MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE *lpdA1* GENE IN *S. meliloti*

R. N. ABBAS[1,2], B. BABIC[2], M. POILLY[2], D. J. MEEK[2], B. T. DRISCOLL[2]

1. *Department of Natural Resources McGill University 21,111 Lakeshore, Ste-Anne de Bellevue, Qc, Canada H9X 3V9*
2. *Microbial Biotechnology Department, Genetic Engineering and Biotechnology institute (GEBRI), Sadat City University, Sadat City, Egypt.*

Many microorganisms, such as *Escherichia coli*, have a single gene encoding dihydrolipoamide dehydrogenase, which can function as the E3 component of different multi-enzyme dehydrogenase complexes involved in carbon metabolism and energy production. In contrast, the genome of the symbiotic N_2-fixing soil bacterium *Sinorhizobium meliloti* encodes three *lpdA* alleles. Based on their position relative to genes encoding the other complex subunits, each of the *lpdA* alleles are predicted to function in a different enzyme complex. The *lpdA1* is presumed to encode the E3 component of pyruvate dehydrogenase (PDH); *lpdA2*, the E3 component of alpha-ketoglutarate dehydrogenase (KGD); and *lpdA3*, the E3 subunit of a branched-chain alpha-ketoacid dehydrogenase (BKD). To date, no functional characterization of these *lpdA* genes has been done in *S. meliloti*. Analysis of the LpdA amino acid sequences revealed conserved functional domains, suggesting that the three *S. meliloti lpdA* alleles encode functional proteins and that each may be specific to the complex encoded by the adjacent genes. To test this hypothesis, insertion mutation was isolated for the *lpdA1* allele. Internal fragment of *lpdA1* allele cloned into plasmid pVIK112 recombined into the *S. meliloti* genome by single cross-over yielded *lpdA1* mutant. Driscoll and Finan (2006)

MATERIALS AND METHODS

1.1. Materials

1.1.1. Bacterial strains, Plasmids and growth conditions

Bacterial strains and plasmids are listed in Table (1) Complex (LBmc) and defined as (M9) media, growth conditions, and antibiotic concentrations were as described by (Finan *et al.*, 1984; Finan *et al*., 1986; Driscoll and Finan, 2006). M9 was supplemented with 0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mg/L biotin, and (Glucose, Arbinose, Pyrophate, Isolucine, lucine, Valine) according to the Mutant test.

2.2. Bioinformatics analysis

The sequence analysis was performed using several tools available at the GenBank (http://www.ncbi.nlm.nih.gov/genbank) notably the BLAST suite (blastn and blastp), using non-redundant nucleotide

and protein sequences (nr) database. The conserved domain investigation was done using the GenBank CD-search, as well the fingerprint Scan (EMBL-EBI). The multiple sequence alignment analysis was done using the CLUSTALW program (Thompson *et al.*, 1994) using Gonnet series for weight matrix for both pairwise and multiple alignment parameters. The rooted phylogram was made using CLUSTALW alignment and PHYLIP's DRAWTREE program (Phylip, 2000). The protein sequences used for the phylogenetic analysis were selected by searching the GenBank database for dihydrolipoamide dehydrogenases identified as being the part of the PDH complexes in bacteria. The identity and similarity between the *lpdA* genes sequences was estimated using the EMBOSS global pairwise alignment algorithm NEEDLE (Rice *et al.*, 2000), and the possibility of the presence of the transmembrane regions within the protein sequences was tested using the TMHMM program (Krogh *et al.*, 2001).

1.3. Enzyme assay

In preparation for the enzyme assays, the cultures were grown in LB_{mc} supplemented with the appropriate antibiotics. After centrifugation and washing of obtained cell pellets with the sterile saline (0.85% NaCl), the cell suspension was used to inoculate M9 arabinose (15 mM) and succinate (15 mM) medium, supplemented with 1% of the LB broth. The cultures were grown in minimal media to minimize the unspecific enzyme activities observed under control conditions with no substrate. Cell growth and preparation of cell-free sonicated extracts were preformed essentially as described (Finan *et al.*, 1988). Cells from late-log phase cultures were washed twice the with 20 mM Tris pH 7.8, and 1 mM $MgCl_2$, resuspended in 4 mL/g cells of sonication buffer containing 20 mM Tris pH 7.8, 1 mM $MgCl_2$, 10% glycerol and 10 mM β-mercaptoethanol, then disrupted by sonication. Protein concentration was determined by the Bradford method (Bradford, 1976), using the BioRad protein assay dye with bovine serum albumin as standard.

