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Bactericidal Effect of 405-nm LED Light against *Aggregatibacter Actinomycetemcomitans* Is Due Primarily to Disruption of Respiratory Chain Terminal Members Cytochrome *bd* Oxidase and Quinol Peroxidase

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Irradiation with 405-nm visible violet LED light without additional photosensitizers decreased the viability of the aggressive periodontopathic bacterium *Aggregatibacter actinomycetemcomitans*. The number of CFU/mL decreased linearly on a logarithm chart versus irradiation time, with a 1-log reduction time of 1.32 min. The antimicrobial photodynamic effect of 405-nm LED light involved inhibition of the activity of membrane-bound cytochrome *bd*, a terminal quinone: oxygen oxidoreductase, and quinol peroxidase, a terminal quinone:H_2O_2 oxidoreductase. The 405-nm LED irradiation reduced minus oxidized difference spectrum showed that the 640-nm peak (α-peak of heme *d*) completely disappeared, and the height of the 556-nm (α-peak of hemes *b* and *c*) and Soret band (425 nm; γ-peak of hemes *b*, *c*, and *d*) was reduced to approximately half of the peak heights of non-irradiated controls. Survival of bacteria-injected silkworm larvae was also examined. Fifth-instar silkworm larvae were almost completely killed by approximately 40 h after bacterial injection, but almost all silkworm larvae irradiated with 405-nm LED light (20 mW/cm² for 5 min, energy density: 6 J/cm²) survived, similar to controls not injected with bacteria, indicating that 405-nm LED light killed the injected bacteria. The bactericidal effect of 405-nm blue-light on *A. actinomycetemcomitans* is primarily due to disruption of cytochrome *bd* oxidase and quinol peroxidase of the respiratory chain.

Key words aggressive periodontitis, cytochrome *bd*, quinol peroxidase, respiratory chain, *Aggregatibacter actinomycetemcomitans*

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a facultative anaerobic, carbon dioxide–requiring, gram-negative bacterium in the gamma subdivision of the proteobacteria.[1)] This organism is a human pathogen associated with localized aggressive periodontitis (LAP), a severe disease in adolescents characterized by rapid bone and tissue destruction and ultimately loss of teeth.[2)] Moreover, *A. actinomycetemcomitans* is a member of the HACEK group (*Haemophilus* spp., *A. actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*) of gram-negative bacteria that can cause infective endocarditis and is responsible for ~3% of cases of this disease.[3)] The organism also causes abscesses in different parts of the body, including the brain.[4,5)]

Systemic administration of antibiotics confers clinical benefits in the treatment of LAP.[6)] However, many systemic antibiotic therapies are unable to consistently suppress subgingival *A. actinomycetemcomitans* to undetectable levels, and single-agent systemic therapies using metronidazole or tetracycline can markedly reduce but not eradicate oral *A. actinomycetemcomitans*.[6)] Alternative approaches for reducing numbers of oral microorganisms and ameliorating aggressive periodontitis, such as lethal photosensitization using photosensitizers, including methylene blue, toluidine blue, or phenothiazine chloride, in conjunction with visible light (660 nm – 690 nm), have been reviewed.[7)] In this refer, authors suggested that the antimicrobial photodynamic therapy using extrinsic photosensitizer is not significant additional benefit for patients with aggressive periodontitis, if compared to scaling and root planning alone. Recently, Cieplik *et al*. demonstrated that *A. actinomycetemcomitans* can be killed by irradiation with blue light (460 nm) derived from an LED source without the need for an exogenous photosensitizer.[8)]

In this study, we examined the bactericidal effect against *A. actinomycetemcomitans* of irradiation with 405-nm LED light without a photosensitizer. Our results indicate that the terminal quinol oxidase, cytochrome *bd*, and terminal quinol peroxidase (QPO) of the respiratory chain are damaged by irradiation with 405-nm LED light, resulting in a dramatic reduction in bacterial viability.

