Menoufia J. Agric. Biotechnology, Vol. 1 August (2016): 23 - 35

FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF THE *LPDA2* GENE IN *SINORHIZOBIUM MELILOTI*

Rateb N. Abbas[(1)*] and Noha M. Sorour[(2)]

[(1)] Department of Microbial Biotechnology and [(2)] Department of Industrial Biotechnology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt, 2285 7/79

* *E-mail address:* rateb.youssef@gebri.usc.edu.eg

Received: May 5, 2016 **Accepted: Jun. 28, 2016**

ABSTRACT: *Escherichia coli and many microorganisms, have a single gene encoding dihydrolipoamide dehydrogenase, which can function as the E3 subunit dihydrolipoamide (LpdA) component of different multi-enzyme dehydrogenase complexes. In contrast, Sinorhizobium meliloti genome encodes three lpdA alleles, each of the lpdA alleles are predicted to function in a different enzyme complex. The lpdA1 encode the E3 component of pyruvate dehydrogenase (PDH); while the lpdA2 is presumed to encode the E3 component of 2-Oxoglutarate dehydrogenase (OGD) and lpdA3, probable the E3 component of a branched-chain alpha-ketoacid dehydrogenase (BKD). To date, no functional characterization of the lpdA2 and lpdA3 genes has been done in S. meliloti. Analysis of the LpdA amino acid sequences revealed conserved functional domains, suggesting that the S. meliloti lpdA2 allele encode functional protein, which may be specific to the complex of E3 subunit of OGD. To test this hypothesis, insertion mutation was isolated for the lpdA2 allele. Internal fragment of lpdA2 allele was cloned into the plasmid pTH1703 and recombined into the S. meliloti genome by single cross-over which yielded lpdA2 mutant. Results obtained revealed that the LpdA2 mutated strain had greatly diminished OGD activity and wild-type levels of PDH and BKD.*

***Key words**: Sinorhizobium meliloti, lpdA2, TCA cycle, 2-Oxoglutarate Dehydrogenase, Mutant.*

INTRODUCTION

As a free-living soil microbe, *Sinorhizobium meliloti* has access to a wide variety of compounds available as carbon sources. Studies have shown that the preferred carbon source of *S. meliloti* is succinate over other compounds such as glucose, fructose, galactose, lactose, and myo-inositol (Harris and Sokatch, 1988; Jelesko and Leight, 1994; Rice *et al*., 2000 and Meek, 2013). Carbohydrate metabolism occurs mainly through the Entner-Doudoroff and pentose phosphate pathways (Martinaze-De Drets and Arias, 1972; Mulongoy and Elkan, 1977). Arabinose is metabolized in a multistep non-phosphorylative pathway into the tricarboxylic acid (TCA) cycle intermediate 2-Oxoglutarate dehydrogenase (OGD) (Duncan and Fraenkel, 1979). The multimeric enzymes OGD, pyruvate dehydrogenase (PDH) and branched-chain alpha-ketoacid dehydrogenase (BKD) are all similar in that they are composed of three subunits, and each corresponding subunit performs the same basic function and have LPDA as E3 subunit. The OGD complex mediates the oxidative decarboxylation of the tricarboxylic OGD to make dicarboxylic succinyl-CoA. The overall enzyme complex structure and function are similar to the previously described PDH complex (Harris and Sokatch, 1988). The difference between the two is that the first step, decarboxylation of the 2-Oxoglutarate, is mediated by 2-Oxoglotarate dehydrogenase (E1), and that the dihydrolipoamide transacetylase of PDH is replaced with dihydrolipoamide

succinyltransferase (EC 2.3.1.61), transferring the succinyl group instead of acetyl to the CoASH forming the succinyl-CoA (Meek, 2013).

The overall reaction equation is therefore:

2-Oxoglutarate + NAD^+ + CoASH → succinyl-CoA + CO_2 + NADH + H^+

Prior to this work, it was not known if those genes (*lpdA1, 2 and 3*) are actually transcribed and translated into functional proteins, or that they are the part of the associated PDH, OGD, and BKD enzyme complexes. Therefore we intended to examine the functionality of the LpdA2 enzyme and its association with the PDH, OGD, and BKD complexes, through the induction of the knock-out mutations in the *lpdA2* gene (Abbas *et al*., 2013). Moreover examine the possibility of the substitution of the inactive dihydrolipoamide dehydrogenase LpdA2 by the other two (LpdA1 and LpdA3) proteins. The present study also aims to investigate some basic elements of its regulation, and evolutionary origin.