1.4. Molecular biology techniques

Standard techniques were used for alkaline extraction of plasmid DNA, digestion of DNA with restriction endonucleases, DNA ligations, transformation of $CaCl_2$-competent *E. coli* cells, and agarose gel electrophoresis (Sambrook *et al.*, 1989). DNA fragments were isolated from agarose gels using the QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario, Canada). Bacterial genomic DNA was extracted as previously described (Meade *et al.*, 1982). Restriction endonuclease digested genomic DNA was transferred by Southern blot to positively charged nylon membranes as described by (Sambrook *et al.*, 1989). Hybridization was detected using the colorimetric method nitroblue tetrazolium (NBT), and 5-bromo-4-chloro- 3-indolylphosphate (BCIP).

1.5. Plant growth conditions

Alfalfa (*Medicago sativa* var. Iroquois) nodulation experiments in Leonard

jars containing 1:1 (v/v) sand and vermiculite were done as previously described (Yarosh *et al*., 1989). *S. meliloti* strains were inoculated into a minimum of three pots with 10 seedlings each. Controls were inoculated with sterile distilled H_2O alone. Growth chamber (Conviron Inc., Winnipeg, Manitoba, Canada) conditions were 16 h of day (25°C) and 8 h of night (20°C). Shoot dry weights were determined from plant tops cut at the stem-root interface 28 days after inoculation. Shoots from each pot were pooled and dried for 3 weeks at 50°C prior to weighing. Percent nitrogen and carbon content analyses were done by applying 2-mg samples of ground-dried shoots to an NC2500 elemental analyzer (CE Instruments, Milan, Italy).

RESULTS AND DISCUSSION

2.1. Bioinformatics analysis

Initial investigation of the possible functionality of the *lpdA* genes and the corresponding LpdA proteins of *S. meliloti* were done by analyzing their gene and protein sequences that are available at the GenBank database. Comparison of the *lpdA* sequences using NEEDLE showed 38.7% identity and 56.0% similarity between lpdA1 and lpdA2, 31.8% identity and 52.9% similarity between lpdA1 and lpdA3, 42.6% identity, and 60.4% similarity between lpdA2 and lpdA3 protein sequences.

The search of the Genbank nucleotide database using the blastn algorithm revealed significant matches (hi score and low e-value) with the gene sequences from numerous organisms for the *lpdA1* gene sequence and *lpdA* gene(s) sequences from *R. leguminosarum* as plant-microbe associations more than *lpdA* gene(s) sequences from *A. vinelandii* as free living symbiosis organism, and *E. coli* as standard bacterial model (Fig. 1). Phylogenetic analysis of the functionally related sequences using the multiple sequence alignment (CLUSTALW) suggests that the proteins belonging to the same functional group are evolutionary closer to each other than to the other groups (Fig. 2). Interestingly, the *lpdA* of *E. coli* that is shared by both pyruvate dehydrogenase and α-keto glutarate dehydrogenase complexes is evolutionary closer to the LpdA1 and LpdA2 proteins than to the LpdA3. The *S. meliloti* mercuric reductase, MerA1, although in this case used as an out-group, also shares the common ancestry with pyruvate, and α-ketoglutarate dehydrogenase group.

