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Full Length Research Paper

Escherichia coli phoA gene inactivation, insertion and restoration based on the λ red system

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The red recombinase system of bacteriophage lambda has been used to inactivate chromosomal genes in *Escherichia coli* K-12 through homologous recombination using linear PCR products. Here, *E. coli* BW *25113* mutants were created by changing the localization of genes on the genome using a method which contains both inactivation and reinsertion steps. The phoA gene, the structural gene for alkaline phosphatase, was inactivated by having the ORF and its regulator region replaced with a kanamycin cassette flanked by FLP recognition target sites. The site for insertion was selected based on the intergenic distance and non-coding regions, thus the new neighboring genes sequences were not affected. The Insertion was carried out by designing primers armed with homologous sequences to the insertion site; the primers contained the enzyme restriction sites in order to ligate the gene PCR product with the FRT-kan-FRT marker prior to the electroporation. The PCR reactions, cultures in the media supplemented with appropriate antibiotics and comparative gene expression study were carried out to confirm the knockout, the insertion and the expression of the gene in its new location. This procedure and the created *E. coli* mutants can be efficiently used for the study of bacterial genomics, especially in systems biology to understand the relation between the genes loci on the genome and their expression alongside new neighboring genes and others with the same functional group.

Key words: Gene, operon, knockout, insertion, systems biology, electroporation.

INTRODUCTION

Data analyses of both eukaryotic and prokaryotic genomes have been improved in the recent years; especially after first entire genome sequencing the *Haemophilus Influenzae*. Many studies have been done on genome, expression pathways, transcription (monocystronic or polycistronic) and regulation. Thus, genes regulation is highly correlated to environmental composition of nutrients. The availability of the genome organization, biochemical and physiological data for *Escherichia coli*, makes is to be a resourceful tool for post genomic studies. The *E. coli* K-12 MG 1655 which has 4639675 bp (Blattner et al., 1997) and the *E. coli* K-12 W3110 which has 4646332 bp (Hayashi et al., 2006) have been instrumental in these advancements. The number and types of known and predicted genes products of *E. coli* K-12 which have been presented (Riley et al., 2006) show that about 10% of the genes still have unknown functions.

Many systems are present on the *E. coli* chromosome and have clearly been explained; thus, the pho regulon, arginine regulon and glycerol regulon have been singled out as systems which have sets of genes and operons which work together in response to the nutriments concentration in the environment. In particular, the pho regulon in *E. coli* is a set of more than 50 genes which respond to the Pi concentration in the environment. They are regulated by a two-component regulatory system composed of products from the phoB and the phoR genes and by the pst operon (Wanner, 1996). When P_i is available in the environment medium, *E. coli* uses the pit genes or pst operon for the uptake of the P_i from the medium (Harris et al., 2001; Hoffer et al., 2001) but when

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P_i is not available or is limited as it activates the Pho regulon adaptive response. The Pho regulon is also implicated in the recovery of other forms of phosphates such as organic phosphates to meet its nutritional requirements (Wanner and Boline, 1990). This regulatory system consists of the sensory histidine kinase PhoR and the transcriptional activator PhoB, which binds to PhoB boxes in the promoters of genes belonging to the pho regulon.

PhoR senses phosphate levels outside the cell and has an amino terminus which is associated with the bacterial membrane (Makino et al., 1989). Under reduced phosphate conditions, PhoR phosphorylates, the transcriptional activator PhoB, enhances the DNA binding activity of this protein (Makino et al., 1989). Active PhoB directly binds the promoter region of PhoB-regulated genes and often activates gene expression (Makino et al., 1986).

One of the genes which are expressed under low phosphate conditions is the phoA, located, precisely at 8.6 min on the *E. coli* W3110 genome (Bachmann and Low, 1980; NAIST, 2006) and encodes bacterial alkaline phosphatase. The alkaline phosphatase of *E. coli* is synthesized under low phosphate conditions and is secreted across the inner membrane to the periplasmic space where it plays a central role in the breakdown of organic phosphate esters (Wanner, 1987).

There are still a lot of investigations being conducted to identify the unknown genes and their products of *E. coli* k-12 (Serres et al., 2001, 2004; Hayashi, 2006) and pathogenic strains of *E. coli.* But, until now, all genetic and metabolic studies have been performed with the wild types of bacteria strains. Thus, using the λ red recombination system, we created mutants which can be used as new tools for systems biology and gene function studies. We obtained these mutants by changing the gene location on the chromosome.

This paper describes the delocalization of the phoA gene from the position 8.6 min to the position 97.2 min (insL -yjhV (1103 bp)) that we made using a series of PCR reactions, antibiotic supplemented cultures and gene expression tests to check the new construction.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and chemicals

The *E. coli* strains and plasmids used in this study were obtained from *E. coli* genetic stock center and from our laboratory collection, they are listed in Table 1 and 2 respectively. Routinely, bacteria were grown in Luria-Bertani (LB); LB Agar plates, and when required, they were isolated with ampicillin 100 µg/ml or kanamycin 50 µg/ml, at 30 or 37°C. LB was used as a high-phosphate medium and the Low-P_i (LP) used was the MOPS (morpholinepropanesulfonic acid) medium as described by Neidhardt (Neidhardt et al., 1974) which contained 40 mM MOPS (pH 7.4), 4 mM Tricine plus 10 µM $FeSO_4$, 86 mM NaCl, 9.5 mM NH_4Cl, 5 mM K_2HPO_4 and 20 mM glucose (final pH 7.2). SOB and SOC media were prepared as described by Hanahan (1983) and Miller (1992) and were used only after the electroporation steps. All liquid cultures were growth monitored by measuring the optical density at 600 nm (OD_{600}).

Molecular biology methods

Plasmids were routinely isolated using TIANGEN Mini Plasmid Kit; the genomic DNA was extracted using a bacteria genomic DNA purification kit and .DNA fragments extracted and purified from agarose gels using a TIANgel Midi Purification Kit (Tiangen Biotech, Beijing, China). Restriction enzyme digests were performed using buffers and enzymes from Promega Corp. Ligations were performed using T_4 DNA ligase (Promega) by incubating it at 16°C for 4 h to overnight. Agarose gel electrophoresis, transformation of *E. coli* cells, electroporation and PCR were carried out as described previously by Sambrook et al., 1989. All PCRs were carried out using Taq DNA polymerase (Tiangen Biotech, Beijing, China) and by using buffers and instructions as supplied by the manufacturer. Others enzymes and molecular size markers were from Promega and all procedures were performed according to the manufacturer's instructions.