MATERIALS AND METHODS

1. Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids are listed in Table (1); Complex LBmc, M9 media, growth conditions, and antibiotic concentrations were as previously described (Duncan and Fraenkel, 1979; Finan *et al.*, 1984; Finan *et al*., 1986; Finan *et al*., 1988; Driscoll and Finan, 1997). M9 medium was supplemented with 0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3mg/L biotin, and (glucose, arabinose, pyruvate, isolucine, lucine, valine) according to the mutant growth test (Ucker and Singer, 1978).

2. Sequences 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 progé *Sinorhizobium meliloti, lpdA,* TCA cycle*, 2-Oxoglutarate Dehydrogenase,* Mutation ram (Phylip, 1989). 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 OGD 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) as shown in Figures 1 and 2.

3. 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 (Maniatis, 1989). DNA fragments were eluted from agarose gels using the QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario, Canada). Bacterial genomic DNA was extracted as previously described by Meade *et al.* (1982). lpdA2 primers sequences used for lpdA2 fragment isolation are shown in Table (2). A 363bp fragment of lpdA2 gene was cloned into the suicide vector pTH17013 generating the pRA2

plasmid. Single homologous recombination between the cloned fragment and genomic DNA of *S. meliloti* resulted in integration of the entire pRA2 plasmid into the genome, producing the lpdA2 mutant strain RN002 (Figure 3).

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, Cm^r	Finan et.al.1986.
EcR006	DH5α λpir pTH1703 carrier	Kalogeraki and Winans. 1997
EcR007	DH5α λpir pTH1703 carrying 363 bp lpdA2 fragment, Gm^r (pRN2) Lac^-	This study
Sinorhizobium meliloti		
RmG212	Rm1021, Smr, Lac-	Wild-type strain
RN002	RmG212:pRA2,lpdA2,Sm^r,Gm^r,Lac^-	This Study
Plasmids		
pTH1703	Suicide cloning vector, Gm^r	Kalogeraki and Winans. 1997
pRA2	pTH1703 carrying 363 bp *lpdA2* fragment Gm^r Lac^-	This Study

Table (2.) PCR primers used for the amplification of *lpdA2* gene and its fragment.

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 fragment of the gene.

3.1. Polymerase Chain Reaction (PCR)

All the PCR amplifications were done using the PTC-100 thermocycler (MJ Research, Watertown, MA, USA). The reaction conditions were the same for all the primers that were used, except that the extension time was adjusted according to the length of the targeted amplicon. The initial denaturation step was done at 95 °C for 5 min, after which the Taq polymerase was added, and the reaction continued for the next 25 cycles of 30 sec of denaturation at 95 °C, 30 sec for the annealing at 62 °C, and the extension at 72 °C for 1min. Final extension was done for 5 min at 72 °C, after which the samples were retrieved and kept on ice, or in a freezer at -20 °C until needed (Invitrogen® Taq DNA polymerase kit).

3.2. Plasmid Construction

Primers Δ*lpdA2*-FW (TTCTGAATTCTTTCCGCCTTCGCCCGTAAG) and Δ*lpdA2*-REV (AATGTCTAGACGACATTGGTCTTTCCGTAGCC) were used to amplify a 363 bp internal fragment of lpdA2 and ligated to the cloning vector pGEM-T easy (pJM01). The plasmid pRN2 was generated from cloning the double digest *lpdA2* fragment from pJM01into pTH1703 using XhoI/NsiI restriction enzymes.

3.3. Conjugation (Tri-Parental Mating)

To mobilize plasmid pRN2, cultures of the recipient (*S. meliloti* Rm G212), donor *E. coli* (EcR007) and mobilizer strain (MT616) were grown overnight (O/N) in LB with appropriate antibiotic and washed 2x in sterile saline, then the three cultures mixed in a 1:1:1 ratio and spotted onto LB agar plates. Controls were the pure cultures. Following O/N incubation, the spots were scraped with a sterile stick, suspended in saline and 100 µL spread onto LB agar containing the appropriate selective antibiotics (Abbas *et al*., 2013). This produced the lpdA2 mutant strain RN002 (Figure 3).

4. Enzyme Assays

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 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 non-specific enzyme activities observed under control conditions with no substrate. Cell growth and preparation of cell-free sonicated extracts were preformed essentially as described by Finan *et al*. (1988). Cells from late-log phase cultures were washed twice 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. The MDH assay was used as control, while PDH, OGD, and BKD assays (Table 4) were used to test the effects of respective mutation on the abilities of mutated strains to metabolize target compounds, thus to confirm the inactivation of the alleged components of the three enzyme complexes.