2.2. Induction of the lpdA1 mutant

To examine the hypothesis that *lpdA1* gene encodes a functional protein as dihydrolipoamide dehydrogenase E3 and to incorporate a reporter gene expressed from the *lpdA1* promoters it was necessary to make mutant in *S. meliloti* that produce non-functional LpdA1 proteins and have integrated *lacZ* reporter gene. To make the *lpdA1* mutant, PCR products of the amplification of the corresponding *lpdA1* gene fragment of 263 bp long were inserted directly into the pVIK112 suicide vector (Table 2). The

vectors were constructed to contain the forward orientated gene fragment insertion, with respect to the target gene and vector's reporter gene (*lacZ*) and kmr as antibiotic resistant marker. The incorporation of the modified vector DNA into *S. meliloti* G212 genome was performed using bacterial conjugal (triparental) mating during which a single cross-over recombination of the introduced plasmid to the genome of the recipient producing the *lpdA1* mutants. Both resistance marker and expression of the *lacZ* were used to verify the correct insertion of the modified vectors into the *S. meliloti* RmG212 genome lpdA1 mutnat was confirmed by southern plot using DIG label system as shown in Fig. (3).

2.3. Growth phenotypes

The growth phenotypes of the mutant strains (Table 3) were determined by incubating cultures on solid M9 medium supplemented with acetate, arabinose, glucose, glutamate, malate, pyruvate, succinate, and leucine. The choice of carbon sources was based on predicted inability of mutants to metabolize certain carbon sources, availability and suitability of other compounds to be used for the growth evaluation. The effect of the mutation in the *S. meliloti lpdA1* gene was evident from the total inability of the organism to grow on pyruvate as a sole carbon source, which was expected due to the block in the PDH. Similarly to the *B. subtilis* PDH mutants that were unable to grow on glucose (Gao and Jiang, 2002), and the PDH deficient *P. aeruginosa* (Jeyaseelan and Guest, 1980) that failed to grow on malate, succinate, glucose, and glutamate, the *S. meliloti lpdA1* mutant was unable to metabolize those compounds growing on solid media. The inability to grow on pyruvate is different from the ability of the previously reported putative *S. meliloti* arylesterase mutant (Soto *et al.*, 2001) in which the mutation presumably had an effect on *lpdA1* transcription due to the polar effects of the Tn5 insertion.

2.4. Dihydrolipoamide dehydrogenases and associated enzyme complexes in S. meliloti

Dihydrolipoamide dehydrogenases occur as functional constitutive elements of several different multienzyme complexes, such as the pyruvate dehydrogenase complex (PDH), α-ketoglutarate complex (KDH), and branched-chain α-ketoacid dehydrogenase complex (BKD). In these complexes the LPDs act as the E3 subunits, catalyzing oxidation of dihydrolipoamide to lipoamide while reducing FAD^+ cofactor, and ultimately NAD^+ to NADH. LPD also participates in the glycine cleavage multienzyme system (GCV), in which it functions as the L protein (Wilson *et al.*, 1993). The results in Table (3) indicate that disruption of *lpdA1* gene resulted in undetectable activity of the PDH, while the KDH activity was reduced to 39%, and MDH to 79% of the activity observed for the reference RmG212 strain. The MDH and the BKD

activity remained comparable to the activity observed for the RmG212 (Table 4).

