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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Reduction of hydrogen peroxide stress derived from fatty acid beta-oxidation improves fatty acid utilization in *Escherichia coli*

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Abstract Fatty acids are a promising raw material for substance production because of their highly reduced and anhydrous nature, which can provide higher fermentation yields than sugars. However, they are insoluble in water and are poorly utilized by microbes in industrial fermentation production. We used fatty acids as raw materials for L-lysine fermentation by emulsification and improved the limited fatty acid-utilization ability of *Escherichia coli*. We obtained a fatty acid-utilizing mutant strain by laboratory evolution and demonstrated that it expressed lower levels of an oxidative-stress marker than wild type. The intracellular hydrogen peroxide (H_2O_2) concentration of a fatty acid-utilizing wild-type *E. coli* strain was higher than that of a glucose-utilizing wild-type *E. coli* strain. The novel mutation *rpsA*^{D210Y} identified in our fatty acid-utilizing mutant strain enabled us to promote cell growth, fatty-acid utilization, and L-lysine production from fatty acid. Introduction of this *rpsA*^{D210Y} mutation into a wild-type strain resulted in lower H_2O_2 concentrations. The overexpression of superoxide dismutase (*sodA*) increased intracellular H_2O_2 concentrations and inhibited *E. coli* fatty-acid utilization, whereas overexpression of an oxidative-stress regulator (*oxyS*) decreased intracellular H_2O_2 concentrations and promoted *E. coli* fatty acid utilization and L-lysine production. Addition of the reactive oxygen species (ROS) scavenger thiourea promoted L-lysine production from fatty acids and decreased intracellular H_2O_2 concentrations. Among the ROS generated by fatty-acid β-oxidation, H_2O_2 critically affected *E. coli* growth and L-lysine production. This indicates that the regression of ROS stress promotes fatty acid utilization, which is beneficial for fatty acids used as raw materials in industrial production.

Keywords *Escherichia coli* · Fatty acid metabolism · Reactive oxygen species · Oxidative stress · Hydrogen peroxide stess · L-Lysine production

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Introduction

Fatty acids are stored as triglycerides within organisms and are an important source of energy as they are both highly reduced and anhydrous. Indeed, the energy yield from 1 g fatty acid is more than twice that from carbohydrate. However, industrial production by fermentation mainly uses sugars such as glucose and sucrose as raw materials, as fatty acids are insoluble in water and are poorly utilized by producer strains. Recently, biodiesel production from microalgae as a renewable energy source has received considerable attention (Chisti 2007). Commercial microalgae cultures for fatty acid production and the bioconversion of fatty acids to fuels and chemicals by microorganisms are attractive alternative carbon resources for substance production (Chen et al. 2011; Dellomonaco et al. 2010; Hu et al. 2008; Rosenberg et al. 2008; Service 2009). High-yield aerobic fermentation by *Escherichia coli* from fatty acids to renewable fuels and chemicals such as ethanol, acetate, acetone, butanol, and propionate has previously been proposed (Dellomonaco et al. 2010), suggesting that fatty acids could become an effective carbon source for industrial production.

E. coli is used to produce several industrial primary metabolites, amino acids, and organic acids (Leuchtenberger et al. 2005; Wendisch et al. 2006). Among these, L-lysine is used in food and feed additives and is produced worldwide at quantities of over 1,500,000 metric tons per year. The use of

this bacterium has economic advantages because of its fast growth and substrate consumption rates. In addition, more biochemical, molecular biological, and post-genomic data are available for these model organisms than for most others.

Fatty acids are assimilated and degraded to acetyl-CoA in *E. coli* by the β-oxidation-pathway proteins FadL, FadD, FadE, FadB, and FadA under both aerobic and anaerobic conditions (Cronan and Subrahmanyam 1998). All of the *E. coli* fatty acid β-oxidation pathway genes (*fadL*, *fadD*, *fadE*, *fadB*, *fadA*, *fadI*, and *fadJ*) and short-chain fatty acid utilizing genes (*atoD*, *atoA*, and *atoB*) have been identified (Jenkins and Nunn 1987), and most β-oxidation pathway genes are regulated by FadR (Cronan and Subrahmanyam 1998). FadR also upregulates *fabA*, *fabB*, and *iclR* genes and downregulates *fad* genes (*fadL*, *fadD*, *fadE*, *fadB*, *fadA*, *fadI*, and *fadJ*) and the *uspA* gene. *fabA* and *fabB* are involved in fatty acid synthesis (Magnuson et al. 1993), whereas IclR regulates acetyl-CoA metabolism through *aceBA*, which encodes glyoxylate shunt-pathway enzymes (Resnik et al. 1996). The *uspA* gene is induced by various stresses, including heat and oxidative (Nachin et al. 2005), and *fabA* overexpression decreases the monounsaturated fatty acid content of *E. coli* cell membranes, leading to increased cell resistance to oxidative stress or stress caused by reactive oxygen species (ROS)-generating compounds (Pradenas et al. 2012).

Here, we examined the mechanism of fatty acid degradation by *E. coli* to promote fatty acid utilization. The *E. coli* genome evolves and adapts to laboratory cultivation conditions (Fong et al. 2005; Herring et al. 2006). We therefore initiated wild-type *E. coli* cultivation for fatty acid utilization on minimal medium supplied with sodium oleate as the sole carbon source. Oleate was used because it is common in vegetable oils and is relatively easy to handle experimentally. We analyzed the physiological phenotype of the *E. coli* mutant obtained that could utilize oleate efficiently and investigated the effects of oxidative stress, especially those caused by ROS-generating compounds, on cell growth and lysine production.

Materials and methods

Bacterial strains and plasmids All strains, plasmids, and primers used are listed in Table 1. The *oxyS* gene encoding an oxidative-stress regulator and its promoter region was amplified by the polymerase chain reaction (PCR) using the *E. coli* MG1655 genome and the primer set oxyS1 (5′-TACCCGGG GATCCTCTAGAGTTCCGCGAGGCGCACCATATTGTT GGTGAA-3′) and oxyS2 (5′-TTGCATGCCTGCAGGTCG ACAGAAACGGAGCGGCACCTCTTTTAACCCT-3′).