4.1. Malate Dehydrogenase

Malate dehydrogenase (EC1.1.1.37) (MDH) assay was performed as described previously (Englard and Siegal, 1969). For the oxidation of malate reaction, each cuvette contained 100 mM glycine-NaOH (pH 10), 85 mM L-malate, 2.5 mM NAD^+ and

ddH_2O to 1 mL. For the reduction of oxaloacetate, each cuvette contained 100 mM glycine-NaOH (pH 10), 200 µM NADH, 3 mM oxaloacetate and ddH_2O to 1 mL. Reactions were monitored spectrophotometrically at wavelength 340 nm using the Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Reactions were initiated by the addition of 0.1 mg crude cell extract, extinction coefficient $\varepsilon = 6.22 \times 10^{-3}$ $nmol^{-1}$.

4.2.2-Oxoglutarate dehydrogenase assay

2-Oxoglutarate dehydrogenase (EC1.2.4.2) (OGD) assay was performed as previously described (Reed and Mukherjee, 1969). To a cuvette 50 mM phosphate buffer (pH 8), 1 mM $MgCl_2$, 2 mM NAD^+, 3 mM cysteine-HCl, 0.2 mM TPP (Thiamine Pyrophosphate), 0.1 mg crude cell extract and ddH_2O to 1 mL were added. Reactions were initiated by the addition of 60 µM Na-CoA and 1 mM 2-Oxoglutarate and monitored at wavelength 340 nm, extinction coefficient $\varepsilon = 6.22 \times 10^{-3}$ $nmol^{-1}$.

4.3. Pyruvate dehydrogenase assay

Pyruvate dehydrogenase (EC1.2.4.1) activity was done as previously described for 2-Oxoglutarate dehydrogenase (Reed and Mukherjee, 1969) by substituting 2-Oxoglutarate with Na-pyruvate (Abbas *et al*., 2013).

4.4. Branched-chained keto-acid dehydrogenase assay

Branched-chained keto-acid dehydrogenase (BKD) (EC1.2.4.4) assay was done using the modified method of Harris and Sokatch (1988). To a cuvette 30 mM potassium phosphate buffer (pH 8), 2 mM $MgSO_4$, 2 mM DTT, 0.1% Triton X-100, 0.56 mM TPP, 0.56 mM CoA, and 1.4 mM NAD^+, 0.1 mg of crude cell extract and ddH_2O were incubated at 37°C for one h and added up to the final volume of 1 mL. Reaction started with the addition of 0.28 µM keto-leucine and followed spectrophotometrically at wavelength 340 nm, extinction coefficient $\varepsilon = 6.22 \times 10^{-3}$ $nmol^{-1}$.

RESULTS AND DISCUSSION

1. Sequences Analysis

Preliminary investigation of the probable functionality of the *lpdA* genes and the corresponding LpdA proteins of *S. meliloti* were done by analyzing their genes 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 as reported by (Abbas *et al*., 2013). 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. Interestingly, the lpdA of *E. coli* that is shared by both pyruvate dehydrogenase and 2-Oxoglutarate dehydrogenase complexes is evolutionary closer to the LpdA1 and LpdA2 proteins than to the LpdA3 as shown in Figure (1).

The search of the Genbank nucleotide database using the blastn algorithm revealed significant matches (hi score and low e-value) with the *lpdA*2 gene(s) sequences of the following microorganisms *S. meliloti* , *Rhizobium etli* as plant-microbe associations symbiotic organisms, *lpdA2* gene sequences of *Azospirilum lipoferum* as free living symbiotic organism, and *E. coli* as standard bacterial model (Figure 2). On the other hand, The phylogenetic analysis of the LPD sequences indicates that the similarity between the proteins from evolutionary

distant bacteria participating in the same enzyme complexes is greater than that detected between the LpdA sequences of *S. meliloti*, suggesting that the *lpdA* genes were most probably not due to gene duplication within this species, but rather horizontal gene transfer (Jelesko and Leight, 1994; Abbas *et al*., 2013).