2.5. Gene expression study

The pVIK112 plasmid used for the site-targeted mutagenesis carries the promoterless *lacZ* gene that, once recombined with the targeted gene, creates a transcriptional *lpdA1:lacZ* fusion which can be used as a reporter for the examination of the expression of the *lpdA1* gene. To test the potential up- or down-regulation of the *lpdA1* gene the cultures were grown overnight in the minimal media supplemented with different carbon sources, and their effects on the transcription of the *lpdA1* gene were measured indirectly through the activity of the β-galactosidase. Test tubes containing the LB broth, and M9 minimal media supplemented with 15 mM arabinose, glutamate, glucose, leucine, malate, pyruvate and succinate were chosen to take into account the ability of the mutants to grow in the presence of the particular carbon source. Particular interest was examining the effect of the possible *lpdA1* inducers such as pyruvate, arabinose, glutamate, and leucine, which could provide an insight into the regulation of the PDH, KDH, and BKD enzyme complexes. The results (Table 3) indicate that the expression of *lpdA1* was somewhat higher under the influence of the complex medium, arabinose, malate, and succinate. It seems that both glucose and pyruvate do not have direct effect on the expression of the PDH component. The lack of substantial growth on malate and succinate can be explained by the interruption in the anaplerotic reaction in which the acetyl-CoA is replenished through the malic enzyme conversion of malate to pyruvate, and subsequently by the action of the PDH to obtain acetyl-CoA from pyruvate. Obviously, the *lpdA1* mutant's inactivated PDH is not able to complete the final conversion to the acetyl-CoA. The inability of the LpdA1 to metabolize glutamate is also not unusual, since glutamate enters the TCA cycle by the action of the glutamate synthase that converts glutamate to α-ketoglutarate, so the block in the PDH again affects the anaplerotic pathway in which the malate is used to provide the acetyl-CoA for the continuation of the TCA cycle. However, the ability to grow on arabinose is quite surprising. In rhizobia, arabinose can enter the TCA cycle through pyruvate in slow growing rhizobia, or α-ketoglutarate in fast growers, such as *R. leguminosarum* and *S. meliloti* (Duncan, 1979; Duncan and Fraenkel, 1979; Sarma and Emerich, 2006). If it is true that the arabinose enters the cycle as it was published, it is not clear why the mutant would grow better on arabinose than on the malate, succinate, and glutamate, especially knowing that it enters into the cycle at the same place as glutamate. Although much has been done to examine the ways arabinose is metabolized, some of the genes, notably arabinose dehydrogenase, and arabinolactonase, have yet to be identified in the *S. meliloti* genome (Poysti and Oresnik, 2007).

It appears that in the *lpdA1* mutant rate limiting step in functioning of TCA

cycle is the formation of the acetyl-CoA, as seen inability of the mutant to grow on acetate, leucine and beta hydroxybuyrate (data not shown) all of them entering the cycle as acetyl-CoA. Unlike the decreased activity of PDH in putative arylesterase mutant (Soto *et al.*, 2001), that might have been the result of the "leaky" Tn5 transcription terminator, the activity of PDH in *lpdA1* mutant was completely absent, similarly to the activity of PDH in *B. subtilis* LPD mutant (Gao and Jiang, 2002). The absence of detectable PDH activity, inability of the *lpdA1* mutant to grow on pyruvate, and inability to fix atmospheric nitrogen strongly indicate that it is highly unlikely that any of the other two LpdA enzymes can substitute defective LpdA1 in *S. meliloti*. Contrary to the expression of the *pdhA* gene (Cabanes *et al*., 2000) in *S. meliloti* grown in M9 minimal media with succinate or pyruvate as the sole carbon sources, the investigation of the expression of the *lpdA1* indicated that the transcription was increased in presence of succinate and not affected by pyruvate, under the same conditions. This finding gives more credibility to the hypothesis that *lpdA1* is expressed from the promoter separate from the one that regulates the *pdhABC* cluster.

2.6. Plant assay

To test the ability of *S. meliloti* strains to form a functional symbiotic relationship with the plant hosts, the plant growth evaluation was performed by infecting alfalfa seedlings (*Medicago sativa var. Iroquois)* with selected bacterial strains. The average mass of the plants inoculated with the wild type RmG212 and the *lpdA* mutants varied greatly (Table 5). The lowest mass per plant was measured for the plantsinoculated with *lpdA1* mutant. The average mass per plant was just above the uninoculated control, with several plants appearing Fix^+ (Fig. 4), however, analysis of variance (ANOVA, $F(4, 10) = 147.793$, $P<0.001$) indicated that the difference between the average plant weights of the plants inoculated with the *lpdA1* mutant, and the controls was not statistically significant at $P=0.05$. This strain produced fewer number of pink root nodules, compared to the wild-type RmG212. The phenotype of the *lpdA1* mutant was clearly Fix^- four weeks post-inoculation.

This study clearly confirmed the theoretical probability that the *S. meliloti lpdA1* gene encode functional protein, established its association with PDH enzyme complexes. However further studies for *lpdA2* and *lpdA3* genes would need to examine possible substitution of inactive *lpdA2* with any of the other two *lpdA*, or yet unknown functionally similar enzymes. It would be also interesting to investigate possibility of existence of an alternative arabinose metabolism pathway.

