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Activation mechanism for *N*-nitroso-*N*-methylbutylamine mutagenicity by radical species

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ABSTRACT

N-Nitrosodialkylamines are known to be potent indirect-acting mutagens/carcinogens, which are activated by cytochrome P450. The reaction product of *N*-nitroso-*N*-methylbutylamine (NMB) with modified Fenton's reagent supplemented with copper salt (Fe^{2+}–Cu^{2+}–H_2O_2) was reported to be mutagenic in *Salmonella typhimurium* TA1535 without S9 mix. In this study, the NMB activation mechanism was investigated by ESR spectroscopy with radical trapping agents to detect radical species and also by observing changes in mutagenic potency with a *Salmonella* strain in the Ames assay in the presence of radical trapping agents. In ESR spectroscopy experiments, the hydroxyl radical generated from the modified Fenton's reagent was detected using the hydroxyl radical trapping agent 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). Since the amount of the DMPO–OH adduct decreased with the addition of NMB, hydroxyl radical was presumed to react with NMB followed by the generation of nitric oxide (NO), which was detected as CarboxyPTI through reaction with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (CarboxyPTIO). The mutagenicity of the reaction extract decreased following the addition of DMPO or CarboxyPTIO. Furthermore, the mutagenicity of the reaction product in the presence of DMPO was enhanced by the addition of NO. The reaction product from NMB with Fe^{2+}–Cu^{2+}–NO in the absence of H_2O_2 was mutagenic, and this activity increased with the introduction of additional NO. These findings suggest that hydroxyl radical takes part in the generation of NO from NMB and that NO plays an important role in NMB activation in the presence of Fe^{2+} and Cu^{2+}.

1. Introduction

N-Nitrosodialkylamines are found in food, beverages, tobacco, cosmetics, and other sources.[1–6] *N*-Nitrosodialkyamines also form easily within the human body by the reaction of secondary and tertiary amines in foods and drugs with nitrite, derived from nitrate in saliva under the acidic conditions of the stomach,[7–10] and with nitric oxide, formed from activated macrophages under neutral conditions in vivo.[11] Since many *N*-nitrosodialkylamines induce cancers in experimental animal tests, *N*-nitroso compounds are suspected to be causative agents for human cancer.[12–14] *N*-Nitrosodialkylamines are activated via α-hydroxylation by cytochrome P450, followed by elimination of the corresponding aldehyde. The reaction of the generated alkyldiazonium ions with DNA base nucleophiles causes mutagenicity.[15] The mutagenicity of *N*-nitrosodialkylamines has been detected in *Salmonella typhimurium* or *Escherichia coli* in the presence of a rat liver S9 mix.[16] The metabolism of *N*-nitrosodialkylamines by rat liver microsomes have been reported,[17,18] and short-lived radicals have been detected in the presence of rat liver microsomes.[19] Non-enzymatic degradation of nitrosamines upon their exposure to UV light or Fenton reagent have been also reported;[17,20–23] however, little is known about the biological role of this non-enzymatic activation process. Fenton's reagent with copper ion, herein called modified Fenton's reagent, was used as an oxidant for the activation of the *N*-nitrosodialkylamines. Ethyl acetate extract from the reaction mixture which included Fe^{2+}–Cu^{2+}–H_2O_2 and *N*-nitrosodialkylamines; *N*-nitrosodimethylamine (NDM), *N*-nitrosodiethylamine (NDE), *N*-nitrosodipropylamine (NDP), *N*-nitrosodibutylamine (NDB), *N*-nitroso-*N*-methylpropylamine (NMP), *N*-nitroso-*N*-methylbutylamine (NMB), was assayed for their mutagenicity by the Ames assay.[24] The extracts from NDP, NDB, NMP, or NMB with Fe^{2+}–Cu^{2+}–H_2O_2 were mutagenic in *S. typhimurium* TA1535 and *E. coli* WP2 *uvr*A, indicating that the direct-acting mutagen was produced from *N*-nitrosodialkylamines with an alkyl chain longer than propyl by Fe^{2+}–Cu^{2+}–H_2O_2.[24] Since the extract from the reaction mixture with NMB showed the highest mutagenic activity among the *N*-nitrosodialkylamines tested, NMB activation mechanism by the modified Fenton's reagent is investigated. We identified radical species in the reaction mixture using a spin-trapping method with ESR spectroscopy, and compared the mutagenic activity of reaction extracts of NMB and Fe^{2+}–Cu^{2+}–H_2O_2 with or without the trapping

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agents. We also investigated the role of nitric oxide (NO) and metal ions in the formation of direct-acting mutagens.