MATERIALS AND METHODS

Bacterial Growth Conditions *Aggregatibacter actinomycetemcomitans* (American Type Culture Collection ATCC29522, purchased from Summit Pharmaceuticals International, Tokyo, Japan) was cultured in minimal broth containing 3% polypeptone (Becton Dickinson, Franklin Lakes, NJ, USA), 1.2% yeast extract (Becton Dickinson), 0.04% NaH-

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CO_3, and 1% fructose at 37°C in an atmosphere containing 5% CO_2. Brain-heart infusion (BHI, Becton Dickinson) agar plates containing 0.5% yeast extract were used to determine the number of colony forming units (CFU).

Preparation of Membrane Vesicles Bacterial cells in late exponential phase were collected by centrifugation at 12,000 × *g* for 15 min. All subsequent steps were performed at 4°C. The resulting pellet was washed with 33 mM Tris-HCl (pH 7.5) and then resuspended in fresh 10 mM Tris-HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride. After passage through in a French-press cell destructor (Ohtake Works LTD., Tokyo) at 16,000 psi, unbroken cells were removed by centrifugation at 12,000 × *g* for 15 min. The disrupted lysate was then centrifuged at 60,000 × *g* for 1 h. The resulting pellet was washed twice with 10 mM Tris-HCl (pH 7.5) and finally resuspended in the same buffer. The membrane vesicle preparation was used for subsequent experiments.

Assays of Viability of Intact Bacteria and Activity of Membrane Vesicles After 405-nm LED Light Irradiation Bacterial cells in late exponential phase were collected by centrifugation at 12,000 × *g* for 15 min, washed twice with phosphate-buffered saline (PBS), and finally suspended with PBS. The resulting bacterial suspension was added to the wells of a 96-well microplate (200 μL per well) (multiple-well plates, polystyrene flat-bottom) and subjected to LED light irradiation. As indicated in Figure 1A, the LED (405 nm) irradiator (THORLABS M405 L3, Thorlabs Inc., NJ, USA) used in the study has a 0.6-cm-diameter hole in the top black acrylic board. The LED light radiates to the bottom of the microplate through the hole, and the power density of the LED light was set to 20 mW/cm^2 at the top board upper surface by use of a power sensor (THORLABS S302C, Thorlabs Inc.). A non-contact thermometer was used to confirm that there was no temperature increase in the samples during irradiation. The LED-treated bacterial suspension in each well was appropriately diluted with PBS and spread into BHI agar plates, and the number of CFU/mL was determined after overnight cultivation. For the irradiation of membrane vesicles (200 μL each sample), the same method was used as indicated in Fig. 1A. For the irradiation of growing bacteria, the absorbance at 650 nm of the growth medium (200 μL per well) in a 96-well microplate was measured after a 1-min exposure to 405-nm LED light (20 mW/cm^2 for 1 min; energy density: 1.2 J/cm^2) every hour.

Respiratory Chain of *A. Actinomycetemcomitans* In our previous report,[9] we speculated that type 2 NADH dehydrogenase (NDH-2) and Na-translocating NADH quinone oxidoreductase (NQR) would be present as NADH:quinone oxidoreductase in the electron-input side of the respiratory chain. Furthermore, cytochrome *bd* and QPO would be present as quinone:O_2 oxidoreductase and quinone:H_2O_2 oxidoreductase, respectively, in the electron-output side of the respiratory chain of *A. actinomycetemcomitans* (Fig. 2).

