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Enhancement of antibacterial effects of epigallocatechin gallate, using ascorbic acid

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Abstract

Although plant polyphenols such as (−)-epigallocatechin gallate (EGCG) have antibacterial activity towards methicillin-resistant *Staphylococcus aureus* (MRSA), such polyphenols are unstable in solution. Because the instability of polyphenols is attributable to their oxidation, we examined the effects of antioxidants and inhibitors of polyphenol oxidation on the maintenance of polyphenol antibacterial activity. The antibacterial activity of EGCG was enhanced in the presence of ascorbic acid, and ascorbic acid was the most effective for retaining the concentration of stable EGCG. On the other hand, the antibacterial activity of EGCG was lowered in the presence of casein in spite of its suppressing effect on the EGCG decrease. The effect of EGCG on the antibiotic resistance of MRSA was also enhanced in the presence of ascorbic acid. The addition of an antioxidant may affect other pharmacological effects of polyphenols in analogous ways, although this does not mean the clinical usefulness of the addition directly.

Keywords: *Camellia sinensis* L. (Theaceae); Antibacterial; Antibiotic resistance; Ascorbic acid; Epigallocatechin gallate; Methicillin-resistant *Staphylococcus aureus*; Polyphenol; Tea

1. Introduction

Infectious diseases caused by bacteria resistant to multiple drugs have become one of the most serious problems in hospitals today. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most frequent causes of community-acquired infections (Halem et al., 2006). Several polyphenols, including the hydrolyzable tannins tellimagrandin I and corilagin (Shiota et al., 2004), the tea polyphenol epicatechin gallate (Shiota et al., 1999), licorice polyphenols (Hatano et al., 2000), and *Zanthoxylum* proanthocyanidins (Kusuda et al., 2006), can suppress the antibiotic resistance of MRSA or act synergistically when used with some antibiotics.

(−)-Epigallocatechin gallate (EGCG) (**1**) is the most abundant polyphenol in tea leaves (*Camellia sinensis* L., Theaceae) and has antibacterial activity against MRSA (Zhao et al., 2002; Hamilton-Miller and Shah, 2000). However, polyphenols are generally unstable in oxidative

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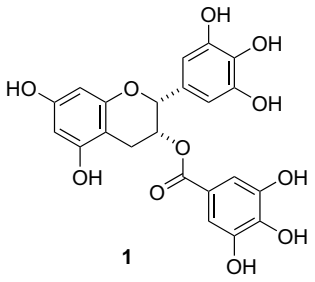

Scheme 1.

conditions, and EGCG (**1**) levels decrease rapidly, even in solutions of neutral pH (Hatano et al., 2003). Although oxidative products of EGCG (**1**), such as theasinensin A, also have suppressive effects on antibiotic resistance, the effects are limited when the compounds are incubated for prolonged periods. The suppressive effects of *Zanthoxylum* proanthocyanidin also decrease when incubated with bacteria for more than 10 h (Kusuda et al., 2006).

Because the decrease in the effectiveness of polyphenols is attributable to an oxidative structural change, as observed for EGCG (**1**), the inhibition of oxidation should enhance or prolong their antibacterial effects. We therefore investigated the effects of ascorbic acid and several other food additives that may suppress polyphenol oxidation. A part of the results for ascorbic acid was shown in a preliminary way (Hatano et al., 2006). In this paper, we describe the enhancement or suppression of the antibacterial effect on antibiotic resistance in the presence of EGCG (**1**) and these additive compounds with the experimental detail (see Scheme 1) .

2. Results and discussion

2.1. Inhibitory effect of ascorbic acid and other food additives on EGCG oxidation

EGCG (**1**) was oxidized even in a weakly acidic solution buffered at pH 6.5; after 5 h, approximately half of the EGCG (**1**) was oxidized (Table 1). The addition of ascorbic acid noticeably suppressed the decrease in active EGCG (**1**) levels. Of the four amino acids tested, cysteine, which has a thiol residue that constitutes its antioxidant effect, had a more potent effect than proline, aspartic acid, or glutamic acid.

Preliminary tests indicated that the presence of bovine serum albumin suppressed the oxidation of EGCG (**1**), suggesting compounds that form complexes with EGCG (**1**) may suppress polyphenol oxidation by decreasing the effects of metal ions on the phenolic hydroxyl groups. We therefore examined the effects of food-additive proteins and oligo- or polysaccharides. We observed weak suppressive effects of proteins such as α-casein and ovalbumin, and of saccharides such as alginic acid.

