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Identification of a Gene for Aerobic Growth with a SoxS Binding Sequence in *Escherichia coli* by Operon Fusion Techniques

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Abstract Eight *Escherichia coli* cells with aerobic growth defects were isolated by the insertion of λ*plac*Mu53, a hybrid bacteriophage of λ and Mu, which created transcriptional fusion to *lacZY*. Two of these mutants, CL10 and CL12, were irradiated with UV to obtain specialized transducing phages. The phages that took out the neighboring chromosomal DNA of the related gene responsible for defective aerobic growth were identified. The *in vivo* cloned chromosomal sequence revealed that the mutated gene of CL10 was located at min 34.5 on the *Escherichia coli* linkage map and 1,599,515 on the physical map. The physical map indicated that there were 7 cistrons in the operon. We named this operon *oxg10*. The promoter sequence of *oxg10* exhibited a possible binding site for SoxS, a transcriptional regulator that activates the transcription of various SoxRS regulon genes. Transferring the *oxg10*::λ*plac*Mu53 mutation into the wild-type strain, RZ4500, resulted in the inhibition of normal aerobic growth, while the same mutation in strain MO inhibited aerobic cell growth completely. The full operon sequences of *oxg10* were cloned from the *Escherichia coli* genomic library. The mutated gene of CL12 was identified to be a *sucA* gene encoding the α-ketoglutarate dehydrogenase E1 component in the TCA cycle.

Key words: Oxygen sensitivity, λ*plac*Mu53, *in vivo* cloning, SoxS binding site

Under aerobic conditions, living things are continuously exposed to reactive oxygen species produced as an inescapable byproduct of normal aerobic metabolism. These reactive oxygen species, for example, superoxide radical anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxy radicals (·OH), result from the imperfect reduction of oxygen molecules used in respiration [8, 10, 11, 14, 16]. However, these byproducts of aerobic respiration damage many biological molecules, including DNA, proteins, and lipids [14]. In order to survive these dangerous species, aerobes have a variety of defense mechanisms and repair mechanisms to protect themselves from oxidative damages. These mechanisms consist of several enzymes, therefore, oxygen toxicity, such as a hyperoxygenation condition, only appears when the degree of oxidative stress exceeds the capacity of the cell defense systems [10]. *Escherichia coli* is a facultative bacterium, which can grow aerobically or anaerobically, and has defense mechanisms against oxidative damage by the reactive oxygen species [3]. The expression of the genes responsible for defense against oxidative stress is regulated by specific regulatory system for the regulon the genes belong to. Recent studies have classified the regulon according to their regulatory protein, SoxRS, OxyR, and ArcAB [12-14].

Until now, many genetic loci responsible for defense against oxidative stress have been identified in *E. coli* [9, 13, 14, 17, 21], and many mutants that cannot grow aerobically have been isolated [15, 22].

The current study created gene-fusion mutants using the operon fusion technique [1, 6] with λ*plac*Mu53 to obtain mutants without any additional oxidative chemicals so as to find the genes needed for normal aerobic metabolism and their phenotypes. Those cells showing the phenotype of a growth defect under aerobic conditions, yet capable of normal growth in anaerobic conditions, were selected. The effects of the mutations were also tested in the wild type strain RZ4500. The sequence data from the chromosomal DNA fragments that were cloned *in vivo* as specialized transducing phages were analyzed.

All bacterial strains used in the experiments were derivatives of *E. coli* K-12, and most of the bacterial strains, phages, and plasmids are described elsewhere [7]. The mutant fusion strains were constructed by infecting

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the *E. coli* MO strain with λp*lac*Mu53 and the helper phage λpMu507 [5]. The infections was performed using the procedure of Bremer *et al.* [5]. The infected cells were spread on an LB plate containing kanamycin (40 μg/ml) and XG (20 μg/ml), then the blue colonies were picked under anaerobic conditions. The cells were tested for growth by incubating for 24 h under anaerobic conditions (An) and for 48h under aerobic conditions (Ox). The mutants that did not form colonies under aerobic conditions, yet formed blue colonies under anaerobic conditions, were selected and purified on an LB-XG plate. For the anaerobic incubation, the plates were placed in a jar with an anaerobic atmosphere made by an H_2 and CO_2 producing pouch pack (BBL GasPak anaerobic System; Becton Dickinson & Co).