Primers and nucleotide sequence

Oligo software was used to design all primers which were later purchased from Shanghai Sangon Biological Engineering technology and Services Co, Ltd and from Takara. Primers and reactions conditions for individual PCRs are listed in Table 3 and 4. All chromosomal, plasmids or genes sequences used in this work were from NCBI database and from *E. coli* K-12 W3110 database (NAIST, 2006).

phoA gene inactivation

Gene inactivation was performed as described previously (Datsenko and Wanner, 2000) with slight modifications. Here, we considered the entire gene ORF and its regulator region. Briefly, we used *E. coli* BW 25113/pkD46, in which the plasmid pKD46 expresses the red recombinase in the presence of L-arabinose and carries the ampicillin resistance marker. The strains were grown in 5 ml LB cultures with ampicillin, overnight at 30°C then 2.5 ml of this culture were inoculated in 250 ml of fresh LB supplemented with 30 mM L-arabinose and ampicillin and incubated with shaking at 30°C. At each 20 min, the optimal density was measured using the 2800 UV/VIS spectrophotometer (UNICO) and when the culture reaches $OD_{600} \approx 0.6$; we prepared the electro competent cells as described by Sambrook et al., 1989.

Previously, the kanamycin gene flanked by FRT sites, from the plasmid pKD13, was amplified by PCR using primers (56 nt) containing 36 nucleotides of homologous sequence to the region 5' and 3' of the target gene. The PCR products were gel-purified using Tiangen gel extraction kit according to the manufacturer's instructions and incubated for 1 h, at 37°C in the presence of the DnpI (Fermentas) in order to remove the parental and methylated DNA.

The electroporation was done using a BioRAd MicroPulser and the appropriate electroporation cups provided by Bio-Rad Laboratories (Richmond, CA), according to the manufacturer's instructions. We used 40 µl of electro competent cells and 10 - 100 ng of PCR product in 2 µl TE. The phage λ recombination genes αβγ carried by the plasmid pKD46 catalyzed the exchange of the target gene with the kanamycin resistance marker flanked by FRT sites. Following electroporation, shocked cells were suspended to 800 µl LB (SOB) without any antibiotic, incubated for 1 h at 30°C, and then we selected the Km^R transformants on the LB agar plates supplemented with 50µl kanamycin at 37°C, overnight.

The global strategy for the gene inactivation is presented in the

Table 1. The bacterial strains and their sources.

Strains	Description	Source
E. coli W 3110	Wild type^{-} λ^{-} IN (rrnD-rrnE)1	Laboratory collection
E. coli BW 25113/pkD46	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78	#
E. coli W3110/pKD46	W3110,pkD46	This work
E. coli BW 25141/pkD13	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 Δ*phoBR580 hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78 *galU95 endA*BT333 *uidA*(Δ*MluI*)::*pir+ recA1*	#
E. coli BW 25113ΔphoAFRT-kan-FRT	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78,ΔphoA	This work
E. coli BT340	*DH5ά carrying pCP20*	#
E. coli BW25113ΔphoAFRT-kan-FRT/pCP20	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78,ΔphoAcarrying pCP20	This work
E. coli BW 25113ΔphoA FRT-FRT	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78ΔphoA	This work
E. coli BW25113ΔphoA FRT-FRT/pKD46	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78ΔphoA,pKD46	This work
E. coli BW25113ΔphoA FRT-FRT/FRT-kan-FRT,phoA*	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78ΔphoA, phoA*	This work
E. coli BW25113ΔphoA FRT-FRT/FRT-kan-FRT, phoA*/pCP20	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78ΔphoA, phoA*,pCP20	This work
E. coli BW25113FRT-FRT/FRT-FRT,phoA*	*E. coli* Bw 25113; phoA* between insL -yjhV	This work

#*E. coli* Genetic Stock Center, Yale University.

Table 2. Different plasmids.

Name	Description	Reference
pKD13	AY048744, 3434 bp DNA, 1.4-kb FRT-Kan resistance gene-FRT	Datsenko and Wanner, 2000
pKD46	AY048746, 6329 bp DNA, Red recombinase plasmid	Datsenko and Wanner, 2000
pCP20	bla cat p repast, express the recombinase Flp	Cherepanov and Wackernagel, 1995; Datsenko and Wanner, 2000

Figure 1.

This intermediary strain carries the kanamycin marker in place of the target gene. The construction was checked by PCR of the entire target gene region. We removed the kanamycin resistance cassette by performing the electroporation (25 µF; 2.5 kV; 200 Ω or 15 µF; 1.8 kV; 335 Ω) of this bacterium with the plasmid pCP20, that carries ampicillin and chloramphenicol resistances genes (Cherepanov and Wachernagel, 1995). The ampicillin-resistant transformants were selected at 30°C. We incubated a colony in LB without any antibiotics at 43°C for 10 min to induce the flp recombinase expression. The bacteria lose all resistances by the destruction of plasmids. At the end of this procedure, the target gene was deleted, generating the *E. coli* BW 25113 Δ phoA FRT-FRT.

phoA gene restoration on the genome

E. coli BW 25113Δ phoA FRT-FRT was used to make the new mutants carry the reinserted phoA gene to a preselected position, the insL – yjhV intergenic distance is located at the 97.1 - 97.2 min. The insertion site was selected according to the intergenic distances length between the new neighboring genes. In this new construction, the target gene was placed between the genes insL - yjhV (1103 bp) (NAIST, 2006). To achieve this, PCR reactions were performed for the phoA gene and the kanamycin marker using two sets of specific primers armed by the homologous extensions to the insertion region. The strategy we used to perform the insertion is presented in Figure 2.

The two PCR products were gel-purified and incubated with Dnpi enzyme as described above and were digested by the Hind III restriction enzyme (Fermentas) as described in (Sambrook et al., 1989) and according to the manufacturer's instructions. Reactions were incubated for 16 h at 37°C. Digested PCR products were then ligated with T4 DNA ligase and recovered as described by Sambrook et al., 2001. *E. coli* BW25113Δ phoA FRT-FRT was transformed with the plasmid pKD46. The bacterium that carries the pKD46 was then made electro competent as described

Table 3. Primers used in this work.

