Effect of Dihydrolipoamide Dehydrogenase *LpdA3* Gene Knockout in *Sinorhizobium meliloti* Metabolism

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ABSTRACT

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 is predicted to function in a different enzyme complex. The *lpdA1* is encoding the E3 component of pyruvate dehydrogenase (PDH);*lpdA2*, the E3 component of 2-Oxyglutarate dehydrogenase (OGD), while the *lpdA3* is presumed to encode the E3 subunit of a branched-chain alpha-ketoacid dehydrogenase (BKD). To date, no functional characterization of *lpdA3* gene has been done in *S. meliloti*. Analysis of the LpdA3 amino acid sequences revealed conserved functional domains, suggesting that the *S. meliloti lpdA3* allele encode functional proteins, as well as, each may be specific to the complex encoded by the adjacent gene. To test this hypothesis, insertion mutation was induced in the *lpdA3* allele. Internal fragment of *lpdA3* allele cloned into plasmid pVIK112 recombined into the *S. meliloti* genome by single cross-over yielded *lpdA3* mutant. The resulting mutant carried transcriptional *lpdA:lacZ* fusions. The carbon and amino acids utilization phenotype of *lpdA3* mutant was found to be distinct and indicative of the enzyme complex rendered non-functional by the mutation, BKD is almost demolished in the *lpdA3* mutant and both the PDH and OGD activities were similar to wild-type (WT) level.

Keywords:Sinorhizobium meliloti, lpdA3,TCA cycle, Branched-chain alpha-ketoacid dehydrogenase, insertmutation.

INTRODUCTION

Only one gene encodes for E3 subunit dihydrolipoamide dehydrogenase (*lpdA*) in *E. coli* (Guest *et al* . 1981). Located immediately downstream of the genes encoding for E1 (*aceE*) and E2 (*aceF*) subunits of the PDH complex, *lpdA*can be expressed with *aceEF*as one polycistronic message from the promoter (P_{ace}) upstream of *aceE*or as a single transcript from its own promoter (P_{lpd}). Expression via two promoters explains how a single *lpdA*gene product can be used in more than one enzyme complex(Eley*et al*., 1972). Having one gene for the E3 subunit of multiple enzymes appears to be quite common in the literature with examples including the bacteria *Azotobacter vinelandii*(Westphal and de Kok, 1988) and *Bacillus subtilis*(Lowe *et al*. 1983), *Saccharomyces cerevisiae*(Dickinson et al . 1986), and *Homo sapiens* (Otulakowskiet al . 1988), all of which have one *lpdA*allele expressed from its own promoter.

Members of the order Rhizobiales seem to be an exception; many species of this order have multiple copies of *lpdA*. *S. meliloti*has three *lpdA*alleles in its genome (Capela*et al*. 2001) with each gene found in close proximity to, or as part of, an operon with the other subunits of pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGD), or branched-chain alpha-ketoacid dehydrogenase (BKD). The locus for *lpdA1* is two open reading frames (ORFs) downstream of an operon encoding for the E1 and E2 subunits of PDH (*pdhABC*), while *lpdA2* is three ORFs downstream of the operon for the other subunits of OGD (*sucAB*). The operon for E1 and E2 subunits of BKD includes, and terminates with *lpdA3*. The hypothesis is that each *lpdA*gene product is specific to the complex of which it is in proximity and are not interchangeable.

The multimeric enzymes PDH, OGD and BKD are all similar in that they are composed of three subunits, and each corresponding subunit performs the same basic function. In *E. coli* the core of the PDH complex, to which the E1 and E3 subunits are non-covalently attached, comprises twenty-four E2 subunits. To these are bound 12 copies of two identical subunits of E1, and 6 copies of two identical subunits of E3 (Eley*et al*. 1972). This large complex is approximately 45 nm in diameter making it visible under electron microscope. The aim of this work is to study the function of *lpdA3*gene in *S. meliloti*e.To achieve this goal,insert mutation was induced for the *lpdA3* allele. Internal fragment of *lpdA3* allele was cloned into the plasmid pVIK112, recombined into the *S. meliloti* genome by single cross-over to yield *lpdA3* mutant, and then tested for three suggested enzyme activities, PDH, OGD and BKD.

MATERIALS AND METHODS

1-Bacterial Strains, Plasmids and Growth Media

Bacterial strains and plasmids used in this study 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, 1993). 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.