Assay of Oxidase Activity Ubiquinol-1:O_2 oxidoreductase (Q_1H_2 oxidase) activity was assayed spectrophotometrically as described previously.[10] The activity of NADH:O_2 oxidoreductase (NADH oxidase) was measured according to the method of Kasahara and Anraku[11] using a Clark-type oxygen electrode (Rank Brothers, Cambridge, England). The reduced form of ubiquinone-1 (ubiquinol-1) was prepared as described by Rieske.[12]

Assay of NADH Dehydrogenase Activity NADH: ubiquinone-1 oxidoreductase (NADH-Q_1 dehydrogenase) activity was assayed according to the method of Hatefi,[13] with slight modifications. The buffer used to assay the total activity of NDH-2 and NQR was 50 mM Tris-HCl (pH 7.5) containing 2 mM KCN (inhibitor of cytochrome *bd*) and 300 mM NaCl. NQR activity is enhanced in 300 mM NaCl, but NDH-2 activity is not affected by NaCl. Therefore, only NDH-2 activity was measured in the absence of NaCl, and the total NADH dehydrogenase activity of NDH-2 and NQR was measured in the presence of 300 mM NaCl.

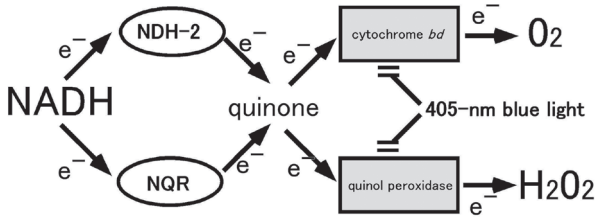

Fig. 1. Proposed Model of the Electron Flow and Sites of the Respiratory Chain of *A. Actinomycetemcomitans* That Are Inhibited by Irradiation

The distribution of NADH:quinone oxidoreductase and terminal quinol:oxygen oxidoreductase was deduced from data obtained from the KEGG GENOME Database, as indicated in reference.[9] The enzymes inhibited by LED (405 nm) blue light include cytochrome *bd* and quinol peroxidase, as determined in this study. Quinol peroxidase (QPO) was purified and characterized by the authors in a previous study.[14] NDH-2:type 2-NADH dehydrogenase, NQR: Na-translocating NADH quinone oxidoreductase.

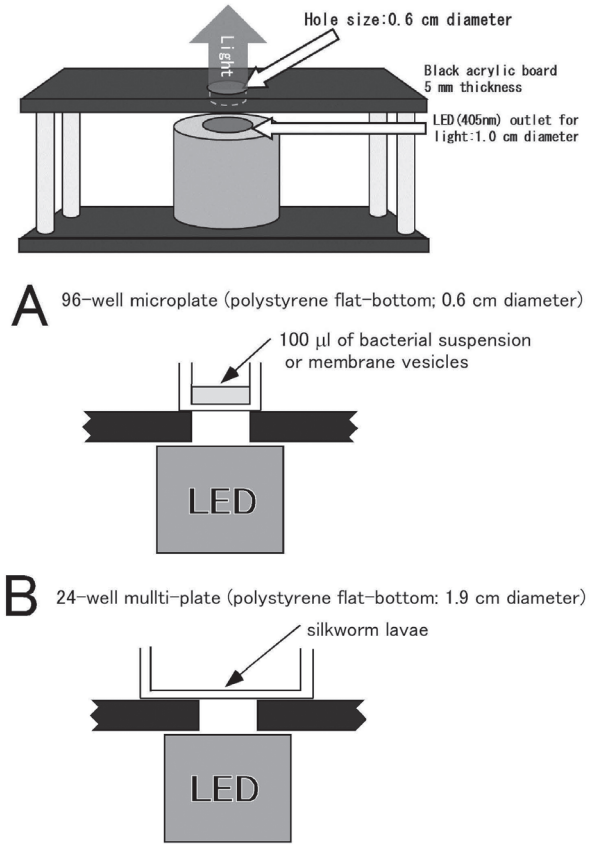

Fig. 2. Overview and Cross-Sectional Schematic Diagram of The Experimental Set-Up for 405-nm Blue-Light Irradiation of Bacterial Suspensions or Membrane Vesicles (A) and Silkworm Larvae (B)

Analysis of Redox Difference Spectra Reduced minus oxidized difference spectra of French press membrane vesicles were measured at 25°C in a quartz cell with a 1-cm light path using a DW-2 UV-VIS spectrophotometer (AMINCO, Silver Spring, MD, USA). Full reduction was carried out with sodium dithionite ($Na_2S_2O_4$).