Primers name	Sequence	Target	Reference
phoA1	5'-gct-gtc-ata-aag-ttg-tca-cgg-3'	phoA gene	This work
phoA2	5'-tct-gcc-att-aag-tct-ggt-tgc-3'		
Ph1P1	5'-AAA-GTT-AAT-CTT-TTC-AAC-AGC-TGT-CAT-AAA-GTT-GTCgtgtaggctggagctgcttc -3'	FRT-kan-FRT for inactivation	This work
Ph2P2	5'-TAT-GCG-CCC-GTG-ATC-TGC-CAT-TAA-GTC-TGG-TTG-CTA-attccggggatccgtcgacc -3'		
k1	5'-CAGTCATAGCCGAATAGCCT-3'	Kan gene fragment	Datsenko and Wanner, 2000
k2	5'-CGGTGCCCTGAATGAACTGC-3'		
Kt	5'-CGGCCACAGTCGATGAATCC-3'		
A1	5'-CAGCATTCCTGACGACGATAC-3'	phoA gene region	This work
A2	5'-AAAACCAGACCGAAAAGCAAG-3'		
H1phoA1	5'-CGTCTCATTCAAAAAACCTCCGCAACCCCATGTTT gctgtcataaagttgtcacgg -3'	phoA gene for insertion	This work
PhoA2M1'	5'-TCTTCATGTTTTAAGCTTGAGCGTATGCGCCCGTGA tctgccattaagtctggttgc-3'		
M1FRT1	5'-TCACGGGCGCAAGCTTCATGGTTAAAACATGAAGA gtgtaggctggagctgcttc -3'	FRT-kan-FRT for insertion	This work
FRT2H2	5'-GCGGTCTTAGTGCGCTGTAATATCCAACGATATAGT attccggggatccgtcgacc-3'		
Ir1	5'- TCAAAAACAGAGGCTTTTTCC-3'	Insertion region	This work
Ir2	5'- TCGGTTTTCTGATTCGTTTGG-3'		
PhoAex1	5'-AAAGCACTATTGCACTGGCAC-3'	RT-PCR	This work
PhoAex2	5'-GCGGCTTTCATGGTGTAGAAG-3'	RT-PCR	This work

* restriction sites are underlined

above. Electroporation was done as described above but in this case, we used the ligated phoA gene - FRT-kan- FRT DNA fragment. Km transformants were selectedselected and the kananmycin gene removed as described above.

PCR verification.

We used PCR reactions to show that in the wild type and in the mutants nucleotides, sequences of interest have the correct structure and position. The primers we used are listed in the Table 3 and the conditions of each reaction in Table 4.

Alkaline phosphatase assay

Running AP assays gives a numerical value of the amount of alkaline phosphate being produced. If the mutant cells were functional, they would have low AP units while growing in a high-phosphate media (LB), and would have high AP units while growing on low-phosphate media (MOPS). To assay the function of the phoA gene, a comparative alkaline phosphatase (AP) assays was performed using strains BW25113 and *E. coli* BW25113FRT-FRT/FRT-FRT, phoA*. The strains BW25141 and BW26337 were tested as positive and negative controls, respectively. For these assays, triplicate overnight cultures in 3 ml of high-phosphate medium and low-phosphate medium were grown at 37°C.

The OD_{600} of the cultures were measured. 1 ml of cells was collected by centrifugation. Cells were diluted 1:2 in 1 M Tris HCl pH 8.2; two drops of chloroform and one drop of 0.1% SDS were added. Cells were vortexed for 1 minute. 100 µl of 20 mM para-nitrophenyl phosphate were added and reactions were incubated at 37°C until the solution turned yellow. Reactions were stopped with 400 µl of 1 M KH_2PO_4 and then placed on ice. Tubes were centrifuged for 1 min and the OD_{420} of the supernatant was measured. AP units are arbitrary units that equal (OD_{420} x 2000)/(OD_{600} x incubation time). The average of the three samples is reported.

RNA isolation and RT-PCR

Strains were grown in high-phosphate or low-phosphate medium and were harvested during the mid-log growth phase. An RNeasy mini kit from QIAGEN was used for the isolation of total RNA from bacteria. Quantification of the total amount of RNA was achieved by spectrophotometry. A Bioer two Steps RT-PCR kit was used for RT-PCR according to the manufacturer's instructions. Two primer pairs (PhoAex1 and phoAex2) were used to target phoA expression. Two negative controls samples were also made without RNA template and reverse transcriptase, respectively. PCR amplification was performed; the primers

Table 4. PCR conditions and product sizes.

Reaction name	PCR conditions	Product size
phoA region wild type	94ºC5',94ºC30",57ºC30",72ºC1',"72ºC10',30 cycles	1789 bp
phoA wild type*	94ºC5',94ºC30",55ºC30",72ºC1',"72ºC10',30 cycles	1566 bp (80up+1416phoA+70down)
FRT-kan-FRT inactivation	94ºC5',94ºC30",57ºC30",72ºC1',"72ºC10',30 cycles	1394 bp
Kan gene fragment	94ºC5',94ºC30",57ºC30",72ºC30'',"72ºC10',30 cycles	490 bp
phoA region after FRT-kan-FRT electroporation	94ºC5',94ºC30",57ºC30",72ºC1',"72ºC10',30 cycles	1541 bp(73+1395+73)
phoA region after kanamycin removing	94ºC5',94ºC30",57ºC30",72ºC30'',"72ºC10',30 cycles	746 bp (1541bp-795bp)
Insertion region wild type	94ºC5',94ºC30",55ºC30",72ºC1',"72ºC10',30 cycles	1181 bp(43+1103+35)
phoA insertion	94ºC5',94ºC30",55ºC30",72ºC1',"72ºC10',30 cycles	1602 bp (36+1566+36)
FRT-kan-FRT insertion	94ºC5',94ºC30",57ºC30",72ºC1',"72ºC10',30 cycles	1394 bp
Insertion fragment**	-	2997 bp (H1+*+36+1323+H2)
Insertion region after electroporation	94ºC5',94ºC30",57ºC30",72ºC2',"72ºC10',30 cycles	3095 bp (35+2997+43)
Insertion region after kanamycin removing	94ºC5',94ºC30",57ºC30",72ºC2',"72ºC10',30 cycles 50℃ for 30 min	2290 bp (3085 bp-795 bp)
RT-PCR	94ºC5',94ºC30",55ºC30",72ºC1',"72ºC10',30 cycles	

**ligation product between phoA insertion and FRT-kan-FRT insertion, H1 and H2 are homologous sequence from the insertion region and have respectively, 36 bp.

