The importance of preventing infectious disease for public health continues to increase, and effective disinfectants are needed to inactivate pathogenic microorganisms. Chlorine dioxide (ClO₂) is known as one of the most efficient disinfectants. We studied the inhibitory effect of a novel disinfectant, MA-T, for three species of bacteria (Escherichia coli, Staphylococcus aureus, and Aggregatibacter actinomycetemcomitans). We found that NADH:O₂ oxidoreductase activity (NADH oxidase activity) was markedly decreased in all three species, corresponding to the decrease in colony-forming units following treatment with MA-T. In E. coli, NADH:ubiquinone-1(Q₁) oxidoreductase (NADH-Q₁ dehydrogenase; NDH) activity was decreased following MA-T exposure, indicating that both the NDH-1 and NDH-2 enzymes were targets of this disinfectant. The activity of ubiquinol-1 (Q₃H₂): O₂ oxidoreductase (Q₃H₂ oxidase) also was decreased, indicating that cytochromes b₅₃ and bd were damaged by MA-T. In S. aureus, NADH-ferricyanide dehydrogenase activity and Q₃H₂ oxidase activity were strongly decreased, suggesting that NDH-2, cytochrome bd, and cytochrome aa₃ were targets of MA-T in this species. In A. actinomycetemcomitans, only Q₃H₂ oxidase activity was decreased, indicating that in this species, only cytochrome bd was impaired by MA-T treatment. NADH oxidase activity in membrane vesicles prepared from untreated E. coli was not markedly affected by treatment with MA-T, suggesting that MA-T may attack components of the respiratory chain only in live bacteria (i.e., those possessing a membrane potential), because the membrane vesicles cannot produce the membrane potential.

Key words respiratory chain, disinfectant, terminal oxidase, NADH dehydrogenase

INTRODUCTION

The importance of mitigating the spread of infectious disease for public health has continued to increase over the last several decades. Many examples of emerging and re-emerging infections have appeared, including methicillin-resistant Staphylococcus aureus-derived diseases, drug-resistant tuberculosis, cholera by Vibrio cholerae, hemorrhagic colitis, and drug-resistant Salmonella typhi. In the food industry, drug-resistant Salmonella typhi and Escherichia coli are potent chlorinating agents, and chlorine dioxide, a reagent with strong oxidizing activity, is one of the most efficient disinfectants. The use of chlorine dioxide as a preoxidant, instead of chlorine, is known to have the benefit of minimizing trihalomethane production following chlorination. The solubility of chlorine dioxide is five times that of chlorine and the oxidation activity of chlorine dioxide is about three-fold that of chlorine gas. The ClO₂ radical is a powerful one-electron oxidant, possessing a redox potential of 936 mV, and is known for its ability to oxidize both inorganic and organic species. Chlorine dioxide also is known for its anti-bacterial and anti-viral properties. Chlorine dioxide is capable of triggering the denaturation of enzymes and proteins. ClO₂ destroys the anabolic pathways of protein and thus kills microorganisms, including bacteria, viruses, and fungi.

MA-T (α or γ; A2-care, Co., Ltd., Tokyo), a commercially available disinfectant, is a stable and mild ClO₂-generating reagent. MA-T contains sodium chlorite, in combination with one of two kinds of cationic detergents that serve as the Lewis acid catalyzing the generation of ClO₂ (the Lewis acidity of both were (α: LUMO (Lowest Unoccupied Molecular Orbital) = -4.12 eV) and (γ: LUMO = -4.02), measured according to the method of reference), in unpublished observation), in a buffer stabilizing the solution at a neutral pH. Notably, ClO₂ is not detected in MA-T during storage or before use. Live bacteria, the targets of this disinfectant, would induce the production of ClO₂ by MA-T. The mechanism by which this chlorine dioxide-generating disinfectant, MA-T, destroys bacteria is of great interest, but is currently unclear. In this paper, we report data indicating that one of the targets of MA-T is the respiratory chain of bacteria.

