Metabolic Roles of Carotenoid Produced by Non-Photosynthetic Bacterium 
*Gordonia alkanivorans* SKF120101

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Carotenoids produced by non-photosynthetic bacteria protect organisms against lethal photodynamic reactions and scavenge oxygenc radicals. However, the carotenoid produced by *Gordonia alkanivorans* SKF120101 is coupled to reducing power generation. SKF120101 selectively produces carotenoid under light conditions. The growth yield of SKF120101 cultivated under light conditions was higher than that under dark condition. In the cyclic voltammetry, both upper and lower voltammograms for neutral red (NR) immobilized in intact cells of SKF120101 were not shifted in the condition without external redox sources but were commonly shifted downward by glucose addition and light. Electric current generation in a biofuel cell system (BFCS) catalyzed by harvested cells of SKF120101 was higher under light than dark condition. The ratio of electricity generation to glucose consumption by SKF120101 catalyzes production of reducing power from light energy, first evaluated by the electrochemical technique used in this research.

**Keywords:** Carotenoid, *Gordonia alkanivorans*, cyclic voltammetry, biofuel cell, neutral red

The *Gordonia* genus is known to be widely distributed in natural ecosystems such as soil, water, estuary sand, and mangrove rhizosphere, and in artificially operated systems including oil-producing wells, sewage sludge, activated sludge foam, and clinical samples [1, 10]. Some of the species belonging to the *Gordonia* genus partially or completely degrade various xenobiotic compounds and natural macromolecules [5, 15, 18, 21]. *Gordonia alkanivorans* was first isolated from tar-contaminated soil. The species name originated from its alkane-degrading function [3, 17]. This catabolic function of *G. alkanivorans* is useful for biodegradation of organic contaminants and bioremediation of contaminated environments. However, the anabolic function of *Gordonia* species for carotenoid biosynthesis is not known for its competitiveness and usefulness, because some plants produce much more carotenoid than bacteria. *G. jacobaea* MV-1 was reported to be the first *Gordonia* strain characterized with regard to carotenoid biosynthesis [9]. Carotenoids are best known as auxiliary pigments of the photosynthetic light-harvesting apparatus in photosynthetic organisms. Carotenoids synthesized by non-photosynthetic bacteria and fungi are also reported to protect against lethal photodynamic reaction, and function as scavengers of oxygenc radicals [24, 33, 36]. The carotenoid produced in the non-photosynthetic microorganisms was found to be not coupled to energy metabolism but was an antioxidative compound to scavenge the reactive oxygen species [31]. The metabolism for light-induced carotenoid production in the non-photosynthetic bacterium *Myxococcus xanthus* was studied in detail [6]. *G. alkanivorans* SKF120101 also is a light-induced carotenoid producer, based on the selective production of reddish color under light condition, whose growth and glucose consumption increased more under light than dark condition [29].

Electrochemistry is a useful tool to study bacterial physiology coupled with the metabolic redox reaction generated by catabolism and anabolism. Biochemical reducing power generated by bacterial catabolism can be measured by electrochemical techniques of cyclic voltammetry and biofuel cell system. The BFCS has been employed to produce electricity from the coupling redox reaction of...
electron mediator and biochemical reducing power. The biochemical reducing power is not enough to produce applicable electricity, because the bacterial catabolism generated in the BFCS is much slower than the chemical reaction generated in chemical fuel cell systems for production of electron-driving force [14, 16]. However, the BFCS is useful to electrochemically analyze and compare reducing power generated by bacterial catabolism under a specific condition. Biochemical reducing power (NADH) is converted to electrochemical reducing power via a specific electron mediator (neutral red). The electrochemically reduced electron mediator on an anode surface is oxidized in coupling with reduction of oxygen (electron acceptor) on a cathode surface in the BFCS, by which electron-driving force (voltage) is generated in coupling with electron transfer (current) from anode to cathode [23, 28]. The amount of electrons transferred from the anode to the cathode is proportional to the catabolic activity of bacteria for generation of biochemical reducing power [4, 22].

In this research, we isolated a light-induced carotenoid-producing bacterium, *G. alkanivorans* SKF120101, and analyzed the metabolic function of the carotenoid using the electrochemical techniques of cyclic voltammetry and BFCS. Electrochemical reducing power generated by coupling with catabolism of SKF120101 was selectively increased by light. Electricity generation in the BFCS catalyzed by SKF120101 was significantly increased by light. On the basis of the light-induced metabolic activation of SKF120101, we suggest that the carotenoid produced by strain SKF120101 may catalyze regeneration of biochemical reducing power with light energy.