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. The multiple sequence alignment analysis was done using the smartblastalignprogram (Thompson *et al.*, 1994). The rooted phylogram was made using smartblast alignment and PHYLIP's DRAWTREE prog. *Sinorhizobium meliloti, lpdA,* TCA cycle*, BKA,* Mutationram (Phylip, 1989 and 2000). The protein sequences used for the phylogenetic analysis were selected by searching the GenBankdatabase 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).

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). Restriction endonuclease digested genomic DNA was transferred by Southern blot to positively charged nylon membranes as described by Roche DIG-system (Roche, Laval, Canada). Hybridization was detected using the colorimetric method nitro-blue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolylphosphate (BCIP). *lpdA3* primers sequences used for *lpdA*3 fragment isolation are shown in table (2).

Polymerase chain reaction (PCR)

All the PCR amplifications were done using the PTC-100 thermocycler. The reaction conditions were the same for all the primers, 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 5min, after which the Taq polymerase was added, the reaction was continued for the next 30 cycles of 30 sec of denaturation at 95 °C, 30 sec for the annealing at 62 °C, and the extension at 72 °C for 2 min for the complete *lpdA3*gene and for 45sec for the *lpdA3*internal fragmentamplification. 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.(Meek 2013, Abbas *et al* ., 2013, Abbas and Sorour 2016) The PCR primers [Invitrogen Taq polymerase kit]used in this study are listed in Table 2.

Plasmid construction

Primers ∆*lpdA3*-FW (GGCGCTGATTTT CGTTGAAGGA) and ∆*lpdA3*-REV (CGGTGAA TCCGGGATTCAGTT) were used to amplify a 492 bp internal fragment of lpdA3 and ligated to the cloning vector pGEM-T easy (pJM01). The fragment from a double digest using *EcoR*1 and *Kpn*I was ligated to similarly digested pVIK112 resulting in pRN1 plasmid(Meek 2013, Abbas *et al* ., 2013, Abbas and Sorour 2016).

Table 1. Bacterial strains and plasmids.

Strains and plasmids	Relevant characteristics	Reference
Escherichia coli		
DH5α	endA1 hsdR17 (rk- mk-) supE44 thi-1recA1 gyr96 relA1 ∆(argF-lacZYA) U169 Φ80dlacZ ∆M15λ	BRL Inc.
DH5α λpir	DH5α λpir+	Lab strain
MT616	DH5α, mobilizer strain, Cm^r	Finan et.al.1986.
EcR001	DH5α λpir+ pVIK112 carrier	Kalogeraki and Winans.1997
EcRoo2	DH5α λpir pVIK112 carrying 492 bp lpdA3 fragment, Km^r(pRA1)	This Study
Sinorhizobiu mmeliloti		
RmG212	Rm1021, Smr, Lac-	Lab strain
RN001	RmG212:pRA3, lpdA3-, Sm^r, Nm^r, Lac+	This Study
Plasmids		
pVIK112	Suicide cloning vector, Km^r	Kalogeraki and Winans.1997
pRA1	pVIK112 carrying 492 bp lpdA3 fragment, Km^r	This Study

Table 2. PCR primers used for the amplification of *lpdA3*gene and it's fragment, and for the synthesis of the Southern blot probe.

Primers	Sequence
Lpd3-FW-WhG	GGCGCTGATTTTCGTTGAAGGA
lpdA3-RW-WhG	CGGTGAATCCGGGATTCAGTT
∆*lpdA3*-FW	TCTAGAATTCTTCGTAGACGGCAAGACGGTGG
∆*lpdA3*-RW	TCAATCTAGACGATTTCCTCCAACCCCCAG

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

Conjugation (tri-parental mating)

To mobilize plasmid pRN3, cultures of the recipient (*S. meliloti*Rm G212), donor *E. coli* (EcR001) and mobilizer strain (MT616) were grown overnight (O/N) in LB with appropriate antibiotic and washed 2x in sterile saline, then the three cultures were 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. This produced the

lpdA3 mutant strain RN001(Abbas *et al* ., 2013, Abbas and Sorour 2016).

4. Enzyme assays

In preparation for 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) , M9Pyruvate (15 mM), M9 Succinate(15 mM) and M9 Lucine 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 mMTris pH 7.8, and 1 mM MgCl$_2$, resuspended in 4 ml/g cells of sonication buffer containing 20 mMTris 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 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.

