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Antimicrobial Activity of Aminoreductone against *Helicobacter pylori*

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Anti-*Helicobacter pylori* (*H. pylori*) effects of aminoreductone (AR), a Maillard reaction product, were evaluated in this study. AR effectively inhibited the growth of all 24 strains (19 clinical isolates and 5 isogenic mutants) irrespective of susceptibility to antibiotics and clinical manifestation. The minimum inhibitory concentration (MIC) of AR ranged from 0.5 to 5 mM. A killing assay with multiples of MIC was performed, demonstrating that the killing activity of AR was significantly higher than that of its derived melanoidin, an inhibitor of *H. pylori* urease−gastric mucin adherence, formed in the final stage of the Maillard reaction. These significant effects of AR on *H. pylori* were observed even in acidic conditions (pH 3). At most, 25 mM AR effectively exhibited bactericidal activity in all strains. These results rise up the possibility that foods containing AR, such as milk and dairy products, are valuable sources for preventing colonization of *H. pylori* in the stomach and its associated tissue damages.

KEYWORDS: *Helicobacter pylori*; inhibitory effects; MIC; aminoreductone; Maillard reaction; dairy product; milk

1. INTRODUCTION

Helicobacter pylori (*H. pylori*), a Gram-negative spiral microaerophilic bacterium, is one of the most common chronic bacterial pathogens of humans (*1*, *2*), colonizing in the stomach of up to half the world's population (*3*). The prevalence of *H. pylori* infection is about 40% in developed countries and 80−90% in the developing world (*4*). *H. pylori* plays a major role in the development of gastroduodenal diseases, including chronic gastritis, peptic ulcer, low-grade gastric mucosal associated lymphoid tissue lymphoma, and gastric cancer (*5*−*7*). There is an effective regimen, a triple therapy for *H. pylori* eradication, which usually consists of a proton pump inhibitor (*8*) and a combination of two antibiotics, amoxicillin and clarithromycin or amoxicillin and metronidazol in Japan. However, increased unsuccessful cases by eradication therapy are of great concern in Gastroenterology due to the increased occurrence of drug-resistant *H. pylori* (*9*), which prompt us to seek alternative agents instead of antibiotics. The effective anti-*H. pylori* agents that can eradicate or decrease the quantity of *H. pylori* colonized in the stomach with no adverse effect are necessary as alternative ingredients to suppress the harmful condition (*9*−*11*).

Maillard reaction, a chemical reaction between amino groups and reducing sugars, is very significant for foods because it strongly affects the quality (*12*). The Maillard reaction starts with the condensation of protein amino groups and reducing sugars and leads to the final polymerized products via formation of numerous intermediated products (*12*, *13*). In previous reports, we proposed an assay for determining an ability of milk to reduce a tetrazolium salt XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrezolium-5-carboxanilide) as a method of evaluating the extent of the Maillard reaction (*14*−*16*). It was also shown that aminoreductone (AR: 1-butylamino-1,2-dehydro-1,4-dideoxy-3-hexulose), which was formed during the Maillard reaction in a model solution of lactose and butylamine, was mainly responsible for the reducibility of XTT (*17*). Because the formation of AR was obtained as an indicator for the extent of the Maillard reaction, the discovery about the role and characteristic of AR should be an interesting topic for food scientists. In the field of food technology, both consumers and regulatory organizations demand high quality, healthy, and safe food. Food scientists always attempt to develop new processes to achieve these things. Throughout investigating the Maillard reaction in food, especially in milk, we have been always considering many factors that can contribute to a good and health beneficial product.

The effects of Maillard reaction products, concretely melanoidin, on microorganisms have been investigated. Several reports highlighted the role of melanoidin in vivo and in vitro against *Bacillus stearothermophilus*, a high thermoresistant food-degradative microorganism (*18*−*20*), and against some pathogenic and spoilage-causing bacteria frequently found in food such as *Escherichia coli* (*19*−*24*), *Staphylococcus aureus* (*22*), *Salmonella enteritidis* (*23*), and *Bacillus subtilis* (*19*, *25*). Rurián-Henares et al. stated that the antimicrobial activity of melanoidins could be

