, ArPanK and ArPPCS/PPCDC were cloned from genomes of three different archaea. The proteins were expressed, purified and tested for the enzymatic activity.

This work demonstrated that the putative MmPS and MmPPCS/PPCDC are really the pantothenate synthetase and the PPCS/PPCDC enzymes in this organism. They present similarity with the *E. coli* enzyme but these similarities are not so stringent.

This was the first work in which an archaeal pantothenate synthetase was overexpressed and tested.

13. Future works

Both parts of this work could be continued by a future PhD.

For what concern the part on the plants, it could be interesting to quantify not only CoA and PA but also the other intermediates in the pathway like P-PA and P-pantetheine.

Another possibility to find some correlation between metabolites is then to quantify the amount of ACP that are present into the developing seeds.

For the archaeal project, the future target is to express an archaeal PanK, perhaps changing expression vector and/or organism. Once the ArPanK is purified, it could be interesting study if it forms a complex together with the ArPS and, in case how they interact. Together with these studies, it should be important to calculate the Km values for both enzymes and clarify the mechanism of the reaction.

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