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$_2$O 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$_2$O 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 ε = 6.22 x 10^{-3} nmol^{-1}.

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$_2$O 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 ε = 6.22 x 10^{-3}nmol^{-1}.

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).

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$_2$O 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 μMketo-leucine and followed spectrophotometrically at wavelength 340 nm, extinction coefficient ε = 6.22 x 10^{-3} nmol^{-1}.

RESULTS AND DISCUSSION

Sequence analysis

Initial investigation of the possible functionality of the *lpdA* genes and the corresponding LpdA proteins of *S. meliloti* was done by analyzing their gene and protein sequences that are available at the GenBank database. Comparison of the LpdA sequences using BLAST between LpdA1 and LpdA2 protein showed 39% identity, 32% between LpdA1 and LpdA3, and 43% identity, 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 all of the three gene sequences. Similar results were obtained using the blastp search against non-redundant protein sequences database (nr) (Figure 1). Fingerprint search using blast proteinidentified several different motifs in lpdA3 and the lpdA1 and lpdA2, notably a complete pyridine nucleotide-disulphide oxidoreductase class-I, and FAD-dependent pyridine nucleotide reductase signature, but also some other partial signatures such as mercuric reductase class II signature. Also conserved domain like pyridine nucleotide-disulphide oxidoreductase, dimerization domain, and one complete, and one incomplete domain belonging to NAD(P)-binding Rossman-fold protein superfamily were found (Figure 2) chain Alpha- ketoacids. Unlike the *lpdA1* and *lpdA2* gene position in relation to the rest of the genes from the associated complexes, the close association of the *lpdA3* sequence with the *bkd*sequence cluster strongly suggest that *lpdA3* is expressed from the same promoter as the rest of the BKD genes(Figure 3).The phylogenetic analysis of the LPD amino acids 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 (Jelesko and Leight, 1994; Abbas et al ., 2013, Abbas and Sorour, 2016)
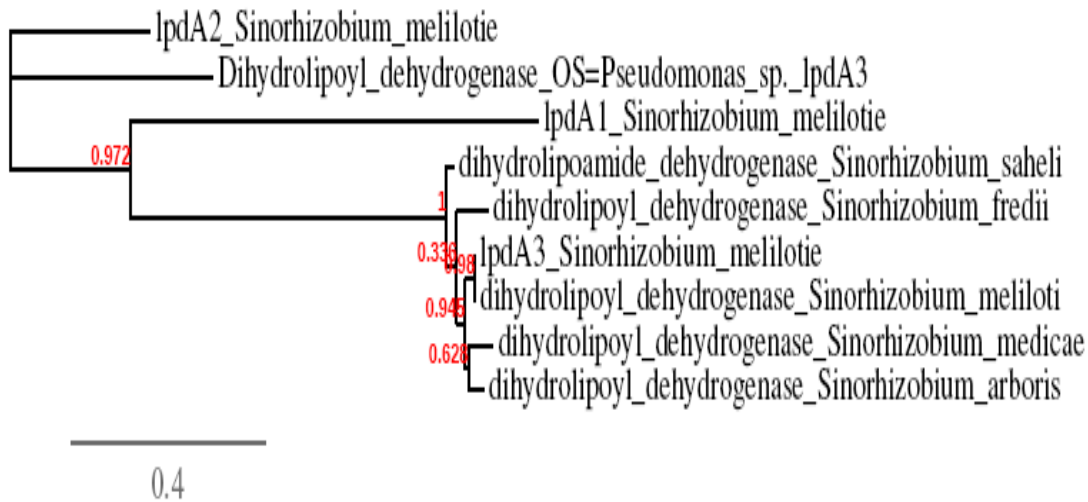
Figure 1. LPDA Rooted Phylogram representingthe evolutionary relationship of selected functionally similar protein sequences in some microorganisms.
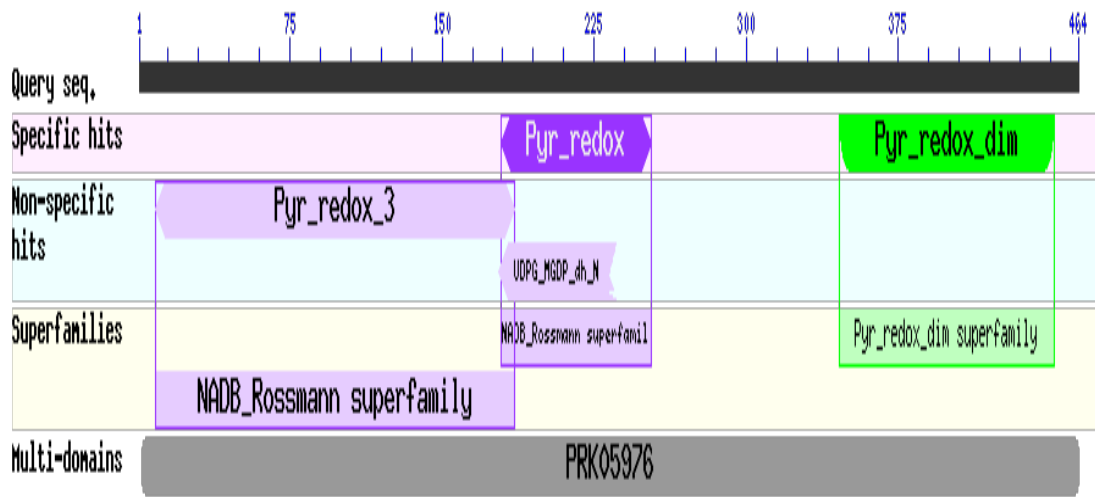

