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Identification of Genes for Growth with Oxygen in *Escherichia coli* by Operon Fusion and Southern Blot Techniques

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Abstract Seven *Escherichia coli* cells defective with aerobic growth were isolated by the insertion of λp*lac*Mu53, a hybrid bacteriophage of λ and Mu, which created a transcriptional fusion to *lacZY*. These insertion mutant cells were tested on an XG (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) medium for anaerobic expression of *lacZ* by fusion to a promoter. The chromosomal DNA from these strains were digested by *Eco*RI, and the *Eco*RI fragments that contained the fused gene and *lacZ* sequence were identified by Southern hybridization, using *lacZ* containing plasmid as a probe. The *Eco*RI fragment from each strain was cloned and sequenced. The sequence data were compared with the GenBank database. The mutated gene of three strains, CYT4, CYT5, and OS11, was found to be identical, and it was *nrdAB* that encoded ribonucleoside diphosphate reductase. The gene *nrdAB* was at min 50.5 on the *Escherichia coli* linkage map and 2,348,084 on the physical map, and is involved in heme-related reduction-oxidation reaction. OS6 and OS14 mutant strains had insertion at min 8.3 and the mutated gene was *hemB*. The *hemB* encodes 5-aminolevulinate dehydratase or porphobilinogen synthase. The OS3 mutant had insertion in *cydB* at min 16.6. The *cydAB* encodes cytochrome *d* oxidase. In the case of OS1, the fusion was made with *sucA*, the E1 component of α-ketoglutarate dehydrogenase.

Key words: Oxidative stress, λp*lac*Mu53, *nrdAB*, *hemB*, *cydA*B

Oxidative damage to DNA, RNA, proteins, and cell membrane occurs when the cellular concentration of reactive oxygen species exceeds the capacity of the cell to eliminate them. Reactive oxygen species such as superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxy radical (OH·) naturally arise during normal metabolism in aerobically growing cells as a result of the incomplete reduction of molecular oxygen. They also arise from a variety of environmental sources such as ionizing radiation and redox-cycling agents (e.g. paraquat) [10, 18, 20]. Aerobic prokaryotic and eukaryotic organisms have developed a set of cell defense systems to mitigate the damaging effects of reactive oxygen species. To alleviate the toxicity of these compounds, *Escherichia coli* possess several enzymes that prevent oxidative damage (alkyl hydroperoxidase, catalases, superoxide dismutase, and glutathione reductase) and repair DNA lesions resulting from oxidative damage (e.g. exonuclease III, RecBC nuclease, and endonuclease III) [29]. These enzymes are controlled by the regulatory proteins SoxRS, OxyR, Fnr, and ArcAB. The regulatory system responds to growth conditions as determined by the availability of oxygen, and controls the expression of proteins that function in aerobic respiration, anaerobic metabolism, and defense against reactive oxygen species [2, 16]. The response to oxidative stress conditions overlaps with other stress responses such as heat shock, starvation, and the SOS response [21]. However, these defense enzymes are not always necessary for the cell to grow under aerobic condition. The mutant strain of one of these defense enzymes can grow aerobically, as the defective mechanism can be complemented with another mechanism.

The *soxRS* regulon is triggered by superoxide-generating agents or by nitric oxide [23, 32]. Upon exposure to redox-cycling compounds, *E. coli* induces the synthesis of about 40 proteins [22]. At least nine of these proteins are produced by a SoxRS regulon. The products of the genes known to be regulated by *soxRS* include *nfo*, *zwf*, *sodA*, *fumC*, *micF*, *acnA*, and *fpr* [18]. The predicted SoxR (17 kDa) and SoxS (13 kDa) proteins each have a predicted helix-turn-helix motif that may mediate specific DNA binding [31]. SoxR protein is a homodimer, and each monomer (M.W. ~17,000) contains one redox-active [2Fe-2S] cluster [4, 9, 23, 31, 38]. The induction of the *soxRS* regulon occurs in two steps, and SoxR is most likely the sensor: SoxR is first activated by superoxide and enhances

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the production of SoxS, which in turn activates the transcription of the target genes. SoxS recognizes the consensus binding motif, AN_2GCAYN_7CWAC, where "N" is any base, "Y" is a pyrimidine, and "W" is A or T [24].

The *arcA* (aerobic respiration control) gene, encoding a pleiotropic transcriptional regulator, belongs to a two-component regulatory system [12]. ArcB functions as a sensor-transmitter and communicates with ArcA, the receiver-regulator. *In vitro* studies demonstrate that ArcB is autophosphorylated and can subsequently transfer phosphate via its kinase activity to Arc. It has been suggested that the phosphorylated form of ArcA binds DNA and mediates transcription [13, 14]. When activated anaerobically by the membrane sensor protein encoded by *arcB*, ArcA can then bind to regulatory DNA sites to mediate both positive and negative control of gene expression [7, 15, 25, 33, 37].

The Fnr protein appears well poised as a regulator of anaerobic respiration and fermentation pathways [25]. *fnr* (fumarate nitrate reduction) is a pleiotropic regulator that controls the transcription of numerous target operons of anaerobic respiration [12]. Fnr is a transcriptional repressor of the aerobic respiratory genes and a transcriptional activator of the anaerobic respiratory pathway genes. The mechanism by which Fnr senses the anaerobic state is still unclear, although its cysteine-rich N-terminal domain is essential for this process [37].