Table 1
Effects of food additives on the decrease in EGCG (**1**) concentration in solution

Compound added	Concentration of additive (mg mL^{-1})[a]	Concentration of EGCG (**1**) (mg mL^{-1})[b]
None	–	0.049 ± 0.0013
Ascorbic acid	0.1	0.098 ± 0.0014
Proline	0.1	0.056 ± 0.0035
Cysteine	0.1	0.093 ± 0.0088
Aspartic acid	0.1	0.058 ± 0.0023
Glutamic acid	0.1	0.055 ± 0.0029
Casein	0.625	0.057 ± 0.0016
Ovalbumin	0.625	0.061 ± 0.0022
β-Cyclodextrin	1.25	0.051 ± 0.0021
Trehalose	2.0	0.047 ± 0.0025
Pectin	0.1	0.052 ± 0.0002
Alginic acid	0.5	0.055 ± 0.0038

[a] EGCG (**1**) (0.1 mg mL^{-1}) was incubated in 0.05 M phosphate buffer, pH 6.5, at 37 °C for 5 h in the presence of the indicated concentrations of the above compounds.

[b] The concentration of EGCG (**1**) remaining in the solution immediately after the incubation was estimated from the peak area on high-performance liquid chromatography. Values indicate the mean ± standard deviation of 3–6 experiments.

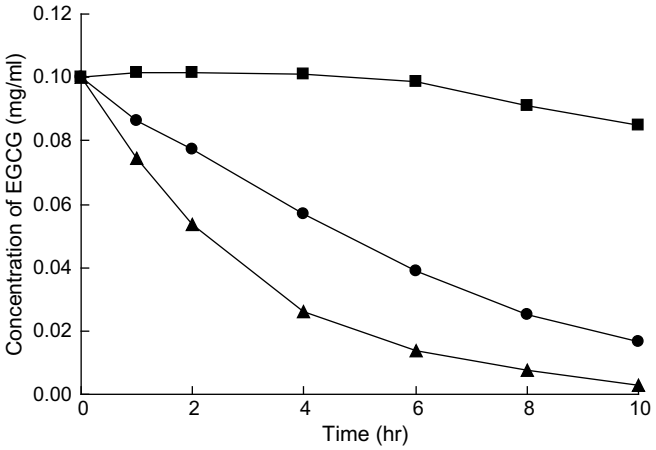

Fig. 1. Changes in the concentrations of EGCG (**1**) and ascorbic acid following incubation. Solutions containing EGCG (**1**) (0.1 mg mL^{-1}) in the absence or presence of ascorbic acid (0.1 mg mL^{-1}) were incubated at 37 °C for 10 h in 0.05 M phosphate buffer, pH 6.5, and their concentrations at various times were estimated from the peak area on high-performance liquid chromatography. Concentration of EGCG (**1**) (solid circle) in the absence of ascorbic acid, EGCG (**1**) (solid square) in the presence of ascorbic acid, and ascorbic acid (solid triangle) in the absence of EGCG (**1**).

Of the compounds tested, ascorbic acid had the most potent effect on inhibiting/preventing the decrease in active EGCG (**1**) levels; this was attributed to its reductive capacity, i.e., its antioxidant effects. In a solution containing both ascorbic acid and EGCG (**1**), the degradation of ascorbic acid preceded that of EGCG (**1**) (Fig. 1). When the concentration of ascorbic acid reached 20% of that at

Table 2
Effects of food additives on the minimum inhibitory concentrations (MICs)

Compound added	Concentration of additive (μg mL^{-1})[a]	MIC of EGCG (**1**) (μg mL^{-1})				
		MRSA				MSSA
		OM481	OM505	OM584	OM623	209P
None	–	64	64	64	64	64
Ascorbic acid	64	32	32	32	32	64
Cysteine	64	32	32	32	32	64
Aspartic acid	64	64	64	64	64	64
Glutamic acid	64	64	64	64	64	64
Proline	64	64	64	64	64	64
Ovalbumin	200	64	64	64	64	64
Casein	200	128	128	128	128	128
β-Cyclodextrin	400	64	128	128	64	64
Alginic acid	80	64	64	64	64	64
Trehalose	320	64	64	64	64	64
Pectin	64	64	64	64	64	64

MICs of EGCG (**1**) against strains of methicillin-resistant and -sensitive *Staphylococcus aureus* (MRSA and MSSA, respectively).