SUMMARY

The alfalfa-Sinorhizobium symbiosis is one of the best studied for plant-microbe associations. The pyruvate dehydrogenase complex catalyzes the key metabolic step connecting the glycolysis to the Tricarboxylic acid (TCA) cycle. The PDH also participates in anaplerotic

synthesis of acetyl-CoA in bacteroids, or when grown in the presence of dicarboxylic acids as the sole carbon sources. The complex consists of multiple copies of the three subunits, pyruvate dehydrogenase (EC 1.2.4.1), that acts as the E1 component of the complex, dihydrolipoamide transacetylase (EC 2.3.1.12), the E2 component, and dihydrolipoamide dehydrogenase (EC 1.8.1.4), that participates as the E3 subunit. The lpdA1 gene was mutated through the site-directed, single cross-over recombination, using modified pVIK112 suicide plasmids. The lpdA1 mutant failed to grow on pyruvate, and was significantly delayed on other tested carbon sources. The activity of the pyruvate dehydrogenase complex was not detected in this mutant, and the regulation of the lpdA1 was not dependent on the presence of pyruvate. This mutant was Nod+, but appeared Fix^-.

REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Cabanes, D., P. Boistard and J. Batut (2000). Symbiotic induction of pyruvate dehydrogenase genes from *Sinorhizobium meliloti*. Mol. Plant Microb. Interact., 13: 483-493.

Driscoll, B. T. and T. M. Finan (2006). NAD^+ dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. Mol. Microbiol., 7: 865-873.

Duncan, M. J. (1979). L-arabinose metabolism in rhizobia. J. Gene. Microbiol., 113: 177-179.

Duncan, M. J. and D. G. Fraenkel (1979). Alpha-Ketoglutrate dehydrogenase mutant of *Rhizobium-Meliloti*. J. Bacteriol., 137: 415-419.

Finan, T. M., E. Hartweig, K. LeMieux, K. Bergman, G. C. Walker and E. R. Signer (1984). General transduction in *Rhizobium meliloti*. J. Bacteriol., 159: 120-124.

Finan, T. M., B. Kunkel, G. F. De Vos and E. R. Signer (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol., 167: 66-72.

Finan, T. M., I. Oresnik and A. Bottacin (1988). Mutants of *Rhizobium meliloti* defective in succinate metabolism. J. Bacteriol., 170: 3396-3403.

Gao, H. X. and K. P. Jiang (2002). The E1 beta and E2 subunits of the *Bacillus subtilis* pyruvate dehydrogenase complex are involved in regulation of sporulation. J. Bacteriol., 184: 2780-2788.

http://www.ncbi.nlm.nih.gov/genbank

Jeyaseelan, K. and J. R. Guest (1980). Isolation and properties of pyruvate dehydrogenase complex mutants of *Pseudomonas aeruginosa* PAO. J. Gene Microbiol., 120: 385-392.

Kalogeraki V. S. and S. C. Winans (1997). Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene, 188, Issue 1, 25 March 1997, p. 69-75.

Krogh, A., B. Larsson, G. Von Heijne and E. L. Sonnhammer (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol., 305: 567-580.

Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown and F. M. Ausubel (1982). Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobiumme liloti* induced by transposon TnS mutagenesis. J. Bacteriol., 149: 114-122.

Phylip, (2000). Phylogeny Inference Package Version 3.2. Cladistics, 1989: 164-166.

Poysti, N. J. and I. J. Oresnik (2007). Characterization of *Sinorhizobium meliloti* triose phosphate isomerase genes. J. Bacteriol., 189: 3445-3451.

Rice, P., I. Longden and A. Bleasby (2000). EMBOSS: the European molecular biology open software suite. Trends in Genetics, 16: 276-277.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

Sarma, A. D. and D. A. Emerich (2006). Comparative proteomic evaluation of culture grown vs nodule isolated *Bradyrhizobium japonicum*. Proteomics, 6: 3008-3028.