2. Results

2.1. Generation of radical species from metal ions and H_2O_2

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), which reacts with hydroxyl radical to form a DMPO–OH adduct, has been used to detect hydroxyl radical in ESR spectroscopy experiments.[25] Hydroxyl radical induces a characteristic four-line (1:2:2:1, a^N = 14.8 G) DMPO–OH signal. The relative intensity, a ratio of the height of the lowest peak in the DMPO–OH signal to the height of a manganese marker, was used to quantify hydroxyl radical formation. A DMPO–OH ESR signal was detected from the reaction mixture of DMPO and Fe^{2+}–Cu^{2+}–H_2O_2 (Fig. 1).

The relative intensity of DMPO–OH decreased significantly with the addition of NMB into the reaction mixture of Fe^{2+}–Cu^{2+}–H_2O_2 and DMPO (Fig. 2). This data indicated that NMB reacted with hydroxyl radical derived from Fe^{2+}–Cu^{2+}–H_2O_2.

To confirm the production of nitric oxide (NO) via the oxidation of NMB by Fe^{2+}–Cu^{2+}–H_2O_2, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (CarboxyPTIO) was also used. The ESR signal of CarboxyPTIO is characterized by five lines (1:2:3:2:1, a^N = 8.2 G), and CarboxyPTI forms after reaction with NO, and seven line signals in the ESR spectrum are observed.[26] CarboxyPTIO was detected in the reaction mixture of Fe^{2+}–Cu^{2+}–H_2O_2 and NMB (Fig. 3A). A decrease in the signals for CarboxyPTIO was observed concomitantly with an increase in the signals for CarboxyPTI (Fig. 3B). The signal intensities for CarboxyPTI correlated with the amount of NMB added, indicating that NO was generated from NMB and Fe^{2+}–Cu^{2+}–H_2O_2.

2.2. Effect of radical species on NMB mutagenicity in the presence of Fe^{2+}–Cu^{2+}–H_2O_2

To investigate the involvement of radical species in NMB mutagenicity, DMPO or CarboxyPTIO was added to the reaction mixture of NMB and modified Fenton's reagent, and then extracts of the reaction mixture in the presence or absence of NO were evaluated for their mutagenicity in the *S. typhimurium* TA1535 assay.

With the addition of NO to the reaction mixture of NMB and Fe^{2+}–Cu^{2+}–H_2O_2, the increase in the mutagenicity of the extract was proportional to the amount of NO added (Fig. 4A). In contrast, the addition of CarboxyPTIO to the reaction mixture of NMB and Fe^{2+}–Cu^{2+}–H_2O_2 apparently decreased the mutagenicity of the extract (Fig. 4B).

The mutagenicity of the extract was inhibited by the addition of DMPO to the reaction mixture of NMB and the modified Fenton's reagent (Fig. 5A). When NO was introduced to the reaction mixture of NMB and the modified Fenton's reagent in the presence of DMPO, the increase in the mutagenicity of the extract was proportional to the amount of NO (Fig. 5B).

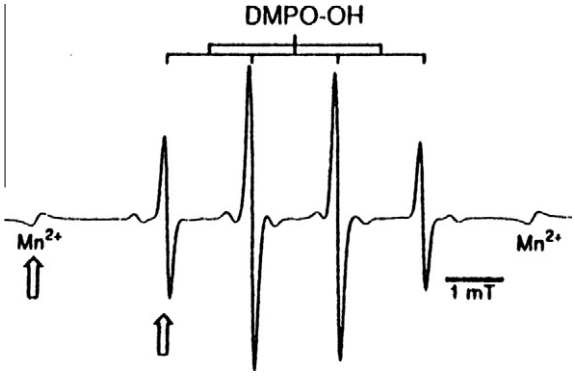

Figure 1. ESR spectra of the reaction product of modified Fenton's reagent with DMPO.