[a] The MRSA and MSSA strains were incubated in the presence of the indicated concentrations of the additives. At these concentrations, the additives did not show any inhibitory effects on the bacterial growth.

the start of the incubation, the concentration of active EGCG (**1**) began to decrease.

2.2. Antibacterial activity of EGCG (*1*) following the addition of compounds that suppressed the decrease in active EGCG (*1*) levels

The effect of ascorbic acid and other food-additives on the antibacterial activity of EGCG (**1**) against MRSA was examined. The four MRSA strains tested were obtained from clinical isolates. The minimum inhibitory concentrations (MICs) of EGCG (**1**) for the four strains of MRSA and methicillin-sensitive *S. aureus* (MSSA) strain 209P were all 64 μg mL^{-1}. The addition of ascorbic acid and cysteine to EGCG (**1**) enhanced the antibacterial activity, indicated by the decrease in the MIC to 32 μg mL^{-1} (Table 2). In contrast, the addition of casein and β-cyclodextrin increased the resistance of all or some of the MRSA strains tested. The addition of the other compounds had no effect on the MICs (Table 2).

We examined the effects of ascorbic acid and casein on bacterial resistance during incubation by monitoring changes in turbidity. An increase in turbidity was suppressed in the presence of EGCG (**1**), and the addition of ascorbic acid enhanced the effect of EGCG (**1**) (Fig. 2a). In contrast, the addition of casein decreased the antibacterial activity of EGCG (**1**) (Fig. 2b).

The enhancing effect of ascorbic acid on the antibacterial activity of EGCG (**1**) was further confirmed by monitoring changes in the bacterial quantities, in terms of colony forming units (CFU). EGCG (**1**) inhibited the increase of MRSA, and the addition of ascorbic acid further enhanced the antibacterial activity of EGCG (**1**) (Fig. 3).

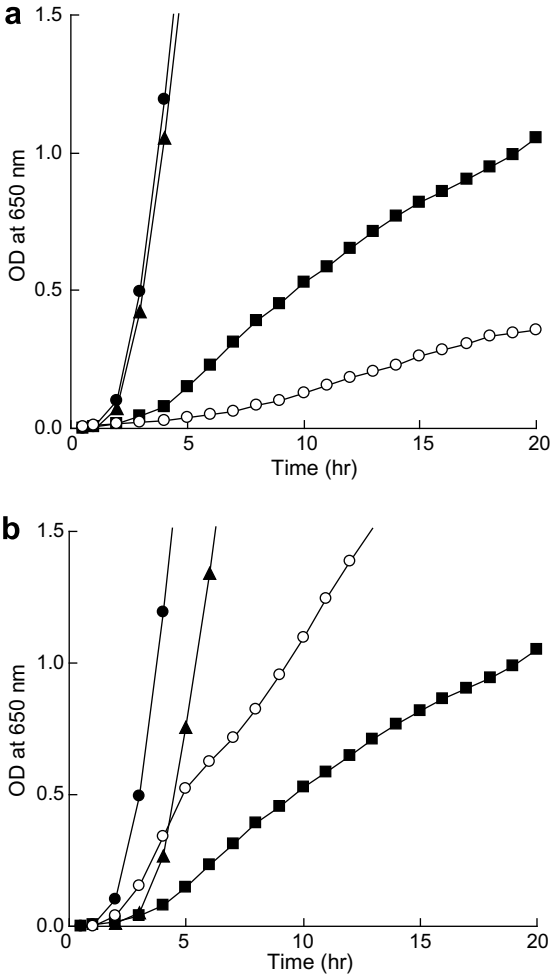

Fig. 2. Effects of the addition of ascorbic acid and casein to a solution containing EGCG (**1**) on the increase in methicillin-resistant *Staphylococcus aureus* strain OM505. (a) Turbidity (OD at 650 nm) of solutions in the absence of EGCG (**1**) and ascorbic acid (control, solid circle), in the presence of EGCG (**1**) (solid square, 48 μg mL^{-1}), in the presence of ascorbic acid (solid triangle, 16 μg mL^{-1}), and in the presence of both EGCG (**1**) and ascorbic acid (open circle). (b) Turbidity (OD at 650 nm) of solutions in the absence of EGCG (**1**) and casein (control solid circle), in the presence of EGCG (**1**) (solid square, 48 μg mL^{-1}), in the presence of casein (solid triangle, 200 μg mL^{-1}), and in the presence of both EGCG and casein (open circle).