Assay of Quinol Peroxidase The ubiquinol-1:H_2O_2 oxidoreductase activity of QPO was measured at 25°C under anaerobic conditions.[14]

LED Light Irradiation of Silkworms The effect of bacterial injection on silkworm growth was examined using a previously described method, with slight modifications.[15,16] Eggs of silkworms (*Bombyx mori*, Hu · Yo × Tukuba · Ne) were purchased from Ehime Sanshu (Ehime, Japan). Silkworm larvae were maintained on an artificial diet (Silkmate 2S; Nihon Nosan, Yokohama, Japan) at 27°C. On the first day of the fifth-instar stage, the larvae were fed for 1 day with an artificial, antibiotic-free diet (Silkmate; Katakura Industries Co., Ltd., Tokyo). The hemolymph of day 2 larvae was injected through the dorsal surface with 50 µL of a suspension of *A. actinomycetemcomitans* (1×10^8 /mL) in PBS(−) containing 100 µM ammonium ferric citrate and 2.5% mucin. After 1 h, larvae were irradiated with 405-nm LED light for 5 min at 20 mW/cm^2 (dose: 6 J/cm^2 ; this dose should have no influence for the viability of silkworm larvae, selected by preliminary experiments) in a 24-well multi-well plate instead of 96-well plate, as shown in Fig. 2B. The larvae were maintained at 37°C in plastic Tupperware tray and not fed after the injection. Survival was monitored at indicated times.

Statistical Analysis All assays except the silkworm viability assay were performed as two independent experiments (biological replicates), each consisting of duplicate reactions (technical replicates; total n = 4) or triplicate reactions (total n = 6). Unless otherwise indicated, values are presented as the mean ± standard deviation (SD). The statistical significance of differences was determined using two-tailed, non-paired Welch's *t* tests. LED treatment values were compared with those of non-treated controls. Significant differences ($p<0.05$) are indicated by an asterisk (*). Data regarding LED light irradiation of silkworm larvae were plotted using the Kaplan-Meier method and tested for significance using the log-rank test. All data were examined using StatMate software (Atms Co. Ltd., Tokyo, Japan).

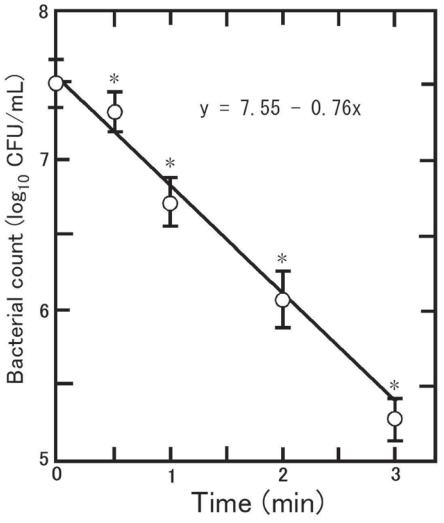

Fig. 3. Inactivation of *A. Actinomycetemcomitans* Suspended in PBS by Irradiation with 405-nm Blue Light at an Irradiance of 20 mW/cm^2 as a Function of Time

Asterisks indicate statistically significant differences compared with non-exposed control bacteria ($p<0.05$). Each data point represents the mean ± SD (n=4). The D value (decimal reduction value, or required time for a 1-$\log_{10}$ reduction) was 1.32 min, as determined by linear regression analysis.