Soto, M. J., J. Sanjuan and J. Olivares (2001). The disruption of a gene encoding a putative arylesterase impairs pyruvate dehydrogenase complex activity and nitrogen fixation in Sinorhizobium meliloti. Mol. Plant-Microbe Interact., 14: 811-815.

Thompson, J. D., D. G. Higgins and T. J. Gibson (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673-4680.

Wilson, R. L., P. S. Steiert and G. V. Stauffer (1993). Positive regulation of the *Escherichia coli* glycine cleavage enzyme system. J. Bacteriol., 175: 902-904.

Yarosh, O. K., T. C. Charles and T. M. Finan (1989). Analysis of C4-dicarboxylate transport genes in *Rhizobium meliloti.* Mol. Microbiol., 3: 813-823.

Table (1): Bacterial strains and plasmids.

Strain, Plasmid and Transposons	Relevant Characteristics	Reference
• *Escherichia coli*		
DH5α	endA1 hsdR17 (rk- mk-) supE44 thi-1recA1 gyr96 relA1 Δ(argF-lacZYA) U169 Φ80dlacZ ΔM15λ	BRL Inc.
DH5α λpir	DH5α λpir+	Lab strain
MT616	DH5α, mobilizer strain, Cmr	Finan et.al.1986.
Ec10251	pVIK112 carrier	Kalogeraki and Winans. 1997
Ec10484	DH5α λpir pVIK112 carrying 263 bp lpdA1 fragment, Kmr	This Study
• *Sinorhizobium meliloti*		
RmG212	Rm1021, Smr, Lac-	Lab strain
Rm30282	RmG212 lpda1::pBB1, lpdA1-, Smr, Tcr, Lac+	This Study
• Plasmids		
pVIK112	Suicide cloning vector, Kmr	Kalogeraki and Winans. 1997
pDM48	pVIK112 carrying 263 bp lpdA1 fragment, Kmr	This Study

Table (2): PCR primers used for the amplification of *lpdA* genes and their fragments, and for the synthesis of the Southern blot probes.

Primers[a]	Sequence
lpdA1-FW-WhG	CGAAGACAGCAGAAAACACGACTG
lpdA1-RW-WhG	TGAGAACCTCCCCGCATTGTAG
Δ*lpdA1*-FW	TTCTGAATTCTTTCCGCCTTCGCCCGTAAG
Δ*lpdA1*-RW	AATGTCTAGACGACATTGGTCTTTCCGTAGCC

[a] FW: forward oriented primer; RW: reverse oriented primer;
WhG: the primer amplifies the whole gene;
Δ: indicates that the primers amplify only a fragment of the gene

Table (3): Plate phenotypes of the *lpdA1* mutant in different carbon sources.

Strain	Acetate	Arabinose	Glutamate	Glucose	Leucine	Malate	Pyruvate	Succinate
RmG212	++	+++	++	+++	++	+++	+	+++
Rm30282	+	++	-	-	+	-	-	-

The wild-type strain, RmG212, was used as the reference strain and it was able to grow in the presence of every carbon source that was tested.

Table (4): Characterization of MDH, KDH, PDH and BKDH specific activity in *lpdA1* mutant, and wild-type RmG212 strain.

Strain	MDH			KDH			PDH			BKDH		
	SA	SE	WT%	SA	SE	WT%	SA	SE	WT%	SA	SE	WT%
RmG212	1585.0	77	100	97.5	4.4	100	26.2	0.2	100	16.8	0.4	100
Rm30282	1252.0	35	79	37.9	1.3	39	0.0	0.0	0.0	17.0	0.3	101

Malate dehydrogenase (MDH), α-ketoglutarate dehydrogenase (KDH),
Pyruvate dehydrogenase (PDH), Branched-chain α -ketoacid dehydrogenase (BKDH)

Table (5): Average weight of the individual plants per strain.

Strain	Average Plant Weight (mg)*	WT%
Uninoculated	5.60 ± 0.28	13
RmG212	42.50 ± 1.70	100
Rm30282	24.20 ± 0.90	24

The numbers after the ± sign represent the standard error of the means (n = 3). The plants were grown in groups of 10-12 plants per pot. RmG212 is the wild-type strain, Rm30282 is lpdA1 mutant, * ANOVA: $F(4, 10) = 147.793$, $p<0.001$. The average plant weights with the same letter in the superscript are not significantly different.