λp*lac*Mu53 inserted into the *E. coli* chromosome randomly without any sequence preference. Thirteen aerobic growth defective mutants were selected and tested for a stable phenotype. High frequency reversion was observed in five strains, and one strain grew poorly even under anaerobic conditions. Seven strains showed a stable phenotype and two of these were used in the current study.

Only the aberrant processes induced by UV could excise the inserted λp*lac*Mu53 prophage, which also took out the flanking chromosomal DNA. The inductions of the λp*lac*Mu53 fusion phage were performed according to a previously described procedure [23]. The anaerobic liquid culture (5 ml) for phage induction was made by degassing the inoculated media with an aspirator (20 mmHg), and purging 5 times with 99.99% nitrogen. After 24 h of incubation, the culture was resuspended in 2.5 ml of 10 mM $MgSO_4$. A 0.5 ml suspension was then spread on a petri dish and irradiated for 10 sec 10 cm below a 20 Watt UV lamp without a cover. A 4.5 ml LB medium was added to the irradiated cells, and incubated for 5 h at 37°C under anaerobic conditions. The lysates were plated on an XG-containing agar, and the well-separated blue turbid plaques were then selected and used to analyze the restriction band pattern of the DNA.

The lysates obtained had an average titer of 3.1×10^9 pfu/ml. The ratio of blue to white plaque was about 1/20. The phage plaques showed various phenotypes with respect to color, turbidity, and size. The variation in the phenotypes of the induced phages reflected the fact that the aberrant excision took out various extent of the adjoining chromosomal regions for each excision event and, in some cases, resulted in the loss of the replication control parts of the phage sequences making the plaques clear. To select the phages that took out the chromosomal DNA sequence in the upstream region of the *lacZ* (λp*lac*Mu53), the presence of the *Eco*RI-*Hin*dIII 3.9 kb band was checked and compared with the bands from λp*lac*Mu53. This band comprises the DNA region between the *Eco*RI site of the *exo* gene and the *Hin*dIII site of the Ac end of λp*lac*Mu53 [23]. If the *Hin*dIII site in the Ac end sequence were removed, the 3.9 kb band would be disappeared, and the chromosomal sequences would only be from the upstream [7]. The presence of chromosomal DNA was confirmed by selecting those phages that contained larger DNA fragment than the fragment of maximum size without the chromosomal DNA. Four phages from CL10 and one from CL12 were selected, and each of them contained different amount of chromosomal DNA, as determined by the size of the restriction fragments.

The chromosomal DNA from the phage was subcloned into a plasmid vector in order to do further analysis of the genes. The *Eco*RI fragment containing the chromosomal DNA was ligated with a *Eco*RI cut pMLB524 plasmid vector by T4 DNA ligase [7].

The recombinant plasmids were named pCK1032, 1039, 1060, 1070, and pCL1205, and the DNA sequences of the inserts were determined. To determine the nucleotide sequence of the cloned chromosomal DNA fragments, a 32-mer primer complementary to the sequence of the plasmids was prepared. The primer hybridized with the '*trp*' region, 5 nucleotides upstream from the β-end of the Mu sequence. The sequencing was carried out using an ALFexpress sequencer with a Cy5™ AutoRead™ sequencing kit (Pharmacia Biotech). Over 600 bp DNA sequence data was obtained for each plasmid (Fig. 2). The chromosomal DNA sequences obtained from pCK1032, 1039, 1060 and 1070 were the same. This indicated that the fragments of each plasmid were from the same insertion mutation and had no rearrangement or recombination during the aberrant excision of the phage.

DNA sequence similarity and amino acid sequence similarity searches were performed using the database search programs, BLASTN 2.0.4 and BLASTP 2.0.4 [2]. In the case of CL12, the sequence data showed that the chromosomal DNA was a *sucA* gene encoding α-ketoglutarate dehydrogenase E1 component in the TCA cycle, and it was no surprise that a TCA cycle enzyme gene was found in this study [15, 22]. For the CL10 mutant, the λp*lac*Mu53 fusion point was found at the end of the open reading frame o511 determined by Blattner *et al.* [4]. The mutated gene of CL10 was named *oxg10*. It was also found in Kohara library clone 5F9 as an open reading frame [18], and located at min 34.5 on the linkage map and 1,599,515 bp on the physical map [4]. The *oxg10* has never been reported before to be involved in aerobic growth of the *E. coli*.

