Expression of the *srlI* gene to improve salt tolerance in *Escherichia coli*

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ABSTRACT

In trying to engineer *E. coli* to enhance salt tolerance, this study shows constitutive over-expression of the *srlI* (glucitol-6-phosphate dehydrogenase) gene studying GMO *E. coli*. These genes used by studied salinity stress at five concentrations of NaCl. The *srlI* gene was isolated from *E. coli* top10 and inserted it into *E. coli* DH5 alpha with pET32a^{+} vector was used. The confirmation of transgene integration and expression were made via the optical density (OD_{600}) that is at 250 mmol/L NaCl concentration the *srlI* increase significantly (0.4227) compared with control (0.3564). The bacterial colonies with the recombined plasmid in the presence of IPTG and X-gal are colored white. The genotypic diversity of isolates was done using PCR that shows the similar patterns as the pattern of whole-cell protein for SDS-PAGE. Therefore, we need to attempt to explore the physiological mechanisms of *E. coli srlI* gene to salt tolerance in newly-developed transgenic bacteria lines for comparison of their physiological performance in a salt-stress environment.

Keywords: *E. coli top10*, *E. coli* DH5 alpha, *srlD* gene

Introduction

The suitable model in biochemical genetics, molecular biology, and biotechnology, *E. coli* strain K-12 was suggested as a candidate for whole-genome sequencing[1]. The complete sequence of *E. coli* is gotten the research toward a more complete understanding of this important experimental organism[2] and has led to new ways of observing at the evolutionary history of bacteria[3].

Escherichia coli strains are responsible for gastrointestinal, urinary, pulmonary, and nervous system infections. We chose strain MG1655 as the representative to sequence because it has been maintained as a laboratory strain with minimal genetic manipulation, having only been cured of the temperate bacteriophage lambda and F plasmid by ultraviolet light and acridine orange, respectively[4]. The K-12 derivative, contain MG1655, carry the rf b-50 mutation[5].

In a short period of time, the E. coli used in the experimental organism has been used for many generations and large populations to be studied. By the use of *E. coli* for long time as a principle organism in molecular biology, there are many tools, protocols, and procedures for studying changes at the genetic, phenotypic, and physiological feature[6]. Because the bacteria can be remain alive in frozen. As Lenski describes as a "frozen fossil record" of samples of evolving populations that can be revived and saved at any time. This frozen fossil record allows *E. coli* to be restarted in live as the contamination in the experiment and permits the isolation and comparison of living exemplars of ancestral and evolved clones. Lenski chose an *E. coli* strain that reproduces only asexually, lacks any plasmids that could permit bacterial together. The estimation in the experiment occurs by the propagation processes of mutation, genetic drift, and natural selection. This asexuality means that genetic markers continue in lineages by common descent, but cannot spread in the populations[7].

E. coli genome sequences in the experimental of organisms help to integrate many resources of biological knowledge and serve as a guide for further experimentation. Availability of the complete set of genes enables main project to biological function in living cells[8].

Salt stress tolerance is also induces *srlI* gene expression[9] at the transcriptional level in fungi such as *Aspergillus nidulans*[10]. The *srlI* gene of the oyster mushroom, *Pleurotus sajor*-caju, was isolated and its expression under various abiotic stresses was characterized[11].

Accordingly, the idea of research came about considering *E. coli* top10 bacteria as a source of target genes that have the ability to transfer them to *E. coli* DH5 alpha in order to improve their characteristics.

Methods

Bacterial strains, plasmid, and media

A pure culture of *Escherichia coli* and plasmid pET32a$^+$, which were obtained from the Provincial Key Laboratory of Plant Molecular Breeding, College of Agriculture, South China Agricultural University, Guangzhou, China were used for constructing the genes. The bacterial strains used in this study were *E. coli* top10 for target gene, and *E. coli* DH5α to competent cell by use CaCl2, which were routinely grown in a Luria broth (LB) medium.