**Materials and Methods**

**Isolation and Cultivation of Bacterial Strain**

The medium used for isolation of SKF120101 was composed of 10 mM K$_2$HPO$_4$, 10 mM NaH$_2$PO$_4$, 1 g NH$_4$Cl, 3 g yeast extract/L, 20 g glucose/L, 0.01 g MgSO$_4$·7H$_2$O/L, 0.01 g FeSO$_4$·7H$_2$O/L and 1 L of tap water, and was also used for successive transfer culture and bacterial cultivation in the BFCS. Glucose and yeast extract were excluded from the medium for autotrophic cultivation of SKF120101 under light conditions. Biomass was evaluated by viable cell count, optical density at 660 nm, and dry cell weight. Seventy-two hour-cultivated bacterial cells were harvested and washed twice with distilled water by centrifugation at 5,000 × g and 4°C for 30 min. The washed cells were dried at 110°C until a constant weight was obtained for determination of dry cell weight, and lyophilized for extraction of reddish compound.

**Spectral Analysis of Carotenoid**

The lyophilized cells of SKF120101 were suspended in acetone and incubated in a 150 rpm shaker at 4°C for 60 min. Acetone-extracted reddish compound was obtained by centrifugation at 10,000 × g and 4°C for 30 min. The supernatant was spectrally analyzed in the range from 800 to 350 nm of wavelength using a spectrophotometer (Shimadzu UV-1601, Japan). Meanwhile, SKF120101 was cultivated on agar plate medium under dark and light conditions to easily compare the color variation induced by the lighting.

**Electrochemical Analysis of SKF120101**

Cyclic voltammetry was employed in order to analyze electrochemical redox reaction between the working electrode and the neutral red (NR) immobilized in bacterial cells. It was conducted using a voltammetric potentiostat (BAS CV50W, USA) linked to a data acquisition system. A glassy carbon electrode (BAS MF2013, USA), a platinum wire, and an Ag/AgCl electrode were utilized as a working electrode, counter electrode, and reference electrode, respectively. The reaction mixture was composed of 25 mM Tris-HCl buffer (pH 7.5), 5 mM NaCl, and intact cells of SKF120101 (OD$_{660}$ = 5.0) modified with NR [27]. Prior to use, the electrodes were cleaned with an ultrasonic cleaner, and the dissolved oxygen in the reaction mixture was purged by argon (99.999%) gassing. The scanning rate was 25 mVs$^{-1}$ over a range of -1200 to 0 mV. During cyclic voltammetry, variations of the upper and lower voltammograms generated by addition of glucose or light were saved in a PC using a data acquisition system.

**Preparation of Cathode**

A cathode (diameter 40 mm) was constructed of a cellulose acetate film (35 μm thickness; Electron Microscopy Science, USA), a ceramic membrane (2 mm thickness), and a porous carbon plate (10 mm thickness) as shown in Fig. 1B, which was prepared according to the methods used in previous research [19]. The cellulose acetate film was attached to the ceramic membrane using acetone vapor. The ceramic membrane and porous carbon plate are permeable to water, ions, and a variety of water-soluble compounds; however, a cellulose acetate film is semipermeable for selective transfer of gas, proton, and water molecule.

**Immobilization of NR in Graphite Felt Anode**

One% of polyvinyl alcohol (PVA, MW 89,000–98,000) solution absorbed into graphite felt (40 × 60 × 8 mm; Electro synthesis GF-S8, USA) was completely dried, by which PVA fiber was directly combined with graphite fiber and the PVA–graphite felt complex

**Fig. 1.** Single-cell-type electrochemical bioreactor (100 ml) for measurement of electricity generation from glucose under light or dark condition. The cathode functions as an electrode and electrolyte by catalyzing reduction of O$_2$ to H$_2$O.
was composed. NR was immobilized in the PVA–graphite felt complex by induction of a covalent bond between the amine of NR and the hydroxyl of PVA using thionyl chloride [13]. Water-soluble PVA was converted to water-insoluble PVNR by substitution of the hydroxyl of PVA by NR. The PVNR–graphite complex is a solid-type electron carrier that mediates electron transfer between bacterial cells and the graphite electrode.