RESULTS

The viability of *A. actinomycetemcomitans* cells suspended in PBS was analyzed by monitoring the colony-forming capacity after blue-light irradiation. As indicated in Fig. 3, the number of CFU/mL of bacterial suspension decreased linearly on a semi-logarithmic plot with time of irradiation with 405-nm LED light at 20 mW/cm^2 irradiance. The D value (decimal reduction value) was 1.32 min as determined by linear regression analysis, indicating that the required energy density for a 1-$\log_{10}$ reduction was 1.584 J/cm^2 ($20 \times 1.32 \times 60/1000$). Bacterial viability was not affected by irradiation with 455-nm or 470-nm LED light at the same energy density as the 405-nm LED light irradiation (data not shown).

The activity of respiratory chain enzymes was examined using membrane vesicles. NADH oxidase activity was significantly reduced after 405-nm LED irradiation compared with the control (Fig. 4A). The Q_1H_2-oxidase activity was dramatically reduced, corresponding with the reduction in NADH oxidase activity (Fig. 4C). Irradiation with 405-nm LED blue light did not affect the activity of the *A. actinomycetemcomitans* respiratory chain enzymes NDH-2 or NQR, however (Fig. 4B). Furthermore, the quinol-1:H_2O_2 oxidoreductase activity of QPO declined dramatically following irradiation with 405-nm LED light (Fig. 4D).

The reduced minus oxidized difference spectra of French press membrane vesicles are shown in Fig. 5. A peak at 640 nm, indicating the α-band of heme *d*, was observed in the spectra of non-irradiated membrane vesicles (Fig. 5A), but this peak was absent in the spectra of membrane vesicles irradiated with 405-nm LED light (20 mW/cm^2 for 3 min; dose: 3.6 J/cm^2) (Fig. 5B). The intensity of peaks at 556 nm (α-band of hemes *b* and *c*) and 425 nm (Soret-band, or γ-band, of hemes *b*, *c*, and *d*) decreased to approximately half of that of these peaks in control vesicles. These suggest that cytochrome *bd* and QPO would be inactivated due to chemical modification of heme *b* and heme *d* of cytochrome *bd* and heme *c* of QPO.

The effect of blue-light irradiation on the growth of cultured *A. actinomycetemcomitans* was also examined. The absorbance of the bacterial culture medium was measured at 650 nm following irradiation with 405-nm LED light (20 mW/cm^2 for 1 min, energy density: 1.2 J/cm^2) at the time points indicated by arrows in Fig. 6. The growth of irradiated bacteria was significantly suppressed in terms of absorbance at 650 nm when compared with non-irradiated controls.

The survival of silkworm larvae injected with *A. actinomycetemcomitans* was also examined (Fig. 7). Immediately after bacterial injection, fifth-instar silkworm larvae were irradiated with 405-nm LED light (20 mW/cm^2 for 5 min, energy density; 6 J/cm^2) or left untreated as a control (9 of 10 silkworms died within 40 h). The survival of silkworm larvae irradiated with 405-nm LED light was similar to that of control larvae not injected with bacteria, indicating that the bacteria injected into the larvae were killed by the 405-nm LED light (6 J/cm^2).

