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The Gene *yggE* Functions in Restoring Physiological Defects of *Escherichia coli* Cultivated under Oxidative Stress Conditions†

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DNA microarray analysis showed that *yfiD*, *yggB*, and *yggE* genes were up-regulated when superoxide dismutase (SOD)-deficient *Escherichia coli* IM303 (I4) was cultivated under the oxidative stress generated by photoexcited TiO_2, and pYFD, pYGB, and pYGE were constructed by inserting the respective genes into a pUC 19 vector. The content of reactive oxygen species (ROS) in IM303 (I4) cells carrying pYGE was reduced to 31% of ROS content in the control cells with pUC 19. In the culture of wild-type strain, *E. coli* MM294, in the medium with paraquat (10 μmol/l), maximum specific growth rate of the cells with pYGE was about five times higher than that of the control cells, with a decreased ROS content in the former cells. The introduction of pYGE also suppressed the occurrence of the cells with altered amino acid requirement in the culture of MM294 cells with paraquat.

Aerobic organisms have high reducing potential of molecular oxygen and preferentially utilize oxygen as a terminal electron acceptor in respiration for effectively generating biochemical energy for their proliferation (9). As a consequence of the aerobiosis, reactive oxygen species (ROS) such as superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) are often formed and can oxidize various biomaterials owing to their high reactivity, which causes DNA breakage, enzyme inactivation, membrane lipid peroxidation, and so on (3, 10, 20).

Several antioxidant systems exist in living cells to avoid cellular damage caused by oxidative stresses (17). Sox regulon is an example of an ROS-scavenging system, and it includes some gene products, for instance superoxide dismutase (SOD), that are induced under oxidative conditions via a SoxRS regulatory mechanism (4, 7, 15). SOD is found in almost all aerobic organisms and catalyzes the conversion of $\cdot O_2^-$ to H_2O_2. Thus, this enzyme is a key component in cellular defense against oxidative stress through reducing the intracellular concentration of $\cdot O_2^-$ to maintain cellular viability (1, 13). It was demonstrated that *Escherichia coli* mutants lacking in SOD displayed several defects in phenotypic features, such as auxotrophy for amino acids and high frequencies of spontaneous mutagenesis, when the cells were exposed to an aerobic condition (5, 16, 18).

In our previous work (12), it was reported that the population of SOD-deficient mutant of *E. coli* IM303 contained a trace of spontaneously derived variant cells which prevailed during the culture under the oxidative stress generated by photoexcited titanium dioxide (TiO_2). This result suggested that the variant cells possibly acquired a certain defense system against the oxidative stress besides the SOD mechanism. In the present study, DNA microarray analysis was performed to find out up-regulated genes in the SOD-deficient mutant of *E. coli* exposed to the oxidative stress produced by photoexcited TiO_2. The SOD-deficient and wild-type strains of *E. coli* were transformed with plasmids carrying the selected genes showing up-regulated expression under the oxidative condition. The transformed *E. coli* cells were cultivated under varied stress conditions to understand biological functions of the genes in terms of cell growth, ROS content, and amino acid requirement.

A mutant of *E. coli*, SOD-deficient strain IM303 (I4), and its wild-type strain MM294 were used throughout the experiments. Strain IM303 (I4) was one of isolates obtained from an original population of the mutant (12). A modified M9 medium containing 8 g/liter glucose and 5 mg/liter thiamine was used with supplementation of amino acid mixture (denoted as 2068 mixture, refer to the catalogue from American Type Culture Collection for an ATCC 2068 synthetic medium; 0.25 g Met, 0.25 g Arg, 0.30 g Tyr, 0.60 g Lys, 0.80 g Ile, 0.40 g Phe, 1.5 g Val, 0.50 g Asp, 2.0 g Thr, 0.80 g Glu, and 3.75 g Ser per liter). IM303 (I4) cells were grown at 37°C in an L-type test tube containing 10 ml of the medium with or without 10 mg/liter TiO_2 particles (Degussa P25; Nippon Aerosil Co., Tokyo, Japan) under a shaking condition of 45 strokes per min. When necessary, the tube was irradiated at an incident light intensity of I = 12.5 W/m^2 with 20-W black light fluorescent lamps

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(Type FL-20S BL-B; Matsushita Electric Industrial Co., Osaka, Japan). These stress conditions were confirmed to be sublethal for the growth of SOD-deficient *E. coli* (see the supplemental material). In the case of MM294 culture, the cells were grown in the test tube containing 10 ml of the modified M9 medium with 10 μmol/liter paraquat (PQ) under the same shaking condition in the absence of TiO_2 and light. Growth of the cells was monitored by measuring an optical density at 660 nm (OD_{660}). Maximum specific growth rate, μ_m, and lag time, t_L, were estimated by fitting the obtained growth-curve data to the modified Gompertz equation (21).