Preparation of Biofuel Cell System
A single-cell-type BFCS with performance verified in previous research was employed [19]. The total and working volumes of the BFCS were 150 ml and 100 ml. The cathode outside (porous carbon plate) was exposed to the atmosphere, by which electrons originated from the PVNR–graphite anode and protons originated from anolyte were effectively reacted with O$_2$. Meanwhile, the cathode inside (cellulose acetate film) was brought into contact with anolyte, by which protons dissolved in anolyte were selectively transferred to the porous carbon cathode, as shown in Fig. 1A. A 15W light bulb was used for lighting the BFCS, and the light intensity was adjusted to ~5,000 lux.

Measurement of Electricity Generated by Harvested Cells
Freshly harvested cells of SKF120101 were used as a catalyst for the BFCS in order to verify results of cyclic voltammetry obtained using the harvested cells, but NR was immobilized in the graphite felt anode instead of bacterial cells. SKF120101 cultivated for 72 h under light condition (~5,000 lux) was harvested by centrifugation at 4°C and 5,000 ×g for 30 min and then suspended in 25 mM phosphate buffer (pH 7) containing 2 g glucose/L. The bacterial cell suspension (OD$_{660}$ = 5) prepared in the BFCS was incubated under light or dark condition at 30°C for 120 min, and the current generation was measured by turning on the switch between anode and cathode (Fig. 1). External resistance and the voltmeter were not employed to selectively compare the current generated under light and dark conditions.

Measurement of Electricity Generated by Growing Cells
Ten ml of SKF120101 culture previously cultivated for 48 h under light condition was inoculated into 90 ml of fresh medium prepared in the BFCS. The effect of lighting (~5,000 lux) on electricity (electric power) generation by catalysis of growing cells was estimated by actual measurement of both current and voltage, but not by calculation using the voltage measured based on the fixed value of external resistance. The BFCSs for dark cultivation of bacterial cells were doubly enveloped with aluminum foil and black plastic paper to completely block light. The anode and cathode were connected via an ammeter and a voltmeter by a closed circuit with 1,000 Ω of external resistance (Fig. 1).

Analysis of Glucose Consumption
Bacterial culture was centrifuged at 12,000 ×g and 4°C for 20 min, followed by filtration through a 0.22 μm membrane filter (Sartorius, Germany). Fifty μl of filtered culture was subsequently injected into the HPLC injector. Glucose was analyzed using an HPLC apparatus (Younglin Instrument, Korea) equipped with an ion exchange column (Bio-Rad Aminex HPX-87H, USA) and a refractive index detector (Younglin Instrument Acme9000, Korea). The column and detector were adjusted to a temperature of 35°C. A 0.008N H$_2$SO$_4$ solution was used as the mobile phase of which the flow rate was adjusted to 0.8 ml/min.

RESULTS
Isolation of Non-Photosynthetic Bacterium Gordonia alkanivorans SKF120101
Gordonia alkanivorans SKF120101 was isolated, using the specified medium, from a heterotrophic bacterial community cultivated in organic fertilizer-manufacturing culture and identified based on 16S rDNA sequence homology, which was registered in the GenBank database system under the accession code JQ964108.

Induction of Reddish Compound and Its Spectral Analysis
The lighting for colonies of SKF120101 growing on agar plate induced bacterial cells to be reddish, as shown in Fig. 2. The reddish pigment extracted from the reddish cell was spectrally analyzed in the range from 800 to 350 nm. The absorption maxima at 452, 478, and 506 nm are characteristic of the typical carotenoid spectrum as shown Fig. 2. Color variation of growing cells of G. alkanivorans SKF120101 cultivated under light (left) and dark (right) conditions. White spots are air bubbles generated inside the agar layer.

Fig. 2. Color variation of growing cells of G. alkanivorans SKF120101 cultivated under light (left) and dark (right) conditions. White spots are air bubbles generated inside the agar layer.

Fig. 3. Spectrum of the reddish compound extracted from lyophilized G. alkanivorans SKF120101, using acetone.
in Fig. 3, which is very similar to the three absorption maxima at 450, 474, and 504 nm of dehydro-β-carotene [12]. The difference of the absorption maxima between the reddish pigment and dehydro-β-carotene may be caused by a difference of solvent used for extraction [26].