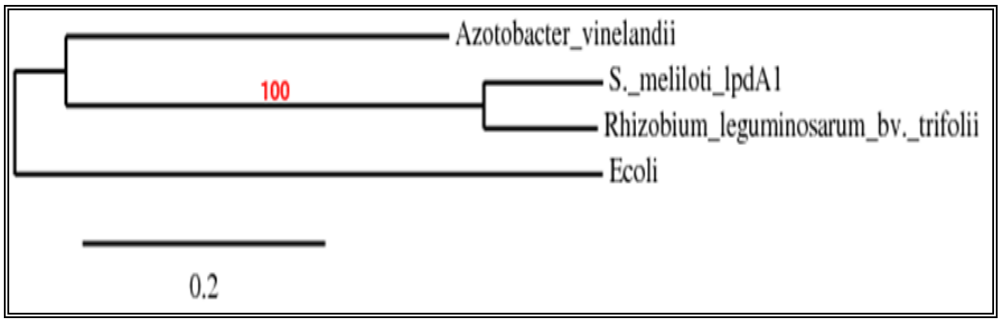

Fig. (1): Phylogenetic tree of the *lpdA1* gene from *S. meliloti* and *lpdA* gene sequences from *A. Vinelandii, R. leguminosarum trifolii* and *E. coli.*

