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In Silico and in Vitro Reverse Genetics of yebV of Escherchia Coli

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Abstract: ***Out of 4300 protein coding genes, 850 still have no attributed function. yebV is one such gene that is highly conserved in bacterial domain and not been characterized yet. It may be due to gene's varying expression levels in different phases or its small size or its non essentiality character. In this paper, we have studied different physiological nature of cell mutated in yebV gene, also tried to model this protein in insilico methods.***

Keywords: *Escherchia coli, yebV,* Bioinformatical tools and Physiological experiments.

1. Introduction

Contemporary strategy announced the systematical approach for cataloging the phenotypes of all the mutation expressed, primed the *Escherichia coli* gene module and transcript. Influence of all genetic loci or products drag the way towards a new extremity in the biology of miniature organisms. Potent mechanism for this exertion is emanating and intention to standardize the *Escherichia coli* community is initiated. The predictable comfort is an operative model of a bacterial cell.

The first complete genomic sequence of *Escherichia coli* (strain k-12, MG1655 derivative) was published in 1997 (Lawrence and Ochman 1998, Merlin, McAteer et al. 2002). *Escherichia coli* can serve the whole genomic and proteomic network, its genome still consist of cryptic, pseudo and unknown function genes. It has an ability to transfer the genetic information horizontally (Ochman, Lawrence et al. 2000). This enhances its speciation by introducing novel genes and thus permits exploitation of competitive environments.

The approach of unknown function of the gene is being encountered by reverse genetics. Through deleting target gene to detect the phenotypic characterization which direct towards its role in bacterial physiology. The deleted strains were obtained from *Escherichia coli* Genetic Stock Center, Yale University. Though the gene *yebV* is well conserved in bacterial domain, it is also disclosed in domain archaea under families like euryarchaeota and thaumarchaeota. Further bacterial domain is conserved in more than one phylum such as eubacteria, firmicutes, actinobacteria and bacteroidetes.

yebV is 237 base pairs long nucleotide sequence encoding a protein of 78 amino acids with molecular weight of 8.75 kDa (Medigue, Viari et al. 1993, Hu, Sherlock et al. 2014). Its isoelectric point is 4.25 indicating it as a mild acidic protein (regulonDB) belongs to putative conserved domains of superfamily DUF (Domain of Unknown Function) 1480. Bioinformatical tools like Multalign and Clustal ω were used to align YebV protein in evolutionary tree. With the help of aligning, 20 highly structurally or functionally conserved residues were spotted (consurf). Being a consensus sequence its structure was adumbrated using homology modeling tool – Modeller 9.12 (Yang, Roy et al. 2013, Yang, Roy et al. 2013). Based on the Bioinformatical results, physiological experiments were designed and carried out. Experiments like fitness assay (Sezonov, Joseleau-Petit et al. 2007, Sakamoto, Terui et al. 2012, Terui, Akiyama et al. 2012), sub - MIC antibiotic inhibition, growth curve with antibiotics, and stress experiments such as heat, osmotic and pH shocks were carried out. These physiological experiments were carried out on the basis of microarray results curated from gene expression omnibus (Chuang, Daniels et al. 1993, Zhou and Yang 2006, Hu, Janga et al. 2009).

2. Materials and Methods

Bioinformatical tools: Bacterial linkage conservation was determined by BLAST. Prior the nucleotide and protein sequences were obtained using Colibri, a database exclusive for escherhia coli. Comparitive studies of conserved residues in the protein sequence and its multiple sequence alignment was performed using Multalign and Consurf. Moddeling of yebv protein was executed using moddeler 9.12. the template was considered by blasting proteining against pdb. The optimum model obtained was conscripted using procheck-online tool and used to assist its appropriate ligand. The various ligands available are interacted to estimate the protein function. The process of docking was facilitated using an online tool COACH. The conscript model was uploaded to obtain the ligand information and docked structure of protein.