DNA Extraction from bacteria

Genomic DNA was isolated from the *E. coli* bacteria top10 as described by the protocol of the Easy Pure DNA MiniPrep Kit, manufactured by the Transgen Company in China, was used to isolate the *E. coli* DNA

PCR

The target gene sequences were collected from NCBI database and the "gi" for the genes and their individual sequence lengths are provided in Table 1

Table-1: NCBI database and the "gi" for the gene *srlI*

Gene	Accession	Sequence length (nucleotide)	Sequence length (amino acid)
srlI	gi/ EU433563.1	780bp	260

For PCR analysis, the 5′ AGA ATT CAT GAA TCA GGT TGC CGT TGT 3′ and 5′: TGT CGA CTC AGA ACA TCA CCT GAC C -3′ primers for *srlI* was used. The amplification reactions consisted of 95°C for 10 minutes (1 cycle), followed by 35 cycles (94°C for 30 seconds , 57°C for 30 seconds, and 72°C for 1 minute); this was followed by an extension cycle of 10 minutes at 72°C. The PCR products were separated by electrophoresis on 1% agarose gel containing 0.1 μg/L ethidium bromide and visualized under UV light. A final concentration of 2.5 μL PCR buffer, 0.1 mM dNTPs and 0.3 μM of each primer and 1 unit of recombinant Taq polymerase were used for each target gene amplification.

Isolation of pET32a$^+$ plasmid

The Easy Pure plasmid MiniPrep Kit, manufactured by the Transgen Company in China, was used to isolate the pET32a$^+$ vector

Cut the *srlI* genes from electrophoresis gel

Cut the *srlI* gene by the Easy Pure Cutting Gel MiniPrep Kit, manufactured by the Transgen Company in China, was used to cut the target gene from electrophoresis gel as described in protocol

Restriction enzymes digestion of *srlI* genes and pET32a+ plasmid

Restriction enzyme digestion of *srlI* gene and pET32a+ plasmid was conducted with *EcoR*I/*Sal*I for *srlI* to transfer *srlI* gene into pET32a+ vector

Digestion *srlI* gene and pET32a+ by digestion enzymes *EcoR*I/*Sal*I at 37°C in water-bath for 3 hours as described as follows:-

Components	*nhaA/srlI Gene*	pET32a+ vector
*EcoR*I 10 U/µL	1.5 µL	1 µL
*sal*I 10 U/µL	1.5 µL	1 µL
DNA sample 100 ng/µL	15 µL	16 µL
10XH buffer	3 µL	2 µL
ddH_2O	30 µL	20 µL

Then connection between *srlI* genes and pET32a+ vector by use T4 DNA ligase at 16°C for 16 hours as described as follows:-

Components	Valium
T4 DNA ligase 1 unit/µL	1 µL
10X buffer	1 µL
srlI gene 98 ng/µL	5 µL
pET32a+ vector 260 ng/ µL	3 µL

Cloning pET32a+ contain the *srlI* gene with *E. coli* DH5α competent cell

Cloning was conducted in a SOC medium by used 10 g of peptone, 10 g of yeast extract, 5 g of NaCl, 20 g of agar per liter, and 50 µg/L of ampicillin.

Transfer *srlI* gene with pET32a+ into *E. coli* competent cell

Transfer 4 µL pET32a+ *srlI* vectors to 100 µL *E. coli* DH5 alpha component cell in 1.5 microcentrifuge tube, ice for 30 minutes, water-bath at 42°C for 70 seconds, ice 3 minutes. Then add the products to 1000 µL SOC medium as described as above, thermo-shaker at 37°C for 50 minutes. This mixture was incubated at 37°C for 24 hours.

IPTG and X-gal screening

IPTG is Isopropyl thiogalactoside, 0.1 M solution. The formula weight is 238.3, so this is 0.238 g in 10 mL of water. Sterilize by filtration, then store in the freezer. And the X-gal is 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside 20 mg/mL solution. It must be dissolved in DMSO (dimethyl sulfoxide) or dimethylformamide, not water! It must be wrapped in foil to protect it from the light, sterilize by filtration, and then stored in the freezer. Putting IPTG and X-gal are on top of pre-made agar plates. Spread 40 µL of IPTG and 40 µL of X-gal on top of the plate with a hockey stick spreader. Then, let the plates dry before you use them. This should take 30 minutes or so if the plate is dry, but up to several hours for freshly made plates.

Measurment cell concentration for *E. coli* top10 culture with spectrophotometer at OD_{600}.

E. coli strain top10, which has been annotated, was chosen for this study. An overnight culture in Luria-Broth (LB) was grown in 250 mL fresh medium in a one-liter Erlenmeyer flask and cultivated in a shaking water bath (130 rpm) at 37°C. The OD_{600} and number of cells per 100 μL were monitored. Seven concentrations 0, 50, 100, 150, 200, 250, and 300 mmol/L NaCl was used in the test of the gene to salt tolerance, select one clone from *E. coli* competent cell that was grown one day before in LB medium with ampicillin and grow again in LB medium liquid for overnight to determinate DNA concentration in LB medium by OD_{600} spectrophotometer.