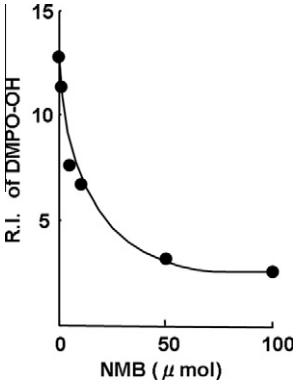

Figure 2. Change of DMPO–OH adduct amount by the addition of NMB in the presence of Fe^{2+}–Cu^{2+}–H_2O_2. Relative intensity (R.I.) of DMPO–OH was plotted against the concentration of added NMB.

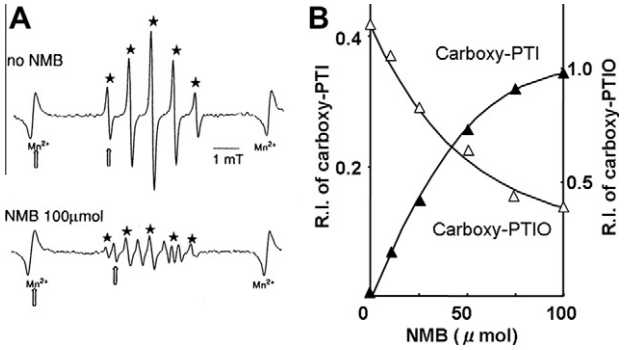

Figure 3. Detection of NO in the reaction of Fe^{2+}–Cu^{2+}–H_2O_2 and NMB. (A) ESR spectrum of CarboxyPTIO in the presence of Fe^{2+}–Cu^{2+}–H_2O_2. (B) Change of the relative intensity for CarboxyPTI and CarboxyPTIO depending on the concentration of added NMB.

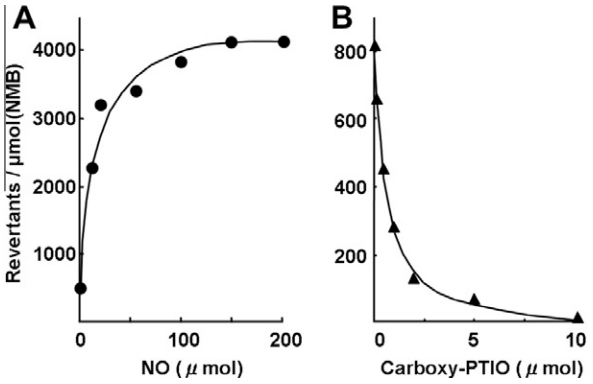

Figure 4. Effect of the addition of NO (A) or CarboxyPTIO (B) on the mutagenicity of the extract from NMB treated with Fe^{2+}–Cu^{2+}–H_2O_2 in a *Salmonella typhimurium* TA1535 assay.

2.3. Effect of metal ion on NMB mutagenicity in the presence of NO

NO was added to the reaction mixture of NMB and the metal ions in the absence of H_2O_2. Extracts from the reaction of NMB with Fe^{2+}–NO, Cu^{2+}–NO or NO alone, were weakly mutagenic, while, an

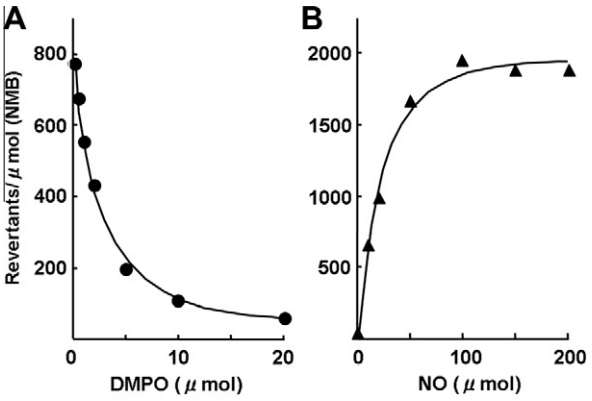

Figure 5. Effect of the addition of DMPO (A) or NO in the presence of DMPO (100 μmol) (B) on the mutagenicity of extracts from NMB treated with Fe^{2+}–Cu^{2+}–H_2O_2 in the *Salmonella typhimurium* TA1535 assay.