Physiological Experiments:

Bacterial Strains: Parent strain BW25113 from Keio collections having Δ*(araD-araB)567* Formally termed as $\Delta araBAD_{AH33}$. This deletion extends from ~25 bp upstream of the *araB* start codon to ~8 bp into the beginning of the *araD* gene, Δ*lacZ4787*(::rrnB-3) Formally termed as $\Delta lacZ_{WJ16}$ or *::rrnB-4.* 4 tandem copies of the *rrnB* transcriptional terminator inserted by gene replacement into the region extending from near the SacII site near N-terminus of *lacZ* through the promoter, λ^-, *rph-1* is a 1 bp deletion that consequncing in frameshift over last 15 codons and has polar effect on *pyre* leading to suboptimal pyrimidine levels on minimal medium. (Jensen 1993 JBact. 175:3401), Δ*(rhaD-rhaB)568, hsdR514*. Mutant strain Δ*yebV* having Δ*(araD-araB)567,* Δ*lacZ4787*(::rrnB-3), λ^-, Δ*yebV761::kan, rph-1,* Δ*(rhaD-rhaB)568, hsdR514*. The

primers used to knockout Δ*yebV761::kan* were : CTGAAAAATGGTGCGATCGATCGGACTGGTCGTAC CACAATCGGCAGCTAAATG**ATTCCGGGGATCCGT CGACC** and ACCCGCCATGCCGACGGGTTCTTTTTGGATCAGGCA AGACGCATAATCCA**TGTAGGCTGGAGCTGCTTCG .** The bolded sequence matches the pKD13 template (Baba, Ara et al. 2006).

Media used: Luria – Bertani (LB) medium – From Hi-media (1% Tryptone, 0.5% Yeast extract, 1% Sodium chloride) (Sezonov, Joseleau-Petit et al. 2007). M9 Medium (M9) – Minimal 9 salt (6g disodium hydrogen phosphate, 3g potassium dihydrogen phosphate, 0.5g sodium chloride, 1g ammonium chloride per liter for 1X), 2mM magnesium sulphate, 0.1mM calcium chloride and 0.2% dextrose were autoclaved separately and mixed as per the requirement (Biriukova, Krylov et al. 2010). Minimal A Medium (MMA) – Minimal A salt (10.5g dipotassium hydrogen phosphate, 4.5g potassium dihydrogen phosphate, 1g ammonium sulphate, 0.5g sodium citrate.$2H_2O$ per liter for 1X) 2mM magnesium sulphate and 0.2% dextrose were autoclaved separately and mixed as per the requirement (Marr 1991).

24 hours CFU in different media: The overnight cultures were diluted 1000 fold in triplicates into fresh tubes containing 3mL of different media (LB, M9 and MMA). The tubes were incubated for 24hours in shaker incubator at 37°C with 180rpm. The samples were serially diluted in 0.9% sodium chloride and 100µL of it was spread plated on LB agar plates. The plates were incubated overnight at 37°C and colonies were counted (de Pedro, Llamas et al. 1975, Marr 1991).

Antibiotic susceptibility test: The overnight cultures were diluted 10 fold into fresh tubes of 3mL LB. 100µL of the diluted culture was spread plated on LB agar plates and kept undisturbed for 15-20. Antibiotics were tested for sensitivity namely tetracycline, ampicillin, rifampicin, ciprofloxacin, erythromycin, chloramphenicol and nalidixic acid with varying concentrations. Discs were placed on the inoculated agar plates and kept for overnight incubation (Kronvall and Ringertz 1991). The zone of inhibition formed around each antibiotic disc was measured. A graph was plotted comparing the mutant strain with the wild type.

Biofilm assay: Overnight culture was diluted 100 fold and loaded into a 96-well microtiter plate. The plate was incubated for 24 hours in non shaking condition at 37°C. The culture from the plate was then discarded and washed with PBS buffer to remove any remaining floating cells. 125µL of 0.1% crystal violet dye was added to each well and incubated for 20 minutes for staining at room temperature. The dye was then discarded and the plate was read at a wavelength of 595nm in ELISA plate reader. The plate was washed twice with water to remove any dye residues and then washed by 90% ethanol to solubilise any dye remaining in the wells. The plate was emptied properly and again read at a wavelength of 595nm.

Heat shock assay: Overnight cultures were diluted 100 fold in 25mL LB flasks and incubated till it reaches midlog (OD 0.45 at 450 nm wavelength) at 37°C in shaker incubator with 180 rpm. The flasks were then kept in waterbath preset at 50°C for 20 minutes. At every 5 minutes samples were taken and plated. The percentage of cells surviving the heat treatment was calculated as the number of CFU/ml remaining after the heat treatment divided by the initial CFU/ml at time zero.

pH assay: Overnight cultures were diluted 100 fold to grow till midlog (OD 0.45 at 450 nm wavelength) in LB flasks at 37°C with 180 rpm. 1 mL midlog culture was then centrifuged and the pellet was resuspended in same volume of three different pH buffer medium of sodium acetate and acetic acid buffer (pH 5), PBS buffer (pH 7) and glycine and sodium hydroxide buffer (pH 8.7). The tube was incubated for one hour and then serially diluted and spread plated on LB agar plates. The plates were incubated overnight at 37°C and colonies were counted.