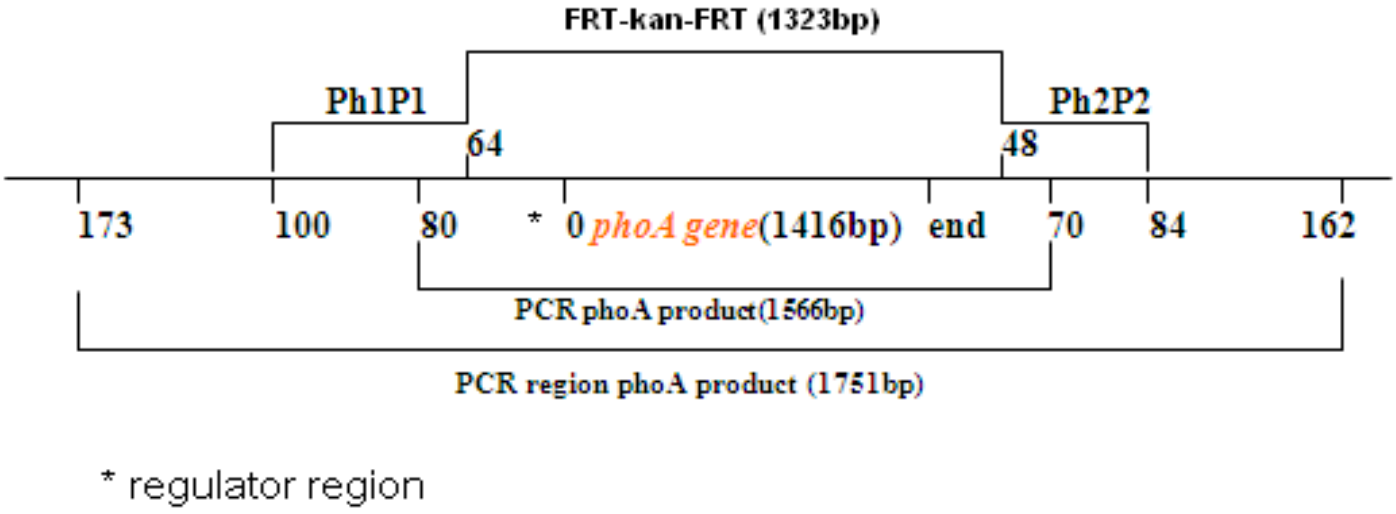

Figure 1. Strategy for phoA gene inactivation from the *E. coli* genome; the phoA gene PCR contains the entire phoA ORF and the regulator region (80 bp upstream) and 70 bp downstream. A kanamycin cassette flanking by two FRT sites was amplified by PCR from the plasmid pKD13 (Datsenko and Wanner, 2000) by using primers containing 36 nt, homology extensions corresponding to the regions 100 – 64 and 48 -84 bp. The electroporation was done and the kanR strains indicated a successful replacement. The PhoA region with a sequence longer than the PCR product of PhoA gene was used to assess the construction.