RNA extraction was conducted as follows, using IM303 (I4) cultures with and without the oxidative stress generated by light-irradiated TiO_2. Cell suspension (18 ml) was withdrawn from the cultures at $OD_{660} = 0.15$ and was quickly mixed with an equal volume of cold ethanol containing 10% phenol to prevent degradation of intracellular RNA. The cells were collected by centrifugation for 5 min at 4°C and 5,000 × *g* and then were washed with the cold phenol-ethanol solution. Total RNA was extracted from the whole cells by employing an RNeasy Mini Kit together with an RNase-free DNase Set (QIAGEN Inc., Valencia, CA) according to the supplier's instruction. Purified RNA was recovered by ethanol precipitation after phenol-chloroform treatment.

Labeled cDNAs were prepared using an RNA Fluorescence Labeling Core Kit (Takara Bio Inc., Otsu, Japan) with dUTP-Cy3 or dUTP-Cy5 fluorescent nucleotide (Amersham Biosciences Ltd., Buckinghamshire, United Kingdom) according to the manufacturer's instruction. Assay of DNA microarray was conducted with an IntelliGene *E. coli* CHIP (Takara Bio Inc.) that contains immobilized cDNAs of 3,437 genes, corresponding to about 80% of predicted open reading frames in *E. coli* K-12. Hybridization was conducted according to the instruction, and fluorescent intensity on the DNA microarray plate was measured by a GMS 418 Array Scanner (Genetic MicroSystems Inc., Woburn, MA), followed by image analysis using an ImaGene (BioDiscovery Inc., Los Angeles, CA). The microarray profiling data were compared between RNA samples from IM303 (I4) cultures with and without the oxidative stress generated by photoexcited TiO_2. For each gene set, the ratio of fluorescent intensities was recorded as a measure of difference in gene expression on the basis of those values from the cells cultivated in the absence of the oxidative stress. In the present study, the genes with the ratios of more than 3 and of less than 0.3 were respectively defined as up-regulated and down-regulated ones in the cells cultivated under the oxidative stress.

The constitutive regions of the selected genes were amplified by PCR using KOD-Dash DNA polymerase (Toyobo Co., Osaka, Japan) from the chromosomal DNA of MM294 cells. A pUC 19 vector was digested with appropriate restriction enzymes, ligated with the amplified DNA fragments with a Ligation-Convenience Kit (Nippon Gene Co., Tokyo, Japan), and then applied for transforming *E. coli* DH5α. The plasmid DNAs multiplied in the cells were extracted and purified by Wizard *Plus* SV Minipreps DNA Purification Systems (Promega Corp., Madison, WI).

IM303 (I4) and MM294 cells carrying pUC 19 vector or plasmid with a selected gene insertion were cultivated under indicated conditions, and an aliquot of culture broth (0.5 ml) was withdrawn from the test tube to determine the intracellular ROS content in a middle exponential growth phase ($OD_{660} = 0.5$). ROS content in the cells was quantified with 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescin diacetate (C-6827; Molecular Probes Inc., Eugene, OR) and was expressed on an H_2O_2 equivalent basis, as described elsewhere (12).

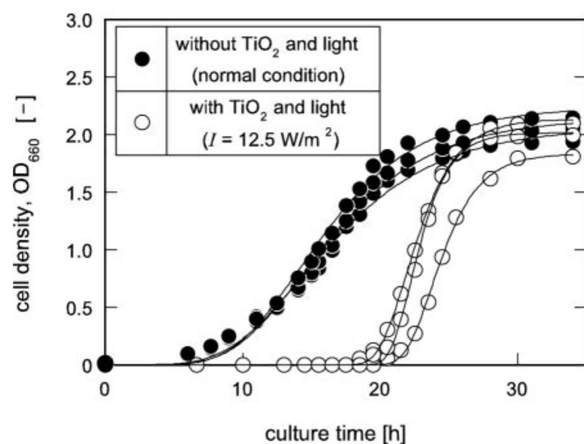

FIG. 1. Growth profiles of a typical isolate, IM303 (I4), from an SOD-deficient mutant of *E. coli*. The cells were grown in the modified M9 medium with 2068 amino acid mixture with and without TiO_2 and light. The solid lines were drawn by fitting the data to the modified Gompertz equation (21).