The PCR product was purified with the Wizard SV gel and PCR clean-up system (Promega, Madison, WI), digested by *Sal*I, and cloned into pTWV229 digested by *Sal*I using the In-Fusion PCR cloning system (Clontech, Mountain View, CA). The resultant plasmid was designated pTWV228-oxyS.

The *sodA* gene encoding the superoxide dismutase overexpressing plasmid pTWV229-sodA was constructed as follows. The *sodA* open reading frame region was amplified using sodA1 (5′-TGATTACGCCAAGCTTAGGAGGTTAA ATGAGCTATACCCTGCCATCCCTGCCGTA-3′) and sodA2 (5′- ATCCTCTAGAGTCGACGCGGCCGCTACT TATTTTTTCGCCGCAAAACGTGCCGCTGC-3′) primers. The PCR product was purified, digested by *Hin*dIII and *Sal*I, and cloned into pTWV229 digested with the same restriction enzymes.

Adaptive evolution and analysis of an effective mutation Minimal medium M9 (Miller 1992) supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.001 % thiamine, 0.5 % Tween80 (polyoxyethylene sorbitan monooleate, CAS:9005-65-6), and 2 g/L sodium oleate was used in adaptive evolution experiments. One loop of *E. coli* MG1655 was inoculated onto an M9 plate and incubated for 20 h at 37 °C. Cells were cultured in L-shaped test tubes using a TN-2612 rocking incubator (Advantec, Tokyo, Japan) at 37 °C with constant shaking at 70 rpm. The optical density at 600 nm of the culture was measured continuously, and test-tube cultivation started at approximately OD_{600} 0.006 and finished at OD_{600} 0.3. The culture broth was transferred into fresh minimal medium, and the test-tube cultivation was repeated 22 times for a total cultivation time of 445 h. A single colony was then isolated from the resultant broth spread onto an M9 plate and designated FitnessOle.

The addition of Tween80 as an emulsifying agent of sodium oleate clarified the medium and allowed us to accurately measure the OD_{600} in fatty acid supplied medium (Suzuki et al., unpublished data). We ascertained that *E. coli* MG1655 and FitnessOle could not grow and utilize Tween80 as a sole carbon source in test-tube and flask cultivation using M9 medium (data not shown).

The FitnessOle genome was analyzed by whole genome sequencing with an Illumina Genome Analyzer II (GAII; Illumina Inc, San Diego, CA). In order to introduce the *rpsA*^{D210Y} mutation into the genomes of other strains, a FitnessOle *ycaI* deletion mutant was constructed by PCR and the λ red deletion method using ycaI1 primer (5′-agacaaccgctcaacaaagttgcacactttccataaacagggaggggtgcTCTA GACGCTCAAGTTAGTATA-3′) and ycaI2 primer (5′-gttgtttgtagtgacgccagatactgtgcacgcaggctacaattcggttcAGATCT TGAAGCCTGCTTT-3′) as

ycaI is located close to *rpsA* in the genome. Because *ycaI* gene is located about 1.7 kbps of *rpsA*^{D210Y} mutation in the *E. coli* genome and no significant phenotypes in this study were observed by *ycaI* gene deletion (data not shown), we can introduce the *rpsA*^{D210Y} mutation with *ycaI* gene deletion by using the phage P1 without phenotypic influence. MG1655

Table 1 Strains and plasmids

Strain or plasmid	Description, genotype, or sequence	Reference
Strains		
MG1655	F-λ-*ilvG rfb-50 rph-1*	CGSC collection number 6300
MG1655 Δ*ycaI*::attR-cat-attL	MG1655 Δ*ycaI* deletion mutant constructed by λ red system	This study
MG1655 *rpsA*^{D210Y} Δ*ycaI*::attR-cat-attL	MG1655 containing *rpsA*^{D210Y} mutation	This study
FitnessOle	High performance fatty acid-utilizing mutant isolated by adaptive evolution	This study
FitnessOle Δ*ycaI*::attR-cat-attL	FitnessOle Δ*ycaI* deletion mutant constructed by λ red system	This study
BW25113	*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1*	Baba et al. (2006), Keio collection
JW4024	BW25113 Δ*oxyR*::FRT-Kan-FRT; *oxyR* deletion mutant in BW25113	Baba et al. (2006), Keio collection
JW3933	BW25113 Δ*soxR*::FRT-Kan-FRT; *soxR* deletion mutant in BW25113	Baba et al. (2006), Keio collection
WC196LC	W3110 NTG mutant (S-aminoethyl-L-cysteine resistant mutant) Δ*ldc* Δ*cadA*	Kikuchi et al. (1997)
WC196LC Δ*ycaI*::attR-cat-attL	WC196LC Δ*ycaI* deletion mutant constructed by λ red system	This study
WC196LC *rpsA*^{D210Y} Δ*ycaI*::attR-cat-attL	WC196LC containing *rpsA*^{D210Y} mutation	This study
Plasmids		
pTWV228	Cloning vector, Ap^r	Takara Bio Inc., Japan
pTWV229	Cloning vector, Ap^r	Takara Bio Inc., Japan
pTWV228-oxyS	*oxyS* gene on pTWV228	This study
pTWV229-sodA	*sodA* gene on pTWV229	This study
pCABD2	pRSF1010 carrying mutated *lysC*, mutated *dapA*, mutated *dapB*, and *C. glutamicum ddh*	Kojima et al. (1994)
pKD46	λ red system helper plasmid	Datsenko and Wanner (2000)
pMW118-λ attL-Cm^R-λ attR	λ red system vector containing Cm^R (cat) gene	Katashkina et al. (2005)

containing the *rpsA*^{D210Y} mutation and WC196LC containing the *rpsA*^{D210Y} mutation were constructed by phage P1 transduction using the phage P1 obtained from the FitnessOle *ycaI* deletion strain.

Statistical testing and estimation of p values The standard error of the mean calculation and a two-tailed unpaired Student's *t* test were performed using Excel software (Microsoft Corporation, Redmond, WA) from more than three independent samples.