In this study, we prepared gene fusion mutants by the operon fusion technique with λp*lac*Mu53 [8], screened the phenotype of these mutants, and selected the cells showing the phenotype of growth defect in aerobic conditions but capable of growing in anaerobic conditions. Observation of growth on the plates led us to conclude that the oxygen was bacteriocidal on these mutants. Among these mutants, we chose seven strains and cloned the chromosomal segments of mutated genes which were identified by Southern blot technique. These genes on the *E. coli* chromosome were physically mapped to find out the location of the genes and to prepare data for further studies. Further study on these genes necessary for the aerobic growth of *E. coli* would clarify defense mechanisms of organisms and reveal new regulatory systems.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids

All the bacterial strains used in the following experiments were derivatives of *E. coli* K-12, and all the bacterial strains, phages, and plasmids are described in Table 1.

Media, Chemicals, and Enzymes

The media used in these experiments have been described previously [27]. Bacto trypton, Bacto yeast extract, and Bacto agar were purchased from Difco and other chemicals from Junsei or Sigma. The antibiotics used in this study are ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (40 μg/ml), and tetracycline (15 μg/ml). For the identification of the expression of the *lac*Z gene and cloning, 40 μg/ml XG (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used. The restriction enzymes, T4 DNA ligase, for the cloning, and *E. coli* DNA polymerase for nick translation were purchased from New England Biolabs, and DNase I and RNase A were from Sigma.

Anaerobic Culture Conditions

For anaerobic incubation, the plates were placed in a jar with an anaerobic atmosphere created by a H_2 and CO_2 producing pouch pack (BBL GasPak anaerobic System; Becton Dickinson & Co), and incubated at 37°C for more than 23 h.

The anaerobic liquid culture was prepared by degassing the inoculated media with an aspirator (20 mmHg) (Maxi-Dry Plus, Heto-Holten Lab. Equipment) and purging 5 times with 99.99% nitrogen, and the culture was incubated in a shaker for more than 24 h at 37°C.

Mutagenesis

Fusion strains were constructed by infecting MO with λp*lac*Mu53 and helper phage λpMu507, according to the procedure of Bremer *et al.* [6]. Mutagenesis was by random insertion. Infected cells were spread on an LB plate containing kanamycin (40 μg/ml) and XG (20 μg/ml), and incubated for 24 h in anaerobic conditions, and for 48 h in aerobic conditions. The mutants that did not form colonies under aerobic conditions but did form blue colonies under anaerobic conditions were selected on the LB-XG plate. For anaerobic incubation of the plates, an anaerobic environment was prepared by an H_2+CO_2 producing pouch pack (BBL GasPak Anaerobic System; Becton Dickinson & Co.).

Reversion Test

Some of the fusion strains have very unstable phenotype and show very high reversion rate. The reversion frequency was checked by counting colonies that can grow aerobically. Thus, anaerobically cultured cells were resuspended in LB medium, and the cells were spread on two LB plates containing kanamycin (40 μg/ml) and XG (20 μg/ml). Then, one plate was incubated for 24 h in aerobic conditions, and another plate was incubated for 24 h in anaerobic conditions. The colony numbers were counted and compared, and the cells that had less than 0.1% of reversion rate were selected for study.

P1 Transduction

For the stable two OS strains, OS4 and OS5, the inserted λp*lac*Mu53 was transferred from the MO into the RZ4500 cell by P1 transduction. P1 transduction was performed as described previously by Miller [27]. After P1 transduction in anaerobic conditions, blue transductants were screened

by replica plating. The cells were incubated for 24 h at 37°C, and the cells that did not form colonies under aerobic conditions but did form blue colonies under anaerobic conditions were selected on the LB-XG-Kan plate.

Preparation of Southern Blot Probe

The following experiments were performed as described previously by Maniatis *et al.* [26]. The plasmid that was used for the preparation of the probe was pRZ5202 [30, 39, 40]. pRZ5202 is a plasmid that has been used as a promoter cloning vehicle, and contains the W209 *trp-lacZ* fusion that contains the full sequence of the *lacZ*. [α-^{32}P]dCTP was used for the labeling of DNA.

Southern Blot Hybridization on *Eco*RI-Digested Chromosomal DNA

Chromosomal DNA was prepared by standard procedures as described previously [35]. The anaerobic culture medium was prepared by degassing in vacuum (Maxi-Dry Plus, Heto-Holten Lab. Equipment) and purging 5 times with 99.99% nitrogen. Each strain was grown in an LB medium containing kanamycin. After 24 h of incubation, the culture was extracted for chromosomal DNA. Ten μg of DNA from each strain was digested with *Eco*RI (Takara), and the DNA fragments were separated on 0.7% agarose gels and transferred to nylon membranes (Nytran-Plus; Schleicher & Schuell) by capillary transfer system (Turboblotter Rapid Downward Transfer System, Schleicher & Schuell).

Southern blot hybridization was carried out according to the procedure previously described [26]. The nylon membrane was used for the hybridization with the *lacZ* probe at 68°C, for 16 h. The detection of hybrid DNA was carried out by Bio-Imaging Analyzer, BAS-2500 (Fujifilm).

Cloning of Chromosomal Region into Plasmid Vector

The *Eco*RI fragment containing the fused gene was purified on 0.7% agarose gel (Sea Plaque GTG agarose, FMC) by

Table 1. *E. coli* strains, phages, and plasmids used in this study.

