

Regular Article

The Respiratory Chain of Bacteria is a Target of the Disinfectant MA-T

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Received September 7, 2020; Accepted October 22, 2020

The importance of preventing infectious disease for public health continues to increase, and effective disinfectants are needed to inactivate pathogenic microorganisms. Chlorine dioxide (ClO_2) 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: O_2 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(Q_1) oxidoreductase (NADH- Q_1 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 (Q_1H_2): O_2 oxidoreductase (Q_1H_2 oxidase) also was decreased, indicating that cytochromes *bo3* and *bd* were damaged by MA-T. In *S. aureus*, NADH-ferricyanide dehydrogenase activity and Q_1H_2 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 Q_1H_2 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 caused by *Escherichia coli*, Coronavirus Infectious Disease of 2019 (COVID-19), and so on.^{1,2} There is a clear need for effective disinfectants able to inactivate microorganisms without adversely affecting the human body. Chlorine dioxide and chlorine, common disinfecting and bleaching chemicals used in the food industry, are potent chlorinating agents, and chlorine dioxide, a reagent with strong oxidizing activity, is one of the most efficient disinfectants.³ The use of chlorine dioxide as a preoxidant, instead of chlorine, is known to have the benefit of minimizing trihalomethane production following post-chlorination.^{4,5} The solubility of chlorine dioxide is five times that of chlorine and the oxidization activity of chlorine dioxide is about three-fold that of chlorine gas.² The ClO_2 radical is a powerful one-electron oxidant, possessing a redox potential of 936 mV,⁶ and is known for its ability to oxidize both inorganic and organic species.^{6,7} Chlorine dioxide also is known for its anti-bacterial and anti-viral properties.⁷ Chlorine dioxide is capable of triggering the denaturation of enzymes and proteins.⁸ ClO_2 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 ClO_2 -generating reagent. MA-T contains sodium chlorite, in combination with one of two kinds of cationic detergents that serves as the Lewis acid catalyzing the generation of ClO_2 (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,⁹ in unpublished observation), in a buffer stabilizing the solution at a neutral pH. Notably, ClO_2 is not detected in MA-T during storage or before use. Live bacteria, the targets of this disinfectant, would induce the production of ClO_2 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 Cul-

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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-T α - or MA-T γ -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 Violamo Sonicater 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 (Q₁H₂ 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 \pm 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* \leq 0.05 was considered significant, with * and ** used to indicate *p* \leq 0.05 and *p* \leq 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 *bcl* (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 α or MA-T γ decreased cell densities from initial values of 2.78 x 10⁸ CFU/mL to 2.60 x 10⁷ (9.35%) and 4.2 x 10⁶ (1.5%) CFU/mL, respectively. NADH oxidase activity fell from 129 nmoles O₂/min/mg protein to 13.0 (MA-T α , 10.1%) or 2.63 (MA-T γ , 2.0%) nmoles O₂/min/mg protein (Table 3). NADH-Q₁ dehydrogenase activity (1.66 μ mol/min/mg, non-treatment) decreased to 0.194 (MA-T α , 11.7%) or 0.073 (MA-T γ , 4.4%) μ mol/min/mg. On the other hand, NADH-ferricyanide oxidase activity was not significantly altered by treatment with MA-T. Q₁H₂ oxidase activity changed from 10.2 μ mol/min/mg to 4.11 (MA-T α , 40.3%) or 3.64 (MA-T γ , 35.7%) μ mol/min/mg following MA-T treatment. However, Q₁H₂ 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-1, complex I, or type I NADH:ubiquinone oxidoreductase) and NADH dehydrogenase-II (NDH-2, 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-1 pumps protons across the membrane using downhill redox energy, and consists of 13 subunits, all encoded by the *nuo* operon.¹⁵⁻¹⁸⁾ A modular structure has been suggested for complex I,^{17,18)} 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

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	NQR ^{a)}	<i>bo3</i>	<i>bd</i>	<i>aa3</i> ^{b)}
	Quinol reductase			Quinol oxidase		
<i>E. coli</i>	Yes	Yes	No	Yes	Yes ^{c)}	No
<i>S. aureus</i>	No ^{d)}	Yes	No	No	Yes	Yes
<i>A. actinomycetemcomitans</i>	No	Yes	Yes	No	Yes	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 (*nqo1*, *nqo2* and/or *nqo3*).

Table 2. Bacterial Viability Following Treatment with MA-T for 30 min

Bacterial species	Concentration of MA-T	CFU/mL \pm SD) $\times 10^8$		
		Non-treatment	MA-T α	MAT γ
<i>E. coli</i>	40 ppm	2.84 \pm 0.139	**0.001 >	**0.001 >
	20 ppm	2.78 \pm 0.146	**0.260 \pm 0.0317	**0.042 \pm 0.0050
	10 ppm	3.55 \pm 0.750	3.21 \pm 0.526	2.95 \pm 0.0724
<i>S. aureus</i>	20 ppm	3.04 \pm 0.549	**0.001 >	**0.001 >
	15 ppm	5.16 \pm 0.843	**0.228 \pm 0.436	**0.897 \pm 0.149
	10 ppm	5.22 \pm 0.793	**1.58 \pm 0.841	**1.71 \pm 0.434
<i>A. actinomycetemcomitans</i>	30 ppm	1.81 \pm 0.417	**0.001 >	**0.001 >
	20 ppm	1.37 \pm 0.224	**0.143 \pm 0.0248	**0.130 \pm 0.0217
	10 ppm	0.710 \pm 0.794	**0.365 \pm 0.0455	*0.590 \pm 0.0732