Culture conditions For test-tube cultivation, *E. coli* MG1655 and its derivative strains were grown overnight at 37 °C on M9 plates supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.001 % thiamine, and 2 g/L glucose. One loop of the grown cells was inoculated into 10 mL minimal medium M9 supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.001 % thiamine, 0.5 % Tween80, and 1 g/L carbon source (sodium oleate or glucose) in L-shaped test tubes and cultivated at 37 °C with constant shaking at 70 rpm using a TN-2612 rocking incubator.

For flask cultivation, *E. coli* MG1655 and its derivative cells grown overnight at 37 °C on M9 plates supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.001 % thiamine, and 2 g/L glucose were inoculated into 20 mL of M9 medium supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.001 % thiamine, 0.5 % Tween80, and 10 g/L carbon source (oleic acid, elaidic acid, acetate, maltose, glycerol, or glucose) in a Sakaguchi flask (500 mL) at an initial OD_{600} of 0.2 and cultivated at 37 °C with reciprocal shaking at 120 rpm. The pH of each component was adjusted to 7.0 before sterilization.

For L-lysine fermentation from fatty acids in flasks, *E. coli* strains derived from WC196LC (Leuchtenberger et al. 2005) were cultivated overnight at 37 °C on LB plates composed of 1.0 % Bacto tryptone, 0.5 % Bacto yeast extract, 1 % NaCl, and 1.5 % agar. Cells were then inoculated into 40 mL flask-fermentation medium comprising 2 g/L yeast extract, 1 g/L $MgSO_4·7H_2O$, 24 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.01 g/L $FeSO_4·7H_2O$, 0.082 g/L $MnSO_4·7H_2O$, 20 g/L PIPES, and 10 g /L sodium oleate in Erlenmeyer flasks (500 mL) at an

initial OD_{600} of 0.25. The pH of the medium was adjusted to 7.0 before sterilization. Fermentation was performed at 37 °C with rotary shaking at 200 rpm.

For L-lysine fermentation from fatty acids in a jar fermenter, *E. coli* WC196LC and its derivative strains grown overnight at 37 °C on LB plates were transferred to 300 mL jar-fermentation medium comprising 2 g/L yeast extract, 1 g/L $MgSO_4 \cdot 7H_2O$, 24 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.082 g/L $MnSO_4 \cdot 7H_2O$, and 10 g/L carbon source (sodium oleate or glucose) in 1-L glass vessels (Able Corporation, Tokyo, Japan) at an initial OD_{600} of 0.04 and subjected to batch cultivation in jar fermenters DPC-2A (Able Corporation, Tokyo, Japan) at 37 °C. The pH of the culture was maintained at 6.7 by adding ammonia gas.

Analytical methods Aggregation indexes of *E. coli* MG1655 and the FitnessOle strain were measured as previously described (Malik et al. 2003). Cell growth was analyzed by measuring the OD_{600} with a spectrophotometer U-2900 (Hitachi, Tokyo, Japan) and by counting the number of living cells. Tween80 solution (10 %) was used for dilution to eliminate the influence of fatty acids on OD_{600nm}. Living cell counting in the fermentation broth was carried out by diluting the broth with saline and counting the number of colonies on LB plates after cultivation for 24 h at 37 °C. The maximum specific growth rate (μ_{max}) and maximum specific substrate-consumption rate (ν_{max}) were calculated by nine-dimension polynomial approximations using the numerical computation software package MATLAB (MathWorks, Natick, MA). R-squared values of the approximations were greater than 0.995.

Carbonylated protein concentrations were measured using a protein carbonyl colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI). To measure cells in the same growth phase, we sampled the cells from the flask-fermentation broth when the residual carbon source concentration reached 1 g/L for the carbonylated protein assay and measured the OD_{600} to confirm that sampled cells were divided a similar number of times. Glucose and L-lysine were assayed by a biotech analyzer AS310 (Sakura Si Co., Ltd., Tokyo, Japan). Glycerol was assayed by an electrochemical biosensor BF-5 (Oji Scientific Instruments, Hyogo, Japan), maltose by an ion chromatography system ICS-3000 (Dionex, Sunnyvale, CA), acetate by a liquid chromatograph LC-10AD (Shimadzu, Kyoto, Japan), and oleic acid and elaidic acid by a gas chromatograph GC-2014 (Shimadzu).

Intracellular hydrogen peroxide (H_2O_2) was measured as previously described (González-Flecha and Demple 1999; Maisonneuve et al. 2008). Briefly, bacterial cells were collected from culture broths by centrifugation (13,800×g) for 2 min at 4 °C and resuspended in phosphate buffer (pH 7.3) at an approximate density of 10^6 cells/mL. After 10 min diffusion of intracellular H_2O_2 into the buffer through cellular membranes, the cells were removed by centrifugation at 13,800×g for 2 min at 4 °C. Then, 10 μL supernatant was suspended separately in solution A (2 μM horseradish peroxidase and 10 μM HPF (Maisonneuve et al. 2008) in 100 mM phosphate buffer (pH 7.3)) and solution B (2 μM catalase and 10 μM HPF (Maisonneuve et al. 2008) in 100 mM phosphate buffer solution (pH 7.3)).

The resusupended samples were incubated at 37 °C for 75 min in the dark, and the emitted fluorescence at 515 nm was measured using excitation at 490 nm. The intracellular H_2O_2 concentration was calculated by subtracting the fluorescence of solution B from that of solution A.

Results

Acquisition of fatty acid-utilizing E. coli mutant strain by laboratory evolution and analysis of physiological phenotypes To improve fatty acid utilization by *E. coli*, we attempted to obtain a mutant with enhanced function. We cultivated the wild-type *E. coli* strain MG1655 in minimal media supplemented with sodium oleate as a sole carbon source for 445 h. We then isolated a mutant with improved utilization of fatty acid and designated it FitnessOle. Figure 1 shows the growth (Fig. 1a) and oleate concentration (Fig. 1b) profiles of the wild-type and FitnessOle strains in flask culture. The FitnessOle strain showed significantly enhanced growth in oleate culture with enhanced consumption of oleate. We ascertained that four other independent colonies isolated from the same broth after 445 h cultivation showed the same enhanced growth phenotype as the FitnessOle strain in oleate culture (data not shown). The FitnessOle strain also showed higher μ_{max} and ν_{max} values when grown on fatty acids (oleic or elaidic acids), glycerol, or acetate as the sole carbon source compared with the wild-type strain (Table 2). The FitnessOle strain also showed increased cell biomass accumulation when grown on oleic acid as the sole carbon source under aerobic conditions compared with the wild-type strain. But the FitnessOle strain showed the same cell biomass accumulation as the wild-type strain when grown on oleic acid under anaerobic conditions (data not shown). Cell aggregation of the FitnessOle strain appeared to be facilitated compared with the wild-type strain. We also measured the aggregation index (Malik et al. 2003) and found the aggregation tendency of the FitnessOle strain to be significantly increased (Fig. 2c).