Name	Genotype	Reference or source
Strains		
MG1655	λ^- F^-	[5]
RZ4500	λ^- F^- *lacZΔ145*	[5]
MO	azi-7 relA1 rpsL100 spoT1 metB1	[5]
CAG12027	MG1655 *zdd-230::*Tn*9*	[36]
CAG18459	MG1655 *zde-234::*Tn*10*	[36]
CT11U	RZ4500 *f308d*::p*lac*Mu53	This study
DH5α	$F^-\lambda^-$ *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1 φ80dlacZΔ* M15	
CTY4	RZ4500 *nrdA*::λp*lac*Mu53	This study
CTY5	RZ4500 *nrdB*::λp*lac*Mu53	This study
OS1	MO *sucA*::λp*lac*Mu53	This study
OS3	MO *cydB*::λp*lac*Mu53	This study
OS6	MO *hemB*::λp*lac*Mu53	This study
OS11	MO *nrdB*::λp*lac*Mu53, *f308d*::λp*lac*Mu53	This study
OS14	MO *hemB*::λp*lac*Mu53, *rpoC*::λp*lac*Mu53, *mhpE*::λp*lac*Mu53	This study
Phage		
P1*vir*		[27]
λp*lac*Mu53	Mu *cIts*62 *ner*$^+$ *A*$^+$ '*uvrD*' Mu*S*' '*trpAB*' '*lacZ*$^+$ *lacY*$^+$ *lacA*'	[35]
λpMu507	*cIts*857 *Sam*7 Mu *A*$^+$ *B*$^+$	[35]
Plasmids		
pRZ5202	Promoter cloning vector with W209 *trp-lac* fusion	[30]
pMLB524	pBR322 derivative '*lacZ* Ap^r	[6]
pCT101	pMLB524, *Eco*RI fragment of chromosomal DNA of *sucA*	This study
pCT301	pMLB524, *Eco*RI fragment of chromosomal DNA of *cydB*	This study
pCT401	pMLB524, *Eco*RI fragment of chromosomal DNA of *nrdA*	This study
pCT501	pMLB524, *Eco*RI fragment of chromosomal DNA of *nrdB*	This study
pCT601	pMLB524, *Eco*RI fragment of chromosomal DNA of *hemB*	This study
pCT1101	pMLB524, *Eco*RI fragment of chromosomal DNA of *nrdB*	This study
pCT1102	pMLB524, *Eco*RI fragment of chromosomal DNA of *f308d*	This study
pCT1401	pMLB524, *Eco*RI fragment of chromosomal DNA of *hemB*	This study
pCT1402	pMLB524, *Eco*RI fragment of chromosomal DNA of *rpoC*	This study
pCT1405	pMLB524, *Eco*RI fragment of chromosomal DNA of *mhpE*	This study

Qiaex Gel Extraction kits (Qiagen). The *Eco*RI fragments containing chromosomal DNA were ligated with *Eco*RI digested pMLB524 plasmid vector [6] with T4 DNA ligase. The *Eco*RI fragment containing the chromosomal DNA has a *lacZ* sequence except the 54 bp at C-terminus, and pMLB524 has a C-terminal 54 bp sequence of the *lacZ* gene. The *lacZ* gene was reconstructed by the ligation of these two *Eco*RI fragments. The recombinant plasmids were used to transform DH5α. The clones were screened by the blue color produced by the reconstructed β-galactosidase. Plasmids containing an appropriate size insert were selected by restriction digestion.

Sequencing and Computer Analysis

To find the cloned chromosomal DNA sequence, we synthesized the 32-mer primer that was able to read the upper strand of the plasmids. The primer was designed to anneal the '*trp*' region, 5 nucleotides upstream from the β end of the Mu sequence. The sequencing was done with an ALFexpress sequencer using the Cy5™ AutoRead™ sequencing kit (Pharmacia Biotech). DNA sequence similarity and amino acid sequence similarity were investigated by using the BLAST algorithm (BLASTN 2.0.4 and BLASTP 2.0.4), database search program [3].

RESULTS AND DISCUSSION

Selection of the Mutants

Although organisms have defense systems, reactive oxygen species have been shown to be involved in oxidative cell damage in physiological and pathological processes such as aging, apoptosis, neurodegenerative diseases, and iron metabolism imbalance. In addition, the possibility exists that other enzymes and nonenzymatic compounds are also important in determining an organisms ability to survive in the presence of oxygen [1]. In order to explore the causes for oxygen sensitivity in bacteria, several approaches have been undertaken to identify proteins and genes induced in oxidative stress conditions. An important approach is to screen random operon fusions to a reporter gene (e.g., *lacZ*) for inducibility by oxidative stress. Mud(Ap*lac*) phage, which randomly inserts into the *E. coli* chromosome, has often been used to create operon fusions [28]. Also, to further investigate the mechanisms of oxygen toxicity, Jamison and Adler [17] isolated oxygen sensitive (Oxy^s) mutants of *E. coli* through UV irradiation. However, the above two approaches had inherent difficulties in their mutant analyses due to the method of mutagenesis. The Mud(Ap*lac*) phage is very prone to making a further transposition after its initial insertion into the chromosome, and the effect of the mutation analyzed could be due to unknown additional insertion of the phage into a chromosome during the analysis. The mutagenesis with UV irradiation also generates multiple mutations that could be analyzed without their being detected by the investigator.