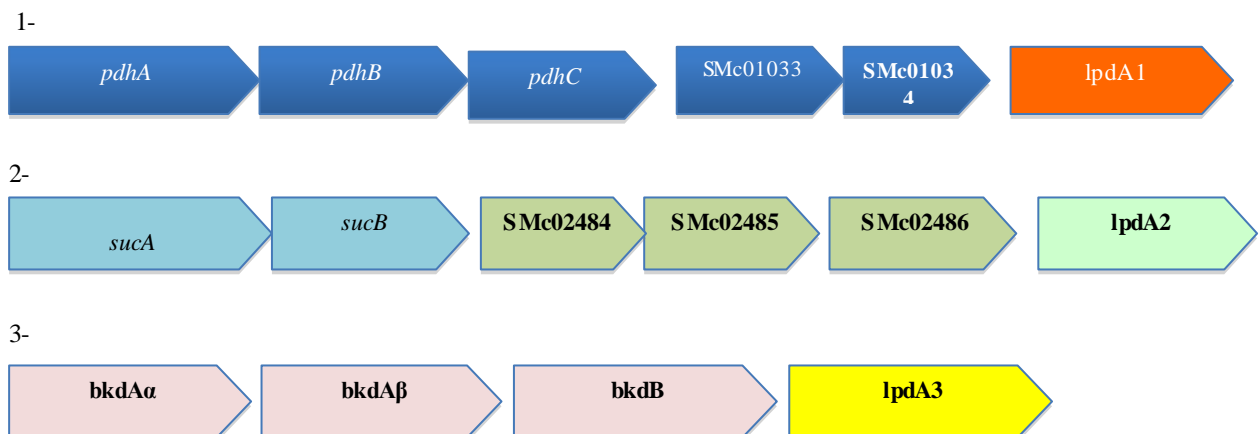**Figure 2.Conserved domains of lpdA3 protein in *S. meliloti*using blast protein program.**

Figure 3. Graphic representation of the 1) pyruvate dehydrogenase, 2) 2-oxyglutarate dehydrogenase, and 3) branched chain α-ketoacid dehydrogenase gene arrangements in *Sinorhizobiummeliloti*.The direction of the transcription is indicated by arrows that depict the genes.

Construction of the *lpdA3* mutant

To examine the hypothesis that *lpdA3* gene encodes a functional protein and to incorporate a reporter gene expressed from the *lpdA3* promoters it was necessary to make *S. meliloti* mutant that produce non-functional LpdA3 protein and have integrated *lacZ* reporter gene. To make the *lpdA3* mutant, PCR product of the amplification of the corresponding gene fragment (493 bp) was inserted directly into the pVIK112 suicide vector. The vector was constructed to contain the forward orientated gene fragment insertion, with respect to the target gene and vector's reporter gene (*lacZ*). 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 cells producing the *lpdA3* mutant. Both resistance markers and expression of the *lacZ*were used to verify the correct insertion of the modified vectors into the *S. meliloti* RmG212 genome. The presence of the targeted insertional mutation in the obtained mutant strains was confirmed using southern blot hybridization. The mutation was confirmed by comparing the hybridization image of wild-type RmG212 genomic DNA restriction pattern and the pattern of the suspected *lpdA3* mutant. Forward and reverse whole gene primers were used for the *lpdA3* DIG-probe synthesis. The lpdA3 probe detected 2 bands in the mutant RN001 digested with *PstI* and one band detected in wild-type (WT) strain RmG112. Results obtained in (Figure 4) confirmed that RN001 has the constructed vectors inserted inside the bacterial chromosome of the *lpdA3* mutant (Abbas *et al.*, 2013).