Growth curve assay: LB with 0.5% glucose was used to prepare different concentrations of antibiotics (1µg/µL, 2µg/µL and 4µg/µL) and to dilute the overnight cultures 1000 fold. The prepared samples were loaded into a 96 - well microtiter plate. One full row was added with LB, to serve as blank. A wavelength of 450nm was used in the ELISA plate reader to measure the optical density of the samples at every 1hour interval to check for growth. The plate was replaced in shaker every time after reading throughout the experiment. A graph is plotted with optical density against time.

3. Results

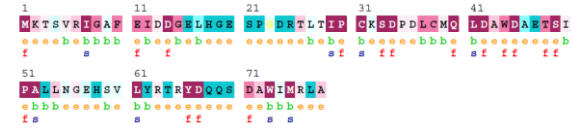
a) Bioinformatical Outcomes

1) Determination of conservation in bacterial lineage:

After selecting the gene, it is checked for conservancy and proved with help of blast results. Both *yebV* nucleotide and protein sequences were blasted against *Escherichia coli* and other organism. When nucleotide BLAST was performed, it was found that the gene *yebV* is well conserved in Enterobacteriacae family. On blasting protein YebV sequence, its occurance was also observed in many genus under Enterobactriaceae family. This protein YebV belongs to DUF (Domain of Unoknow Function) 1480 superfamily. Further when the blast was carried out after excluding Enterobactriaceae family, the gene is also present in *Enterococcus, Streptomyces sp., Bacteroides, Peptoniphilus sps.* and also apart from bacterial kingdom it was also spotted in archeal kingdom in *Halosimplex carlsbadense, Methanosarcina barkeri, Candidatus Nitrososphaera gargensis, Haloferax mucosum, Halophilic archaeon J07HX5 and Haloarcula vallismortis.*

2) Conserved amino acid residues:

After knowing its wide range of occurrence with help of blast, to know its conserved residues, multiple sequence alignment (MSA) was done. The level of conservity is differentiated with darker colour changes.



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e - An exposed residue according to the neural-network algorithm.
b - A buried residue according to the neural-network algorithm.
f - A predicted functional residue (highly conserved and exposed).
s - A predicted structural residue (highly conserved and buried).
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Figure 1: MSA showing conserved residues in the sequence.

On aligning the protein sequence nearly 20 amino acids was reported as 9th level of conservation. Those were M1, I7, D13, I28, P29, C30, S32, D33, Q38, L40, D41, D43, S49, P50, A51, L61, Y66, D67, W73 and M75.

3) Molecular Modelling

Modeling of protein was done using Modeller 9.12. The main aim for modelling protein was to analyze the protein knowing its native (tertiary protein) structure, its stability and its interacting capabilities.

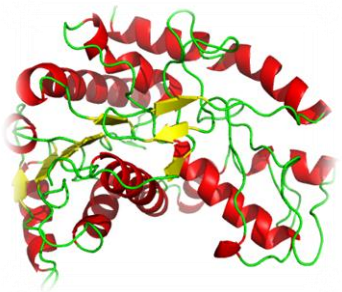


Figure 2: Modeled 3D structure of YebV protein

Four model structures were generated using structure of malate dehydrogenase of *Escherichia coli* (PDB Id: 2PWZ) as a template. This protein has four different chains in it, out of which 'A' chain was used as template. This had a Q score of 0.81 out of 1 and RMSD of 0.60 angstrom, when checked for matching folds.

4) Docking with Ligands

Protein functions by interacting with other molecule. Such protein ligand complexes were found with help of COACH.

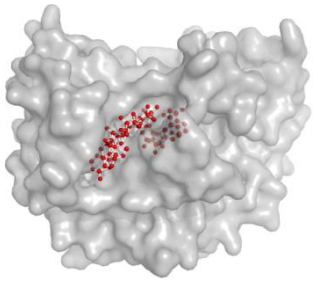


Figure 3: Protein interacting with NAD ligand

In this the binding residues are - I16, W24, P130, D157, G128, S154, H185 and A161