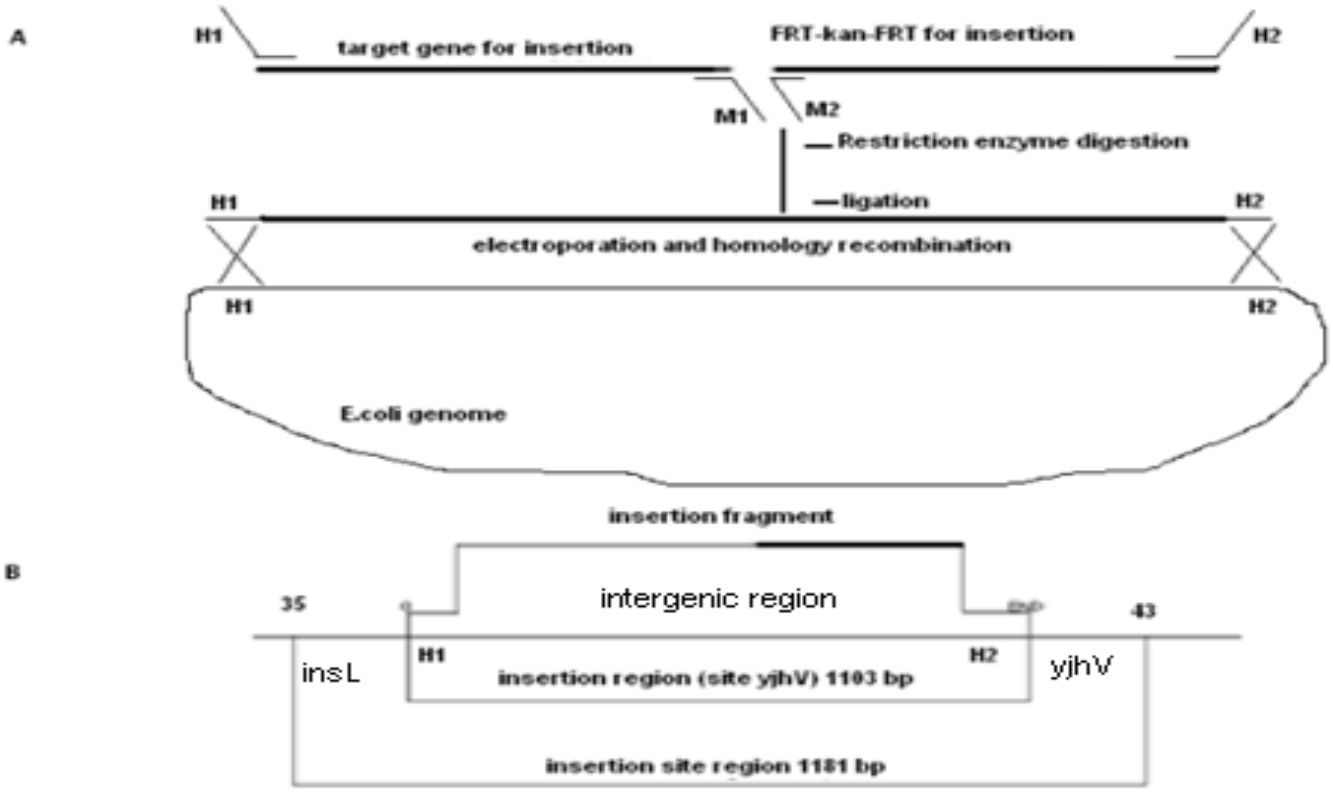

Figure 2. Strategy for gene restoration in the *E. coli* genome. (A) The target gene PCR using specific primers armed by H1 (forward primer), the homologous extension to the insertion region and by M1 (reversed primer), a complementary sequence to M2. M1 and M2 contain the Hind III restriction sites. FRT-kan-FRT fragment PCR using primers M1FRT1 and FRT2H2, in which M2 is a complementary sequence to M1 and H2 is homology extension to the insertion region. FRT1 and FRT2 are specifics to the fragments as designed by Dasenko and Wanner, 2000. The restriction was conducted using the HindIII enzyme and the ligation using T_4 ligase. The electroporation was done and kan resistance strains were selected on LB-agar plates. (B) The intergenic region has a length of 1103 bp. H1 and H2 extensions are homologous to the two ends of the intergenic region, respectively. The ligation fragment between the target gene PCR product and a kanamycin cassette substitute the intergenic region by homology recombination after electroporation. An insertion region was designed; it contains the intergenic region and was used to assess the construction.

and the conditions used are listed in the Table 3 and 4, respectively.

Growth curves and statistical analysis

For growth curves, overnight cultures of mutants and parental *E. coli* strains were diluted into fresh LB media (2.5 ml of bacteria culture into 250 ml of LB) , and the growth was monitored every twenty minutes by measuring the optical density at 600 nm (DO_{600}) using a 2800 UV / VIS spectrophotometer, UNICO. The results were analyzed statistically by studying the correlation between data generated by the wild type and the mutant. Statistical analysis was performed to assess correlation between the wild type and the mutants by comparing data from the growth curves.

RESULTS AND DISCUSSION

Gene delocalization and PCR assessment

The mutants *E. coli* BW25113FRT-FRT/FRT-FRT, phoA* was constructed as presented above for each gene inactivation, gene restoration and verification of all constructions. Figure 3 shows the region containing the phoA gene; it has 1751 bp with 173 bp upstream of the start codon and 162 bp downstream of the stop codon.

The changing of the size of this region allowed us to evaluate the inactivation of the gene and the excision of the resistance marker by the plasmid pCP20.

The *E. coli* genobase (NAIST, 2006) gives the location of the phoA gene at 8.6 min of the *E. coli* W3110 genome, with a sequence of 1416 bp and a protein of 471 aa; Start position (GTG) 400971 and 402386 end position (TAA). With the aim to preserve the regulatory region of the gene, the sequence of 1566 bp was considered: 1416 bp for the phoA gene ORF, 80 bp upstream and 70 bp downstream. The intermediary strain and the mutant contain also the phoA gene sequence as it is illustrated in Figure 4.

The FRT-kan-FRT from the pKD13 played an important role during these experiments for both gene inactivation and gene reinsertion. We only changed the homologous extensions specifics for phoA region in the wild type and

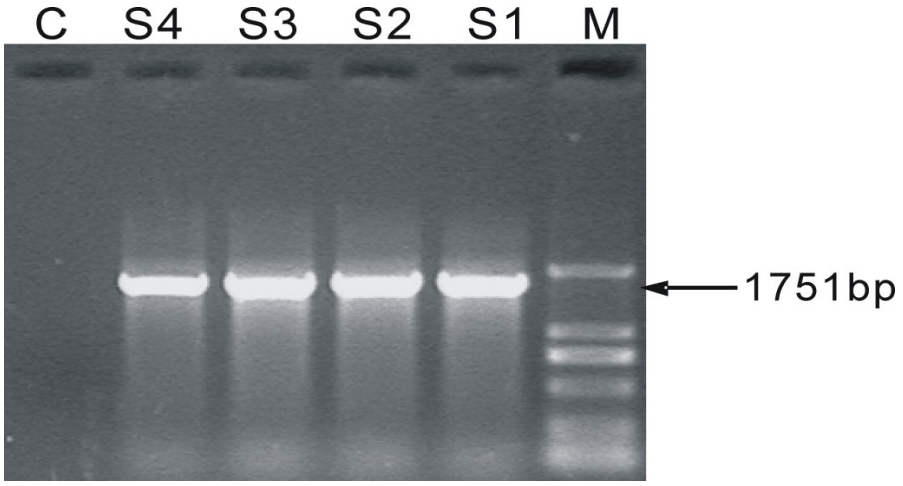

Figure 3. *phoA* gene region. Fragments synthesized by PCR. Electrophoresis of PCR products was performed on a 0.8% agarose gel. Lane M, 2000 bp DNA size markers; Lanes S1, S2, S3, S4, fragments synthesized with the *E. coli BW25113* genomic DNA as a template (phoA gene at 8.6 min) and the lane C, a negative control.