Effect of NaCl salts on growth of *E. coli* and *srlI*.

LB-Agar plates with five concentrations NaCl were coated with *Escherichia coli* DH5 alpha, pET32a-*srlI* with, without IPTG.

The results

Whole genome sequencing (WGS) as a sophisticated molecular diagnostics has shown the emergence of *E. coli* strains. In the *E. coli*. Top 10 (Fig 1), this study because no report was available affected the *srlI* gene (Fig 2) on the salinity tolerance. The salt-tolerant gene *srlI* (Fig 3, 4) showed that were cloned and transferred into *E. coli* competent cell increased tolerance to salt stress.

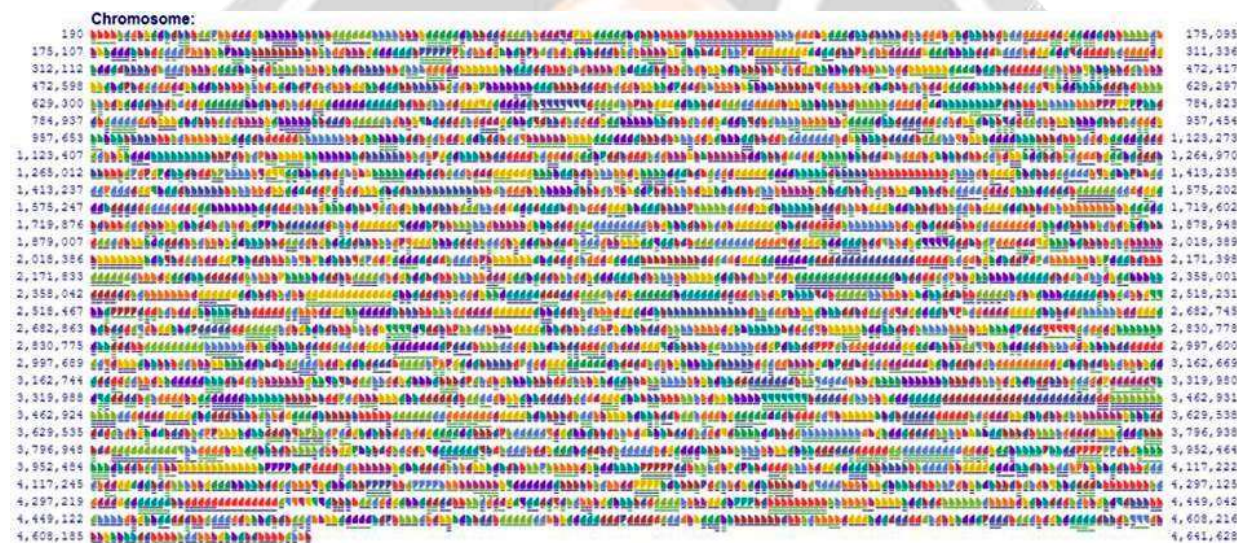

Fig-1. The complete genome of E. coli K12-MG1655 was used as a reference genome. Alignment of all sequences to the reference genome with Snippy produced a full alignment of 4,720,950 bp, 4100 genes which colored that start from *thrL* gene (190-225bp) to *yjtD* gene (4640942-4641628bp).

Fig-2. Select *srlI* (glucitol-6-phosphate dehydrogenase) genes from DNA of whole genome of the *E. coli*. Top10