MATERIALS AND METHODS

Bacterial Growth Conditions Escherichia coli (W3110, derived from the K-12 strain) was shake-cultured at 37°C in BHI (Bacto Brain Heart Infusion; Beckton, Dickinson and Company, MD, USA) broth. Staphylococcus aureus (NDU-112, laboratory stock, derived from a clinical strain), and Aggregatibacter actinomycetemcomitans (American Type Cult-
ture Collection strain ATCC29522; purchased from Summit Pharmaceutical International, Tokyo) were cultured at 37°C in BHI broth in an atmosphere containing 5% CO₂. The bacteria were grown to mid-logarithmic phase, mixed with MA-T solution, and incubated as standing cultures at 25°C for 30 min. The number of CFU (colony-forming units)/mL was measured after MA-T treatment by subjecting the treated cultures to serial dilution with phosphate-buffered saline (PBS), spreading aliquots to BHI agar plates, incubating overnight at 37°C (in the presence or absence of CO₂, as appropriate), and counting the number of colonies per plate.

**Preparation of Membrane Vesicles** The MA-Tα- or MA-Tγ-treated bacteria were pelleted, washed with 10 mM Tris-HCl (pH 7.5) and re-pelleted, and then re-suspended in fresh 10 mM Tris-HCl (pH 7.5). The resulting suspension was sonicated with a Violamino Ultra Sono-cator 85 (As One, Osaka) with cooling in an ice bath. The sonicated lysate was centrifuged at 20,000 x g for 20 min, and the resulting supernatant then was centrifuged at 100,000 x g for 1 h. The precipitate was washed twice with 10 mM Tris-HCl (pH 7.5) by centrifugation at 100,000 x g for 1 h, and suspended in fresh buffer. Above all steps are carried out at 4°C. The resulting membrane vesicle suspension was assayed for the activities of respiratory chain-associated enzymes.

**Assay of Oxidase Activity** Ubiquinol-1:O₂ oxidoreductase (Q₉H₉ oxidase) activity was assayed spectrophotometrically as described previously.10) The activity of NADH:O₂ oxidoreductase (NADH oxidase) was measured according to the method of Kasahara and Anraku,11) using a Clark-type oxygen electrode (Rank Brothers, Cambridge, England).

**Assay of NADH Dehydrogenase Activity** NADH: ferricyanide oxidoreductase (NADH-ferricyanide dehydrogenase) and NADH: ubiquinone-1 oxidoreductase (NADH-Q₁ dehydrogenase) activities were assayed by the methods of Dancey et al.12) and Hatefi,13) respectively.

**Statistical Analysis** All assays were performed as two independent experiments (biological replicates), each consisting of triplicate reactions (technical replicates), for a total n = 6. Unless otherwise indicated, values are presented as mean ± standard deviation (SD). Statistical differences were determined by two-tailed non-paired Student’s t tests. MA-T treatment values were compared with those of non-treated controls. p ≤ 0.05 was considered significant, with * and ** used to indicate p ≤ 0.05 and p ≤ 0.01, respectively. All data were examined using StatMate (Atms Co. Ltd., Tokyo).

RESULTS AND DISCUSSION

Distribution of NADH-quinone dehydrogenases and quinol oxidases among the three bacterial species (E. coli, S. aureus, and A. actinomycetemcomitans) is shown in Table 1 based on the bacterial genome database. These three species appear not to encode enzymes similar to cytochrome bc₁ (complex III) and cytochrome c oxidase (complex IV) of the eukaryotic mitochondrion. As indicated in Table 2, the CFU/mL of E. coli fell by greater than 1000-fold after treatment with 40 ppm MA-T for 30 min at 25°C. Treatment with 20 ppm MA-T or MA-Tγ decreased cell densities from initial values of 2.78 x 10⁹ CFU/mL to 2.60 x 10⁹ (9.35%) and 4.2 x 10⁹ (1.5%) CFU/mL, respectively. NADH oxidase activity fell from 129 nmoles O₂/min/mg protein to 13.0 (MA-Tα, 10.1%) or 2.63 (MA-Tγ, 2.0%) nmoles O₂/min/mg protein (Table 3). NADH-Q₁ dehydrogenase activity (1.66 μmol/min/mg, non-treatment) decreased to 0.194 (MA-Tα, 11.7%) or 0.073 (MA-Tγ, 4.4%) μmol/min/mg. On the other hand, NADH-ferricyanide oxidase activity was not significantly altered by treatment with MA-T. Q₁H₉ oxidase activity changed from 10.2 μmol/min/mg to 4.11 (MA-Tα, 40.3%) or 3.64 (MA-Tγ, 35.7%) μmol/min/mg following MA-T treatment. However, Q₁H₉ oxidase activity was further diminished to almost zero after extension of the MA-T exposure time. Moreover, NADH oxidase activity in membrane vesicles prepared from non-treated E. coli was not markedly affected by treatment with MA-T, suggesting that MA-T may attack the generation of membrane potential in live bacteria.