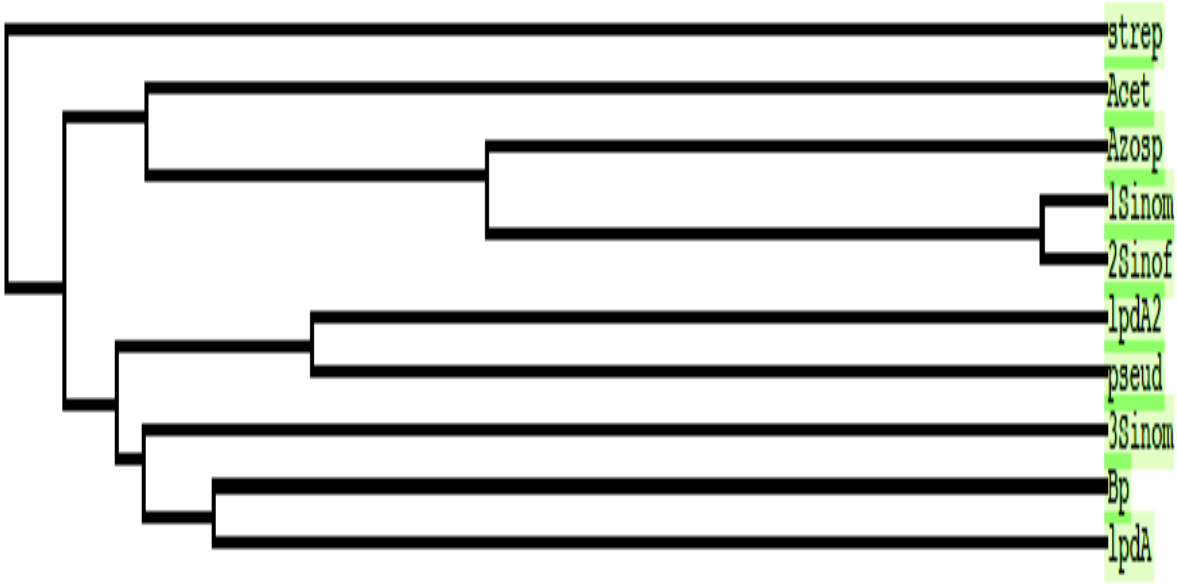

Figure 1. LPD rooted phylogram represents the evolutionary relationship of selected functionally-similar protein sequences. (Strep= lpdA2 protein sequence from Streptomyces sp, Acet =lpdA2 from *Acetobacterium wood*, Azosp=lpdA2 from *Azospirillum lipoferum*, 1Sinom=lpdA1 from *Sinorhizobium meliloti* 1021, 2Sinof= *Sinorhizobium fredii* USDA 257, lpdA2= lpdA2 from *Sinorhizobium meliloti* 1021, Pseud=lpdA2 from Pseudomonas sp. UW4, lpdA=*Escherichia coli* K-12 lpdA, Bp=lpdA2 from *Bacillus pumilus* ATCC 7061 and 3Sinom= lpdA3 from *Sinorhizobium meliloti*.

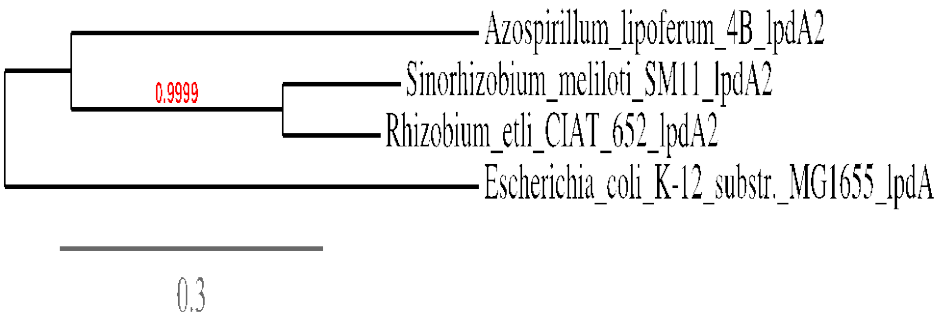

Figure 2. Phylogenetic tree of the *lpdA2* gene sequences from *S. meliloti*, *Azospirilum lipoferum, Rhizobium etli* and *lpdA* from *E. coli.*