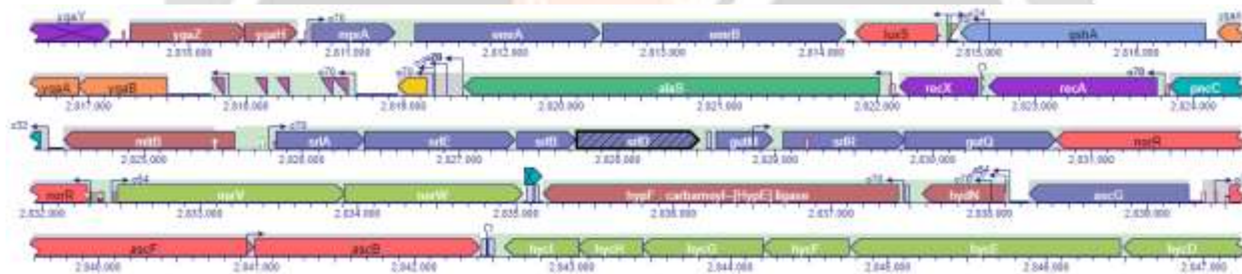

Fig-3. Location of target gene (*srlI*) in *E. coli* DNA sequence

CCGGAATTCAATGAATCAGGTTGCCGTTGTCATCGGTGGTGGGCAAACCTTAGGCGCGTTCCTGTGCCAC
GGTCTGGCTGCCGAGGGGTATCGCGTCGCGGTTGTCGATATTCAGAGCGACAAAGCCGCAAATGTGGCAC
AAGAAATTAACGCCGAATATGGTGAAAGTATGGCGTACGGTTTTGGTGCTGACGCCACTAGCGAGCAAAG
CGTTCTGGCGCTCTCTCGTGGGGTAGATGAAATCTTTGGTCGCGTGGATTTGCTGGTCTACAGCGCCGGA
ATAGCAAAAGCAGCCTTTATCAGCGACTTCCAGCTCGGCGATTTTGACCGTTCGCTACAGGTGAATCTGG
TGGGTTATTTCCTGTGTGCGCGTGAATTTTCGCGTTTGATGATCCGCGACGGGATTCAGGGGCGCATTAT
TCAGATCAACTCGAAATCCGGCAAAGTGGGCAGCAAACACAACTCTGGCTACAGCGCAGCGAAATTTGGT
GGCGTCGGGCTGACTCAATCACTGGCGCTGGATCTGGCGGAGTACGGCATTACGGTGCATTCACTGATGC
TCGGTAACCTGCTGAAATCGCCGATGTTCCAGTCACTGTTGCCACAATACGCGACCAAGCTGGGTATCAA
ACCGGATCAAGTCGAGCAGTATTACATCGACAAAGTACCGCTCAAACGCGGCTGCGATTATCAAGATGTG
CTGAATATGCTGCTGTTCTACGCCAGTCCTAAGGCGTCGTACTGCACCGGACAGTCGATCAATGTCACCG
GCGGTCAGGTGATGTTCTGAAAGCTTGGG

Fig-4. sequence of *srlI* gene

Optical density is a common method for estimating the concentration of bacteria in LB medium liquid (Fig 5). The concentrations of NaCl with the pET32a-*srlI* grow in the LB liquid medium were (0, 50, 100, 150, 200, 250 and 300 mmol/L NaCl) determinate by OD_{600} spectrophotometer. The *srlI* increase significantly in salt tolerance at 250 mmol/L NaCl concentration as increasing the amount 0.4227 compared with control 0.3564. The DH5 alpha

decreases significantly in salt tolerance at 300 mmol/L NaCl concentration by decreasing amount 0.0001 compare with control 0.3585 (Table 2). We have analyzed OD_{600} with concentration NaCl grown in LB liquid. The cell concentrations from the Optical Density (OD_{600}) were reading, taken with a spectrophotometer.

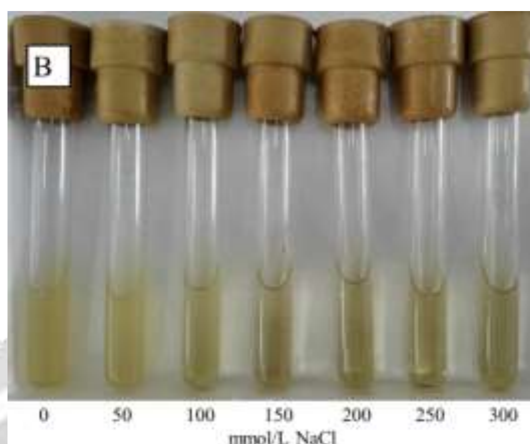

Fig-7. The *srlI* growth in the liquid LB medium at different NaCl concentrations (0, 50, 100, 150, 200, 250 and 300 mmol/L NaCl) to determine with an OD_{600} spectrophotometer

Table-2. The *srlI* and control untransformed DH5 alpha growth in the liquid LB medium at different NaCl concentrations (0, 50, 100, 150, 200, 250 and 300 mmol/L NaCl) determined with an OD_{600} spectrophotometer

NaCl mmol/L	*srlI*	DH5alpha
0	0.3564	0.3585
50	0.3813	0.3001
100	0.4032	0.1860
150	0.4168	0.0623
200	0.4225	0.0203
250	0.4227	0.0001
300	0.4225	0.0001