The aerobic respiratory chain of E. coli can function with either of two different membrane-bound NADH dehydrogenases, including NADH dehydrogenase-I (NDH-I, complex I, or type I NADH:ubiquinone oxidoreductase) and NADH dehydrogenase-II (NDH-II, or type II NADH:ubiquinone oxidoreductase) on the electron input side and with either of two ubiquinone oxidases (bd-type and bo-type).14) E. coli NDH-I pumps protons across the membrane using downhill redox energy, and consists of 13 subunits, all encoded by the nuo operon.15) A modular structure has been suggested for complex I,17) such that the NADH dehydrogenase module has an NADH oxidation site and an artificial electron acceptor (such as ferricyanide) reduction site, and is composed of three subunits, FMN, and six [Fe/S] clusters. The hydrogenase module has a ubiquinone reduction site, and is composed of six subunits and three [Fe/S] clusters. The transporter module has four proton transport sites located within the membrane, and is composed of three subunits. An NADH dehydrogenase fragment consisting of the same subunits and cofactors

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**Table 1. Distribution of NADH-Quinone Oxidoreductases and Terminal Quinol Oxidases among Three Tested Bacterial Species (Data Obtained from KEGG GENOME Database)**

| Organism              | NDH-1 | NDH-2 | NQRa | bo3 | bd | aa3b |
|-----------------------|-------|-------|------|-----|----|------|
| E. coli               | Yes   | Yes   | No   | Yes | No | No   |
| S. aureus             | No    | Yes   | No   | No  | Yes| Yes  |
| A. actinomycetemcomitans | No   | Yes   | Yes  | No  | No | No   |

a) Na⁺-translocating NADH: quinone oxidoreductase
b) The cytochrome aa₃ of S. aureus is not a cytochrome c oxidase contained in eukaryotic mitochondria, but quinol oxidase.
c) Two kind of cytochrome bd (bd-I, bd-II) was reported, but physiological function of bd-II was not known.
d) “No”: there is some genes encoding an NDH-1-like protein, whose electron donor and/or catalytic subunits are unknown, i.e., lack one or more of the genes that code for the flavoprotein subunits (nuo1, nuo2 and/or nuo3).
as the NADH dehydrogenase module has been obtained biochemically by splitting a preparation of the E. coli complex I with the lipid depleting detergent Triton X-100.\textsuperscript{19} This soluble module has NADH-ferricyanide dehydrogenase activity. Notably, site in NDH-I that is targeted by MA-T is contained within the hydrogenase module. In the respiratory chain of E. coli, another dehydrogenase, a NDH-2 (or rotenone-insensitive NADH dehydrogenase), is known, and is composed of a single polypeptide containing FAD as a prosthetic group. NDH-2 catalyzes the transfer of electrons from NADH to ubiquinone and the resulting proteins form a heterodimer.\textsuperscript{24-26} There is a third terminal oxidase present in E. coli; this enzyme, which is encoded by the cyxAB genes, is a bd type oxidase (bd-II), but its function is unclear.\textsuperscript{27,28} Our results indicated that both cytochrome bo3 and bd are damaged by treatment with MAT.

As indicated in Table 2, the CFU/mL of S. aureus decreased by greater than 1000-fold after treatment with 20 ppm MA-T for 30 min at 25°C. Treatment with 15 ppm MA-Tα or MA-Tγ resulted in a decrease of density from an initial value of 5.16 x 10\textsuperscript{8} CFU/mL to 2.28 x 10\textsuperscript{7} (4.4%) or 1.37 x 10\textsuperscript{8} (2.3%) CFU/mL to 1.10 x 10\textsuperscript{7} (6.8%) in treated cultures.