2.3. Effects on the bacterial resistance or suppression of EGCG (*1*) by addition of oxidation-suppressing compounds

Polyphenols, including EGCG (**1**), have suppressive activity on the antibiotic resistance of MRSA, in addition to other antibacterial effects. Therefore, the effects of ascorbic acid, casein, and other compounds that affect the oxidation of EGCG (**1**) were examined in relation to antibiotics and the suppression of antibiotic resistance.

The MIC of oxacillin against the MRSA strains was 128–512 μg mL^{-1}, but decreased to 32–64 μg mL^{-1} in the presence of EGCG (**1**) (Table 3). The addition of ascorbic acid further enhanced the effect of EGCG (**1**), and the MIC of oxacillin was reduced to 16–32 μg mL^{-1} (Table 3). The addition of casein caused an increase in the MIC of oxacillin

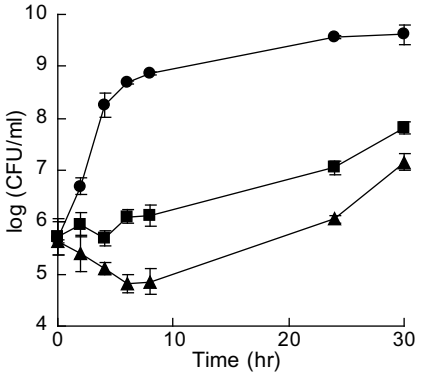

Fig. 3. Changes in the amount of bacteria during incubation, indicating the effect of the addition of ascorbic acid on the antibacterial activity of EGCG (**1**). The amount of bacteria (colony-forming units [CFU] mL^{-1}) in solutions in the absence of EGCG (**1**) and ascorbic acid (control, solid circle), in the presence of EGCG (**1**) (solid square, 48 μg mL^{-1}), and in the presence of both of EGCG (**1**) (48 μg mL^{-1}) and ascorbic acid (32 μg mL^{-1}) (solid triangle).

Table 3
Effects of food additives on the decrease in the minimum inhibitory concentrations (MICs)

Compound added	Concentration of additive (μg mL^{-1})[a]	MIC of oxacillin (μg mL^{-1})				
		MRSA				MSSA
		OM481	OM505	OM584	OM623	209P
None	–	512	128	256	512	<0.5
EGCG (**1**)	16	64	32	32	64	<0.5
EGCG (**1**)	16	32	16	32	32	<0.5
Ascorbic acid	16					
EGCG (**1**)	16	64	32	32	64	<0.5
Cysteine	16					
EGCG (**1**)	16	64	32	16	64	<0.5
Aspartic acid	16					
EGCG (**1**)	16	32	64	64	64	<0.5
Glutamic acid	16					
EGCG (**1**)	16	64	32	32	64	<0.5
Proline	16					
EGCG (**1**)	16	32	32	64	128	<0.5
Ovalbumin	200					
EGCG (**1**)	16	512	128	256	512	<0.5
Casein	200					
EGCG (**1**)	16	32	32	32	64	<0.5
β-Cyclodextrin	200					
EGCG (**1**)	16	64	32	32	64	<0.5
Alginic acid	80					
EGCG (**1**)	16	64	32	64	64	<0.5
Trehalose	320					
EGCG (**1**)	16	64	32	64	64	<0.5
Pectin	16					

MICs of oxacillin against strains of methicillin-resistant and -sensitive *Staphylococcus aureus* (MRSA and MSSA, respectively) in the presence of EGCG (**1**).

[a] The MRSA and MSSA strains were incubated in the presence of the indicated concentrations of the compounds.

(128–512 μg mL^{-1}), and ovalbumin also increased the MIC for two of the bacterial strains. Analogous effects were observed when penicillin G was used as the antibiotic (Table 4). The resistance suppressive activity of EGCG (**1**) was enhanced in the presence of ascorbic acid and weakened in the presence of casein (Table 4).