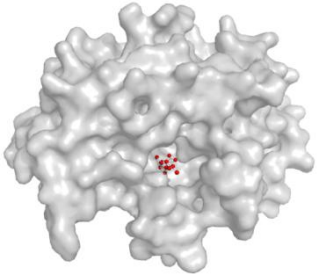


Figure 4: Protein bound with pyruvate as ligand

In this the binding residues are – P130, D157, A161, H185, W229, A24. Pyruvate is an anionic form of pyruvic acid. It is also converted into lactate with help of lactate dehydrogenase (isozyme of malate dehydrogenase) and using NADH

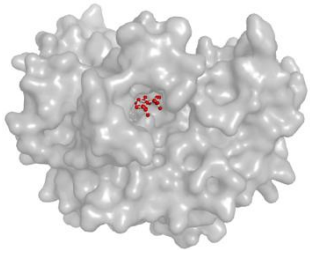


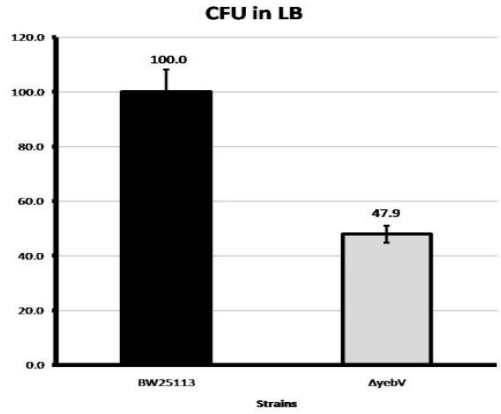
Figure 5: Protein bound with OXQ ligand

Binding residues are: P130, A161, G186, W229, A240, A24. OXQ is nothing but 4-hydroxy-1,2,5-oxadiazole-3-carboxylic acid. It is a small heterocyclic compound which belongs to Furazans with 1 oxygen, 2 nitrogen at 2,1 and 5 positions respectively.

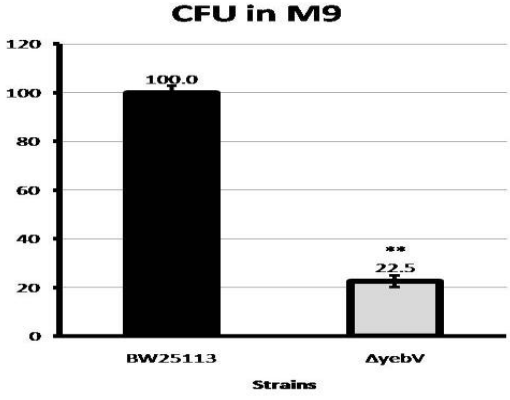
b) Physiological Experiments

1) Growth in LB and Minimal Media

24 hours CFU (Colony Forming Unit) was done using different media, to ensure the strain's capability of being viable in different combinations of nutrient sources. Luria-Bertani (LB) broth and M9 was used for observing its viability after 24 hours.



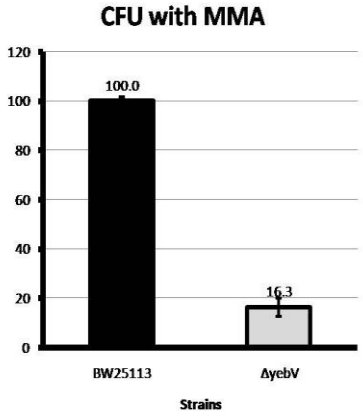
In case of CFU in LB, mutant strain showed less number of viable cells when compared to the wild type BW25113. By performing percentage calculation mutant's growth was seen to be reduced by more than 50 percent.



CFU with M9 also showed retarded growth of mutant than wild type. But on performing student t – test, it was

observed to be extremely statistically significant with p – value of 0.0001. Mutant strain Δ*yebV* showed 22.5 percent growth when compared with the wild type.

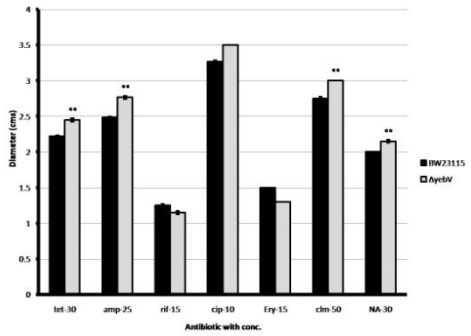
Cells were also grown in Minimal Medium A (MMA), and after 24 hours CFU was performed.



As with other medium, in MMA also mutant showed reduced growth than wild type for which the deletion of gene may be responsible.