In this study we used the λp*lac*Mu53 phage. The λp*lac*Mu53 randomly inserts into the *E. coli* chromosome. We selected thirteen aerobic growth defective mutants, and tested them for stable phenotype. High frequency reversion with a reversion rate higher than 0.1% was observed in six strains (OS1, OS3, OS8, OS9, OS13, OS14). One strain (OS6) grew poorly even in an anaerobic condition, and six strains (OS4, OS5, OS7, OS10, OS11, OS12) showed a stable phenotype with a reversion rate lower than 0.1%. These seven strains, with strong and stable phenotype suitable for handling in further study, were used in this work. The insertion of λp*lac*Mu53 is known to stay fixed in the chromosome without hopping around it, unless the Mu transposase is provided by the helper phage [34] and it is a very stable insertion. The high reversion rate observed, even with this stable λp*lac*Mu53 insertion, was most likely due to complementary mutations on other locations on the chromosome that enabled the cell to bypass the defect, giving a great deal of problems to earlier investigators. This high frequency of the complementary reversion indicates that the *E. coli* has evolved as a result of its creation of a reservoir of stress defense mechanisms that

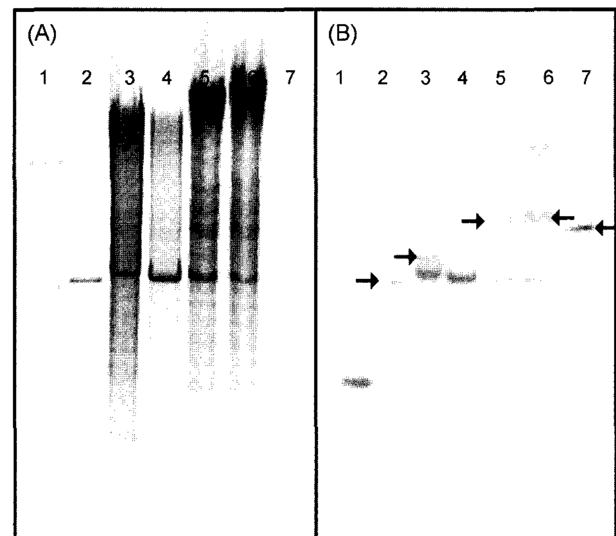

Fig. 1. Southern hybridization of three strains (CTY4, CTY5, OS11).

DNA from each strain was digested with *Eco*RI. The genomic DNA mixed with pACYC184 was digested with plasmid to check the complete digestion. (A) The DNA fragments were separated on 0.7% agarose gels. (B) *Eco*RI-digested genomic DNA was transferred to nylon membranes and hybridized with the *lacZ* probe plasmid (pRZ5202). Lane 1, 1 kb DNA ladder; lane 2, plasmid pACYC184 (100 ng) digested with *Eco*RI; lane 3, CTY4 (10 μg) digested with *Eco*RI; lane 4, RZ4500 (10 μg) digested with *Eco*RI; lane 5, CTY5 (5 μg) digested with *Eco*RI; lane 6, OS11 (5 μg) digested with *Eco*RI; lane 7, pRZ5202 (10 ng) digested with *Eco*RI. The bands of the DNA fragment 8, 7.6, 7.3, 5, and 4.2 kb long (top to bottom) are indicated by arrows.

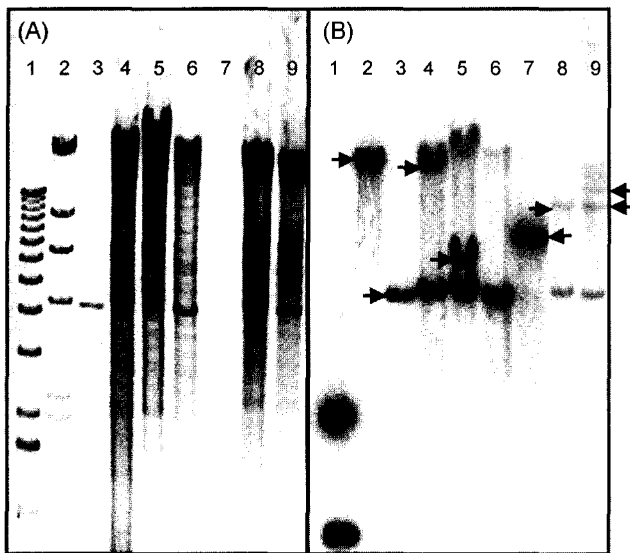

Fig. 2. Southern hybridization of four strains (OS1, OS3, OS6, OS14).

DNA was analyzed in the same way as in Fig. 1. (A) 0.7% agarose gels. (B) Hybridization analysis. Lane 1, 1 kb DNA ladder; lane 2, lamda-*Hin*dIII ladder; lane 3, plasmid pACYC184 (50 ng) digested with *Eco*RI; lane 4, OS1 (5 μg) digested with *Eco*RI; lane 5, OS3 (5 μg) digested with *Eco*RI; lane 6, RZ4500 (5 μg) digested with *Eco*RI; lane 7, pRZ5202 (10 ng) digested with *Eco*RI; lane 8, OS6 (5 μg) digested with *Eco*RI; lane 9, OS14 (5 μg) digested with *Eco*RI. The bands of the DNA fragment 23.1, 18.3, 12.5, 10.5, 10.1, 7.3, 5.6, and 4.2 kb long (top to bottom) are indicated by arrows.

can be easily mobilized in case of a defect of one pathway or another. In the present study the mutants with stable phenotype were used to avoid such ambiguity.