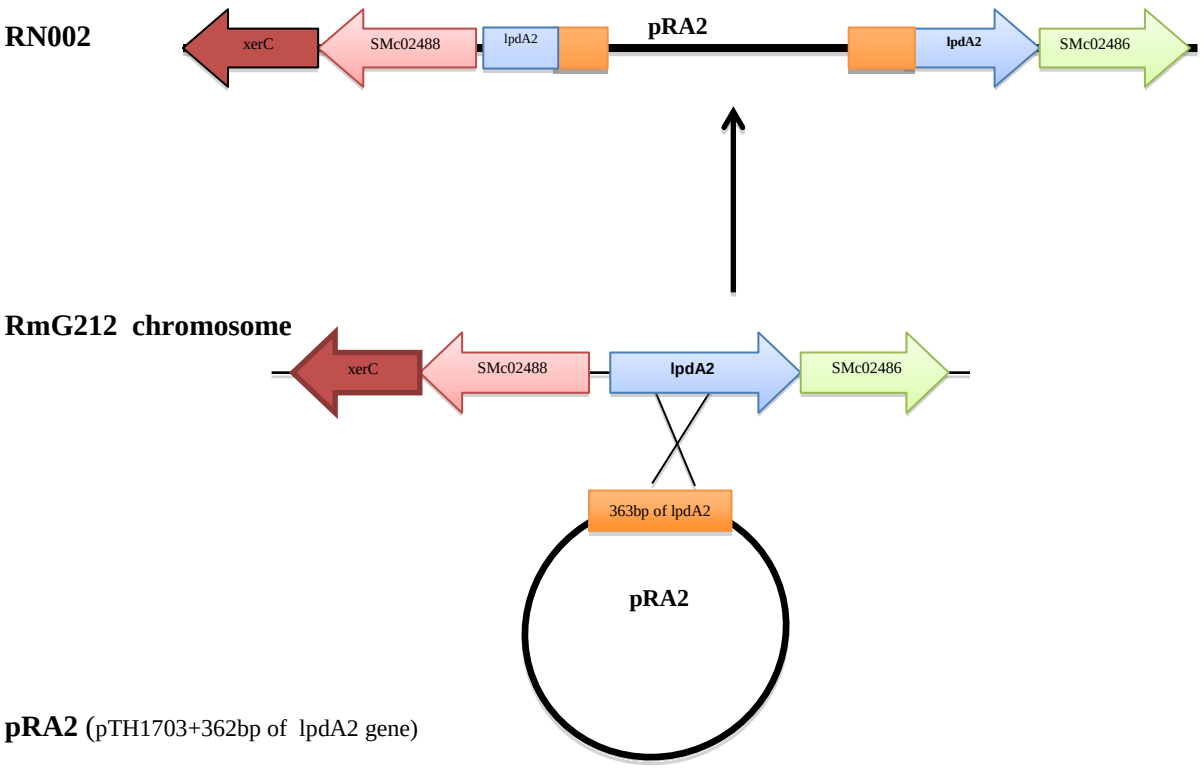

Figure 3. Schematic representation for the lpdA2 mutant RN002 construction.

2. Induction of the lpdA2 Mutant

To examine the hypothesis that lpdA2 gene encodes a functional protein as dihydrolipoamide dehydrogenase E3 componant and to incorporate a reporter gene expressed from the lpdA2 promoter it was necessary to induce mutant in *S. meliloti* that produce non-functional LpdA2 protein. To make the lpdA2 mutant, PCR product of lpdA2 gene fragment of 363 bp long was inserted directly into pTH1703 suicide vector (Tables 1 and 2). The vector was constructed to contain the forward orientated gene fragment insertion, with respect to the target gene and Gmr gene in pTH1703 as antibiotic resistant marker. The incorporation of the modified vectors DNA into *S. meliloti* Rm G212 genome was performed using bacterial conjugation (Tri-Parental Mating) in which a single cross-over of the introduced plasmid to the genome of the recipient to produce the *lpdA2* mutant (Figure 3). 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, *lpdA2* mutant was confirmed by using PCR (Figure 4). The lpdA2 primers for the complete gene were unable to amplify the entire *lpdA2* gene sequences in the mutant strain compared to the wild type (WT) strain (1400bp fragment, Fig. 4). While, the primers for the *lpdA1*-FW-WhG and the 127 lacZ F universal primers were unable to amplify the fragments, in both the WT and lpdA2 mutant strains. On the other hand, the *lpdA1*-RW-WhG and 127lacZ R universal primers were able to produce about 3kbp fragment (Figure 4). This can be explained as follow; since the distance between the

two flanks of the *lpdA2* gene were separated by the insertion of the suicide vector pRN2 (about 10kb) as a result no PCR product can be generated, while the distance between the *lpdA1*-RW-WhG primer sites and 127 lacZ R is short and about 3kb, thus can be amplified easier by the PCR reaction. The overall results indicate that the pRN2 is inserted into the *lpdA*2 gene causing its knockout and generating the *lpdA*2 mutant strain RN002 (Figure 4).