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related to their ability to chelate some metal cations, such as iron and magnesium, which is essential for growth and survival of pathogenic bacteria (*19*−*22*). There is a report concerned with the effect of Maillard reaction products, melanoidin, on *H. pylori*. Melanoidin had been shown to inhibit the adhesion between *H. pylori* urease and gastric mucin, resulting in suppression of the *H. pylori* colonization, anticolonization effect (*13*). However, it is unclear whether melanoidin interferes with the physiological behaviors of *H. pylori* including viability and growth. Moreover, there is little known about whether other ingredients produced during the Maillard reaction process such as AR have direct or indirect anti-*H. pylori* activities. To elucidate the functional properties of AR, one of the Maillard reaction products, we investigated the effect of AR against the gastric pathogenic microorganism, *H. pylori*.

2. MATERIALS AND METHODS

2.1. Reagents. Brucella broth was from Becton Dickinson Co. (Cockeysville, MD). Lactose monohydrate and urea were from Nacalai tesque, Inc. (Kyoto, Japan). *n*-Butylamine and agar were obtained from Wako Pure Chemical Industries (Osaka, Japan). Horse serum was purchased from Gibco (Auckland, New Zealand). All other reagents were of the highest grade commercially available. Milli-Q water or sterilized water was used in all procedures.

2.2. Preparation of AR and the Degradation Product of AR. Purified AR was prepared according to our previous reports (*17*, *26*). Briefly, lactose monohydrate (262 mM) and butylamine (1.16 M) were dissolved in 1.28 M phosphate buffer (pH 7.0). The sample solution (10 mL) was heated at 100 °C for 15 min, immediately cooled on ice, extracted three times with double volume of ethyl acetate, and evaporated. The residue was dissolved in 10 mL of 20% methanol and filtered through a Sep-pak Plus C-18 Cartridge (activated by 5 mL of ethanol and equilibrated by Milli-Q water) to remove brown components (melanoidin). The clear solution was evaporated again and freeze-dried to collect purified AR. In previous work, Shimamura et al. (*17*) reported the ^{13}C and ^{1}H NMR data on this extracted product, and the signals of the extracted compound could be assigned to the AR (1-butylamino-1,2-dehydro-1,4-dideoxy-3-hexulose). The figure of the AR structure was also proposed (*17*). The concentration of AR was calculated with the extinction coefficient of AR (17.98 M^{-1} cm^{-1}) at 320 nm (*26*).

In the final stages of the Maillard reaction, the system containing the brown, high molecular weight of polymerized products is called melanoidins (*12*, *27*). Because melanoidin possessed the antimicrobial activity in several pathogenic and spoilage-causing bacteria (*23*, *24*) and an inhibitory effect of *H. pylori* urease−gastric mucin binding (*13*, *23*), the degradation product of AR (referred to melanoidins) was also used as a reference in this study. The degradation product of AR was prepared as follows: The purified AR solution was kept at room temperature for at least 3 weeks until the remaining amount of AR was less than 0.5%. The remaining amount of AR was confirmed by using the XTT assay (*16*) or by estimating the absorbance at 320 nm (*26*).

2.3. Bacterial Strains and Culture Conditions. Nineteen *H. pylori* isolates randomly chosen among patients suffering with gastro-duodenal diseases were used in this study. In treatment regiments for *H. pylori* eradication, a combination of antibiotics such as metronidazole (MNZ), clarithromycin (CAM), and amoxicillin (AMC) is frequently used (*28*−*30*). As shown in **Table 1**, 24 strains including 5 isogenic mutants of wild-type HPK5 and antibiotic-resistant isolates are as follows: 7 MNZ-resistant; 3 CAM-resistant; 1 MNZ and CAM-resistant; and 13 antibiotic susceptibility strains. *H. pylori* HPK5 was obtained from a Japanese patient with a gastric ulcer (*31*), and its three derivatives such as *cdrA*-disrupted (HPKT510), *ureA*-disrupted (HPT208), and *ureB*-disrupted (HPT209) mutants constructed by insertion mutation with *xylE*-kanamycin (*kan*) resistance cassette (*32*, *33*) and two more derivatives, HPK5BA4 and HPK5SA4 mutants, were also used in this study. In addition, four isolates frequently used worldwide, NCTC11637 (derived from USA), SS1 (derived from Australia) (*28*), 26695 (derived from United Kingdom) (*34*, *35*), and J99 (derived from USA) (*34*) were also employed (*7*, *36*).