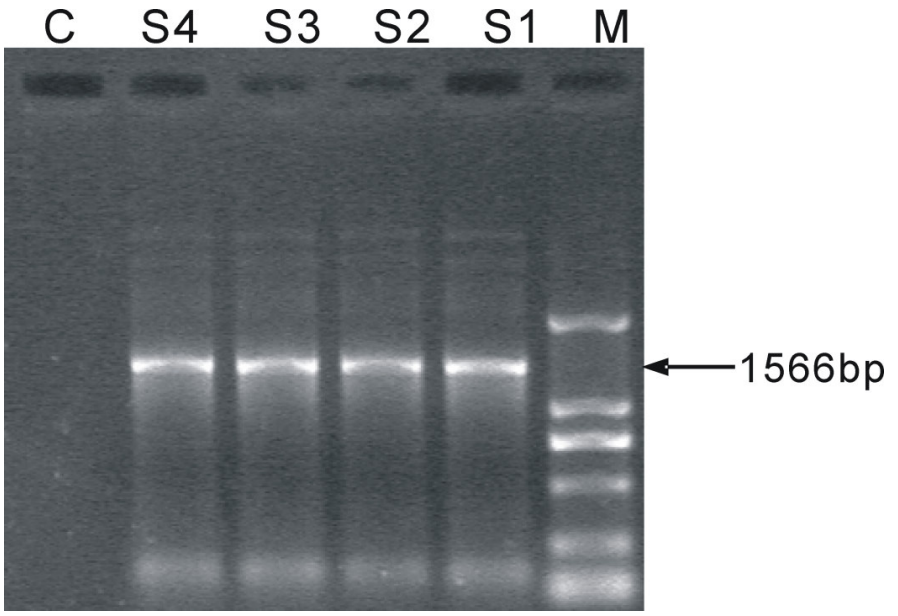

Figure 4. *phoA* gene, Lane M, 2000 bp DNA size markers; Lane S1, fragment synthesized with the *E. coli BW25113* genomic DNA as a template (phoA gene at 8.6 min); Lane S2 fragment synthesized with the intermediary strain *E. coli BW25113ΔphoAFRT-FRT/FRT-kan-FRT,phoA** genomic DNA as a template and Lane S3 and S4 with the mutant (phoA gene at 97.1-97.2 min) *E. coli BW25113ΔphoAFRT-FRT/FRT-FRT* , *phoA** genomic DNA as template; Lane C, a negative control.

for the insertion site in the mutant. The primers used in this step had 56 nt (36 nt for homology extension and 20 nt for the priming site). All FRT-kan-FRT fragments had a sequence of 1394 bp. Experimentally, we noted that each PCR reaction for getting the FRT-kan-FRT DNA fragment for inactivation or reinsertion was successful with the same PCR conditions, the homologous extensions used in 5' end did not affect the PCR reaction (Figure 5).

The Kan gene, present on the FRT-kan-FRT fragment, was used as marker during both the inactivation and reinsertion steps. To assess all the constructions we made, we used a Kan gene fragment PCR using k2 and kt primers. The Kan gene itself has 795 bp (Plasmid pKD13 Genbank accession number AY048744), using k2 and kt

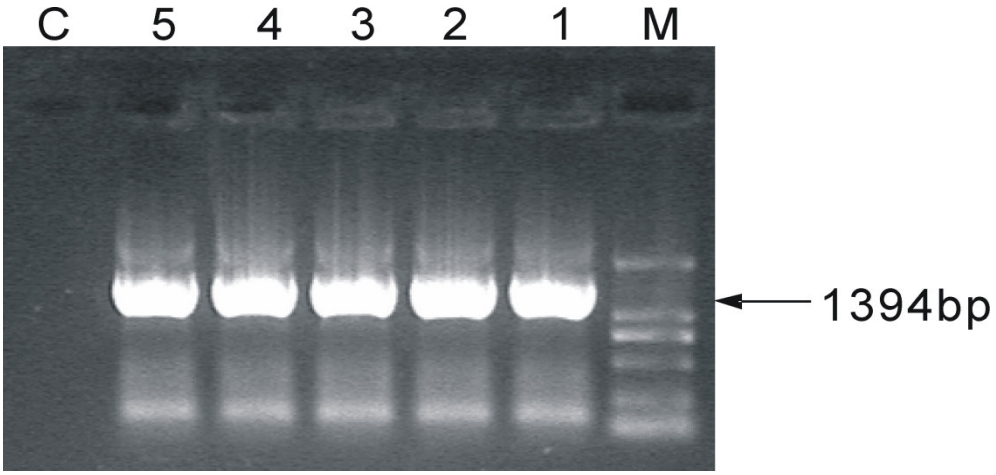

Figure 5. *FRT-kan-FRT.* Lane 1 shows the fragment synthesized with pKD13 plasmid as a template; Lane 2 and 3, fragments synthesized with the intermediary strain *E. coli BW25113ΔphoA/FRT-kan-FRT* genomic DNA as a template; lane 4 and 5, fragments synthesized with the intermediary strain *E. coli BW25113ΔphoAFRT-FRT/FRT-kan-FRT,phoA** genomic DNA as a template; Lane C, a negative control.

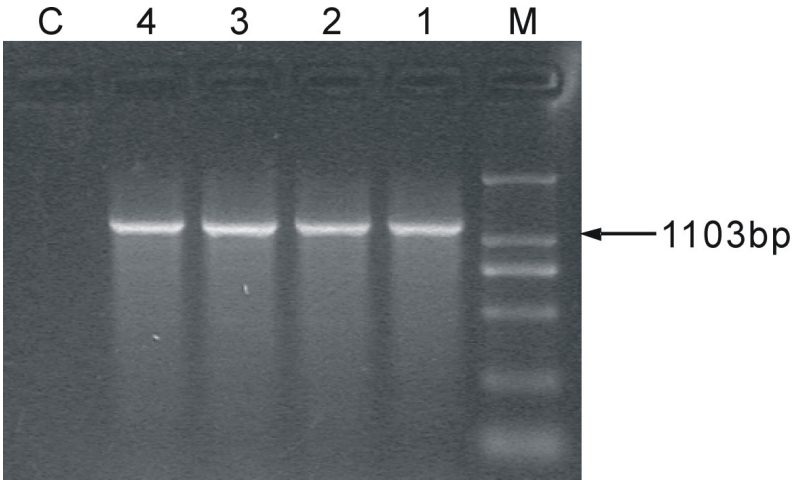

Figure 6. Insertion region *insL- yjhV in the E. coli BW 25113.* Lanes 1, 2, 3, 4 fragments synthesized with *E. coli BW25113* genomic and DNA as a template in lane C, a negative control.

kt primers allowed us to get a PCR product with 489 bp inside the kan gene ORF. Using the *E. coli* BW 25113ΔphoAFRT-kan-FRT genome as template, this PCR helped to visualize the success of the electroporation after the positive growth in LB and on LB-agar supplemented by 50 µg/ml kanamycin. The insertion region we selected is located at 97.1 - 97.2 min on the *E. coli* W3110 chromosome; it is the intergenic distance between the genes *insL and yjhV* and has 1103 bp (Figure 6).