The *uspA* gene, encoding a universal stress protein with unknown functions, has been reported to be a target of FadR that is upregulated when *E. coli* is exposed to oxidative stress (Nachin et al. 2005). FadR functions as switch between fatty acid β-oxidation and fatty acid biosynthesis (Xu et al. 2001). Based on these facts, we investigated the relationship between fatty acid utilization and oxidative stress. We measured the carbonylated protein concentration, a major oxidative-stress

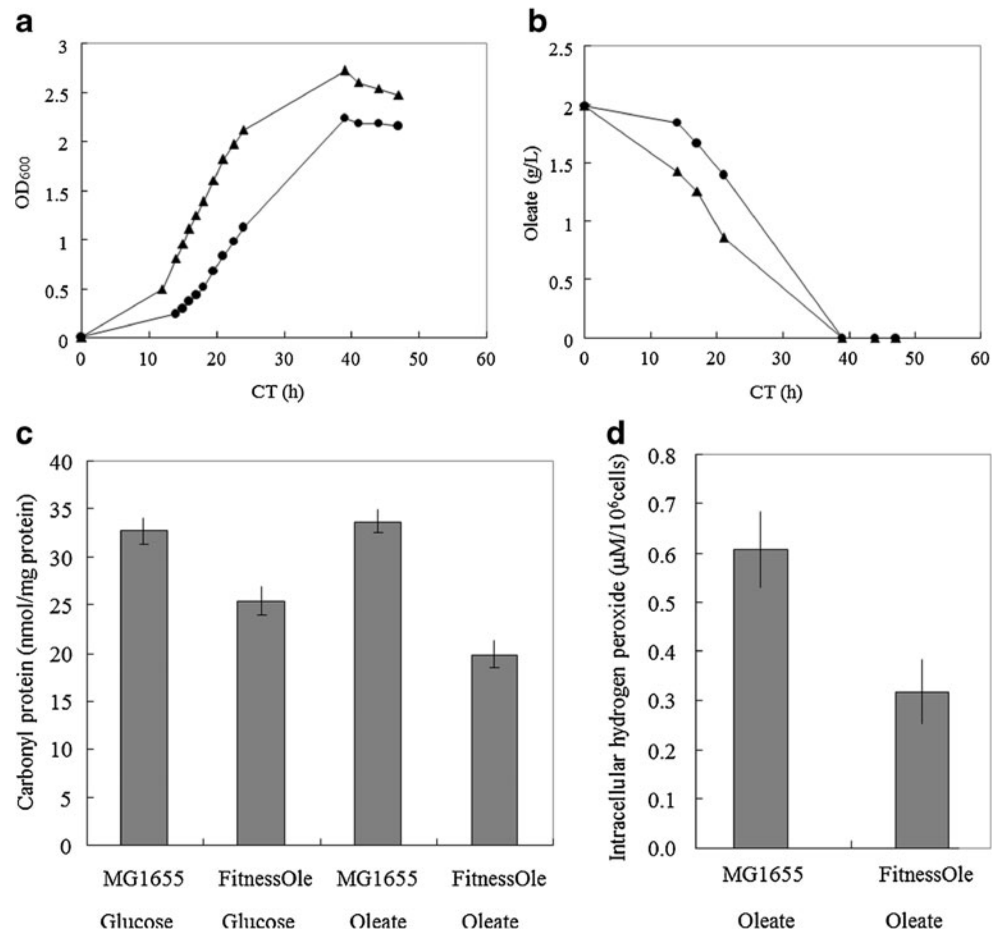

Fig. 1 The physiological phenotypes of the FitnessOle strain cell growth (**a**) and residual oleate concentration (**b**) profiles were measured for MG1655 (*solid circle*) and FitnessOle strains (*solid triangle*). Carbonylated protein content (**c**) and intracellular hydrogen peroxide concentration (**d**) were also measured. Values are the mean of more than three independent samples. *SE bars* represent the standard error of the mean calculated with Excel software

marker, of *E. coli* cells utilizing glucose or oleate as the sole carbon source (Maisonneuve et al. 2008) and found it to be decreased in the FitnessOle strain compared with wild type after a similar number of cell divisions (Fig. 1c), indicating decreased oxidative stress in this strain. We next measured the concentration of intracellular H_2O_2, a major ROS, and also found it to be decreased in the FitnessOle strain compared with wild type when the cells utilized oleate as the sole carbon source (Fig. 1d).

To identify an effective mutation for fatty acid utilization in the genome of the fatty acid-utilizing *E. coli* mutant strain, we carried out whole genome sequencing and discovered the *rpsA*^{D210Y} mutation (Fig. 2a). The *rpsA*^{D210Y} mutation was present in the genomes of four other independent colonies isolated from the broth after 445 h minimal media cultivation. Introduction of this mutation into the MG1655 genome resulted in enhanced cell growth when oleate was used as the sole carbon source (Fig. 2b) and higher μ_{max} and ν_{max} values than those of the wild-type MG1655. Furthermore, introduction of the *rpsA*^{D210Y} mutation caused a decrease in the concentration of intracellular H_2O_2 when the strain utilized oleate as a sole carbon source (Fig. 2d) despite no significant change in aggregation index (Fig. 2c). Introduction of the *rpsA*^{D210Y} mutation into the genome of the *E. coli* L-lysine producer strain WC196LC/pCABD2 (Kikuchi et al. 1997) resulted in increased cell growth and L-lysine accumulation (Table 3). There were, however, no apparent differences in L-lysine production, cell growth, or glucose consumption

Table 2 μ_{max} and ν_{max} values of the FitnessOle strain in flask cultivation with various carbon sources