To examine the alternation in amino acid requirement, the transformed MM294 cells were grown up to $OD_{660} = 0.2$ in the L-tube containing the modified M9 medium with 10 μmol/liter PQ at 37°C. After washing the cells with physiological saline by centrifugation (at 4°C and 10,000 × *g* for 5 min), a series of diluted cell suspensions was plated on the modified M9 solid media containing 2068 amino acid mixture and the mixture of 20 kinds of amino acids (denoted as 1904 mixture, refer to the catalogue from American Type Culture Collection for ATCC 1904 synthetic medium). The plates were then incubated for 48 h at 37°C. The alteration degree of amino acid requirement, A_d, for the transformed MM294 cells was estimated by numerating developed colonies by the equation $A_d = (N_{a20} - N_{a11})/N_{a20}$, where N_{a11} and N_{a20} are the numbers of colonies on the modified M9 plates with 2068 mixture and with 1904 mixture, respectively.

Previous work described that the original population of SOD null mutant of *E. coli*, IM303, contained a trace of spontaneously derived variant cells, and these variant cells were permitted to survive and prevail during the culture with the sublethal oxidative stress generated from light-irradiated TiO_2 (12). As shown in Fig. 1, in the culture of a typically selected isolate, IM303 (I4), with TiO_2 and light (I = 12.5 W/m^2), maximum specific growth rate, μ_m, of the cells was found to be approximately two times higher than that of the cells cultivated in the absence of TiO_2 and light irradiation (normal culture condition), although a prolonged lag time of $t_L \approx 21$ h was calculated in the culture with TiO_2 receiving light.

We then searched the causative genes for physiological changes by means of comparing the difference of total gene expression levels in IM303 (I4) cells cultivated under the conditions with and without the oxidative stress from photoexcited TiO_2. DNA microarray analysis indicated 25 up-regulated and

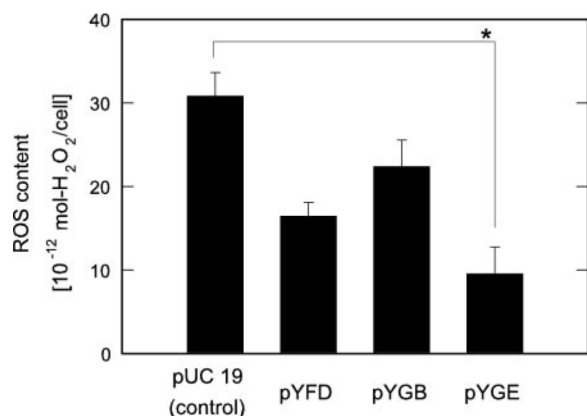

FIG. 2. ROS contents in an SOD-deficient mutant of *E. coli*, IM303 (I4), carrying pUC 19, pYFD, pYGB, and pYGE. The cells were grown in the modified M9 medium with 2068 amino acid mixture as well as 50 mg/liter ampicillin and 10 μmol/liter isopropyl-β-D-thiogalactopyranoside in the absence of TiO_2 and light. The asterisk indicates statistical significance ($P < 0.001$).