The reported *E. coli* whole genome sequence data [4] revealed that there were seven cistrons in *oxg10* (Fig. 1) and also a promoter sequence (Fig. 2) responsible for β-galactosidase expression from the fused λp*lac*Mu53. An interesting finding in the promoter sequence is that the *oxg10* has a SoxS binding consensus sequence. The *soxRS* regulatory system controls the expression of genes that

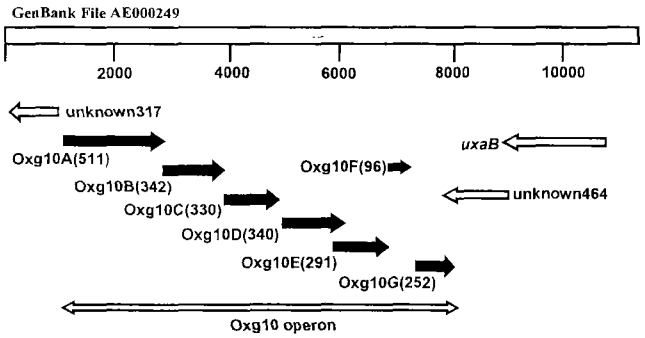

Fig. 1. Physical map of *oxg10* operon and neighboring region.
Seven ORFs at 34.5 min are indicated by the black colored arrows. Oxg10A(511) has the insertion of λ*plac*Mu53. The *uxaB* gene is shown downstream of the operon.

protect the cell from various types of damage, and these genes include *sodA*, endonuclease IV, glucose-6-phosphate dehydrogenase, fumarase C, and NADH:ferredoxin oxidoreductase [14]. In addition, several other genes that could be regulated by the *soxRS* control system were found in the *E. coli* genomic sequence database by searching with the SoxS-binding consensus sequence, AN2GCAYN7CWAC (where N is any base, Y is a pyrimidine, and W is A or T) [19]. The promoter sequences for *oxg10* exhibited the SoxS-binding sequence at two base pairs upstream of the −35 element with three mismatches out of a 18 bp sequence (including nine nonspecific bases) (Fig. 2).

The mutagenesis was performed with the MO strain, which was a very old laboratory strain that had come through more than twenty genetic manipulation steps. It was checked whether there were any differences in the effect of the *oxg10* mutation depending on the *E. coli* strain. The insertion mutation *oxg10*::λ*plac*Mu53 was transferred from the CL10 mutant into the wild-type strain, RZ4500, by P1 transduction [7]. The transductants were selected on an R medium [20] with XG (40 μg/ml) and kanamycin (80 μg/ml). Four blue colonies, CW1, 3, 4, and 5, that showed good phenotypic stability throughout multiple generations, were selected. The growth properties of these strains were compared under aerobic (Ox) and anaerobic (An) conditions. The cells were initially cultured anaerobically in LB liquid medium and the same amounts of cells were plated. Aerobic cultures were carried out on LB plate medium with XG (40 μg/ml) and kanamycin (80 μg/ml), and observed after 12 h and 23 h of incubation. Anaerobic cultures were carried out on the same medium for 23 h in an anaerobic jar (BBL GasPak anaerobic System; Becton Dickinson & Co). The growth of these four mutants, CW1, 3, 4, and 5, were compared with the parent strain RZ4500 (Table 1).

The cell numbers of the mutants and RZ4500 were similar under both aerobic and anaerobic conditions. However, the *oxg10* mutants, CW1, 3, 4, and 5, grew very slowly and made much smaller colonies under aerobic conditions. The colony size of RZ4500 and the mutants was the same under anaerobic conditions. Therefore, *oxg10* mutation caused

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CCGCGCGACC TGTTCTTCTT CACACATTCC CTGTTCTGAA ATTGCCGAAT CGTTGATTGT CATAATTCAT TCTTCACTTT GAACATATTT AAATCTTTAA TGCAATTGTT