Substrate (10 g/L)	μ_{max} of MG1655	μ_{max} of FitnessOle	ν_{max} of MG1655	ν_{max} of FitnessOle
Glucose	0.85	0.85	0.63	0.63
Glycerol	0.62	0.81	0.40	0.59
Maltose	0.44	0.41	0.30	0.29
Oleic acid	0.09	0.24	0.06	0.12
Elaidic acid	0.11	0.23	0.07	0.12
Acetate	0.09	0.11	0.07	0.08

μ_{max} maximum specific growth rate, ν_{max} maximum specific substrate-consumption rate

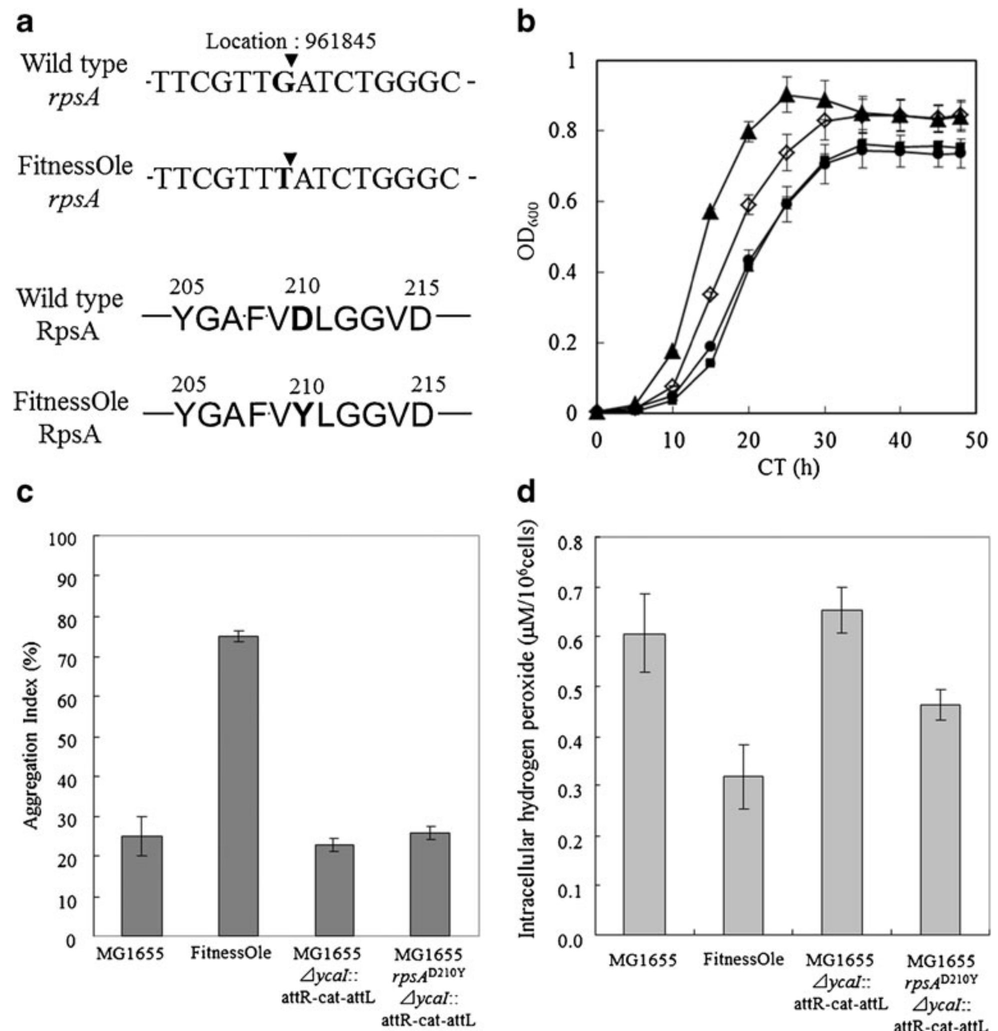

Fig. 2 DNA and amino acid sequence of *rpsA*^{D210Y} mutation (**a**) and its effect on fatty acid utilization. **b** Growth of the *rpsA*^{D210Y} mutant strain in M9 medium test tube cultivation supplemented with sodium oleate as the sole carbon source. Parental strain MG1655 (*solid circle*), FitnessOle strain (*solid triangle*), MG1655 Δ*ycaI*::attR-cat-attL strain (*solid square*) and MG1655 *rpsA*^{D210Y} Δ*ycaI*::attR-cat-attL strain (*empty diamond*)were monitored. Aggregation index (**c**) and intracellular hydrogen peroxide concentration (**d**) of cells cultivated in M9 medium supplemented with sodium oleate as a sole carbon source were also measured. Values are the mean of more than three independent samples. *SE bars* represent the standard error of the mean calculated with Excel software

following introduction of the *rpsA*^{D210Y} mutation when the *E. coli* L-lysine producer strain utilized glucose (Table 3).

Promotion of fatty acid utilization by reducing intracellular H_2O_2 In the wild-type strain MG1655, no carbon source (glucose or oleate)-dependent changes in carbonylated protein accumulation were observed (Fig. 1c), suggesting that the specific ROS stress was decreased only in the fatty acid-utilizing mutant FitnessOle strain. As shown in Fig. 3, the ROS consists of a single oxygen molecule, a superoxide anion (O_2^-), H_2O_2, and a hydroxyl radical (OH) (González-Flecha and Demple 1999; Blanchard et al. 2007; Zheng et al. 1998). The two transcription factors reported to respond to O_2^- and H_2O_2 are also shown (Blanchard et al. 2007; Zheng et al. 1998). SoxR is mainly involved in defense against O_2^- and OxyR mainly against H_2O_2. Thus, we investigated the growth of the *soxR* deletion mutant (JW3933) and the *oxyR* deletion mutant (JW4024) derived from BW25113 (Baba et al. 2006) on glucose or oleate as the sole carbon source. Both the Δ*soxR* and Δ*oxyR* strains showed no significant stationary phase optical density changes compared with their host strain BW25113 when they utilized glucose as the sole carbon source (Fig. 4a). However, the Δ*oxyR* strain showed an apparent cell growth defect and a stationary phase optical density decrease when grown on sodium oleate as the sole carbon source (Fig. 4b).