Figure 4. lpdA2 mutant RN002 detection usin PCR assay. Lane M marker DNA ladder 1Kb lane 1 PCR product of WT RmG212 DNA amplified by *lpdA1*-FW-WhG and *lpdA1*-RW-WhG preimers; lane 2 PCR product of WT RmG212 DNA amplified by *lpdA1*-FW-WhG and *127lacz F* primers; lane 3 PCR product of WT RmG212 DNA amplified by *lpdA1*-RW-WhG and *127lacz R* primer, lane 4 PCR product of RN002 DNA amplified by *lpdA1*-FW-WhG and *lpdA1*-RW-WhG primers; lane 5 PCR product of RN002 DNA amplified by *lpdA1*-FW-WhG and *127lacz F* primers; lane 6 PCR product of RN002 DNA amplified by *lpdA1*-RW-WhG and *127lacz R* primers.

3. Growth phenotypes

The growth phenotypes of the *lpdA2* mutant and WT strains (Tables 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 *lpdA2 mutant*, as expected, did not grow in the presence of arabinose as a sole carbon source on solid medium (Table 3). It grew relatively well in presence of malate and succinate, but not in the presence of the upstream supplied carbon sources with respect to the mutation position in the TCA cycle. The *lpdA2* mutant grew somewhat slower than the reference strain (RmG212) on solid LBmc.

4. Dihydrolipoamide dehydrogenases and associated enzyme complexes in *S. meliloti*

The results in Table (4) indicate that disruption of the *lpdA2* gene had significantly decreased OGD activity to 3% of WT strain level. The PDH and the BKD activities were at the wild-type levels. This mutant had significantly increased almost 3.5 time of the MDH activity, compared to the WT strain (Table 4). The increase in MDH levels was previously reported in *R. leguminosarum suc* mutants (Green and Emerich, 1997; Gao and Jiang, 2002; Sarma and Emerich, 2006) that have the identical structured *mdh-sucCDAB* operon as *S. meliloti*, as well as in the chemically induced *S. meliloti* OGD mutant in which the activity of MDH was increased 3.7 fold, while it was reported by Duncan and Fraenkel (1979) that succinyl-CoA synthetase (expressed from *sucCD*) was increased 4.7 fold, compared to the wild-type strain (Duncan and Fraenkel, 1979). The higher activity of MDH might be a response to the increased quantities of 2-Oxoglutarate, or some other related compounds or metabolites that accumulate in the cell blocked in OGD (Gao and Jiang, 2002), in an attempt to remove the blockage by increasing the transcription of the OGD genes. As a consequence the *mdh* is upregulated because it is cotranscriped from the same promoter as the *sucAB* genes in both *R. leguminosarum* and *S. meliloti*, the situation that was not encountered in *B. japonicum* (Walshaw *et al.,* 1997) which expresses monocistronic *mdh* from a separate promoter (Poole *et al.,* 1999). As OGD activity was significantly abolished in the LpdA2 mutant strain, it seems that neither LpdA1 nor LpdA3 can replace it.

Table 3. Plate phenotypes of the *lpdA2* mutant using different carbon sources.

Strain	acetate	arabinose	glutamate	glucose	leucine	malate	pyruvate	succinate
RmG212	++	+++	++	+++	++	+++	+	+++
RN002	-	-	-	++	++	+++	-	++

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; RN002: mutant strain.

Table 4. Activity of MDH, OGD, PDH and BKDH enzymes in *lpdA2* mutant RN002, and wild-type RmG212 strain.

Strain	MDH			OGD			PDH			BKDH		
	SA	**SE**	**WT (%)**	SA	SE	WT (%)	**SA**	**SE**	**WT (%)**	SA	SE	WT (%)
RmG212	590.94	15.65	100	97.5	4.4	100	18.16	1.8	100	24.81	1.3	100
RN002	1888.1	27.24	319.5	2.9	0.3	2.97	8.95	2.8	45.5	18.5	0.16	74.5

Malate dehydrogenase (MDH), 2-Oxoglutarate dehydrogenase (OGD), Pyruvate dehydrogenase (PDH), Branched-chain α -ketoacid dehydrogenase (BKDH), SA; Specific Activity, SE; Standard Error, WT; Wild-Type.