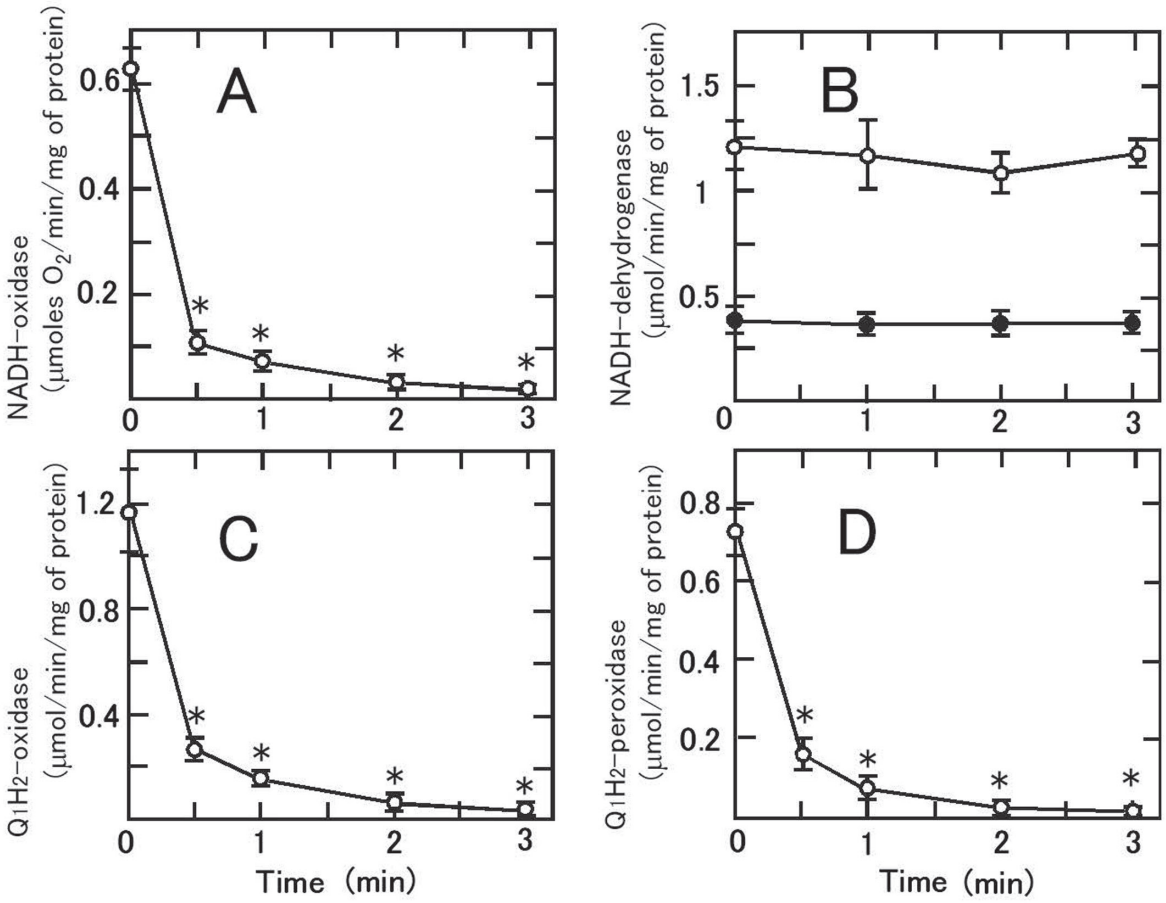

Fig. 4. Inactivation of the Respiratory Activity of Membrane Vesicles Irradiated with 405-nm Blue Light at an Irradiance of 20 mW/cm^2 as a Function of Time

A: NADH oxidase activity. B: NADH-dehydrogenase activity in the presence of 300 mM NaCl (open circles) and in the absence of NaCl (closed circles). C: Q_1H_2-oxidase activity. D: Q_1H_2-peroxidase activity. Asterisks indicate a significant difference in activity following irradiation compared with non-irradiated control activity ($p<0.05$). Each data point represents the mean ± SD (n=6).