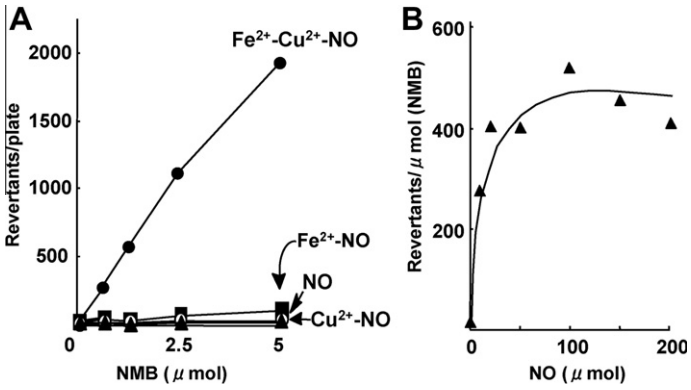

Figure 6. Effect of Fe^{2+} and Cu^{2+} in the presence of NO on the mutagenicity of the extract from NMB treated with Fe^{2+}–Cu^{2+} in the *Salmonella typhimurium* TA1535 assay.

extract from the reaction mixture with Fe^{2+}–Cu^{2+}–NO showed potent mutagenicity, and the increase in the mutagenicity was proportional to the amount of NO added (Fig. 6).

3. Discussion

N-Nitrosodialkylamines are activated to form α-hydroxynitrosamines by cytochrome P450. The α-hydroxynitrosamines in the absence of S9 mix were mutagenic in *S. typhimurium* TA1535, which detects base substituted mutations.[16] We previously reported that modified Fenton's reagent (Fe^{2+}–Cu^{2+}–H_2O_2) activated *N*-nitrosodialkylamines, with alkyl chains longer than propyl, into direct-acting mutagens.[24] Although these direct-acting mutagens were also mutagenic in *S. typhimurium* TA1535, they were not α-hydroxynitrosamines which are unstable in aqueous solution.[16] Based on these results, we hypothesized that another activation mechanism for *N*-nitrosodialkylamines by reactive oxygen species could exist. The current study investigated the mechanism for the formation of direct-acting mutagens derived from *N*-nitrosamines in the presence of modified Fenton's reagent.

Although iron-oxo species can be derived from Fenton's reagent,[22,27–29] hydroxyl radical is produced as the predominant oxidant species under various conditions.[30,31] In this study, Fenton's reagent supplemented with Cu^{2+} generated hydroxyl radical since a DMPO–OH adduct was detected from the reaction mixture of Fe^{2+}–Cu^{2+}–H_2O_2 and DMPO by ESR spectroscopy (Fig. 1). The amount of DMPO–OH adduct decreased as the NMB concentration increased, indicating that NMB reacted with hydroxyl radical (Fig. 2). Keefer et al. reported that *N*-nitrosodimethylamines were oxidized by Fenton's reagent or rat liver microsomes to form NO, which is easily converted to nitrite by oxidizing agents.[18,23] In this study, the presence of NO in the reaction mixture was detected from the CarboxyPTI in the ESR spectra (Fig. 3A). The signals for CarboxyPTI increased as the amount of NMB increased, indicating that NO was generated after reaction of NMB and the modified Fenton's reagent (Fig. 3B). In ESR spectroscopy experiments, the amount of hydroxyl radical formed was almost same among Fe^{2+}–H_2O_2 and Fe^{2+}–Cu^{2+}–H_2O_2, while the amount of hydroxyl radical formed by Cu^{2+}–H_2O_2 was negligible. Furthermore, the amount of NO generation derived from the reaction of NMB and hydroxyl radical was correlated with the amount of hydroxyl radical formed (data not shown). Thus Cu^{2+} was not involved in the formation of hydroxyl radical and NO, but played an important role in mutagen formation.

In order to investigate the effect of NO on the mutagenicity of the extract from NMB and Fe^{2+}–Cu^{2+}–H_2O_2, NO or CarboxyPTIO was added to the reaction mixture. The mutagenicity of the extract increased with the addition of NO to the reaction, and furthermore, the mutagenicity of the extract decreased with the addition of CarboxyPTIO as an NO trapping reagent (Fig. 4). NO is easily oxidized to nitric dioxide (NO_2) by air,[32] and NO_2 is also generated when CarboxyPTIO reacts with NO.[26] However, NO_2 was not involved in the formation of the direct-acting mutagen species since the mutagenicity of the reaction extract decreased with the addition of CarboxyPTIO. The direct-acting mutagen from the Fe^{2+}–Cu^{2+}–H_2O_2 reaction mixture was identical on HPLC with a species formed in the presence of NO (data not shown). These results demonstrated that NO was involved in the formation of the direct-acting mutagen.