Detection of Chromosomal Sequence-*lacZ* Fusion *Eco*RI Fragments

The cloning of the fragment that contains the chromosomal sequence-*lacZ* fusion made it possible to identify the gene that was fused with the *lacZ*. *Eco*RI-digested chromosomal DNA was hybridized with a probe made from *lacZ* containing pRZ5202 by the Southern blot (Figs. 1 and 2). From the Southern blot, the size of the *Eco*RI fragments containing the fused genes of each strain were found to be approximately 5 kb (CTY4), 7.6 kb (CTY5), 7.3 and 8 kb (OS11), 18.3 kb (OS1), 5.6 kb (OS3), 10.1 kb (OS6), and 10.5 and 12.5 kb (OS14) long. The *lacZ* and the other sequences that originated from the λp*lac*Mu53 which was contained in each chromosomal *Eco*RI fragment was 4.3 kb long. From this result it was estimated that each band contained approximately 0.7 kb (CTY4), 3.3 kb (CTY5), 3 and 4.7 kb (OS11), 14 kb (OS1), 1.3 kb (OS3), 5.8 kb (OS6), and 6.2 and 8.2 kb (OS14) long chromosomal DNA sequences of each fused gene, respectively. Each of the chromosomal *Eco*RI fragments was cloned in pMLB524.

Sequencing and Computer Analysis

The recombinant plasmids were named pCT101, pCT301, pCT401, pCT501, pCT601, pCT1101, pCT1102, pCT1401, pCT1402, and pCT1405. We obtained chromosomal DNA sequences longer than 700 bp for each fused gene in the plasmids, which is long enough to definitely identify the gene by sequencing the DNA. The sequencing analysis showed the presence of the intact Mu*S*'-'*trpAB*'-'*lacZ*' sequence originated from λp*lac*Mu53 and the fusion point chromosomal sequence in the cloned *Eco*RI fragments. This indicated that the fused DNA sequence was from the upstream regions of the fusion point, thus eliminating the possibility of the downstream sequence being fused to the *lacZ* by any rearrangement or recombination.

In the case of CTY4 (Fig. 3a), the sequence data showed that the chromosomal DNA was the *nrdA* gene encoding a B1 subunit of ribonucleoside diphosphate reductase. In CTY5 (Fig. 3a) and OS11 (Fig. 3a, 3b), the λp*lac*Mu53 fusion point was the *nrdB* gene encoding a B2 subunit of ribonucleoside diphosphate reductase. The ribonucleoside diphosphate reductase that reduces ribonucleoside for the synthesis of DNA can be an enzyme absolutely required for the reduction of the ribonucleoside under a high level of oxygen stress. The study of the enzyme would help to elucidate an important mechanism underlying the reduction of the ribonucleoside. The sequences analysis (data not shown) showed a SoxS-binding consensus sequence immediately upstream of the -35 element with a few mismatches. This indicates that the *nrdAB* can be a new member of the *soxRS* regulon. A previous study by Li and Demple [24] searching the genomic database with the SoxS-binding consensus sequence reported that the *nrdAB* could be a member of the the *soxRS* regulon, and the present study provides experimental support for the computer database search with the fact that the *nrdAB* is required for the aerobic growth of the cell. OS11 had two genes mutated at min 33.5 (*f308d*) and min 50.5 (*nrdAB*). The gene at min 33.5 is a new gene that has not yet been identified, and it is included in the Kohara library #278 clone [19] and suggested to be an ATP-binding component of a transport system. This gene is not responsible for oxygen sensitivity.

In the case of OS1 (Fig. 3c), the sequence data showed that the chromosomal DNA was the *suc*A gene encoding α-ketoglutarate dehydrogenase E1 component in the TCA cycle. The requirement of *sucA* for aerobic growth is easily comprehensible since it is a component of the TCA cycle that operates mainly under aerobic growth conditions.

In the case of OS3 (Fig. 3d), the sequence data showed that the chromosomal DNA was the *cydB* gene encoding cytochrome *d* oxidase. Expression of the *cydAB* operon is microaerobically controlled by the two ArcA/ArcB component