Growth phenotypes

The growth phenotypes of the mutant strain was determined by incubating cultures on solid M9 media supplemented with acetate, arabinose, glucose, glutamate, malate, pyruvate, succinate and leucine. The choice of carbon sources was based on predicted inability of mutant to metabolize certain carbon sources, and availability and suitability of other compounds to be used for the growth evaluation. The total incubation time was six days. The evaluation of the abilities of the mutant strain to grow on the particular carbon source was performed by comparing the growth characteristics of the mutant strain with the growth characteristics of the wild-type RmG212, and the lpdA3mutant strain on the same m9 medium with deferent carbon source(Table 3). The *lpdA3* mutant grew equally well on all carbon sources on solid media, compared to the reference strain RmG212, except in the presence of leucine. This mutant exhibited similar growth abilities on solid media, compared to the wild-type strain RmG212.(Abbas et al., 2013, Meek 2013, Abbas and Sorour2016).
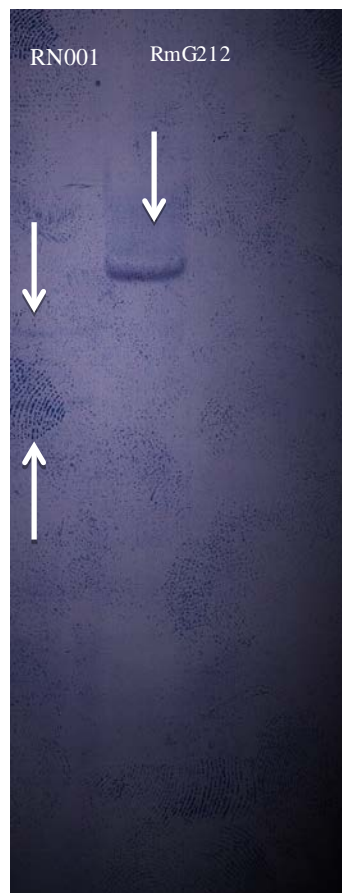
Figure 4.Southern blot hybridization imageof RN001(*LpdA3*) mutant DNA and RmG212 WT DNA digested by *PstI*restriction enzyme.

Enzyme specific activities

Crude cell free extracts of the mutant strains were prepared from the cultures grown in M9 liquid medium, supplemented with succinate, arabinose (both in 15mM concentration) and 1 % of LB broth. The MDH assay was used as a control, while PDH, OGD, and BKD assays were used to test the effects of respective mutation on the abilities of mutated strains to metabolize target compounds, thus to confirm that the inactivation of the alleged components of the three enzyme complexes have indeed effect on the metabolism of the target compounds. The *lpdA3* mutant displayed the MDH, PDH, and OGD enzyme activities at levels similar to the wild type RmG212. However, the activity of the BKD in this mutant was reduced to 4.7% of WT (Table 4).The *lpdA3* mutant was unable to grow on leucine, (Table 3)and had 4.74% of BKD activity which strongly indicates the implication of the LpdA3 and BKD in the metabolism of branched-ketoaminoacids.The disruption in LpdA3 abolished BKD activity but had no effect on PDH or OGD indicating that LpdA1 or LpdA2 cannot compensate for the loss that subunit (Abbas et al., 2013, Meek 2013, Abbas and Sorour2016).

Table 3. Plate phenotypes of the *lpdA3* mutant On different carbon sources.