To determine which ROS has the greatest negative effect on fatty acid utilization in *E. coli*, we constructed expression plasmids harboring the *sodA* gene encoding the O_2^- scavenger dismutase or the *oxyS* gene encoding an oxidative-stress regulator and introduced them into the wild-type strain MG1655. The *oxyS* transcript might be involved in the excretion, rather than removal, of H_2O_2 by catalase peroxidases (González-Flecha and Demple 1999). The resultant strains (MG1655/pTWV229-sodA and MG1655/pTWV228-oxyS) were cultivated on glucose or sodium oleate as a carbon source (Fig. 5). The intracellular H_2O_2 concentration was significantly increased when the *E. coli* MG1655/pTWV228 strain utilized sodium oleate compared with glucose as the sole carbon source (Fig. 5a, b; $p<0.03$,

Table 3 Effect of *rpsA*^{D210Y} mutation on L-lysine production in flask cultivation from fatty acid (sodium oleate)

Strain	Carbon source	OD_{600}	L-Lysine accumulation (g/L)	L-Lysine yield (%)
WC196LC/pCABD2	Glucose	5.5	3.8	39.0
WC196LC Δ*ycaI*::attR-cat-attL/pCABD2	Glucose	5.5	3.8	39.2
WC196LC *rpsA*^{D210Y} Δ*ycaI*::attR-cat-attL/pCABD2	Glucose	5.5	3.8	39.0
WC196LC/pCABD2	Oleate	8.1	4.2	44.9
WC196LC Δ*ycaI*::attR-cat-attL/pCABD2	Oleate	8.3	4.2	44.8
WC196LC *rpsA*^{D210Y} Δ*ycaI*::attR-cat-attL/pCABD2	Oleate	9.3	4.5	47.2

Student's *t* test). Furthermore, overexpression of the *sodA* gene resulted in an increase of intracellular H_2O_2 and a severe growth defect (Fig. 5b, d). Overexpression of the *oxyS* gene decreased intracellular H_2O_2 levels and promoted cell growth (Fig. 5b, d).

Effects of promotion of fatty acid utilization by reducing ROS stress on L-lysine production To investigate the relationship between material production from fatty acids and reduction of ROS stress, we used the *E. coli* L-lysine-producing strain WC196LC/pCABD2 (Kikuchi et al. 1997). Overexpression of *oxyS* in WC196LC/pCABD2 resulted in increased cell growth and L-lysine accumulation (Table 4). This suggests that decreased ROS stress, assumed to be mainly caused by intracellular H_2O_2, promoted fatty acid utilization and L-lysine production. Next, we investigated the effect of the antioxidant reagent thiourea on fatty acid utilization in larger scale fermentation. Thiourea reduces damage caused by H_2O_2 (Blount et al. 1989) and was shown to decrease intracellular H_2O_2 concentrations both in glucose and sodium oleate utilization (Table 5). However, thiourea also reduced the cultivation times required to consume fatty acid and increased cell and L-lysine accumulation when the *E. coli* L-lysine-producing strain utilized sodium oleate (Table 5). No apparent differences in L-lysine production, cell growth, or glucose consumption were observed following thiourea addition when the *E. coli* L-lysine producer strain utilized glucose (Table 5).

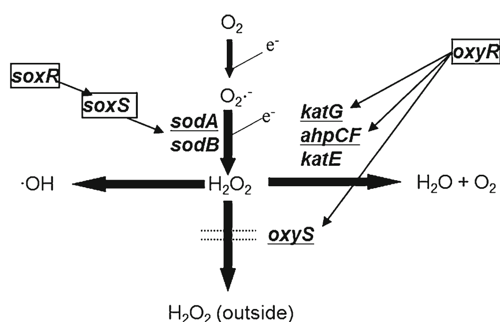

Fig. 3 Schematic representation of ROS generation and elimination, and transcriptional regulation of ROS elimination systems in *E. coli*. Ordinary electron transfer to an oxygen molecule converting into a water molecule is catalyzed by cytochrome oxidases, but incomplete electron transfer to an oxygen molecule generates $O_2^{\cdot -}$, H_2O_2, and OH^-. These ROS molecules cause cell damage so *E. coli* possesses various ROS scavenger genes. Superoxide dismutases (SodA and SodB) convert $O_2^{\cdot -}$ into H_2O_2, which decomposes into harmless H_2O and O_2 with the aid of catalases KatG, KatE, and the alkyl hydroxiperoxide reductase AhpCF. Intracellular H_2O_2 excretion is promoted by small RNA *oxyS*. These ROS-scavenger genes are regulated by SoxR and OxyR. SoxR detects intracellular $O_2^{\cdot -}$ and upregulates SoxS expression. SoxR and SoxS activate $O_2^{\cdot -}$ decomposing genes such as *sodA* and *sodB*. OxyR is an intracellular H_2O_2 sensor and H_2O_2 removal-associated gene regulator. OxyR activates *oxyS* and H_2O_2-decomposing genes such as *katG*, *katE*, and *ahpCF*

Discussion

We predicted that test-tube adaptive evolution would shed light on fatty acid utilization in *E. coli* by comparing physiological phenotype differences between a mutant that can utilize fatty acids efficiently and a wild-type strain. Indeed, the mutant strain FitnessOle showed a higher ability to utilize various fatty acids, including oleic acid, elaidic acid (Table 2), stearic acid, sodium palmitate, myristic acid, and sodium oleate (data not shown), compared with wild type.