Table 1. MICs of Aminoreductone to *H. pylori* Strains[a]

strain	origin	susceptibility	MIC (mM)
NY11	Japan	MNZ	3
NY31	Japan	CAM	1
NY33	Japan	CAM	2
PYI	Japan	-	1
TK1402	Japan	-	1
KMT86	Japan	MNZ, CAM	3
KMT89	Japan	-	4
KMT92	Japan	MNZ	3
KMT93	Japan	MNZ	4
KMT114	Japan	-	5
KMT117	Japan	CAM	3
KMT127	Japan	MNZ	4
KMT129	Japan	-	2.5
KMT130	Japan	-	4.5
NCTC11637	United States	-	3.5
26695	United Kingdom	-	4
SS1	Australia	-	0.5
J99	United States	-	4
HPK5	Japan	-	4
HPKT510	Japan	MNZ	4
HPK5SA4	Japan	MNZ	4
HPK5BA4	Japan	-	4
HPT208	Japan	MNZ	4
HPT209	Japan	-	4

[a] -, antibiotic susceptibility strains; MNZ, metronidazole-resistant strains; CAM, clarithromycin-resistant strains.

All *H. pylori* strains were grown on the Brucella agar plate supplemented with 10% horse serum (HS) and 1.4% agar at 37 °C under microaerobic conditions (10% CO_2) (SANYO CO_2 incubator, Osaka, Japan) for 48 h. Whenever appropriate, Brucella medium supplemented with 10% HS (Brucella-serum medium) was used in this study.

Construction of HPK5BA4 and HPK5SA4 Isogenic Mutants of HPK5. On the basis of wild-type HPK5 strain, *babA*-disrupted (HPK5BA4) and *sabA*-disrupted (HPK5SA4) mutants were constructed by an insertion mutation with *kan* cassette (1.3-kb) of pUC4K (GE Healthcare Bio-Science Corp., Princeton, New Jersey) into corresponding genes (*babA* and *sabA* genes, respectively), according to a previous report (*32*). Two isogenic mutants, HPK5BA4 and HPK5SA4, were confirmed by PCR and RT-PCR and then determined to functionally adhere to the target molecules such as fucosylated Lewis b blood group antigen (Le^b) and the sialic acid, respectively. No binding activity of HPK5BA4-Le^b and HPK5SA4-sialic acid was confirmed.

2.4. Determination of Minimum Inhibitory Concentrations (MIC). The MIC of AR was determined using an agar dilution method according to that previously described (*37*, *38*). Briefly, 750 μL of AR solutions at given concentrations was separately added into the dishes containing 14.25 mL of yet-not-solidified Brucella agar supplemented with 10% of HS. The final concentrations of AR in the agar plates were set to range from 0 to 8 mM. Subsequently, each 10 μL of *H. pylori* suspensions (OD_{600} = 0.5−0.6) was serial diluted and inoculated onto the surface of the sample-supplemented agar plates, followed by being kept at 37 °C for 48 h under microaerobic conditions. The addition of sterilized water was used as a control for all experiments. The number of colony forming units (CFU) was determined for the bacterial viability (*31*). The MIC was defined as the lowest AR concentration to inhibit the 1×10^4 of CFU compared to that of control. In addition, the degradation product of AR (melanoidin) was utilized in this assay to compare with AR in this study. All tests were performed in duplicate at least.

2.5. Killing Assay. To determine the bactericidal activity of AR to *H. pylori*, killing experiments were performed in the presence of 1, 2, or 5 × MIC of AR, according to the methods previously described (*7*, *37*). Bacteria grown well at 24 h after being cultured in Brucella-serum medium, corresponding to the late exponential phase, were harvested and washed by Brucella-serum medium, and then the supernatants were removed after centrifuging at 8000*g* for 1 min (KUBOTA 1120, Kubota Corp., Kanagawa, Japan). In 1.5 mL centrifuge tubes, 0.4 mL of the bacterial

suspensions (OD_{600} = 0.5−0.6) by Brucella-serum medium with or without AR (control) were incubated at 37 °C under microaerobic conditions with shaking (Bio shaker BR-40LF, Taitec Co., Ltd., Kanagawa, Japan) for 3 h. At 1 and 3 h after incubation, each 10 μL of the suspensions was serial diluted and inoculated onto the Brucella agar plate and cultured for 3 days under microaerobic conditions to determine the viability. In addition, the degradation product of AR, melanoidin, was used in this assay. The killing ability of AR and melanoidin to *H. pylori* strains was evaluated by CFU counts and its comparisons with control. All examinations were performed in duplicate at least.