For insertion, we made a new phoA gene PCR by using primers (H1phoA1 and PhoA2M1') with homology extensions with the insertion region. This PCR product was designed to contain the phoA ORF, its regulator region and the insertion region homology extension on one side. We made also a new FRT-kan-FRT PCR by using primers (M1FRT1 and FRT2H2) with homology sequences with the insertion region. The fragment for insertion was constituted by a ligation product between the phoA for insertion and FRT-kan-FRT for insertion. After the insertion of the ligated fragment; we made the PCR to check the change on the insertion region using the same primers ir1 and ir2, phoA1 and phoA2, k2 and kt, respectively. We noted that the insertion region's length was increased, due to the homology recombination of the insertion fragment. Finally, after the pCP20 transformation and Kan gene excision, we got the *E. coli* BW25113FRT-FRT/FRT-FRT, phoA*; a bacteria mutant

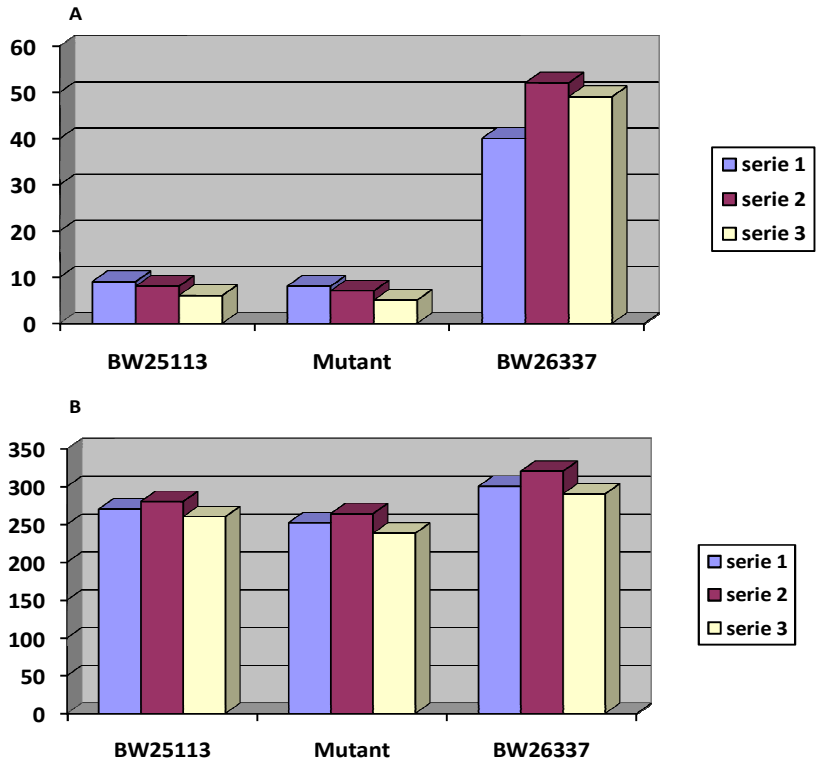

Figure 7. AP assays results of mutant *E.coli BW25113ΔphoAFRT-FRT/FRT-FRT, phoA**. This graph contains the results of an AP assay performed using the mutant *E. coli BW25113ΔphoAFRT-FRT/FRT-FRT, phoA**(phoA gene at 97.1-97.2 min). The positive BW 25113 showed repression of the phoA gene in LB and loss repression in MOPS media. The mutant was tested and the phoA gene was expressed but the positive control appeared to maintain repression in the rich media and loss the repression in the minimal medium. The BW 26337(ΔpstSCAB-phoU) was used as house-counting control because it expresses phoA in both media conditions.

which has two remaining fragments FRT-FRT at the 8.6 min and the 97.1 - 97.2 min.

AP activity assay

An alkaline phosphatase assays was conducted to assess the phoA gene expression in the mutant (97.1 - 97.2 min). The results showed that the phoA was expressed in the mutant both in the high and low phosphate media (Figure 7). The phoA expression in the mutant was lower than in the wild-type but the difference was not significant.

RT-PCR test

Data from RT-PCR confirmed the results collected from the AP assays, the mutant phoA mRNA was present in both high and low phosphate media. Figure 8 shows a fragment from the phoA gene sequence obtained from cDNA obtained from the mRNA in the two environments.

Growth curve

The growth curve study shows also that the mutant has a growth rate similar to the wild type (Figure 9).
Statistics test showed good correlation between the two growths curves. The correlation (r_{yx1}) (Salvatore and Reagle, 2002), was 0.99759916 (y is the wild type and x_1 the mutant).

$$r_{yx_1} = \frac{\Sigma x_1 y}{\sqrt{\Sigma x_1^2} \times \sqrt{\Sigma y^2}}$$

Finally, we constructed a mutant *E. coli* by the relocation of a gene on the same chromosome, so as to have a new tool to study gene function. The post-genomic studies require the availability of various biological tools. However, much remains to be done on the field of genome sequencing; for the determination of functions of each gene and the functional implications of genes, the identification of functional networks of bacterial genomes based on data already available in many gene banks.