Table 4
Effects of food additives on the decrease in the minimum inhibitory concentrations

Compound added	Concentration of additive (μg mL^{-1})[a]	MIC of penicillin G (μg mL^{-1})				
		MRSA				MSSA
		OM481	OM505	OM584	OM623	209P
None	–	32	32	32	32	<0.25
EGCG (**1**)	16	8	16	8	8	<0.25
EGCG (**1**)	16	4	8	4	8	<0.25
Ascorbic acid	16					
EGCG (**1**)	16	8	16	8	8	<0.25
Cysteine	16					
EGCG (**1**)	16	8	16	8	8	<0.25
Aspartic acid	16					
EGCG (**1**)	16	8	16	8	8	<0.25
β-Cyclodextrin	200					
EGCG (**1**)	16	32	32	32	32	<0.5
Casein	200					

(MICs) of penicillin G against strains of methicillin-resistant and -sensitive *Staphylococcus aureus* (MRSA and MSSA, respectively) in the presence of EGCG (**1**).

[a] The MRSA and MSSA strains were incubated in the presence of the indicated concentrations of the compounds.

We also examined the effect of ascorbic acid on the increase in bacteria during incubation (Fig. 4). In the presence of EGCG (**1**), a delay in the time of the increase in MRSA strains relative to that in the absence of EGCG (**1**) was observed, and the addition of ascorbic acid further delayed the increase.

3. Conclusion

Although some natural polyphenols have antibacterial effects and some also have suppressive effects on antibiotic-resistant bacteria, polyphenols are generally unstable, even in neutral solutions. Therefore, we examined the enhancement or prolongation of the effects of EGCG (**1**) in the presence of food-additives such as ascorbic acid, using EGCG (**1**) as a representative polyphenol. A decrease in EGCG (**1**) concentration in the solution was most potently suppressed in the presence of ascorbic acid compared to that with the other compounds examined. Therefore we found an enhancement or prolongation of the effects on MRSA of EGCG (**1**) in the presence of ascorbic acid. In contrast, the presence of proteins such as casein only weakly suppressed the decrease in EGCG (**1**) concentration, and the antibacterial or related effects of EGCG (**1**) also decreased. These findings may be relevant to either many pharmacological or biological effects that have been shown for EGCG (**1**) and other polyphenolic compounds. On the other hand, re-increase of the bacterial amounts after incubating several hours as shown in Fig. 3 showed that the effects of the combination EGCG–ascorbic acid was bacteriostatic, indicating its limitation for usage, especially in clinical applications.

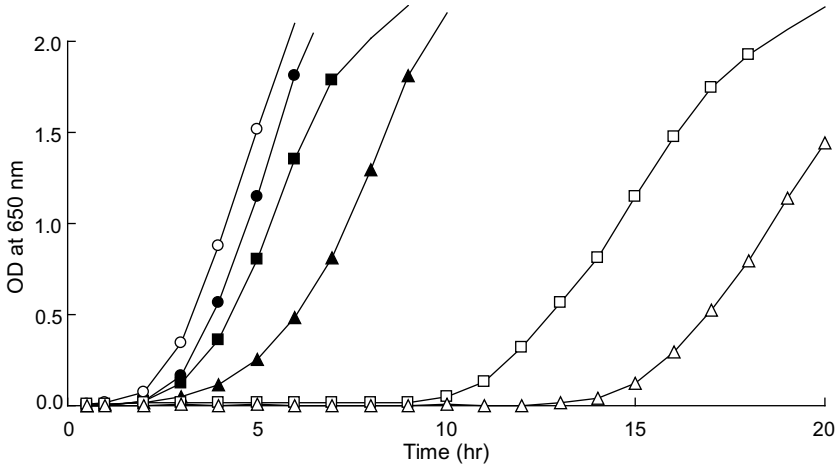

Fig. 4. Effects of the addition of ascorbic acid to a solution containing EGCG (**1**) on the increase in methicillin-resistant *Staphylococcus aureus* strain OM505. Turbidity (OD at 650 nm) of solutions without oxacillin, EGCG (**1**), or ascorbic acid (control, solid circle); in the presence of oxacillin (solid square, 4 μg mL^{-1}); in the presence of EGCG (**1**) (solid triangle, 16 μg mL^{-1}); in the presence of ascorbic acid (open circle, 16 μg mL^{-1}); in the presence of both oxacillin and EGCG (**1**) (open square); and in the presence of oxacillin, EGCG (**1**), and ascorbic acid (open triangle).