CAGTTCTTGC TCATTTATAT CTGTGATGGC AACCACAGTT TGACTCTACG AGCATGAACA AACGCAACCG TGAAAATCAA AATAGCATAA ATTGTGATCT ATTCGTCGGA
           <- Unknown protein f497 start point
AATATGTGCA ATGTCCACCT AAGGTTATGA ACAAATTAAA AGCAGAAATA CATTTGTTCA AAACTCACCT GCAAAACTGA ACGGGGGAAA TATGCAAACG AGTGATACCC
   soxS binding site      -35                    -10                          oxg10 start point
GCGCGTTACC GCTACTTTGC GCCCGCTCGG TTTATAAACA GTATTCAGGG GTCAATGTCC TGAAAGGCAT CGATTTTACG TTGCATCAGG GGGAGGTCCA CGCCCTGCTC

GGCGGCAATG GTGCCGGTAA ATCGACGTTA ATGAAGATTA TTGCCGGTAT TACCCCTGCT GATAGCGGTA CGCTGGAGAT TGAGGGCAAC AACTACGTCA GATTAACGCC

AGTTCATGCT CATCAGCTGG GTATTTATCT CGTTCCCCAG GAACCGCTGC TTTTCCCAAG CCTGTCGATA AAAGAAAACA TCCTGTTTGG GCTGGCAAAA AAACAGCTCT

CCATGCAGAA AATGAAGAAC TTGCTGGCGG CGCTGGGCTG CCAGTTTGAT CTGCATAGTC TGGCAGGATC GCTGGATGTC GCCGATCGCC AAATGGTGGA AATCCTCCGC

GGGCTGATGC GCGACTCGCG GATTCTGATC CTCGATGAAC CTACCGCCTC GCTTACCCCT GCGGAAACCG AACGCTTGTT TAGTCGCTTG CAAGAGCTGC TTGCTACTGG

CGTGGGTATT GTTTTTATCT CGCATAAGCT GCCGGAAATT CGCCAGATTG CCGATCGAAT TAGCGTGATG CGCGACGGAA CCATCGCCTT AAGCGGCAAA ACCAGCGAAC

TGTCTACCGA CGACATTATT CAGGCCATCA CCCCAGCGGT ACGGGAAAAA TCGCTCTCTG CCAGCCAAAA ATTATGGCTG GAGTTACCTG GTAACCGCCC ACAACATGCC

GCCGGAACGC CGGTGCTGAC ACTGGAAAAT CTGACCGGCG AAGGTTTCAG GAATGTCAGC CTGACGCTCA ATGCCGGAGA AATTCTGGGC CTGGCTGGGC TGGTGGGGGC

CGGACGCACA GAACTGGCCG AGACGCTCTA TGGTCTGCGT ACTTTGCGTG GCGGACGCAT TATGCTGAAT GGTAAAGAGA TCAATAAATT ATCCACTGGA GAACGTTTAC

TGCGCGGTCT GGTTTATCTG CCGGAAGATC GCCAGTCATC CGGACTGAAT CTCGATGCTT CGCTGGCCTG GAACGTCTGC GCCCTTACTC ATAACCTTCG TGGATTCTGG

GCGAAAACCG CGAAAGATAA TGCCACCCTG GAACGTTATC GTCGGGCGCT GAATATTAAA TTCAACCAAC CGGAACAAGC TGCACGGACA TTATCCGGTG GCAACCAGCA
           ^ λplacMu53 fusion point
AAAAATCCTC ATTGCCAAAT
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Fig. 2. λ*plac*Mu53 fusion point and predicted −10 and −35 regions.
The −10 region and −35 region are underlined. The fusion point of λ*plac*Mu53 to the chromosomal DNA is indicated with the vertical arrow. The predicted start codon of the newly found Oxg10A protein and another upstream unknown protein are indicated by the horizontal arrows heading right and left, and the putative soxS binding site is double underlined.

Table 1. Comparison of aerobic and anaerobic growth of CW1, 3, 4, 5, and RZ4500.