| Bacterial species | Concentration of MA-T | Non-treatment | MA-Tα | MA-Tγ |
|-------------------|------------------------|---------------|--------|--------|
| E. coli (MA-T:20 ppm) | 40 ppm | 2.84 ± 0.139 | "0.001 >" | "0.001 >" |
|                   | 20 ppm | 2.78 ± 0.146 | "0.260 ± 0.0317" | "0.042 ± 0.0050" |
|                   | 10 ppm | 3.55 ± 0.750 | 3.21 ± 0.526 | 2.95 ± 0.0724 |
|                   | 20 ppm | 3.04 ± 0.549 | "0.001 >" | "0.001 >" |
| S. aureus (MA-T:15 ppm) | 15 ppm | 5.16 ± 0.843 | "0.228 ± 0.436" | "0.897 ± 0.149" |
|                   | 10 ppm | 5.22 ± 0.793 | "1.58 ± 0.841" | "1.71 ± 0.434" |
|                   | 30 ppm | 1.81 ± 0.417 | "0.001 >" | "0.001 >" |
| A. actinomycetemcomitans (MA-T:20 ppm) | 5.11 ± 0.073 | "4.11 ± 0.170" | "3.64 ± 0.233" |

a) NADH: O\textsubscript{2} oxidoreductase (nmol O\textsubscript{2}/min/mg protein)
b) NADH: ferricyanide oxidoreductase (μ mol/min/mg protein)
c) NADH: ubiquinone-1 oxidoreductase activity (μ mol/min/mg protein)
d) The membrane vesicles of E. coli were prepared from non-treated bacteria similar to “Preparation of membrane vesicles” in “Materials and Methods” section. Membrane vesicles were incubated with MA-T at 25°C for 30 min in the presence of 1 mM NADH for energization. The assay was carried out after washing the membrane vesicles with the centrifugation for exclude the MA-T.
e) ubiquinol-1: O\textsubscript{2} oxidoreductase activity (μ mol/min/mg protein)
dehydrogenase activity, because the limiting step is the electron transfer from FAD to ubiquinone.10 We therefore tested only ferricyanide as an electron acceptor for NADH dehydrogenase activity. QH2 oxidase activity changed from 1.74 μmol/min/mg to 0.240 (MA-Tα, 13.8%) or 0.684 (MA-Tγ, 39.3%) μmol/min/mg following MA-T treatment. QH2 oxidase activity fell to almost zero after an extension of the incubation time. In the respiratory chain of S. aureus, reduction of the terminal electron acceptor oxygen is accomplished by two terminal quinol oxidases: (i) the cytochrome aa3 oxidase,9,10,31 a proton-translocating oxidase that usually works under aerobic conditions; and (ii) the cytochrome bd oxidase, which is expressed under microaerobic conditions and does not translocate protons.31,32

As indicated in Table 2, the CFU/mL of A. actinomycetemcomitans fell by greater than 1000-fold after treatment with 30 ppm MA-T for 30 min at 25°C. Treatment with 20 ppm MA-Tα or MA-Tγ resulted in a decrease in cell density from an initial value of 1.37 x 106 CFU/mL to 1.43 x 103 (10.4%) or 1.30 x 102 (9.5%) CFU/mL, respectively. MA-Tα oxidase activity fell from 110 nmoles O2/min/mg protein to 11.5 (MA-Tα, 10.5%) or 17.1 (MA-Tγ, 15.5%) nmoles O2/min/mg protein (Table 3). However, NADH-Q1 dehydrogenase activity and NADH-ferricyanide dehydrogenase activity were not significantly altered by treatment with MA-T. QH2 oxidase activity changed from 7.66 μmol/min/mg to 0.418 (MA-Tα, 5.5%) or 0.591 (MA-Tγ, 7.7%) μmol/min/mg following MA-T treatment. As shown in Table 1, A. actinomycetemcomitans may possess NDH-2, cytochrome bd, and rotenone-sensitive Na+-translocating NADH:ubiquinone oxidoreductase (NQR) enzymes. Actually, rotenone-sensitive NADH-Q1 dehydrogenase activity was enhanced about three fold in the presence of 300 mM NaCl, indicating that this additive dehydrogenase activity may be derived from NQR. However, the NQR activity was not sensitive to MA-Tα or ρ (data not shown).

Taken together, we indicated that one of the targets of MA-T is the respiratory chain of three kinds of bacteria.

**Conflict of interest**  The authors declare no conflict of interest.

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