Introduction of the hydroxyl radical trapping agent DMPO also decreased the mutagenicity of the NMB reaction extract; however, mutagenic activity increased with the addition of NO even in the presence of DMPO (Fig. 5). This result demonstrated that hydroxyl radical was not necessary to activate NMB when NO was also present in the reaction mixture.

With the addition of NO, an extract of the reaction mixture of NMB with or without Fe^{2+} or Cu^{2+} in the absence of H_2O_2, was mutagenic in the *S. typhimurium* TA1535 assay (Fig. 6). Although NO alone, and NO with one of the metals did not activate NMB, both Fe^{2+} and Cu^{2+} were necessary to form the direct-acting mutagen in the reaction. Fenton's reagent with Cu^{2+} was reported to give oxidation products from alcohols that differed from the oxidation reaction without Cu^{2+}.[33] The isolation and structure elucidation of the direct-acting mutagen derived from NMB will be reported soon.

A possible pathway for formation of the direct-acting mutagen from NMB and Fe^{2+}–Cu^{2+}–H_2O_2 is shown in Figure 7. Hydroxyl radical formed from the metal ion and H_2O_2, oxidizes NMB to generate NO. If NO is present in the reaction, H_2O_2 is not necessary for NMB activation. NO reacts with another NMB molecule in the presence of Fe^{2+} and Cu^{2+}, followed by the formation of the direct-acting mutagen. NO, Fe^{2+} and Cu^{2+} play key roles in the formation of

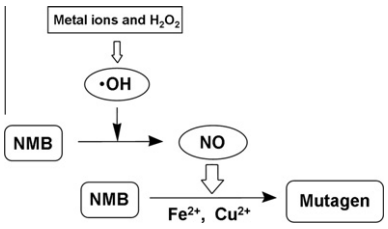

Figure 7. Proposed pathway for direct-acting mutagen formation derived from NMB in the presence of Fe^{2+}–Cu^{2+}–H_2O_2.

the direct-acting mutagen from NMB in the presence of the modified Fenton's reagent.

4. Conclusion

N-Nitrosodialkylamines are known to be potent indirect-acting carcinogens, and the reaction product of *N*-nitroso-*N*-methylbutylamine (NMB) with modified Fenton's reagent supplemented with copper salt (Fe^{2+}–Cu^{2+}–H_2O_2) was mutagenic in *S. typhimurium* TA1535. The NMB activation mechanism was investigated by ESR spectroscopy with radical trapping agents, also by observing changes in mutagenic potency with *Salmonella* strain in the presence of radical trapping agents. Hydroxyl radical generated from the modified Fenton's reagent was detected using the hydroxyl radical trapping agent DMPO. Since the amount of the DMPO–OH adduct decreased with the addition of NMB, hydroxyl radical was presumed to react with NMB followed by the generation of NO, which was detected as CarboxyPTI through reaction with CarboxyPTIO. The mutagenicity of a reaction mixture extract decreased following the addition of DMPO or CarboxyPTIO. Furthermore, the mutagenicity of the reaction product in the presence of DMPO was enhanced by the addition of NO. The reaction product from NMB with Fe^{2+}–Cu^{2+}–NO in the absence of H_2O_2 was mutagenic, and this activity increased with the introduction of additional NO. These findings suggest that hydroxyl radical takes part in the generation of NO from NMB and that NO plays an important role in NMB activation in the presence of Fe^{2+} and Cu^{2+}. *N*-Nitrosodialkylamines are known to be metabolically activated through α-hydroxylation, and the present result suggested a possibility of another activating pathway.