**Effect of Light on Growth of *Gordonia alkanivorans* SKF120101**

The selective reddish color of SKF120101 cultivated under light condition (Fig. 2) may be coupled to primary metabolism, because its growth and glucose consumption were greatly activated under the light condition as shown in Table 1. The viable cell number was about 10 times higher and growth yield was about 1.4 times higher under light than dark conditions. SKF120101 was not autotrophically grown in the medium without glucose and yeast extract under light condition. It may be presumed that the growth yield of SKF120101 may be lower under light conditions than the dark, because extra energy may be consumed for biosynthesis of carotenoid coupled to scavenging oxygenic radicals. The growth yield was different from the presumption. The differences of growth yield is a clue that carotenoid produced in SKF120101 may catalyze extra reducing power production from light energy.

**Effect of Light on Metabolic Reaction of SKF120101**

In cyclic voltammetry for NR immobilized in bacterial cells, upper and lower voltammograms are shifted downward coupled to bacterial catabolism, but are shifted upward coupled to bacterial anabolism [27]. The upper and lower voltammetric peaks for NR immobilized in bacterial cells were not shifted without external energy source (Fig. 4A). Meanwhile, both upper and lower voltammetric peaks were shifted downward by addition of glucose (Fig. 4B) and light (Fig. 4C). These results are a clue that biochemically reduced NR (NRred) coupled to metabolic oxidation of glucose and by the light is electrochemically oxidized to NRox coupled to current generation (Y-axis and arrow marks in Fig. 4). The higher variation of upper voltammetric peaks than of lower ones is caused by the higher biochemical reduction of NRox to NRred than

<table>
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<th>Analytical factors</th>
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<th>Dark</th>
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<td>Dry cell weight (g/L)</td>
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<td>Viable cells (CFU)</td>
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<td>2.8 × 10⁹</td>
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<td>Glucose consumption (mM)</td>
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<tr>
<td>Autotrophic cultivation (w/o glucose and YE)</td>
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<td>Not grown</td>
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Light intensity was adjusted to 5,000 lux with a 15 watt incandescent light, and the bacterial culture was aerated by 150 rpm of rotary shaking.

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**Table 1. Growth of *G. alkanivorans* SKF120101 autotrophically cultivated in GA medium without glucose and yeast extract (YE) or heterotrophically cultivated in GA medium under light or dark conditions for 72 h.**

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Fig. 4. Cyclic voltammetry for NR immobilized in *G. alkanivorans* SKF120101. (A) Cyclic potential scanning from -1,200 to 0 mV under the dark condition. Both upper and lower voltammetric peaks for the NR shift gradually downward (arrow marks) under dark condition with glucose (B) and under light condition without glucose (C).
METABOLIC ROLES OF CAROTENOID PRODUCED BY G. ALKANIVORANS

Electrochemical oxidation of NR_red to NR_ox. Meanwhile, the variation of redox potential (-0.52 V vs. Ag/AgCl) of the voltammetric peaks was not generated in cyclic voltammetry performed under three different conditions. It shows that biochemical and electrochemical redox reactions were definitely generated coupled to redox reaction of NR.

Electricity Generation by Freshly Harvested Cells of SKF120101
Light for intact cells of SKF120101 activated electron transfer from the bacterial cells to the electrode in coupling with the redox reaction of NR immobilized in bacterial cells according to the cyclic voltammetry (Fig. 4C). The current generation in the BFCS (Fig. 1) catalyzed by freshly harvested cells of SKF120101 was an average 1.4~1.6 times higher under the light condition than the dark condition, as shown in Fig. 5. Time-coursed decrease of current is caused by a difference between the biochemical generation and electrochemical consumption of reducing power. The biochemical reducing power is limitedly generated in proportion to metabolic activity, substrate concentration, and metabolite production, but electrochemical reducing power is consumed limitlessly without external resistance for control of electron flow from the anode to the cathode. From this result, it can be assumed that the SKF120101 cultivated under the light condition may produce the extra biochemical reducing power from light energy by catalysis of carotenoid.

Electricity Generation by Growing Cells of SKF120101
The BFCS is generally operated by growing cells of bacteria, because the biochemical reducing power is generated by primary metabolism coupled to reducing power regeneration. The electricity generation was about 2.5 times higher but glucose consumption was about 1.8 times higher under the light than dark condition as shown in Fig. 6. The ratio of electricity generation efficiency (2.5) to glucose consumption activity (1.8) was about 1.4 times, which was similar to the growth yield (Table 1) and current generation by harvested cells (Fig. 5). This result may be an undiscovered biological phenomenon, that light-induced biosynthesis of carotenoid in G. alkanivorans SKF120101 is metabolically coupled not only to additional production of reducing power but also to increase of metabolic activity (Table 1).