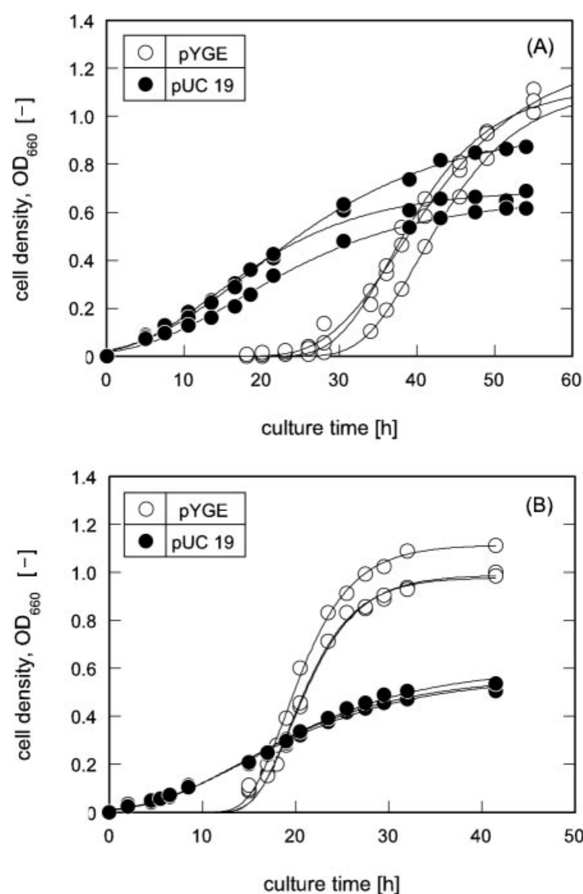

FIG. 3. Growth profiles of *E. coli* cells carrying pUC 19 and pYGE. (A) SOD-deficient mutant of *E. coli*, IM303 (I4). The cells were grown in the modified M9 medium with 2068 amino acid mixture as well as 50 mg/liter ampicillin and 10 μmol/liter isopropyl-β-D-thiogalactopyranoside (IPTG) in the absence of TiO_2 and light. (B) Wild-type *E. coli* MM294 cells. The cells were grown in the modified M9 medium with 2068 amino acid mixture as well as 10 μmol/liter PQ, 50 mg/liter ampicillin, and 10 μmol/liter IPTG. The solid lines were drawn by fitting the data to the modified Gompertz equation (21).

40 down-regulated genes under the oxidative stress (data not shown). Among the 25 up-regulated genes, *yfiD*, *yggB*, and *yggE* genes, which are classified to the genes encoding functionally unknown or hypothetical proteins in GenoBase (http://ecoli.aist-nara.ac.jp/GB5/search.jsp), were focused to be candidates exhibiting the potential to defend bacterial cells. Concerning *yfiD* gene, a few researchers reported that protein YfiD (14.3 kDa) was expressed mainly at low medium pH, and it was predicted that YfiD endows bacterial cells with tolerance to oxidative stresses (6, 19). The two genes *yggB* and *yggE*, which also showed comparatively high expression under the oxidative stress, are functionally still unclear, although Levina et al. (14) suggested that YggB protein, together with MscS, having a mechanosensitive channel activity, may be involved in the regulation of turgor pressure in bacterial cells. As *yggE* is closely located downstream of *yggB*, it is likely that these genes constitute a polycistronic transcription unit having protective functions against cellular stresses. In this context, it was expected from the DNA microarray analysis and several papers (6, 14, 19) that *yfiD*, *yggB*, and *yggE* gene products may function to protect bacterial cells or to recover cellular damage from the oxidative stress. These three genes were selected to be cloned into pUC 19 vector, and the resultant plasmids were named pYFD, pYGB, and pYGE, respectively.

SOD deficiency frequently induces the deteriorated growth of an *E. coli* mutant even in a rich medium with amino acid supplementation and the failure in restoring vitality due to damage to some biosynthetic pathways of amino acids in the cells (1, 5). Thus, it is most likely that ROS such as $\cdot O_2^-$ are accumulated in excess to damage the SOD-deficient cells when cultivated with oxygen supply under an aerobic condition. We investigated the effect of the selected genes on the ROS contents in IM303 (I4) cells cultured under a normal aerobic condition. As shown in Fig. 2, the ROS level in IM303 (I4) cells carrying pYGE appreciably dropped to 31% of the control cells carrying pUC 19 ($P < 0.001$). In the cases of IM303 (I4) cells carrying pYFD and pYGB, on the other hand, the reduction in the ROS contents was not so significant as compared with that in the control cells ($P > 0.002$).

In general, cell growth rate can be linked to an intracellular ROS level, which may be fluctuated in response to oxidative stress against cells, and especially SOD null strains with attenuated DNA-repairing systems exhibit limited growth under oxidative stress in aerobic circumstances (11). As demonstrated in Fig. 3A, IM303 (I4) cells carrying pYGE were remarkably encouraged in their growth activity when compared with that of the control cells carrying pUC 19. The averaged μ_m value of IM303 (I4) cells with pYGE was 0.06 h^{-1}, which was about three times higher than that of the counterpart.