4. Experimental

4.1. Chemicals, antibiotics, and bacterial strains

We used the following commercially available compounds: L-ascorbic acid, β-cyclodextrin, L-glutamic acid, pectin, and L-proline (Nacalai, Kyoto, Japan); alginic acid, L-aspartic acid, α-casein, L-cysteine, and ovalubumin (Sigma, St. Louis, MO, USA). The antibiotics oxacillin sodium salt and penicillin G potassium salt were obtained from Sigma. The four bacterial strains of MRSA, i.e., OM481, OM505, OM584, and OM623, were clinical isolates from Okayama University Hospital. These MRSA strains and MSSA strain 209P were generously supplied by the Department of Microbiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

4.2. Isolation of EGCG (*1*) from green tea leaves

Green tea leaves (73 g) were homogenized in a mixture of acetone and H_2O (7:3, v/v; 700 mL × 3), and the concentrated filtrate (400 mL) from the homogenate was extracted with $CHCl_3$ (400 mL × 3) and EtOAc (400 mL × 6), successively. The EtOAc extract (8.0 g) was subjected to chromatography on a Toyopearl HW-40 column (Tosoh, Tokyo, Japan) eluted with EtOH–H_2O (7:3, v/v), with and the combined fractions containing EGCG (**1**) were subjected to repeated chromatography on MCI gel CHP-20P with increasing concentrations of MeOH in H_2O to obtain EGCG (**1**) (1.15 g).

4.3. Quantitative analysis of the effects of food-additive compounds on the decrease in EGCG (*1*) concentration in solution

A solution of EGCG (**1**) (0.1 mg mL^{-1}) in 0.05 M K_2HPO_4–KH_2PO_4 buffer (pH 6.5) containing 5% EtOH in H_2O was kept at 37 °C for 5 h in the absence or presence of ascorbic acid or other food-additives, and the resulting solution was analyzed using high-performance liquid chromatography (HPLC): column, Cadenza CD-C18, 4.6 × 150 mm (Imtakt, Kyoto, Japan); solvent 0.01 M H_3PO_4:0.01 M KH_2PO_4:CH3CN (17:17:6 by volume); oven temperature of 40 °C; flow rate of 1.0 mL min^{-1}; detection in ultraviolet light at 280 nm. The concentrations of remaining EGCG (**1**) are shown as the means of 3–6 experiments. The time course of changes in the concentrations of EGCG (**1**) and ascorbic acid during incubation for 10 h was also monitored using HPLC.

4.4. Estimation of MICs

The antibacterial effect of EGCG (**1**) against MRSA and MSSA and its suppressive effect on the antibiotic-resistance of MRSA were estimated using a liquid dilution method (Shiota et al., 1999). Briefly, the bacterial strains were grown in a cation-supplemented Mueller-Hinton broth (CSMHB; Difco, Detroit, MI, USA) containing $CaCl_2$ (50 μg mL^{-1}) and $MgCl_2$ (25 μg mL^{-1}), cultured overnight at 32 °C, and then diluted with 0.85% NaCl to 10^4 CFU $well^{-1}$ on 96-well microplates. The solutions in the wells were incubated at 32 °C for 24 h in the absence or presence of serially diluted test compounds. The MICs of the compounds were defined as the lowest concentrations at which the culture lacked turbidity after incubation.

4.5. Effects on the time course of the increase in bacteria

One strain of MRSA, OM505, was cultured overnight in CSMHB; part of the culture was incubated to produce a concentration with an OD_{650} of 0.6–0.7 and then diluted with CSMHB to 1/1000. A 4.5-mL aliquot of the test compound in CSHMB was added to 0.5 mL of the diluted bac-

terial solution, and the mixture was shaken at 30 rpm at 37 °C using a TN-1506 Biophotorecorder (Advantec, Tokyo, Japan) to record the time course of the increase in bacteria at OD_{650}. To estimate the amount of bacteria, the bacterial solution was diluted with 0.85% NaCl solution and plated onto nutrient agar. The number of colonies that formed after 24 h of incubation at 32 °C was counted to obtain the amount of bacteria in CFU mL^{-1}.

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