Strain	Acetate	Arabinose	Glutamate	Glucose	Leucine	Malate	Pyruvate	Succinate
RmG212	++	+++	++	+++	++	+++	+	+++
RN001	++	+++	++	+++	-	+++	+	+++

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.
-= No growth
+= faire growth
++= moderate growth
+++= high growth

Table 4. Characterization of MDH, OGD, PDH and BKDH specific activity in *lpdA3* mutant, and wild-type RmG212 strain

Strain	MDH			OGD			PDH			BKDH		
	SA	SE	% WT	SA	SE	% WT	SA	SE	% WT	SA	SE	% WT
RmG212	662.11	4.1	100	97.5	4.4	100	26.02	0.2	100	24.8	0.3	100
RN001	777.75	1.2	117	90.2	1.1	92	27.9	0.3	107	1.17	0.25	4.75

Malate dehydrogenase (MDH),2-Oxoglutarate dehydrogenase(OGD), Pyruvate dehydrogenase (PDH), Branched-chain α -ketoacid dehydrogenase (BKDH).

Concluding remarks

- The *lpdA3* mutant was unable to grow on leucine, and had very low level of BKD activity which strongly indicates the implication of the LpdA3 and BKD in metabolism of branched-chain Alpha- ketoacids. the

- Unlike the *lpdA1* and *lpdA2* gene position in relation to the rest of the genes from the associated complexes, the close association of the *lpdA3* sequence with the *bkd*sequence cluster strongly suggest that *lpdA3* is expressed from the same promoter as the rest of the BKD genes.

- The disruption in LpdA3 abolished BKD activity but had no effect on PDH or OGD indicating that LpdA1 or LpdA2 cannot compensate for the loss of that subunit.

- Strains with an *lpdA3* mutation had no BKD activity but retained wild-type PDH and OGD levels indicating that this enzyme only interacts with BKD and not the other two enzymes.

REFERENCES

Abbas, R.N, and Sorour, N.M. 2016. Molecular and functional characterization of the lpda2 gene in *S meliloti*. Menoufia Journal of Agricultural Biotechnology. 1:23-35

Abbas, R.Babic, B.Poilly, M. Meek, D.and Driscoll B. 2013.molecular and functional characterization of the lpda1 gene in *S meliloti*. Egypt J Genet Cytol. 42:405-16.

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

Capela, D. BarloyHubler, F. Gouzy, J. Bothe, G. Ampe, F. Batut, J. Boistard, P. Becker, A. Boutry, M. Cadieu, E. Dreano, S. Gloux, S. Godrie, T. Goffeau, A. Kahn, D. Kiss, E. Lelaure, V. Masuy, D. Pohl, T. Portetelle, D. Puhler, A. Purnelle, B. Ramsperger, U. Renard, C. Thebault, P. Vandenbol, M. Weidner, S. and Galibert, F.2001.Analysis of the chromosome sequence of the legume symbiont Sinorhizobium meliloti strain 1021. Proc. Natl. Acad. Sci. U S A. 98: 9877-9882.

Dickinson, J.R. Roy, D.J. and Dawes, I.W.1986.A mutation affecting lipoamide dehydrogenase, pyruvate dehydrogenase and 2 oxoglutarate dehydrogenase activities in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 204; 103 107.

Driscoll, B.T. and Finan, T.M.1993. NAD^{+} dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. Molecular microbiology7:865-73.

Duncan, M.J. and Fraenkel, D.G.1979.Alpha-Ketoglutarate dehydrogenase mutant of *Rhizobium meliloti*.Journal of Bacteriology.137:415-9.

Eley, M.H. Namihira, G. Hamilton, L. Munk, P. and Reed, L.J. 1972.Alpha keto acid dehydrogenase complexes. 18. Subunit composition of the *Escherichia coli* pyruvate dehydrogenase complex. Arch. Biochem. Biophys. 152: 655 669.

England, S.and Siegel, L.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.Hartweig, E.LeMieux, K. Bergman, K. Walker, G.C and Signer, E.R.1984.General transduction in *Rhizobium meliloti*. J Bacteriol 159:120-4.

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

Finan, T.M.Oresnik, I.V. andBottacin, A.N.1988.Mutants of *Rhizobium meliloti* defective in succinate metabolism. J Bacteriol. 170:3396-403.

Guest, J.R. Cole, S.T. and Jeyaseelan, K.1981. Organization and expression of the pyruvate dehydrogenase complex genes of *Escherichia coli* K12. J. Gen. Microbiol. 127: 65-79.

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

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

Jelesko, J.G.and Leigh J.A. 1994. Genetic characterization of a *Rhizobium meliloti* lactose utilization locus. Molecular microbiology.11:165-73.