The FitnessOle strain showed enhanced cell aggregation as well as lowered ROS stress (Fig. 2). Microorganism aggregation can be quantified by measurement of the aggregation index and is positively correlated with membrane hydrophobicity (Malik et al. 2003). Thus, our results indicate that the FitnessOle strain possesses increased membrane

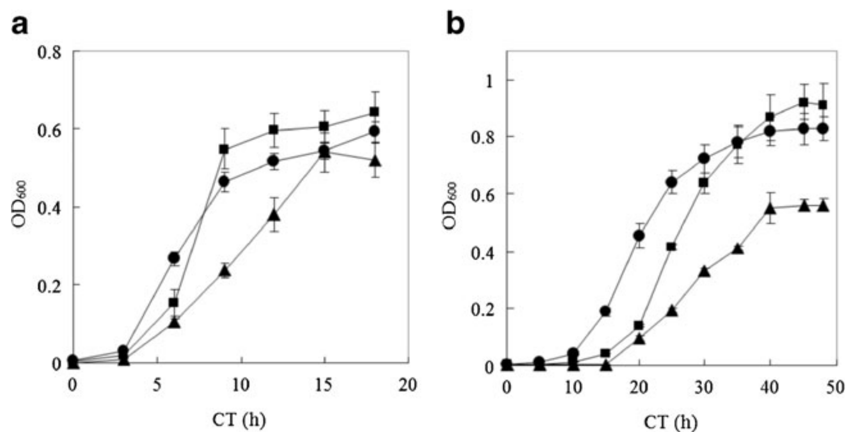

Fig. 4 The effects of antioxidant transcription factor gene deletion. The growth of Δ*soxR* and Δ*oxyR* strains in M9 medium test tube cultivation supplemented with sodium glucose (**a**) or oleate (**b**) as the sole carbon source. Parental strain BW25113 (*solid circle*), Δ*oxyR* strain (*solid triangle*), and Δ*soxR* strain (*solid square*) were monitored. Values are the mean of more than three independent samples. *SE bars* represent the standard error of the mean calculated with Excel software

hydrophobicity; we will examine this together with its relationship with fatty acid utilization in a future study.

Our main focus here was the promotion of fatty acid utilization in *E. coli*. We discovered a novel fatty acid utilization promoting mutation, *rpsA*$^{\mathrm{D210Y}}$. *rpsA* encodes the 30S ribosomal subunit protein. Introduction of the *rpsA*$^{\mathrm{D210Y}}$ mutation decreased intracellular H_2O_2 concentrations (Fig. 2d) but had no effect on cell aggregation (Fig. 2c). These results suggest that intracellular H_2O_2 concentrations can influence fatty acid utilization. We also found that a decrease in ROS stress, particularly that of H_2O_2, was important to enhance the ability to utilize fatty acid in *E. coli*. However, as the ROS was shown to change (Fig. 3), it is difficult to identify which species affects fatty acid utilization. Nevertheless, our research revealed that it was mainly inhibited by H_2O_2 rather than $O_2^{\,-}$ (Figs. 4 and 5). There were no apparent changes for the carbonylated

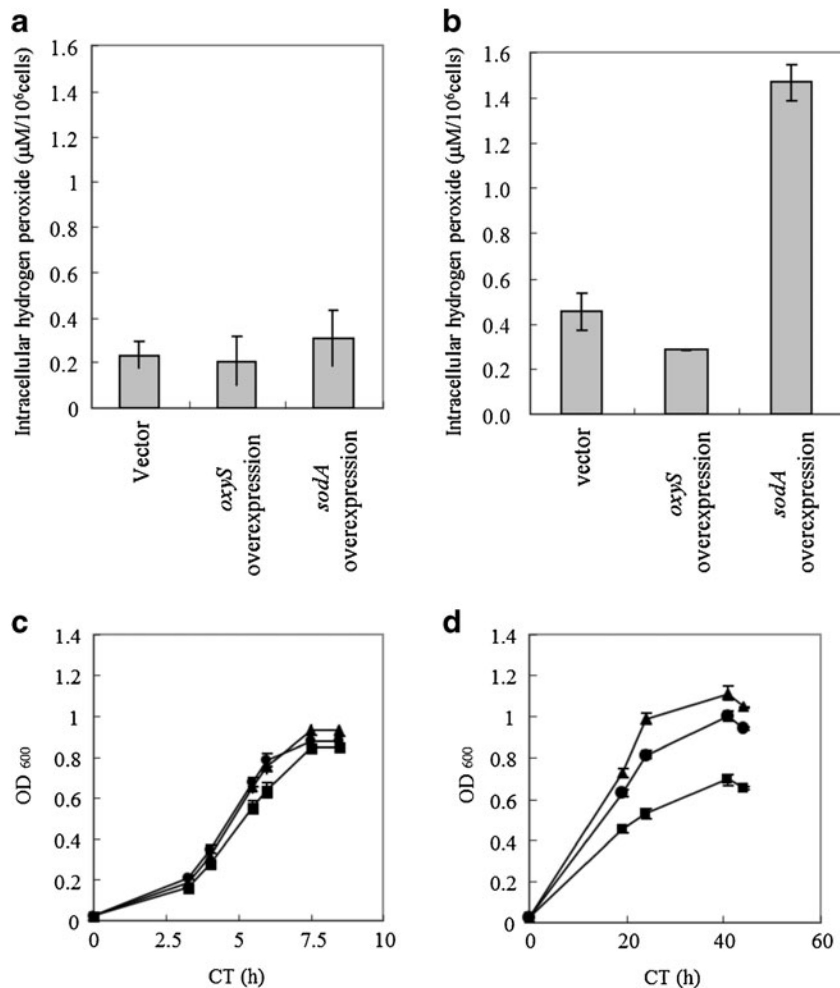

Fig. 5 Effects of overexpression of *sodA* or *oxyS* genes. Intracellular hydrogen peroxide concentration of cells grown in M9 medium supplemented with glucose (**a**) or sodium oleate (**b**) as the sole carbon source. Cell growth using glucose (**c**) or sodium oleate (**d**) as the sole carbon source. Vector control MG1655/pTWV228 (*solid circle*), *oxyS* overexpressing strain MG1655/pTWV228-oxyS (*solid triangle*) and *sodA* overexpressing strain MG1655/pTWV229-sodA (*solid square*) were studied. Values are the mean of more than three independent samples. *SE bars* represent the standard error of the mean calculated with Excel software. The Student's *t* test between the intracellular hydrogen peroxide concentration of vector control samples and that of *oxyS* overexpression samples when grown on sodium oleate gave a *p* value of 0.024

Table 4 Effect of *oxyS* gene overexpression on L-lysine production in flask cultivation from fatty acid (sodium oleate)