5. Material and methods

5.1. Reagents

NMB was synthesized as described.[34] Crude NMB was dissolved in methanol saturated with sodium hydroxide and the whole solution was stirred overnight at room temperature, then purified by fractional distillation [b.p. 87 °C/18 mm Hg]. Iron (II) sulfate heptahydrate and hydrogen peroxide were purchased from Wako Chemical Co (Tokyo, Japan). Copper (II) acetate monohydrate was obtained from Kanto Chemical Co. Ltd (Tokyo, Japan). High purity NO gas was obtained from Takachiho (Nagoya, Japan). DMPO and CarboxyPTIO were purchased from Dojindo Laboratories (Kumamoto, Japan). The *S. typhimurium* TA1535 used was kindly provided by Professor B. N. Ames (University of California, Berkeley, USA).

5.2. Detection of DMPO–OH and CarboxyPTI adducts by ESR spectroscopy

ESR spectra were obtained with a JEOL X-band spectrometer (JES-RE1X) under nonsaturating microwave power conditions. $FeSO_4{\cdot}7H_2O$ (0.25 μmol), $Cu(OAc)_2{\cdot}H_2O$ (0.25 μmol), NMB (0–100 μmol), DMPO (25 μmol) or CarboxyPTIO (2 nmol). Then H_2O_2 (25 μmol) were mixed in 1 M acetate buffer pH 4.5 (1 mL) at 25 °C. After 3 min, the ESR was taken using the following parameters: magnetic field 335.3 ± 5.0 mT, microwave power 1.0–8.0 mW, modulation frequency 100 kHz, modulation width 0.063–0.079 mT, sweep time 2.0 min, response time 0.3 s, receiver gain 250–1000.

5.3. Reaction of NMB and modified Fenton's reagent in the presence of DMPO or CarboxyPTIO

To a solution of NMB (100 μmol) in acetate buffer (pH 4.5, 2 mL) were added $FeSO_4{\cdot}7H_2O$ (100 μmol), $Cu(OAc)_2{\cdot}H_2O$ (100 μmol), DMPO (0–20 μmol) or CarboxyPTIO (0–10 μmol). Then H_2O_2 (100 μmol) was added to the reaction mixture under nitrogen gas. After incubation for 2 h at 37 °C, the reaction mixture was extracted three times with ethyl acetate, and the combined organic phases were dried over Na_2SO_4, filtered, and then evaporated in vacuo to give a yellow oil.

5.4. Reaction of NMB and modified Fenton's reagent in the presence of NO and DMPO

To a solution of NMB (100 μmol) in acetate buffer (pH 4.5, 2 mL) were added $FeSO_4{\cdot}7H_2O$ (100 μmol), $Cu(OAc)_2{\cdot}H_2O$ (100 μmol), DMPO (0 or 100 μmol), and H_2O_2 (100 μmol). Then NO (0–200 μmol) was added to the mixture under nitrogen gas. After incubation for 2 h at 37 °C, the reaction mixture was extracted three times with ethyl acetate, and the combined organic phases were dried over Na_2SO_4, filtered, and then evaporated in vacuo to give a yellow oil.

5.5. Reaction of NMB with Fe^{2+}, Cu^{2+} and NO

To a solution of NMB (100 μmol) in acetate buffer (pH 4.5, 2 mL) were added $FeSO_4{\cdot}7H_2O$ (0 or 100 μmol) and $Cu(OAc)_2{\cdot}H_2O$ (0 or 100 μmol). Then NO gas (100, or 0–200 μmol) was introduced to the reaction under nitrogen gas. After incubation for 2 h at 37 °C, the reaction mixture was extracted three times with ethyl acetate, and the combined organic phases were dried over Na_2SO_4, filtered, and then evaporated in vacuo to give a yellow oil.

5.6. Bacterial mutation assay

Bacterial mutation assays were performed based on the Ames test.[35] The yellow oil obtained above was dissolved into DMSO (2 mL) to give a solution containing the amount of the original *N*-nitrosodialkylamine used in the reaction (5.0 μmol/100 μL). This DMSO solution was diluted to concentrations of 0.6, 1.3, and 2.5 μmol/100 μL. Each concentration of DMSO solution was put into a test tube with 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4), 0.1 mL of a culture of tester strain, and 2 mL of top agar. The mixture was then poured onto a minimal-glucose agar plate. After incubation for 44 h at 37 °C, the colonies were counted. All plates were prepared in duplicate, and the experiments were repeated at least twice.

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