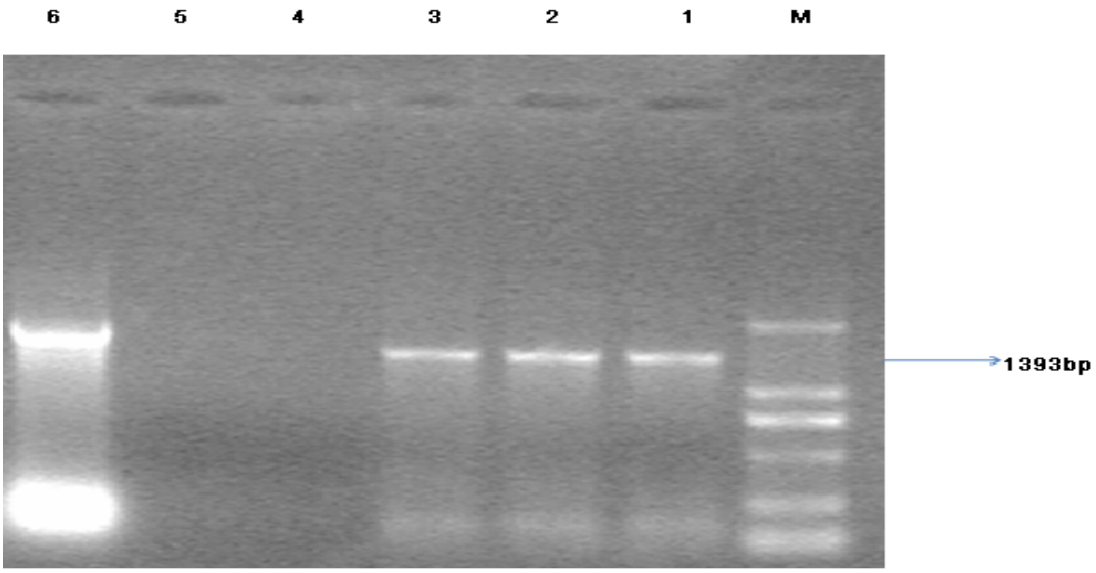

Figure 8. phoA gene expression using reverse transcriptase-PCR. Reverse-transcribed cDNA from the mutant *E. coli* BW25113ΔphoAFRT-FRT/FRT-FRT, phoA*(phoA gene at 97.1-97.2 min) total RNA was analyzed for the presence of a 1393-base pair (lanes 1, 2 and 3). The PCR fragment, a sequence from phoA gene was obtained with the primers PhoAex1 and PhoAex2. Two negatives controls were conducted (lanes 4, 5) without the total RNA as template and without the reverse transcriptase in the RT reaction, respectively. The lane 6 represented the phoA gene PCR product (1566 bp) using phoA1 and phoA2 primers using the *E. coli* BW25113ΔphoAFRT-FRT/FRT-FRT, phoA*(phoA gene at 97.1 - 97.2 min) genome as template.

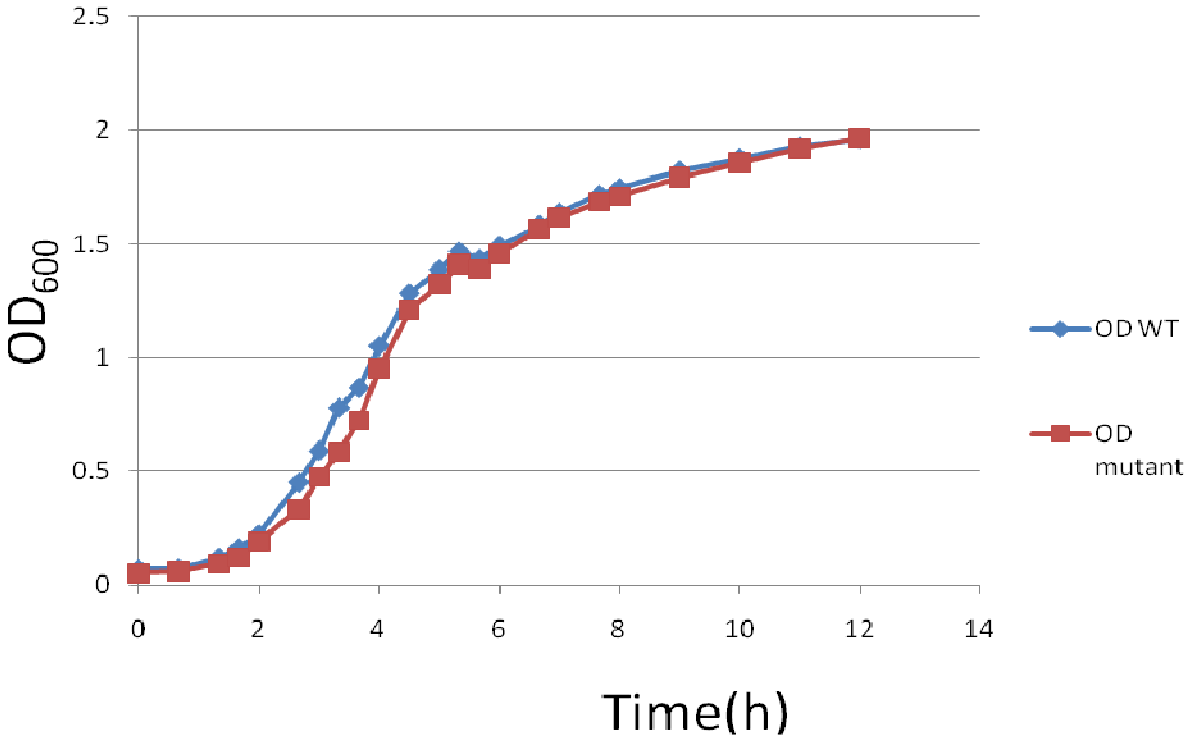

Figure 9. The mutant growth curve of the wild-type BW25113 and its mutant *E. coli* BW25113ΔphoAFRT-FRT/FRT-FRT phoA*(phoA gene at 97.1 - 97.2 min) realized after inoculation of a single colony of each in LB, incubated at 30°C for 16 h; 2.5 ml of these culture were diluted in 250 ml and incubate at 30°C and the OD600 was measured every 20 min. The data for the two strains were statistically analyzed by the correlation test.

The reverse genetics has been of great importance in the determination of gene function using the knockout technique (inactivation), the same for the gene cloning techniques. But until now, all the techniques used have respected the natural order of things; what is known about the expression of a gene, its regulatory network and its functional network is linked to the natural location of genes as it was discovered by genomics. The importance of this work is to create mutants by the relocation of genes; operons and finally to detect changes in the expression of all genes. Thus, the development of this approach will provide tools for systems biology studies with the objective to understand the governing rules of the bacterial genes and the genome organization.

The approach used in this work was based on the method developed by Dasenko and Wanner in 2000, which uses the inactivation of genes using fragments frt-kan-frt with homologous extensions, the inactivation being made mediated by the pKD46 plasmid and the excision by the plasmid pCP20. At the stage of the gene restoration, introduction of restriction sites in the primers is made necessary by the need for ligation prior to electroporation, which make this step very random because of a double electroporation, for both the gene and the fragment contain the resistance marker. Mutants created by the inactivation and reinsertion of a gene contains the frt-frt residues both at the inactivation and the reinsertion regions, which in our opinion will not affect future studies because they do not interrupt any sequence of genes as the AP assays and RT-PCR results showed above.

The choice of phoA gene for this study was guided by a dual purpose; first the phoA gene is a membership of a functional network, the pho regulon which can make easier to study the various implications due to its delocalization on the expression of all pho genes. Second, the phoA gene (1416 bp) has a length size close to the size of the DNA fragment (frt-kan-frt (1394 bp) used for inactivation. That similarity has captured our attention but the method was also successful for the genes or operon with larger size. The choice of region of insertion was made in order not to interrupt the sequence of the new neighboring genes. Thus, the intergenic distances were targeted. In this case, the distance between genes and insl yjhV (1103 bp) located at 97.1 - 97.2 min was chosen.

The described procedure and the created *E. coli* mutants can be efficiently used for the study of bacterial genomics, especially in systems biology to understand the relation between the genes loci on the genome and their expression alongside new neighboring genes and others with the same functional group.

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