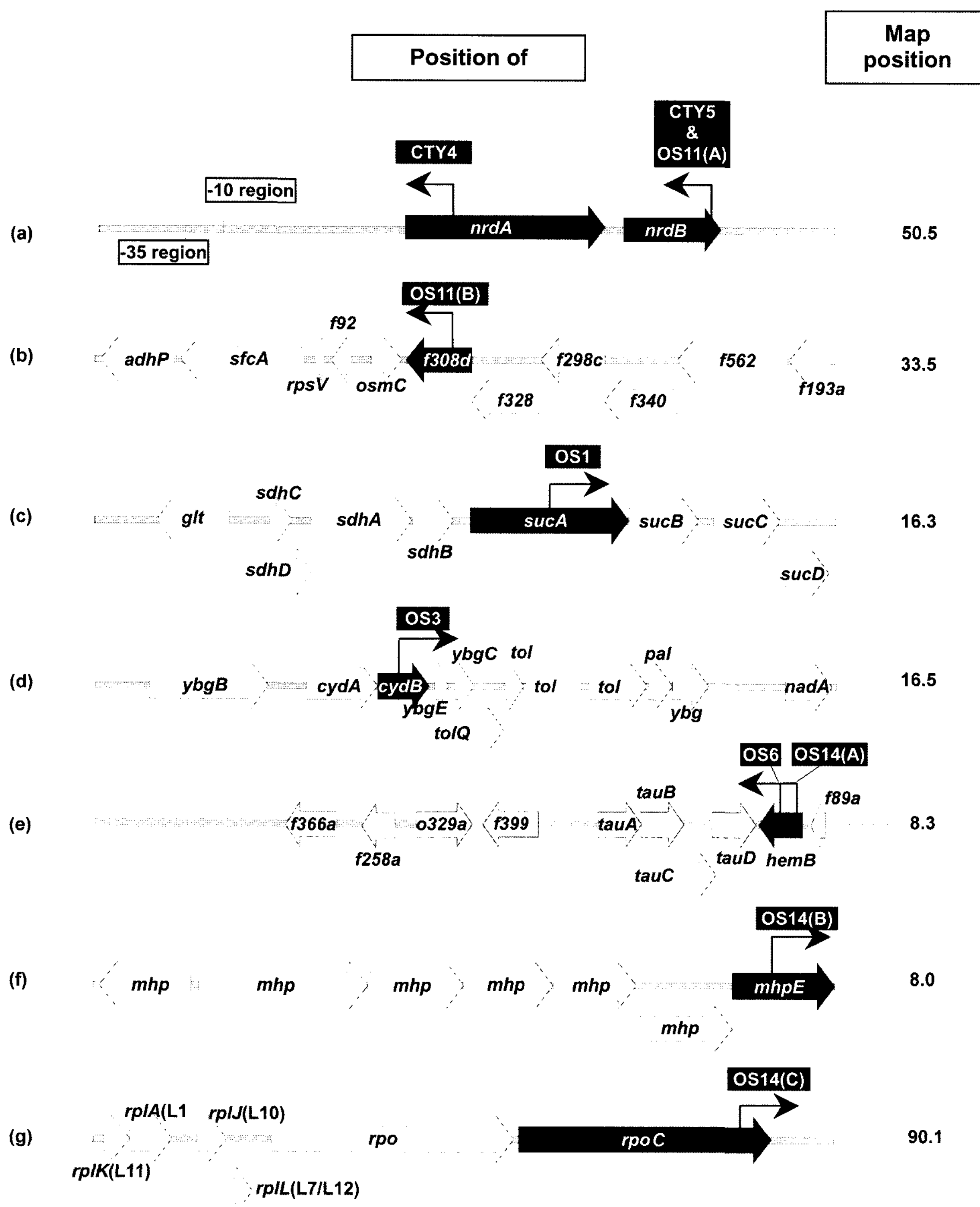

Fig. 3. Location of insertion points of transcriptional fusions identified in this study.
(a) GenBank File, K02672 (8,554 bp). (b) GenBank File, AE000245 (10,437 bp). (c) GenBank File, J01619 (13,063 bp). (d) GenBank File, D90713 (16,419 bp). (e) GenBank File, D85613 (16,048 bp). (f) GenBank File, D86239 (7,294 bp). (g) GenBank File, V00339 (12,337 bp). Arrows indicate the direction of transcription of genes. The solid arrows represent genes identified in this study. The drawings are not to exact scale.

regulatory systems and the pleiotropic regulatory gene Fnr [12, 37].

In OS6 (Fig. 3e) and OS14 (Figs. 3e, 3f, 3g), the λp*lac*Mu53 fusion point was the *hemB* gene encoding 5-aminolevulinate dehydratase or porphobilinogen synthase. It is easily understandable that the *hemB* gene is responsible for oxygen sensitivity. However, it was a surprise to find that *E. coli* contained only the pathway, and that there was no other alternative pathway for the production of the heme component that is very important for the cell

Table 2. The mutant strains, the fused genes, the plamids clones, the locations of the fused genes on linkage map, the functions of the fused genes, and the requirement of the fused gene for the aerobic growth of the *E. coli* cell.

Strain	Fused gene	Plasmid clone	Location on linkage map	The function of the gene	Required for aerobic growth
CTY4	nrdA	pCT401	min 50.5	B1 subunit of ribonucleoside diphosphate reductase	yes
CTY5	nrdB	pCT501	min 50.5	B2 subunit of ribonucleoside diphosphate reductase	yes
OS1	sucA	pCT101	min 16.3	The E1 component of α-ketoglutarate dehydrogenase	yes
OS3	cydB	pCT301	min 16.6	Cytochrome *d* oxidase	yes
OS6	hemB	pCT601	min 8.3	5-Aminolevulinate dehydratase or porphobilinogen synthase	yes
OS11	nrdB	pCT1101	min 50.5	B2 subunit of ribonucleoside diphosphate reductase	yes
OS11	f308d	pCT1102	min 33.5	Suggested to be an ATP-binding component of a transport system	no
OS14	hemB	pCT1401	min 8.3	5-Aminolevulinate dehydratase or porphobilinogen synthase	yes
OS14	rpoC	pCT1402	min 90.1	RNA polymerase beta'-subunit	no
OS14	mhpE	pCT1405	min 8.0	4-Hydroxy-2-ketovalerate aldolase	no

physiology. The regulation of the *hemB* gene for aerobic growth has not yet been studied. This study will serve as the starting point for the study of *hemB* gene oxygen sensitivity.