DISCUSSION

The antimicrobial effects of 405-nm blue light have been increasingly reported over the last two decades, and numerous studies have examined the potential of this light technology for decontamination and infection control applications (see reviews[17,18]). In those reviews, almost all bacteria should be reduced by 405 nm irradiation. The 470 nm irradiation is also appropriate for photoinactivating all bacteria species investigated so far but compared to 405 nm illumination it is less effective by a factor between 2 and 5. Authors suggested that intrinsic photosensitizers would be porphyrins such as cytochromes for 405 nm light, and flavins such as FAD for 470 nm light.[17,18] Bactericidal violet-blue 405-nm light is reportedly safer for mammalian cells such as osteoblasts,[19] fibroblasts,[20] keratinocytes,[21] and HaCat cells,[22] and it is also reportedly safe for mice.[21,22] Visible light irradiation without photosensitizers has also been applied for the control of oral periodontopathic bacteria. Non-coherent visible light LED (400-520 nm) exhibits phototoxic effects against *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.[23–25] Soukos *et al*. reported that broad-band light (380 to 520 nm) killed oral black-pigmented bacteria, including *P. gingivalis* and *Prevotella intermedia*, in pure cultures and in dental plaque samples obtained from human subjects with chronic periodontitis.[26] The growth of *P. gingivalis* was specifically suppressed by irradiation with 405-nm blue light.[27,28] The inactivation of bacteria by irradiation with visible light is caused by singlet oxygen produced by the photo-stimulation of endogenous intracellular porphyrin molecules, as reported for bacteria such as *Propionibacterium acnes*, *Helicobacter pylori*, and some black-pigmented bacteria.[29–32] Cieplik *et al*. demonstrated that *A. actinomycetemcomitans* could be killed by irradiation with blue LED light (460 nm) without an exogenous photosensitizer.[8] The authors speculated that blue light (460 nm) induces the photosensitization of intracellular flavins as intrinsic photosensitizer, the stimulation of which leads to the production of reactive species, predominantly singlet oxygen.

In this study, we found that irradiation with 405-nm blue light decreases the viability of *A. actinomycetemcomitans* without the need for an exogenous photosensitizer. The mechanism of the antimicrobial photodynamic effect of 405-nm LED blue-light irradiation involves the inhibition of a membrane-bound terminal oxidase, cytochrome *bd*, and a terminal peroxidase, QPO, both of which are members of the respiratory chain of *A. actinomycetemcomitans*. Cytochrome *bd* is expressed by many bacteria, and the properties of the enzyme of *Escherichia coli* have been well described.[33,34] Cytochrome *bd* is composed of 3 polypeptides, 2 heme *b* and one heme *d* as the prosthetic group. The QPO of *A. actinomycetemcomitans* was characterized in our previous study.[14] QPO is essential for the secretion of leukotoxin, which destroys leukocytes

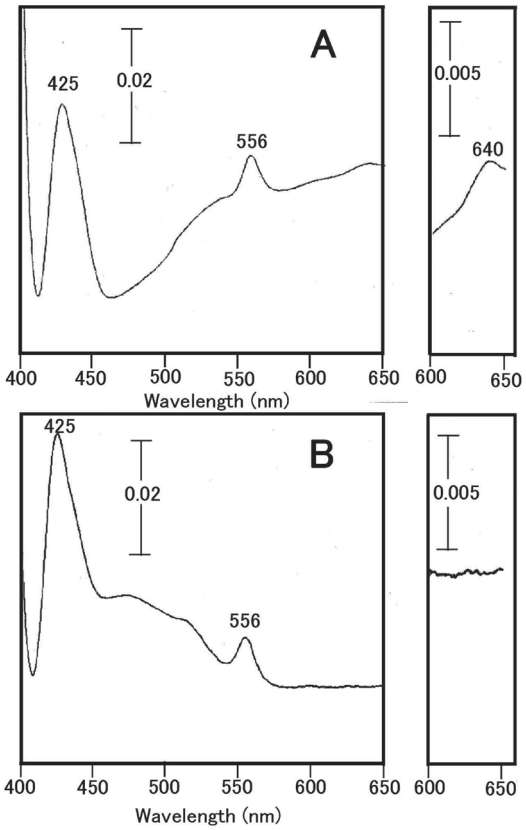

Fig. 5. Comparison of Reduced Minus Oxidized Difference Spectra for the Inactivation of Membrane Vesicles Irradiated with 405-nm Blue Light at an Irradiance of a 20 mW/cm² for 3 min (Energy Density: 3.6 J/cm²) (4.8 mg/mL, B), as Compared with Control Reduced Minus Oxidized Difference Spectra for Non-Irradiated Vesicles (2.4 mg/mL, A)

As the intensity of the α-peak (640 nm) of cytochrome *d* was extremely low, twice as much blue-light irradiated sample relative to the control was added to establish the disappearance of the α-peak of heme *d*.