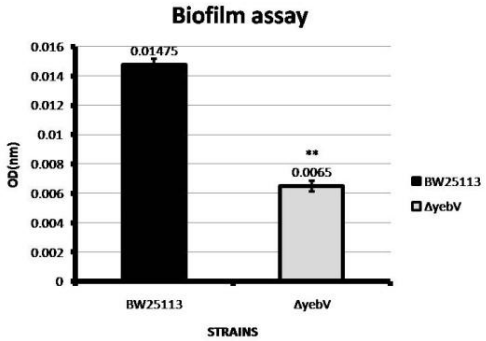
2) Zone of Inhibition

This test was performed using various antibiotic discs with varying concentrations. Antibiotics like tetracycline, ampicillin, rifampicin, ciprofloxacin, erythromycin, chloramphenicol, and nalidixic acid were used



Except for rifampicin and erythromycin, wild type is sensitive for others. In contrast in a way or other presence of gene *yebV* might be helping to resist against antibiotics like tetracycline, ampicillin, ciprofloxacin, chloramphenicol, and nalidixic acid. Statistically this result was found to be significant for all except ciprofloxacin.

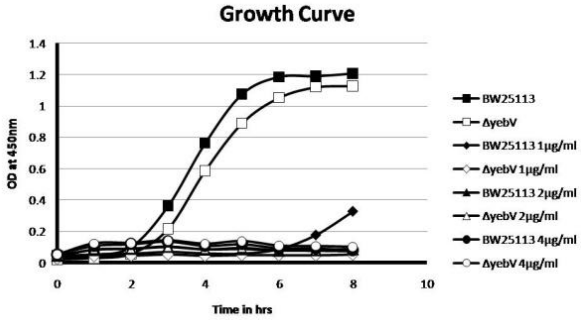
3) Biofilm



From the graph, it can be explained as the ability of forming biofilm is more for wild type when compared to mutant. Statistically it was proven as very significant by t – test with p value < 0.005. The deleted strain showed nearly less than half of the optical density than that of the wild type.

4) Growth in Antibiotics

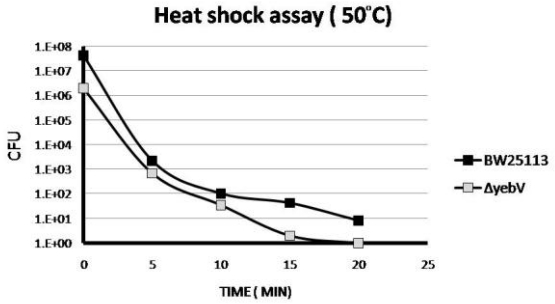
Cells were grown with and without antibiotic in LB broth. Growth curve was performed using various sub MICs (Minimum Inhibitory Concentrations) of chloramphenicol (Koikov 1974, Yourassowsky, Van der Linden et al. 1989). Chloramphenicol exhibits visible changes to cell number in short period of time.



In the absence of antibiotic, wild type showed slightly elevated curve when compared to the mutant. Concentrations of chloramphenicol, such as 1µg/ml, 2µg/ml, and 4µg/ml were used. With the least concentration of chloramphenicol used that is 1µg/ml, wild type is observed to show marginal exalted growth in later hours.

5) Heat Shock

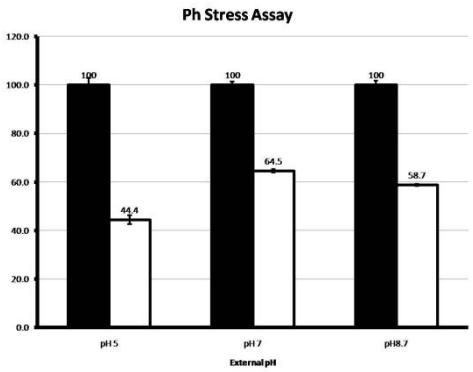
The increase in temperature causes pores in cell membrane and leads to cell death (Russell 2003). A heat exposure of 50 degree Celsius was given and CFU was performed.



The mid log cells concentration decreased gradually by extending heat treatment of cells at 50 degree Celsius. Wild type was comparatively resistant to heat when compared to the mutant strain.

6) pH stress assay:

To study the effect of different pH on *Escherichia coli* strains, pH stress assay was performed. Mid log cells were introduced to three widely varied pH buffers of pH 5.0, 7.0, and 8.7. CFU was done after incubating the cells for an hour in different pH buffers.



Mutant strain showed remarkable decline in number of cells in different pH. Especially with acidic pH 5.0 mutant was least in number thus showing, it might be due to its slight acidic character, which could have been altered the internal pH of the cell.