For the extended examination of *yggE* gene function to reduce intracellular ROS and recover cell growth, the wild-type strain, MM294 cells transformed by pYGE, was cultivated in the medium with 10 μmol/liter PQ, at which the growth rate of the cells was suppressed to about a quarter of that in PQ-free culture (data not shown). As shown in Fig. 3B, MM294 cells with pYGE displayed the profile of significantly recovered growth when compared with the suppressed proliferation of the control cells carrying pUC 19. The averaged μ_m value of MM294 cells influenced by the *yggE* gene was 0.1 h^{-1}, which

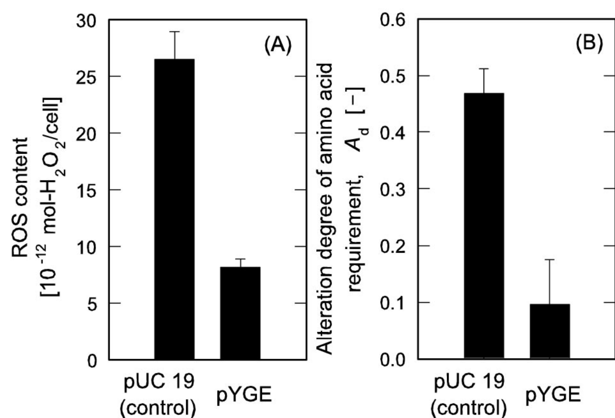

FIG. 4. Influence of the *yggE* gene on ROS content and alteration degree of amino acid requirement in wild-type *E. coli* MM294 cells. (A) ROS content. (B) Alteration degrees of amino acid requirement. The cells were grown in the modified M9 medium with 2068 amino acid mixture as well as 10 μmol/liter PQ, 50 mg/liter ampicillin, and 10 μmol/liter IPTG.

was about five times higher than that of the control cells. We also compared the intracellular ROS contents between the transformants of MM294 cells carrying pUC 19 and pYGE cultivated in the presence of PQ. As indicated in Fig. 4A, the ROS level in the cells with pYGE was approximately 31% of that in the control cells ($P < 0.001$). It is worth noting that the introduction of pYGE led to a decrease in the ROS content in the wild-type cells cultivated with PQ as a generator of oxidative stress, in accordance with the enhancement of the μ_m value. These results suggest that *E. coli* cells get relief from the oxidative stress at an intracellular level and that increased ROS level in the cells by exogenous stress generator can be moderated by *yggE* gene product.

It is known that SOD-deficient mutants are highly sensitive to oxidative stress and mutagenic DNA lesions are promoted in an aid-of-Fenton reaction in the cells, enhancing the frequency of oxygen-dependent mutagenesis (1, 2). Several researchers reported that the SOD deficiency imposed upon auxotrophies of *E. coli*, especially for some amino acids, when the cells suffered from oxidative stress under an aerobic condition (5, 8, 18). Therefore, we investigated the occurrence of altered amino acid requirement by estimating the A_d value in the culture of MM294 cells under an oxidative stress. As seen in Fig. 4B, the A_d value of MM294 cells with pYGE cultivated with 10 μmol/liter PQ was notably lowered at a level of $A_d = 9.6\%$, the value of which corresponded to one-fifth reduction, as compared with the control cells with pUC 19 cultivated with 10 μmol/liter PQ ($P < 0.001$). Under the same culture condition, MM294 cells without any plasmid showed the value of $A_d = 40\%$, which was comparable to the cells with pUC 19. These results demonstrated that the introduction of *yggE* gene effectively suppressed the emergence of the cells with strict amino acid requirement. It is considered that the biosynthetic pathways of amino acids in MM294 cells may be progressively deteriorated under an oxygenated condition, and some of the cells may lose their ability to proliferate under insufficient nutrient conditions. In this viewpoint, it is likely that the changes in amino acid requirement can be responsible for spontaneous occurrence of mutagenic alterations. The result shown in Fig. 4B can support the consideration that *yggE* gene product protects the genes concerning amino acid biosyntheses from oxidative damage through reducing intracellular ROS to a moderate harmless level.

With respect to *yggE* gene product, however, details of an antioxidant mechanism are still unclear. Further examinations will be undertaken to elucidate a working mechanism of *yggE* gene product and to understand a new strategy of living cells to fight against oxidative stress.

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