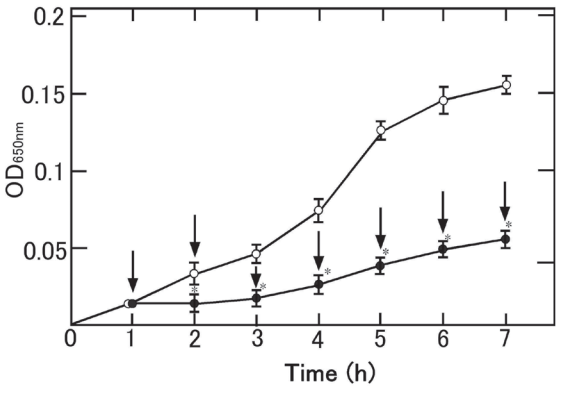

Fig. 6. Absorbance at 650 nm of Growing *A. Actinomycetemcomitans* Following Irradiation with 405-nm Blue Light (20 mW/cm² for 1 min at The Time-Point Indicated by The Arrow; Closed Circles) or without LED Irradiation (Open Circles)

Asterisks indicate a significant decrease in absorbance reading compared with the equivalent non-exposed control bacterial sample. Each data point represents the mean ± SD (n=6).

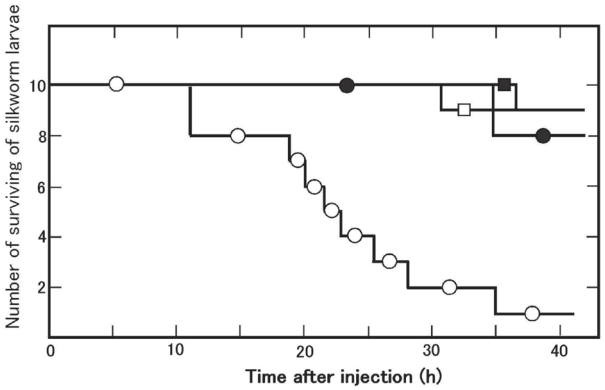

Fig. 7. Survival of Silkworm Larvae Injected with *A. Actinomycetemcomitans*

Fifth-instar larvae of silkworms (n=10) were irradiated with 405-nm LED blue light (5 min, 20 mW/cm²; closed circles) or not irradiated (open circles) immediately after bacterial injection. The survival of silkworm larvae irradiated with LED blue light but without bacterial injection (closed squares) or without LED irradiation with PBS injection (open square) was examined as a control.

and erythrocytes in humans and is a major LAP-associated virulence factor of *A. actinomycetemcomitans*.[35,36] We hypothesize that these two cytochrome-containing enzymes absorb the 405-nm blue light via the hemes, the endogenous auto-photosensitizer, and produce singlet oxygen, which in turn destroys the cytochromes. This results in inhibition of the bacterial respiratory chain and the production of reactive oxygen species that diminish bacterial growth and viability. Using silkworm larvae as an animal model, we also found that LED blue-light irradiation efficiently kills bacteria within the larvae. Taken together, we indicated that periodontopathic bacteria are much susceptive to 405 nm-LED light compared to the results of review references.[17,18] The bacteria infected-animal (silkworm larvae) is recovered by 405 nm illumination from outside of animal, indicating that blue-light can pass through the organs or cells in the presence of much biological materials, and finally killed *A. actinomycetemcomitans*. This result is to be expected to adapt to the therapy for brain abscesses and endocarditis, in addition to aggressive periodontitis, by use of 405 nm LED attached-catheter.

Conflict of interest The authors declare no conflict of interest.

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