Primer design for salt tolerance gene have been cloned and transferred into *E. coli* DH5 alpha competent cell and it showed increased tolerance to salt stress. Salt tolerance was tested in *E.coli* DH5 competent cell overexpression *srlI*. DNA extraction that was helped us to sequence analysis of *srlI* gene along with cDNA (available at NCBI website) and two primers were designed to match to the segment of DNA. Cloning screened by isolation plasmid, the analysis of the published microarray data and select tow primers in order to clone the gene via PCR confirmed the actual expression of *srlI* gene and PCR assay (Fig 6). These primers were designed to ensure compatibility with the $pET32a^+$ vector (Fig 7) and PCR assay (Fig 8). Positive clones were first screened by PCR using gene specific primers and then by restriction digestion with *SalI/EcoRI* for *srlI* gene. The target genes were cloned into $pET32a^+$ vector by use Easy Pure Plasmid Mini Prep Kit to isolate plasmid as described the protocol above.

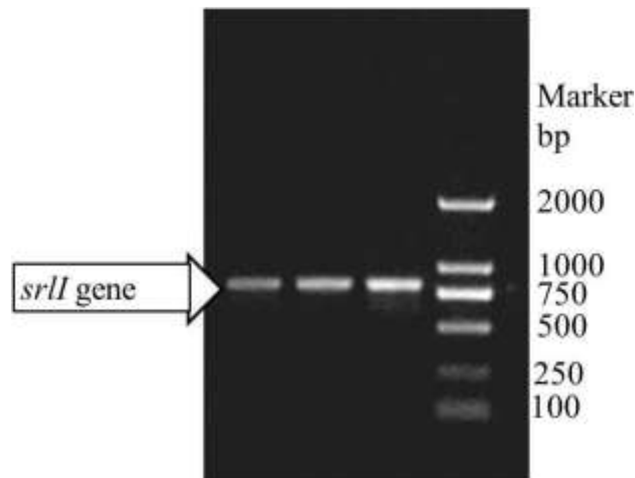

Fig-6. PCR assay with 1% agarose gel containing 0.1 µg/L ethidium bromide and visualized with UV light for *srlI*, 780bp genes. M, marker, 2000bp.