Strain	Ox[a]				An[b]		Colony number ratio, Ox/An
	12 h		23 h		23 h		
	Number	Colony size	Number	Colony size	Number	Colony size	
RZ4500	15	Large	15	Very large	17	Medium	0.88
CW1	NDc	ND	33	Medium	42	Medium	0.79
CW3	ND	ND	15	Medium	29	Medium	0.52
CW4	ND	ND	28	Medium	37	Medium	0.76
CW5	ND	ND	58	Medium	76	Medium	0.76

[a]Aerobic conditions.
[b]Anaerobic conditions.
[c]ND: Not discernible. Because the colonies were very small, it was difficult to count the colony numbers.

cells to grow more slowly under aerobic conditions in the RZ4500 genetic background. Containing a SoxS-binding site, *oxg10* can be assigned to the SoxRS regulon and may be involved in the system for eliminating oxygen toxicity. It is also possible that *oxg10* is a part of the nutritional or electron transfer pathway for normal aerobic growth.

Analytical studies about the other mutant strains still remain to be undertaken.

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References

1. Aliabadi, Z., F. Warren, S. Mya, and J. W. Foster. 1986. Oxygen-regulated stimulons of *Salmonella typhimurium* identified by Mud(Ap*lac*) operon fusions. *J. Bacteriol.* **165:** 780–786.
2. 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.
3. Bae, W., T. Kang, J. Jung, C. Park, S. Choi, and B. Jeong. 2000. Purification and characterization of NADH-dependent Cr(VI) reductase from *Escherichia coli* ATCC33456. *J. Microbiol. Biotechnol.* **10:** 580–586.
4. Blattner, F. R., G. Plunkett, 3rd, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277:** 1453–1474.
5. Bremer, E., T. J. Silhavy, and G. M. Weinstock. 1988. Transposition of lambda *plac*Mu is mediated by the A protein altered at its carboxy-terminal end. *Gene* **71:** 177–86.
6. Choe, M. H. and W. S. Reznikoff. 1991. Anaerobically expressed *Escherichia coli* genes identified by operon fusion techniques. *J. Bacteriol.* **173:** 6139–6146.
7. Choe, M. H. and W. S. Reznikoff. 1993. Identification of the regulatory sequence of anaerobically expressed locus *aeg-46.5*. *J. Bacteriol.* **175:** 1165–1172.
8. Demple, B. 1991. Regulation of bacterial oxidative stress genes. *Annu. Rev. Genet.* **25:** 315–337.
9. Demple, B. 1996. Redox signaling and gene control in the *Escherichia coli soxRS* oxidative stress regulon - a review. *Gene.* (Review) **179:** 53–57.
10. Farr, S. B. and T. Kogoma. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* **55:** 561–585.
11. Fridovich, I. 1978. The biology of oxygen radicals. *Science* **201:** 875–880.
12. Greenberg, J. T., J. H. Chou, P. A. Monach, and B. Demple. 1991. Activation of oxidative stress genes by mutations at the *soxQ/cfxB/marA* locus of *Escherichia coli*. *J. Bacteriol.* **173:** 4433–4439.
13. Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple. 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **87:** 6181–6185.
14. 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.
15. Jamison, C. S. and H. I. Adler. 1987. Mutations in *Escherichia coli* that effect sensitivity to oxygen. *J. Bacteriol.* **169:** 5087–5094.
16. Kim, J. S. and J. K. Lee. 2000. Cloning, DNA sequence determination, and analysis of growth-associated expression of *sodF* gene coding for Fe- and Zn-containing superoxide dismutase of *Streptomyces griseus*. *J. Microbiol. Biotechnol.* **10:** 700–706.
17. Kogoma, T., S. B. Farr, K. M. Joyce, and D. O. Natvig. 1988. Isolation of gene fusions (*soi::lacZ*) inducible by oxidative stress in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **85:** 4799–4803.
18. 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.

19. Li, Z. and B. Demple. 1996. Sequence specificity for DNA binding by *Escherichia coli* SoxS and Rob proteins. *Mol. Microbiol.* **20:** 937–945.
20. Miller, J. H. 1992. *A Short Course in Bacterial Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Cold Spring Harbor, NY, U.S.A.
21. 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.
22. Schellhorn, H. E. and H. M. Hassan. 1988. Isolation and characterization of respiratory-deficient mutants of *Escherichia coli* K-12. *J. Bacteriol.* **170:** 78–83.
23. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. *Experiments with Gene Fusions.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.