Concluding remarks

- The phylogenetic analysis of the LPD sequences indicates that the similarity between the proteins from evolutionary distant bacteria participating in the same enzyme complexes is greater than between the LpdA sequences of *S. meliloti*, suggesting that the *lpdA* genes were most probably not a product of the gene duplication within this species, but rather horizontal gene transfer.
- Enzyme activity from the LpdA2 mutant had greatly reduced OGD activity and increased MDH activity. OGD activity may not completely abolished in this strain due to background activity from the non-specific dehydrogenase present in all crude extracts.
- As OGD activity was significantly abolished in the LpdA2 mutant strain, it seems that neither LpdA1 nor LpdA3 can replace it.

Acknowledgments

Thanks to Dr. Brian Driscoll (Department of Natural Resources, McGill University, Montreal, Canada) for bacterial strains (RmG212 and *E. coli* strains) and for the time I spent in his lab.

Conflict of Interest Statement

All authors declare that there are no financial/commercial conflicts of interest.

REFERENCES

Abbas, R., B. Babic, M. Poilly, D. Meek and B. Driscoll (2013). molecular and functional characterization of the lpda1 gene in *S meliloti*. Egypt J Genet Cytol, 42:405-416.

Bradford, M.M. (1976). A. rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry; 72:248-54.

Cabanes, D., P. Boistard and J. Batut (2000). Symbiotic induction of pyruvate dehydrogenase genes from *Sinorhizobium meliloti*. Molecular Plant-Microbe interactions;13:483-93.

Charles, T.C., R.S. Singh and TM. Finan (1990). Lactose utilization and enzymes encoded by megaplasmids in *Rhizobium meliloti* SU47: implications for population studies. Microbiology, 136:2497-2502.

Driscoll, B.T. and T.M. Finan (1993). NAD+-dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. Molecular microbiology, 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-Ketoglutarate dehydrogenase mutant of *Rhizobium meliloti*. J of Bacterio, 137:415-419.

Englard, S. and L. Siegel (1969). Mitochondrial l-malate dehydrogenase of beef heart:[EC 1.1. 1.37 l-Malate: NAD oxidoreductase]. Methods in enzymology, 13:99-106.

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

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.

Gao, H., X. Jiang, K. Pogliano and AI. Aronson (2002). The E1β and E2 subunits of the *Bacillus subtilis* pyruvate dehydrogenase complex are involved in regulation of sporulation. J Bacteriol, 184:2780-2788.

Green, L.S. and D.W. Emerich (1997). The formation of nitrogen-fixing bacteroids is delayed but not abolished in soybean infected by an [alpha]-ketoglutarate dehydrogenase-deficient mutant of *Bradyrhizobium japonicum*. Plant physiology, 114:1359-1368.

Green, L.S., J.K. Waters, S. Ko and D.W. Emerich (2003). Comparative analysis of the *Bradyrhizobium japonicum* sucA region. Canadian journal of microbiology, 49:237-243.

Harris, A. and R. Sokatch (1988). Branched-Chain amino acid. Methods Enzymol 166:181-182.

Hornez, J.P., M. Timinouni, C. Defives and JC. Derieux (1994). Unaffected nodulation and nitrogen fixation in carbohydrate pleiotropic mutants of *Rhizobium meliloti*. Current Microbiology, 28:225-229.

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

Jelesko, J.G. and JA. Leigh (1994). Genetic characterization of a *Rhizobium meliloti* lactose utilization locus. Molecular microbiology, 11:165-173.

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, 188:69-75.

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. Journal of molecular biology, 305:567-580.

Maniatis, T. Molecular cloning: a laboratory manual. In: Sambrook, J., *et al (*1989). (Eds.) Cold Spring Harbor Laboratory P*ress,* New York;.

Martinez-De Drets, G. and A. Arias (1972). Enzymatic basis for differentiation of Rhizobium into fast-and slow-growing groups. J Bacteriol, 109:467-470.

Meade, H.M., S.R. Long, G.B. Ruvkun, S.E. Brown and FM. Ausubel (1982). Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. J Bacteriol, 149:114-122.

Meek, D. (2013). The biochemical and genetic characterization of the TCA cycle in *Sinorhizobium meliloti*. Ph.D. Thesis, Department of Natural Resource Sciences, McGill University, Montreal, Canada;

Miller, H. (1972). Experimental in molecular genetics. Cold Spring laboratory, Cold Spring Harbor, New York; p. 352-359.

Mulongoy, K., G.H. Elkan (1977). The role of 6-phosphogluconate dehydrogenase in Rhizobium. Canadian journal of microbiology, 23:1293-1298.

Phylip, F. (1989). Phylogeny Inference Package (Version 3.2). Cladistics; 5:164-166.