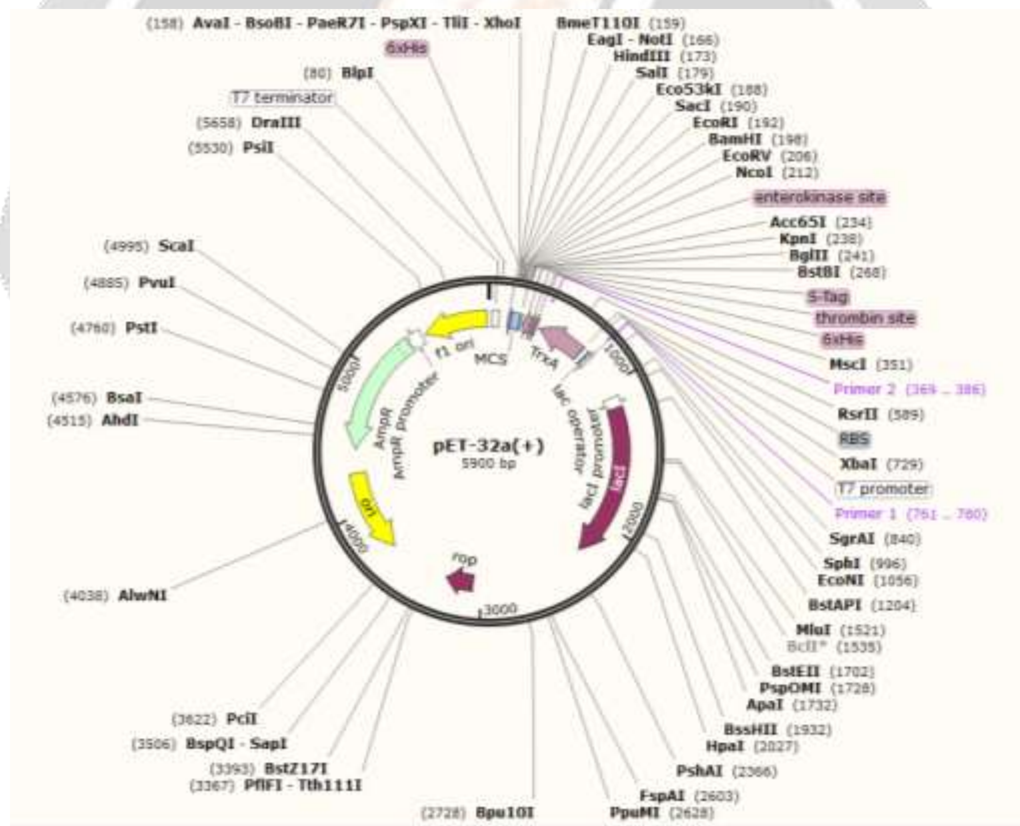

Fig-7. pET32a^{+} map sequences according to NCBI webside.

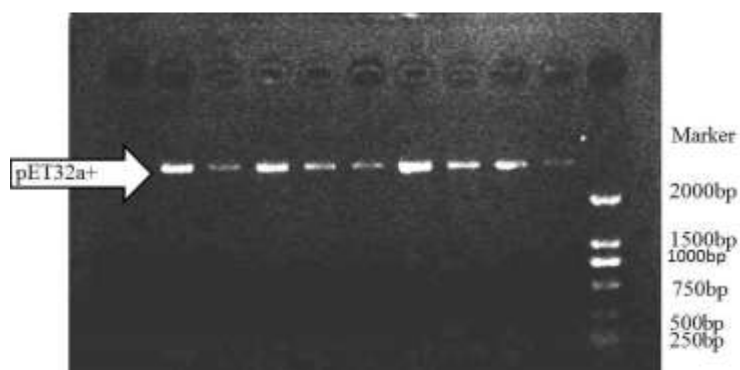

Fig-8. The pET32a^{+} assayed with 1% agarose gel containing 0.1 μg/L ethidium bromide and visualized with UV light.

On the selection plates (IPTG isopropyl beta-D-thio galactopyranoside and X-gal 5-bromo-4-chloro-3-indolyl-beta-D-galacto pyranoside with ampicillin), on which bacteria from the transformation approach, only bacteria with the original plasmid or with a recombined Plasmid can grow. On the plasmid is the gene for the ampicillin. Bacterial colonies with a recombined plasmid (also contain the LacZ gene) are colored white. IPTG and X-gal as the colored white that indicated bacterial colonies with pET32a- *srlI* were recombined (Fig 9). The bacterial colonies with the recombined plasmid in the presence of IPTG and X-gal are colored white, indicating recombination of *srlI* gene within *E. coli* competent cell colonies. The correct type of vector and competent cells are important considerations when planning a blue white screen. The *E. coli* cell should contain the new gene and some of the commonly used cells with such genotype DH5α -Blue.

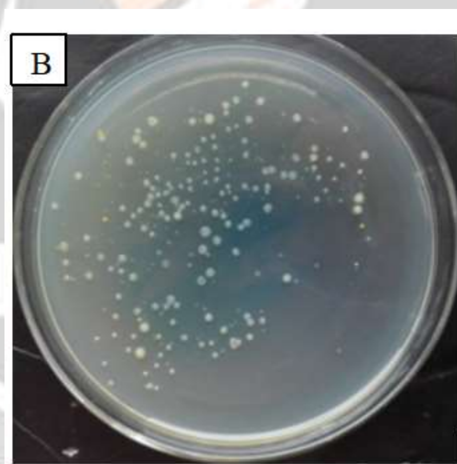

Fig-9. IPTG/ X-Gal LB agar plates for *srlI* blue/white colony Screening

At 37°C, to confirm their abilities to tolerate high concentration of salt. Streaked *E. coli* cells with pET32a-vector on LB with 0, 50, 150, 200, and 250 mmol/L NaCl. To confirm the effective contribution of bacterial survival against NaCl. Additionally, the gene transformed in *E. coli* DH5α were also grown with different concentrations NaCl but without IPTG. Plasmids from these genes positive colonies were success. Effect of NaCl salts on growth of *E. coli* DH5α, the *E. coli* DH5α (control) displayed a growth inhibition when LB medium used a NaCl containing plates. These inhibitory effected increased progressively with the increasing NaCl concentration in the plates. The *srlI* was unaffected by up to 200 mmol/L NaCl and *E. coli* development was not compromised, as the *E. coli* growth in all salt treatment. However, *E. coli* DH5α grown at 200 mmol/L NaCl displayed a reduction in growth (Fig 10).