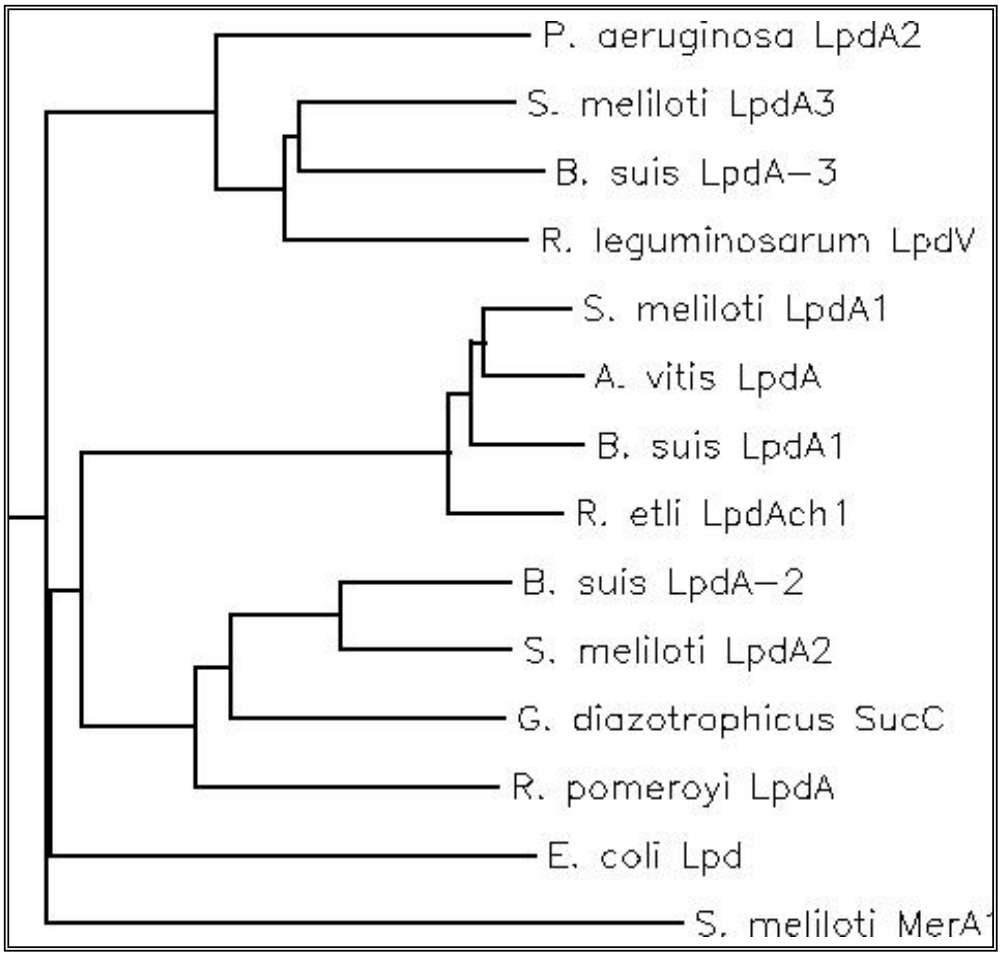

Fig. (2): LPDA rooted phylogram represents the evolutionary relationship of selected functionally-similar protein sequences. The lengths of the individual branches indicate the relative evolutionary distance between the proteins.

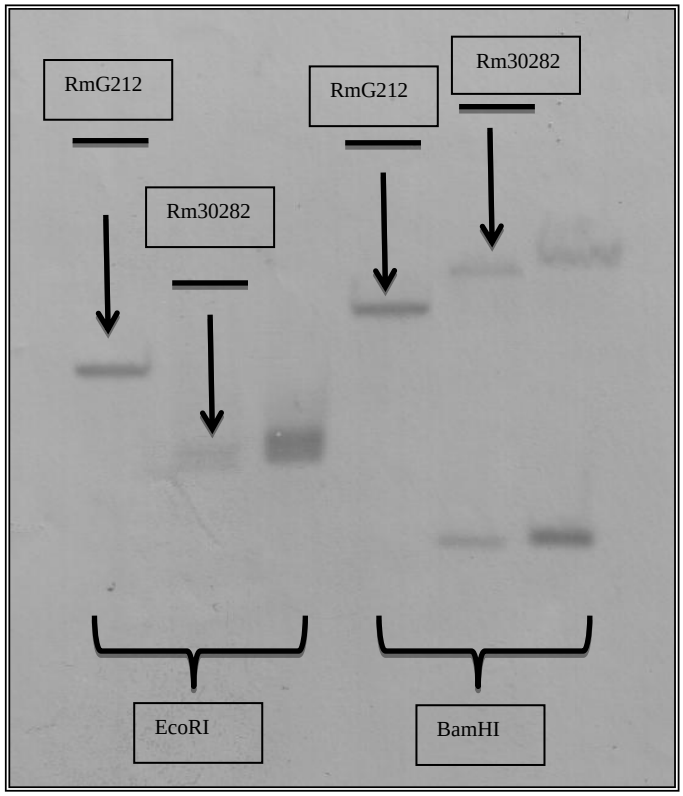

Fig. (3): Southern blot hybridization images of (A) Rm30282 (*lpdA1)*, mutant DNA. The restriction enzyme(EcoRI and BamHI) used are marked at the bottom part of the images. The unmarked lanes belong to the strains that were tested but not kept or used in this study.

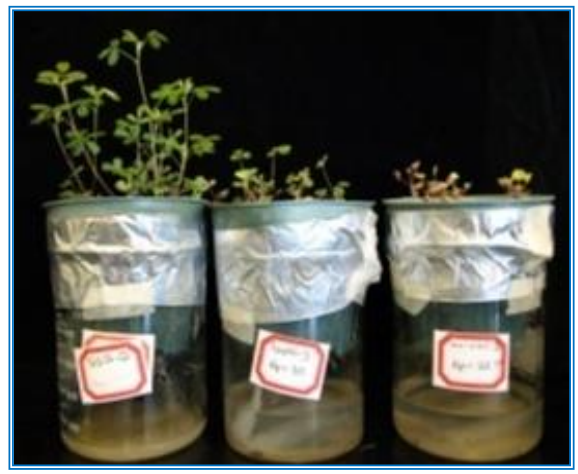

Fig. (4): The comparison of the LpdA1^{-} (Rm30282) - inoculated plants (middle) with the uninoculated control (right), and the plants inoculated with the wild-type RmG212 (left). The photograph was taken 28 days post-incubation.