It was of interest to observe that OS14 had three genes mutated at min 8.3, min 8.0, and min 90.1. It is known that the genes located at min 8.0 and min 90.1 are *mhpE* (encodes 4-hydroxy-2-ketovalerate aldolase) [11] and *rpoC* (RNA polymerase beta'-subunit), respectively, and that these two genes are not responsible for oxygen sensitivity.

The New Gene f308d of the OS11 Is Not Necessary for Aerobic Growth

OS11 had two genes mutated at min 33.5 (unknown gene *f308d*) and min 50.5 (*nrdB*). We separated *f308d* and *nrdB* fusion in OS11 by transducing RZ4500 with the P1 lysate prepared from OS11 and by selecting the kanamycin resistant colonies. Several candidate colonies were tested by P1 transduction using a P1*vir* lysate prepared from CAG12027 (MG1655 *zdd-230::Tn9*) and CAG18459 (MG1655 *zde-234::Tn10*) [5, 36]. The cell that has only *f308d* fusion showed both white and blue colonies after P1 transduction, since the P1 transduction could replace the *f308d*::λp*lac*Mu53 fusion on the chromosomal sequence of the transduced RZ4500. The cell that gave the white transductants was named CT11U. CT11U was tested for growth with oxygen by aerobic and anaerobic plating, and it was found that it grew very well under aerobic conditions. The *f308d* gene is not responsible for oxygen sensitivity. Stable insertion of the λp*lac*Mu53 made it easy to separate multiple insertions of the λp*lac*Mu53, and to identify the mutation that was responsible for oxygen sensitivity.

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REFERENCES

1. Adler, H., R. Mural, and B. Suttle. 1992. Oxygen sensitivity of an *Escherichia coli* mutant. *J. Bacteriol.* **174:** 2072-2077.
2. Ahn, J. B., H.-J. Hwang, and J.-H. Park. 2001. Physiological responses of oxygen-tolerant anaerobic *Bifidobacterium longum* under oxygen. *J. Microbiol. Biotechnol.* **11:** 443-451.
3. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25:** 3389-3402.
4. Amabile-Cuevas, C. F. and B. Demple. 1991. Molecular characterization of the soxRS genes of *Escherichia coli*: Two genes control a superoxide stress regulon. *Nucleic Acids Res.* **19:** 4479-4484.
5. Bachmann, J. B. 1987. Derivations and genotypes of some mutant derivatives of *E. coli* K-12, pp. 1201. *In* F. C. Neidhardt, (ed.). *Escherichia coli and Salmonella typhimurium.* American Society for Microbiology: Washington, DC, U.S.A.
6. Bremer, E., T. J. Silhavy, and G. M. Weinstock. 1985. Transposable lambda placMu bacteriophages for creating lacZ operon fusions and kanamycin resistance insertions in *Escherichia coli. J Bacteriol.* **162:** 1092-1099.
7. Chao, G., J. Shen, C. P. Tseng, S. J. Park, and R. P. Gunsalus. 1997. Aerobic regulation of isocitrate dehydrogenase gene (icd) expression in *Escherichia coli* by the arcA and fnr gene products. *J. Bacteriol.* **179:** 4299-4304.
8. Choe, M. H. and W. S. Reznikoff. 1991. Anaerobically expressed *Escherichia coli* genes identified by operon fusion techniques. *J. Bacteriol.* **173:** 6139-6146.
9. Chun, G.-T. and S. N. Agathos. 2001. Application of oxygen uptake rate measured by a dynamic method for analysis of related fermentation parameters in cyclosporin a fermentation: suspended and immobilized cell cultures. *J. Microbiol. Biotechnol.* **11:** 1055-1060.
10. Farr, S. B. and T. Kogoma. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium. Microbiol. Rev.* **55:** 561-585.