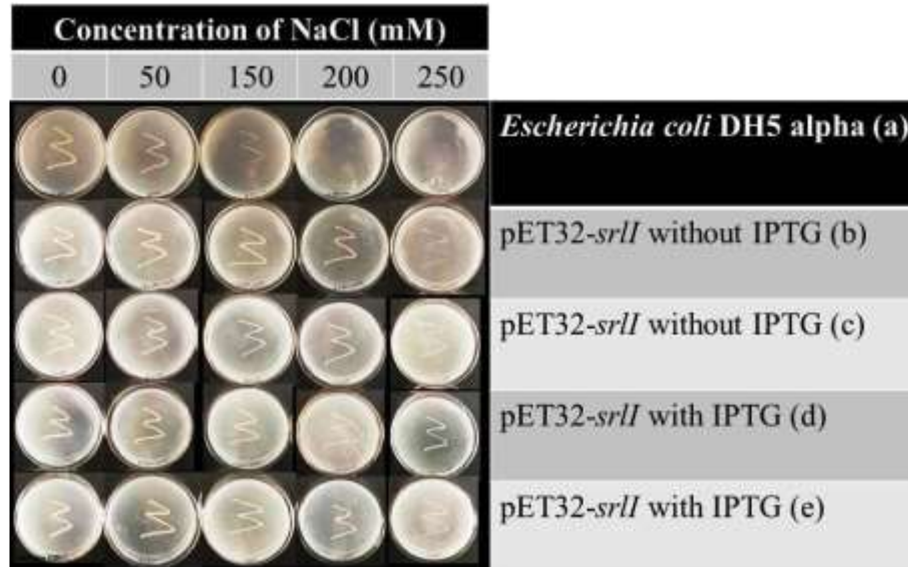

Fig-10. Optical pictures of LB-Agar plates with five concentrations NaCl were coated with *Escherichia coli* DH5α (a), pET32a-*srlI* without IPTG (b, c), and pET32-*srlI* with IPTG (d, e).

This gene is in accordance with the protein electrophoretic patterns (Fig 11). Transcription of the free RNA nucleotides pair with the cDNA of the template strand was used. Moreover, the genotypic diversity of isolates was done using PCR that shows the similar patterns as the pattern of whole-cell protein for SDS-PAGE. The genes represented by *srlI* compared with *E. coli* compenent cell as control. The results from the analysis showed the same level of similarity existed between the genes and control done by SDS-PAGE and PCR DNA fingerprinting. The correlation of these results confirmed the formation of isolated two genes in each step. Protein yielded a single band on SDS-PAGE gels stained with coomassie blue.

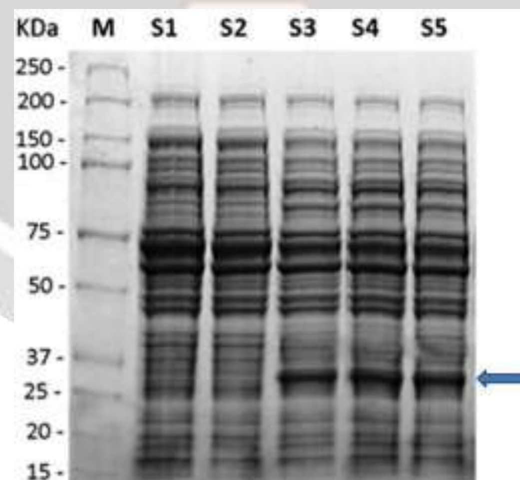

Fig-11. Whole cell protein profile of genes isolated strains showed by SDS-PAGE. Lanes: M, molecular-mass standards; lanes S1, S2) control, S3, S4, S5) srlI, KDa, Kilo Daltons.

Discussion

The suitable model in biochemical genetics, molecular biology, and biotechnology, *E. coli* strain K-12 was suggested as a candidate for whole-genome sequencing[12]. *E. coli* is a most important among other bacteria, so that