4. Discussion

Out of numerous unknown function genes in *Escherichia coli*, one of the conserved gene is *yebV*. *yebV* belongs to DUF1480 super family. This protein is mainly found in Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae. *yebV* is 237 base pairs long which transcripts and translates to form a protein polypeptide of 78 amino acids long. BLAST (Basic Local Alignment Search Tool) is a tool frequently used for calculating sequence similarities (Madden, Tatusov et al. 1996, Salgado, Gama-Castro et al. 2006, Mackie, Paley et al. 2014). This may help to know about new members of gene family and thus explores evolutionary relationships (Zhu, Hu et al. 2004, Madan Babu, Teichmann et al. 2006, Rani and Bapi 2009). BLAST results show that the gene *yebV* is well conserved under Enterbacter family among various genus like *Shigella*, *Salmonella*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Cronobacter*, etc. Both nucleotide and protein BLAST showed high similarity lines from various organisms. This shows that the gene is wide spread among the bacterial species.

The protein sequence was identified with nearly 20 conserved residues. Those were M1, I7, D13, I28, P29, C30, S32, D33, Q38, L40, D41, D43, S49, P50, A51, L61, Y66, D67, W73 and M75. These were further classified as structurally and functionally conserved residues. Secondly to know about the transcription unit, sites like regulonDB, Ecocyc were referred (Holmes and Bruno 2001, Fukami-Kobayashi and Saito 2002, Edgar and Batzoglou 2006, Goldenberg, Erez et al. 2009, Ashkenazy, Erez et al. 2010). This gene has its own independent transcriptional unit with conserved -35 and -10 sequences for promoter yebVp6, regulated by σ70. The gene upstream to it is *rsmF* that is methyltransferase producing gene and *yebW* as downstream gene with unknown function.

Protein molecular modeling was done to analyze its behavior in detail with other ligands (Levefelt and Lundh 2006, Naznin, Sarker et al. 2011, Frost and McCray 2012). Proteins perform their function by interacting with other macro or micro molecules such as nucleotides, proteins, ions, etc (Descorps-Declere, Barba et al. 2008, Frost and McCray 2012, Xu and Zhang 2013). Structure of malate dehydrogenase of *Escherichia coli* (PDB Id: 2PWZ) was used as a template for modeling protein. This had a Q score of 0.81 out of 1 and RMSD of 0.60 angstrom, when checked for matching folds. The validation of occurrence of these amino acids in this conformation was checked using ramachandran plot. This came out to have 88.4% residues in most favoured region. With help of modeled structure its gene ontology terms like cellular component, biological process and biochemical function were predicted. It was found that this protein may be a part of cytoplasm, which plays a role in primary cellular metabolic process. The biochemical function was predicted as catalytic activity like oxidoreductase that may act on CH – OH group of donors and NAD or NADP as acceptors (Laskowski, Watson et al. 2005).

With the help of docking, NAD (Nicotinamide adenine dinucleotide) was identified as ligand with highest confident score of 0.89 on 1.00, this rating was followed by pyruvate, then 4-hydroxy-1,2,5-oxadiazole-3-carboxylic acid (OXQ) after that. NAD is an coenzyme having a role in various metabolic reactions of cell (Beckett 2005). For performing physiological experiments, assays were chosen on the basis of significance in microarray experiments provided in gene expression omnibus. Experiments like fitness, growth curve, sub – mic antibiotic disc zone of inhibition, heat and pH stress, etc (Baba, Ara et al. 2006, Biriukova, Krylov et al. 2010). By growing the wild and mutant strain in different medium like LB, minimal 9 media and minimal media A, it was observed that mutant's growth was retarded than the wild type.

In antibiotic susceptible test, Except for rifampicin and erythromycin, wild type is sensitive for others. In contrast in a way or other presence of gene *yebV* might be helping to resist against antibiotics like tetracycline, ampicillin, ciprofloxacin, chloramphenicol, and nalidixic acid. Statistically this result was found to be significant for all except ciprofloxacin. By performing crystal violet biofilm assay, the deleted strain showed nearly less than half of the optical density than that of the wild type. This remained in the same manner with heat shock also (Russell 2003). In case of pH stress the results came out to be extremely significant which has mutant with less persistence in acidic and alkaline conditions (Tucker, Tucker et al. 2002, Mates, Sayed et al. 2007, Oberto, Nabti et al. 2009, Ramos-Morales 2012).

Finally, these experiments lead broadly towards the function of the specified gene. This may be further narrow down by performing more molecular and physiological experiments.

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