11. Ferrandez, A., J. L. Garcia, and E. Diaz. 1997. Genetic characterization and expression in heterologous hosts of the 3-(3-hydroxyphenyl)propionate catabolic pathway of *Escherichia coli* K-12. *J. Bacteriol.* **179:** 2573-2581.
12. Fu, H. A., S. Iuchi, and E. C. Lin. 1991. The requirement of ArcA and Fnr for peak expression of the cyd operon in *Escherichia coli* under microaerobic conditions. *Mol. Gen. Genet.* **226:** 209-213.
13. Iuchi, S., D. C. Cameron, and E. C. Lin. 1989. A second global regulator gene (arcB) mediating repression of enzymes in aerobic pathways of *Escherichia coli. J. Bacteriol.* **171:** 868-873.
14. Iuchi, S., D. Furlong, and E. C. Lin. 1989. Differentiation of arcA, arcB, and cpxA mutant phenotypes of *Escherichia coli* by sex pilus formation and enzyme regulation. *J. Bacteriol.* **171:** 2889-2893.
15. Iuchi, S. and E. C. Lin. 1992. Purification and phosphorylation of the Arc regulatory components of *Escherichia coli. J. Bacteriol.* **174:** 5617-5623.
16. Iuchi, S. and L. Weiner. 1996. Cellular and molecular physiology of *Escherichia coli* in the adaptation to aerobic environments. *J. Biochem. (Tokyo).* **120:** 1055-1063.
17. Jamison, C. S. and H. I. Adler. 1987. Mutations in *Escherichia coli* that effect sensitivity to oxygen. *J. Bacteriol.* **169:** 5087-5094.
18. Koh, Y. S. and J. H. Roe. 1995. Isolation of a novel paraquat-inducible (pqi) gene regulated by the soxRS locus in *Escherichia coli. J. Bacteriol.* **177:** 2673-2678.
19. Kohara, Y., K. Akiyama, and K. Isono. 1987. The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50:** 495-508.
20. Lee, J.-H., J.-H. Kim, M.-R. Kim, S.-M. Lim, S.-W. Nam, J.-W. Lee, and S.-K. Kim. 2002. Effect of dissolved oxygen concentration and pH on the mass production of high molecular weight pullulan by *Aureobasidium pullulans. J. Microbiol. Biotechnol.* **12:** 1-7.
21. Lee, S.-J., S.-C. Lee, S.-H. Choi, M.-K. Chung, H.-G. Rhie, and H.-S. Lee. 2001. Effect of ArsA, arsenite-specific atpase, on inhibition of cell division in *Escherichia coli. J. Microbiol. Biotechnol.* **11:** 825-830.
22. Lee, Y. C., H. B. Kwon, S.-H. Lee, H. W. Kwon, H.-C. Sung, J. Kim, and M. H. Choe. 2001. Identification of a gene for aerobic growth with a SoxS binding sequence in *Escherichia coli* by operon fusion techniques. *J. Microbiol. Biotechnol.* **11:** 1115-1119.
23. Li, Z. and B. Demple. 1994. SoxS, an activator of superoxide stress genes in *Escherichia coli*. Purification and interaction with DNA. *J. Biol. Chem.* **269:** 18371-18377.
24. Li, Z. and B. Demple. 1996. Sequence specificity for DNA binding by *Escherichia coli* SoxS and Rob proteins. *Mol. Microbiol.* **20:** 937-945.
25. Lynch, A. S. and E. C. Lin. 1996. Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: Characterization of DNA binding at target promoters. *J. Bacteriol.* **178:** 6238-6249.
26. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y., U.S.A.
27. Miller, J. H. 1992. *A Short Course in Bacterial Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, U.S.A.
28. Mito, S., Q. M. Zhang, and S. Yonei. 1993. Isolation and characterization of *Escherichia coli* strains containing new gene fusions (soi::lacZ) inducible by superoxide radicals. *J. Bacteriol.* **175:** 2645-2651.
29. Mukhopadhyay, S. and H. E. Schellhorn. 1997. Identification and characterization of hydrogen peroxide-sensitive mutants of *Escherichia coli*: Genes that require OxyR for expression. *J. Bacteriol.* **179:** 330-338.
30. Munson, L. M., W. Mandecki, M. H. Caruthers, and W. S. Reznikoff. 1984. Oligonucleotide mutagenesis of the lacPUV5 promoter. *Nucleic Acids Res.* **12:** 4011-4017.
31. Nunoshiba, T., E. Hidalgo, C. F. Amabile Cuevas, and B. Demple. 1992. Two-stage control of an oxidative stress regulon: The *Escherichia coli* SoxR protein triggers redox-inducible expression of the soxS regulatory gene. *J. Bacteriol.* **174:** 6054-6060.
32. Park, S.-H., D.-H. Lee, K.-H. Oh, K. Lee, and C.-K. Kim. 2002. Detection of aromatic pollutants by bacterial biosensors bearing gene fusions constructed with the *dnaK* promoter of *Pseudomonas* sp. DJ-12. *J. Microbiol. Biotechnol.* **12:** 417-422.
33. Shin, J.-H., D.-H. Roh, G.-Y. Heo, G.-J. Joo, and I.-K. Rhee. 2001. Purification and characterization of a regulatory protein XylR in the D-xylose operon from *Escherichia coli. J. Microbiol. Biotechnol.* **11:** 1002-1010.
34. Silhavy, T. J. and J. R. Beckwith. 1985. Uses of lac fusions for the study of biological problems. *Microbiol. Rev.* **49:** 398-418.
35. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. *Experiments with Gene Fusions.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., U.S.A.
36. Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross. 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli. Microbiol. Rev.* **53:** 1-24.
37. Tseng, C. P., J. Albrecht, and R. P. Gunsalus. 1996. Effect of microaerophilic cell growth conditions on expression of the aerobic (cyoABCDE and cydAB) and anaerobic (narGHJI, frdABCD, and dmsABC) respiratory pathway genes in *Escherichia coli. J. Bacteriol.* **178:** 1094-1098.
38. Wu, J. and B. Weiss. 1992. Two-stage induction of the soxRS (superoxide response) regulon of *Escherichia coli. J. Bacteriol.* **174:** 3915-3920.
39. Yu, X. M., L. M. Munson, and W. S. Reznikoff. 1984. Molecular cloning and sequence analysis of trp-lac fusion deletions. *J. Mol. Biol.* **172:** 355-362.
40. Yu, X. M. and W. S. Reznikoff. 1984. Deletion analysis of the CAP-cAMP binding site of the *Escherichia coli* lactose promoter. *Nucleic Acids Res.* **12:** 5449-5464.