Kalogeraki V.S andWinans S.C.1997.Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene. 188:69-75.

Lowe, P.N. Hodgson, J.A. and Perham, R.N. 1983. Dual role of a single multienzyme complex in the oxidative decarboxylation of pyruvate and branched chain 2 oxo acids in *Bacillus* subtilis. Biochem. J. 215: 133 140.

Maniatis, T.1989. Molecular cloning: a laboratory manual. In: Sambrook, J., et al . (Eds.) Cold SpringHarbor Laboratory Pr*ess,* New York.

Meade, H.M. Long, S.R., Ruvkun, G.B. Brown, S.E andAusubel, F.M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. J Bacteriol. 149:114-22.

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..

Otulakowski, G. Robinson, B.H. and Willard, H.F.1988. Gene for lipoamide dehydrogenase maps to human chromosome 7. Somat. Cell Mol. Genet. 14: 411-414.

Phylip F. Phylogeny Inference Package.1989. (Version 3.3). Cladistics; 5:164-6.

Phylip, F. Phylogeny Inference Package (Version 3.3). Cladistics 2000; 5:164-6.

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

Rice, P.Longden, I.and Bleasby, A. 2000. EMBOSS: the European molecular biology open software suite. Trends in Genetics.16:276-7.

Thompson, J.D. Higgins, D.G. and Gibson, T.J1994.CLUSTAL W.. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research. 22:4673-80.

Westphal, A.H. and de Kok, A. 1988. Lipoamide dehydrogenase from *Azotobacter vinelandii*. Molecular cloning, organization and sequence analysis of the gene. Eur. J. Biochem. 172: 299-305.

تاثير اقصاء جين lpdA3على التمثيل الغذائى فى السيانوريزوبيم ميليلوتى
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علي عكس بكتريا القولون و العديد من الكائنات الدقيقه الاخري و التي تحتوي علي جين وحيد من الــ lpdA فإن بكتريا السيانوريزوبيم ميليلوتي تحتوي علي ثلاثه اليلات من جين وحيد من الــ lpdA يعتقد ان lpdA۱ يشفر لـمكون الـ E۳ الخاص بانزيم هدرجه البيروفيات (E3 component of pyruvate dehydrogenase) وان lpdA۲ يشفر لـمكون الـ E۳ الخاص بانزيم هدرجه ۲الدياوكسي جلوتاريت 2- of E3 component (Oxoglutarate dehydrogenase) وانlpdA3 يشفر لـمكون الــ E۳ الخـاص بـانزيم هدرجـه الفـا ـ كيتواسـيد ذو السلسله المتقرعـه (E3 component of a branched-chain alpha-ketoacid dehydrogenase). حتي الان لم يتم عمل التوصيف الوظيفي lpdA۳ و بدراسه جين الـ lpdA۳ وجد انه يشفر لتتابع من البروتين الوظيفي يعتقد انه الـ E۳ الخاص بانزيم هدرجه الفا ـ كيتواسيد ذو السلسله المتقرعه (E3 component of a branched-chain alpha-ketoacid dehydrogenase) وللتأكد من تلك الفرضيه تم عمل طفره لـجين الـ lpdA۳ من خلال استنساخ قطعه من الجين واضافتها الي البلازميد pVIK112 و اعادته الي الجينوم الخاص ببكتريا السيانوريزوبيم ميليلوتي مما ادي الي وقف عمل الجين و الحصول علي سلاله طافره في جين الـlpdA۳ من بكتريا السيانوريزوبيم ميليلوتي و التي اظهرت الاختبارات الخاصـه بالنشـاط الانزيمي لها مستوي شبه منعدم لانزيم هدرجه الفا ـ كيتواسيد ذو السلسله المتقرعه (E3 component of a branched-chain alpha-ketoacid dehydrogenase)ومستوي مماثل للطرز البري بالنسبه لانزيمي هدرجه البيروفات و هدرجه ۲الدياوكسي جلوتاريت. مما يؤكد عدم قدره اي من الأليلين الـ lpdA۱ وlpdA۲ من تعـويض غيـاب الأليـل lpdA۳ و انـه مخـتص منفـردا بالتشـفير لـمكون الـ E۳ الخـاص بـانزيم هدرجـه الفـا ـ كيتواسـيد ذو السلسـله المتقرعـه E3 component of a branched-chain alpha-ketoacid·(dehydrogenase)