Phylip, F. (2000). Phylogeny Inference Package (Version 3.3). Cladistics; 5:164-166.

Poole, P., C. Reid, A.K. East, D. Allaway, M. Day and M. Leonard (1999). Regulation of the mdh-sucCDAB operon in *Rhizobium leguminosarum*. FEMS microbiology letters;176:247-255.

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

Reed, L.J. (1969). Mukherjee BB. α-ketoglutarate dehydrogenase complex from *Escherichia coli*. Methods in enzymology, 13:55-61.

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

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

Thompson, J.D., D.G. Higgins, T.J. Gibson and W. Clustal (1994). 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.

Trottier, O. (2008). Genetic characterization of gamma-aminobutyrate metabolism in *Sinorhizobium meliloti*. MSc. Thesis, Department of Natural Resource Sciences, McGill University, Montreal, Canada.

Ucker, D.S. and E.R. Signer (1978). Catabolite-repression-like phenomenon in *Rhizobium meliloti*. J Bacteriol, 136:1197-1200.

Walshaw, D.L., A. Wilkinson, M. Mundy, M. Smith and P.S. Poole (1997). Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*. Microbiology, 143:2209-2221.

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

التوصيف الوظيفي و الجزيئي لجين lpdA2 في السيانوريزوبيم ميليلوتي

راتب نبيل عباس[١] ، نهي محمد سرور[٢]

[١] قسم البيوتكنولوجيا الميكروبيه معهد بحوث الهندسه الوراثيه والتكنولوجيا الحيويه . جامعه مدينه السادات

[٢] قسم البيوتكنولوجيا الصناعيه معهد بحوث الهندسه الوراثيه والتكنولوجيا الحيويه . جامعه مدينه السادات

الملخص العربي

علي عكس بكتريا القولون و العديد من الكائنات الدقيقه الاخري و التي تحتوي علي جين وحيد من الـ lpdA فآن بكتريا السيانوريزوبيم ميليلوتي تحتوي علي ثلآثه اليلات من جين وحيد من الـ lpdA يعتقد ان lpdA يشفر لمكون الـ E3 الخاص بانزيم هدرجه البيروفيات (E3 component of pyruvate dehydrogenase) وان 2lpdA يشفر لمكون الـ E3 الخاص بانزيم هدرجه ٢الديآوكسي جلوتاريت

(E3 component of 2-Oxoglutarate dehydrogenase) وان3lpdA يشفر لمكون الـ E3 الخاص بانزيم هدرجه الفا . كيتواسييد ذو السلسله المتفرعه. (E3 component of a branched-chain alpha-ketoacid dehydrogenase) . حتي الان لم يتم عمل التوصيف الوظيفي لكل من الـ lpdA2 وlpdA3 . و بدراسه جين الـ dA2lp وجد انه يشفر لتتابع من البروتين الوظيفي يعتقد انه الـ E3 الخاص بانزيم الديآوكسي جلوتاريت- ٢ديهيدروجينياز . وللتآكد من تلك الفرضيه تم عمل طفره لجين الـ lpdA2 من خلال استنساخ قطعه من الجين واضافتها الي البلازميد PTH1703 و اعادتها الي الجينوم الخاص ببكتريا السيانوريزوبيم ميليلوتي مما ادي الي وقف عمل الجين و الحصول علي سلاله طافره في جين lpdA2 من بكتريا السيانوريزوبيم ميليلوتي و التي اظهرت الاختبارات الخاصه بالنشاط الانزيمي لها مستوي شبه منعدم لا نزيم هدرجه ٢الديآوكسي جلوتاريت

ومماثل للطرز البري بالنسبه لانزمي هدرجه البيروفيات و هدرجه الفا- كيتواسييد ذو السلسله المتفرعه. مما يؤكد عدم قدره اي من الاليلين الـ lpdA1 وlpdA3 من تعويض غياب الاليل lpdA2 و انه مختص منفرداً بالتشفير لمكون الـ E3 الخاص بانزيم هدرجه ٢الديآوكسي جلوتاريت.

"Functional and Molecular Characterization of the *lpdA2* gene in *Sinorhizobium meliloti*"

Rateb Nabil Abbas* [(1),] Noha Mohamed Sorour[2]

[1] * Department of Microbial Biotechnology,
[2] Department of Industrial Biotechnology,
Genetic Engineering and Biotechnology
Research Institute,
University of Sadat City,
Sadat City,
Egypt, 22857/79