DISCUSSION

Carotenoid is an accessory light-harvesting pigment located in the cell membranes of all photosynthetic organisms and is essential for the survival of photosynthetic organisms [8, 20]. Carotenoid excited by absorption of blue light energy (Fig. 3) induces excitation of chlorophyll by rearrangement of electronic levels in the light-harvesting complex, and protects chlorophyll from photodynamic reaction by transferring its excitation energy to singlet oxygen in plants, algae, and photosynthetic bacteria [2]. The carotenoid of the non-photosynthetic bacteria was seen to be maintained by evolutionary conservation of enzymes catalyzing the earliest biosynthetic reactions [30]. The physiological function of carotenoid synthesized by non-photosynthetic bacteria has been known to be an oxygenic radical scavenger and protector against toxic molecules generated by photodynamic reaction [34]. The cellular location of carotenoid pigments produced by the non-photosynthetic bacteria was identified to be the cell wall and membrane, which has structural similarity with those of the

Fig. 5. Electricity generation by freshly harvested cells of G. alkanivorans SKF120101 in 25 mM phosphate buffer containing 10 mM glucose under light (solid symbol) and dark conditions (open symbol). The anode and cathode were connected via an ampere meter by closed circuit without external resistance.

Fig. 6. Electricity generation by anaerobically growing cells of G. alkanivorans SKF120101 in M9 medium supplemented with glucose coupled to glucose consumption under light (solid symbol) and dark conditions (open symbol).
photosynthetic bacteria, although this is not useful for understanding the metabolic function of carotenoid [25].

The metabolic function of carotenoid in photosynthetic organisms is to catalyze transfer of the excitation energy to chlorophyll in the light-harvesting complex, by which electron-driving force is generated in the electron transport system coupled to the photosynthetic reaction center [35]. The linear system of conjugated C=C bonds of carotenoid provides high reducing potential, making itself potent to be electrochemically excited. The biochemical reducing power generated in the photosynthetic reaction center can be converted to electrochemical reducing power via a proper electron mediator by using a BFCS [32]. This is a useful technique to evaluate the metabolic function of light-harvesting pigments such as are chlorophylls and carotenoids. The electrochemical reducing power generated in a BFCS catalyzed by photosynthetic bacteria can be measured using an amperemeter (current). Practically, the electric current is produced in the BFCS catalyzed by photosynthetic bacteria and the current production is more increased under light than dark conditions [7]. Light-activated increase of electric current is caused by the coupling redox reaction of electron carriers and photosynthetic pigments excited by light energy. This is a very reasonable phenomenon based on the light-harvesting function of photosynthetic bacteria, but is an unusual phenomenon for non-photosynthetic bacteria without the systematic light-harvesting apparatus.

The redox reaction of NR immobilized in intact cells of SKF120101 may not be induced by light, considering the non-photosynthetic metabolism (non-autotrophic growth) of the SKF120101. However, the light for intact cells of SKF120101 induced electron flow from bacterial cells to the working electrode (Fig. 4) and activated electricity generation in the BFCS (Fig. 5, 6). Theoretically, metabolic oxidation of glucose is coupled to regeneration of NAD(P)H in glycolysis and the TCA cycle, and the regenerated NAD(P)H may be oxidized in coupling with biochemical reduction of NR [27], which is converted to electrochemical reducing power by anodic reaction in the BFCS. Accordingly, the carotenoid selectively produced by SKF120101 under light condition may be a light-induced pigment capable of catalyzing the generation of auxiliary reducing power from light energy. It is completely different from the light-harvesting metabolism of purple and green bacteria. Electron-driving force is generated from photosynthetic pigments excited by electromagnetic energy of light, and the electron donors for regeneration of reducing power (NADPH) are hydrogen, ammonium ion, and ferrous ion in the electron transport system of purple and green bacteria. In this study, we evaluated the metabolic function of carotenoid produced by G alkanivorans SKF120101 by using cyclic voltammetry and the BFCS, and obtained a positive result that a carotenoid may catalyze regeneration of auxiliary biochemical reducing power with light energy under only heterotrophic growth condition.

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