Strain	OD_{600}	L-Lysine accumulation (g/L)	L-Lysine yield from oleate (%)	Intracellular H_2O_2 (μM/10^6 cells)
WC196LC/pCABD2, pTWV228	4.3	3.8	40.9	0.52
WC196LC/pCABD2, pTWV228-oxyS	6.2	4.2	45.3	0.26

protein accumulation in wild-type *E. coli* cells grown in M9 media with glucose or oleate (Fig. 1c). On the other hand, the intracellular H_2O_2 concentration was significantly increased when the wild-type strain utilized sodium oleate compared with glucose as the sole carbon source (Fig. 5a, b). Carbonylated protein content indicates total ROS stress including , H_2O_2, $O_2{}^{-}$, and OH (Fig. 3). These data show that the specific ROS stress is H_2O_2 stress when *E. coli* utilized sodium oleate among the various ROS. Our preliminary microarray research revealed that *katG*, *ahpC*, *ahpF*, and *oxyR* transcripts were increased in FitnessOle strain compared with the wild type when they utilized sodium oleate (Doi et al., unpublished data). In addition, our preliminary microarray research also revealed that *sodA*, *sodB*, *soxS*, and *soxR* transcripts were decreased in FitnessOle strain compared with the wild type when they utilized sodium oleate (Doi et al., unpublished data). These results indicate the independence of H_2O_2 and $O_2{\cdot}^{-}$. No significant phenotype was previously reported following the overexpression of *oxyS* when *E. coli* was cultivated on LB, in which the main carbon source was amino acids not fatty acids (González-Flecha and Demple 1999).

Similarly, no significant phenotype was observed by the overexpression of *oxyS* when *E. coli* utilized glucose as the main carbon source (Fig. 5a, c). However, we did observe the promotion of fatty acid utilization following *oxyS* overexpression, presumably because of reduced H_2O_2 levels (Fig. 5b and d) and L-lysine production (Table 4). We assumed that this effect was a result of H_2O_2 excretion by the *oxyS* transcript (González-Flecha and Demple 1999). These results demonstrate a specific phenotype after *oxyS* overexpression and suggest that more H_2O_2 is generated when utilizing fatty acid compared with glucose and amino acids supplied in LB medium. We presume that H_2O_2 is generated by flavin adenine dinucleotide ($FADH_2$) during the fatty acid β-oxidation pathway.

This autoxidation of $FADH_2$ is a well-known phenomenon that occurs, for example, in glucose concentration analysis by glucose oxidase (Raba and Mottola 1995). Free $FADH_2$ was previously shown to be reduced by cytosolic enzymes such as L-aspartate oxidase and was autoxidized to generate endogenous *E. coli* H_2O_2 (Korshunov and Imlay 2010; Messner and Imlay 2002). We assumed that $FADH_2$ reduced by FadE, the acyl-CoA dehydrogenase in the fatty acid β-oxidation pathway, would be the endogenous H_2O_2 source in the present study. We are currently investigating the effects of $FADH_2$ oxidation by electron-transfer-flavoprotein (ETF) or ETF dehydrogenase (EC 1.5.5.1). Our preliminary research revealed that the overexpression of these homologous genes in *E. coli* results in the decrease of intracellular H_2O_2 and increased L-lysine accumulation during the utilization of fatty acid as a carbon source (Hoshino et al., unpublished data), supporting our hypothesis of endogenous H_2O_2 generation by $FADH_2$ autoxidation.

In jar fermentation, thiourea addition decreased intracellular H_2O_2 concentrations, lowered cultivation time, and increased L-lysine production when *E. coli* utilized fatty acid (Table 5). However, when glucose was utilized, the total cultivation time and L-lysine production remained the same, even though intracellular H_2O_2 concentrations decreased (Table 5). This

Table 5 Effects of ROS-scavenger addition on L-lysine production

Supplied carbon source and antioxidant	Cultivation time (h)	OD_{600}	L-Lysine accumulation (g/L)	Intracellular H_2O_2 (μM/10^6 cells)
Glucose 10 g/L	16.0	5.3	4.7	0.14
Glucose 10 g/L Thiourea 1 mM	16.0	5.3	4.7	0.11
Glucose 10 g/L Urea 1 mM (mock control)	16.0	5.2	4.6	0.14
Sodium oleate 10 g/L	41.5	6.8	4.8	0.17
Sodium oleate 10 g/L Thiourea 1 mM	33.0	7.3	5.2	0.11
Sodium oleate 10 g/L Urea 1 mM (mock control)	41.5	6.8	4.8	0.17

The L-lysine producing strain, WC196LC/pCABD2, was cultivated in a jar fermenter, and the intracellular H_2O_2 concentration was measured immediately after the exhaustion of carbon sources

suggests that higher ROS stress was generated following fatty acid utilization, which inhibited growth. The addition of thiourea, a common antioxidant molecule, to reduce ROS stress is a promising approach for fatty acids used on an industrial scale as raw materials for fermentation. Thiourea is used as a building material because it is inexpensive compared with antioxidants such as vitamin C or tocopherol.

The present study focused only on the physiological phenotype of the FitnessOle strain and identified a novel mutation, *rpsA*^{D210Y}. We are currently using this mutation to understand the mechanism of decreasing intracellular H_2O_2 concentrations. Our preliminary data revealed that superoxide dismutase SodB protein expression decreased following the introduction of the *rpsA*^{D210Y} mutation, as shown by 2-D electrophoresis and liquid chromatography–mass spectrometry analysis (Doi et al., unpublished data). However, the *rpsA*^{D210Y} mutation alone could not achieve the increased cell growth and decreased intracellular H_2O_2 concentration shown by the FitnessOle strain. Therefore, we are now investigating the other mutations of the FitnessOle genome, which should reveal more information concerning *E. coli* fatty acid utilization. We expect this to clarify the relationship between the reduction of ROS and changes in membrane hydrophobicity.

Fatty acids are a promising raw material for substance production, and we have shown that they can be used as such for amino acid fermentation by means of emulsification, despite their insolubility in water (Suzuki et al., unpublished data). This report is the first to show the bioconversion of fatty acid into L-lysine by obtaining a fatty acid-utilizing mutant, the FitnessOle strain. H_2O_2 generated by fatty acid β-oxidation was revealed to have a critical effect on growth and lysine production when *E. coli* utilized fatty acid as a carbon source. This will be useful for future industrial production using fatty acids as substrates, and we hope to identify further useful insights to help in the realization of this process.

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