Table 3. Bacterial Respiratory Chain Activities Following Treatment with MA-T for 30 min

Bacterial species	Activity	Non-treatment	MA-T α	MAT γ
<i>E. coli</i> (MA-T:20 ppm)	NADH-O ₂ ^{a)}	129 \pm 7.87	**13.0 \pm 1.26	**2.63 \pm 0.183
	NADH-ferricyanide ^{b)}	3.34 \pm 0.173	3.29 \pm 0.355	3.35 \pm 0.196
	NADH-Q ₁ ^{c)}	1.66 \pm 0.129	**0.194 \pm 0.0114	**0.073 \pm 0.010
	NADH-O ₂ (membrane) ^{d)}	96.2 \pm 5.03	96.5 \pm 2.92	**66.9 \pm 5.31
	Q ₁ H ₂ -O ₂ ^{e)}	10.2 \pm 0.930	**4.11 \pm 0.170	**3.64 \pm 0.233
<i>S. aureus</i> (MA-T:15 ppm)	NADH-O ₂	316 \pm 12.9	**7.26 \pm 0.767	**29.5 \pm 3.45
	NADH-ferricyanide	5.11 \pm 0.073	**0.382 \pm 0.0125	**1.97 \pm 0.0933
	Q ₁ H ₂ -O ₂	1.74 \pm 0.155	**0.240 \pm 0.0225	**6.84 \pm 0.0254
<i>A. actinomycetemcomitans</i> (MA-T:20 ppm)	NADH-O ₂	110 \pm 10.9	**11.5 \pm 0.881	**17.1 \pm 1.38
	NADH-ferricyanide	5.11 \pm 0.115	4.95 \pm 0.105	5.01 \pm 0.0844
	NADH-Q ₁	4.43 \pm 0.239	4.48 \pm 0.293	4.32 \pm 0.142
	Q ₁ H ₂ -O ₂	7.66 \pm 0.251	**0.418 \pm 0.0167	**0.591 \pm 0.0143

a) NADH: O₂ oxidoreductase (nmoles O₂/min/mg protein)

b) NADH: ferricyanide oxidoreductase (μ mol/min/mg protein)

c) NADH: ubiquinone-1 oxidoreductase (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: O₂ oxidoreductase activity (μ mol/min/mg protein)

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-100.¹⁹⁾ 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 but, unlike complex I, does not couple the reaction to proton transfer.²⁰⁾ Our results indicated that NDH-2 also is targeted by MA-T. NDH-2 does not have a modular structure, and the MA-T target region may be proximal to the ubiquinone binding site rather than the FAD site. NDH-2 is the dominant entry point for electrons into the respiratory chain under aerobic conditions, but NDH-1 is the preferred NADH dehydrogenase under anaerobic growth conditions.^{21,22)} In other words, NADH dehydrogenase, especially NDH-1, is used in anaerobic electron transport, and MA-T therefore would inhibit bacterial growth under anaerobic conditions.

Cytochrome *bo3* oxidase is a quinol oxidase that accepts electrons from quinol and transfers these electrons to molecular oxygen. This enzyme contains a heme-Cu cofactor and is essential when oxygen is present at high concentrations. Cytochrome *bo3* oxidase is a protein complex containing

four subunits.²³⁾ Under conditions of low aeration, the oxygen high-affinity cytochrome *bd* oxidase is the preferred terminal reductase. The structural components are encoded by *cydAB*, and the resulting proteins form a heterodimer.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.^{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-T α or MA-T γ resulted in a decrease of density from an initial value of 5.16 $\times 10^8$ CFU/mL to 2.28 $\times 10^7$ (4.4%) or 8.97 $\times 10^7$ (17.4%) CFU/mL, respectively. MA-T treatment caused a decrease in oxygen consumption activity (NADH oxidase activity) from an initial value of 316 nmoles O₂/min/mg protein to 7.26 (MA-T α , 2.3%) or 29.5 (MA-T γ , 9.3%) nmoles O₂/min/mg protein (Table 3). NADH-ferricyanide reductase activity (5.11 μ mol/min/mg, no-treatment) decreased to 0.382 (MA-T α , 7.5%) or 1.97 (MA-T γ , 38.6%) μ mol/min/mg. NDH-2 is the only enzyme with NADH-ferricyanide dehydrogenase activity present in *Staphylococcus aureus*. NDH-2 uses FAD as a prosthetic group and NADH as a substrate.²⁹⁾ This enzyme has higher NADH:artificial electron acceptor (such as ferricyanide or menadione) oxidoreductase activity, but has much lower NADH-ubiquinone

dehydrogenase activity, because the limiting step is the electron transfer from FAD to ubiquinone.³⁰ We therefore tested only ferricyanide as an electron acceptor for NADH dehydrogenase activity. Q₁H₂ 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. Q₁H₂ 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,^{30,31} 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 10⁸ CFU/mL to 1.43 x 10⁷ (10.4%) or 1.30 x 10⁷ (9.5%) CFU/mL, respectively. NADH 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. Q₁H₂ 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:quinone 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 γ (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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