have many strains, only 20% of *E. coli* is common[13]. The *srlI* (gi/ EU433563.1, 780bp) (Table 1) from *E. coli* top10 (Fig 1) was isolated and used in this research. This study is necessary because there has not been great progress in *E. coli* genetic engineering for salt tolerance. This candidate assumes the existence of transgene cassettes linked on one plasmid (Fig 7). A similar work was in reported in the various literatures for stable integration of two transgenes at 100% frequency [14]. This study showed, significant increasing in growth *E. coli* was detected by spectrophotometer these results would suggest that the induction of *srlI* in response to NaCl stress in requires conditions which are currently unknown. The Optical Density (OD_{600}) (Fig 5) reading taken with a spectrophotometer, the increase in growth *E. coli* was detected induction of *srlI* in response to NaCl stress, to confirm the effective contribution of bacterial survival against NaCl. Quantitation of bacteria is an important tool in molecular biology. In particular, the ability to transfer plasmids into *E. coli* enables production of recombinant proteins. Endpoint quantitation of *E. coli* is used to normalize results to a standard amount of bacteria and inhibitory effects can be studied via growth studies. Absorbance at 600 nm is the recommended wavelength for measuring solutions containing *E. coli* bacterial samples. Research citing the use of microplate readers measuring cell growth at 600 nm is plentiful[15]. This results same Venkata et al. (2021)[16] validated microplate reader 600 nm cell growth reading compared to classical methods for studying post antibiotic effects, while Zahra et al. (2020)[17] used lysogen growth measurement at 600 nm to develop a means to detect sublethal concentrations of antibacterial agents that can interfere with prokaryotic translation. Yangwon et al. (2022)[18] examined the metal-ion tolerance of various strains of *E. coli* by determining the each culture at 600 nm in the presence of several metals after a 24 hours period.

The cloning entailed successful integration between plasmid $pET32a^+$ and the target gene screened, positive clones were first screened with PCR using gene-specific primers. The initial result of the PCR analysis confirms the integration of *srlI* gene generation. PCR techniques are used in many areas of biology, for instance for amplification of foreign genes from plant tissues as an analytical tool for detecting transformed cells[19]. The PCR reaction leads to the amplification of a specific DNA sequence[20].

To confirm *srlI* gene (Fig 10) survival in the presence of high concentration NaCl, the plasmids were purified and reintroduced into an *E. coli* competent cell and streaked in LB plates containing IPTG and different NaCl concentrations. The optimum IPTG concentration was 1mM and time of maximum induction in this reference study was different found to be the same as observed by Ajamaluddin et al. (2016)[21].

The analysis of the *srlI* gene transformed in *E. coli* competent cell was grown with different concentrations NaCl with/ without IPTG. These results indicated thriving bacterial colonies with these genes. The growth efficiency of microorganisms is traditionally evaluated in two ways: by evolutionary principles which are based on fitness measurements, usually division rate, in a competitive environment, and by the demands of biotechnology, which is focused mostly on the growth yield of bioreactors, in terms of product or biomass obtained per unit of substrate. However, optimum fitness and growth yields are not always compatible[22]. Bacterial clones which were able to grow on LB plates supplementing with IPTG, the optimum IPTG concentration was 1mM[23].

In batch culture, where nutrients are abundant, even in exponential phase, which is often assumed to be balanced growth, it has been shown that growth is not optimized in the same way as in chemo stats[22].

The gene represented by *srlI* was compared with *E. coli* competent cell as a control. The results from the analysis showed that there exists a same level of similarity between the genes and control done by SDS-PAGE (Fig 11), and PCR (Fig 6). The microorganism Escherichia coli in particular, it has been shown that the bacteria optimize their biomass production based upon the constraints on the fluxes of substrate consumption[24]. Protein composition of different gene template is closely related to those having a certain genetic similarity. This phenomenon has also been verified by SDS-PAGE of protein[25]. However, the results of study conducted by Renata et al. (2018)[26] showed a difference in molecular weight of the protein. Using two different standards, the calculated molecular mass always corresponded to 28 and 29 kDa. Sera recognizing these two bands also recognized the purified 85B protein, with the same estimated molecular weight as the 28 kDa band (data not shown), suggesting that the lower band corresponds

to MPT59, or 85B. Moreover, the N-terminal sequence of the 29 kDa band, purified by elution from acrylamide gels, corresponds to the homologous N-terminal sequence of the three 85 proteins[27] MPT59 manifesting itself as one of the most potent antigens, correspond to 85B. Ag 85B was expressed having 30 kDa size as analyzed on 12% SDS-PAGE analysis. The optimum IPTG concentration was 1mM and optimum time of expression was 3 hours for Ag85B. The optimum IPTG concentration was also found to be the same as observed by Ajamaluddin et al. (2016)[21] whereas; time of maximum induction in this reference study was different. The pET system of expression utilizing pET plasmid was used for expressing proteins. The system is well known and IPTG is used for induction, which is cheap substrate making overall expression process affordable.

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