2.6. AR Activity in Acidic Conditions. To investigate the activity of AR in the acidic conditions, the killing assay except for the use of the phosphate-buffer saline (PBS) instead of Brucella-serum medium was carried out. PBS mimics the buffering capacities of the stomach (*36*). Briefly, the bacteria were harvested and washed once in PBS (pH 7). The cells were suspended in PBS (adjusts to pH 3 with HCl) with or without the presence 10 mM urea (*36*) and incubated for 1 h at 37 °C under microaerobic conditions. The killing ability of AR in the acidic condition to *H. pylori* strains was determined by CFU counts and compared with control (PBS at pH 7). All examinations were performed in duplicate at least.

2.7. Cluster Analysis. In all strains, cluster analysis was performed in the tolerant ability to AR based on MIC value and the multiple of MIC required for bactericidal activity by a tree joining algorithm (single linkage and squared Euclidean distance) (*39*) with STATISTICA software (StatSoft, Inc. Tulsa, Oklahoma, U.S.).

3. RESULTS AND DISCUSSION

3.1. Growth Inhibition of *H. pylori* by AR. The inhibitory effects of AR to 24 *H. pylori* strains were examined by an in vitro assay. All the strains exhibited susceptibility to AR at concentrations lower than 5 mM (**Table 1**). The MIC values ranged from 0.5 to 5 mM (for SS1 and KMT 114, respectively), although the bacterial growth of almost strains, including mutant strains, was inhibited at a MIC value lower than 4 mM.

AR has a labile aminoreductone structure (*40*) so that it can be subjected to the degradation process, followed by the advanced stages of the Maillard reaction, to form melanoidins during storage (*12*, *40*). Melanoidin plays a role of antimicrobial activities in vitro (*19*−*22*, *41*) and interacts with the different microbial species in the hindgut (*41*). Thus, AR derived melanoidins might also be contained in the culture medium and affect the survival of *H. pylori* during the assay. Therefore, to confirm the inhibitory activities of AR in this assay, the degradation products of AR (mainly melanoidins) were employed for the growth inhibition assay at the concentration similar to each MIC value of AR, finding no inhibitory effect of such degradation products (data not shown). These results indicated that the AR itself, but not melanoidin, possessed the potential effectiveness to inhibit the growth of all strains including five mutant strains examined irrespective of the susceptibility to antibiotics (CAM and MNZ), clinical outcomes, and geographical districts. The lack of activity of melanoidins in this work was due to their low concentration with respect to the one of aminoreductone, which seemed to be more active than melanoidins. Because melanoidins showed the antimicrobial activities against many kinds of pathogenic bacteria (*19*−*21*), they might need a higher concentration to perform their mechanism of action against *H. pylori*. MNZ and CAM are valuable agents in the treatment of several protozoal and bacterial infections including *H. pylori* (*7*, *29*, *30*, *42*). However, the presence of antibiotic-resistant *H. pylori* is increasing, which causes the failure of eradication therapy with antibiotics (*6*, *30*, *42*), which forces us to improve these situations with alternative ingredients. Interestingly, AR showed the growth inhibition of the MNZ-resistant and CAM-resistant and MNZ- and CAM-resistant *H. pylori* strains, indicating that AR might be an alternative ingredient as an adjuvant therapy for eradication of *H. pylori* infection.

The inhibitory activity of the Maillard reaction products (melanoidin), ubiquitously found in the heat-treated foods, on the bacterial growth was reported (*23*, *25*). The products formed in the Maillard reaction in a mixture with histidine-glucose and arginine-xylose were suggested to be the inhibitory constituents for both the pathogens and spoilage-causing bacteria in foods (*19*−*23*). The brown Maillard reaction products also showed an inhibitory effect on *B. stearothermophilus*, a high thermoresistant food-degradative microorganism (*18*, *19*). Moreover, concretely, melanoidin has antimicrobial activity against pathogenic microorganisms in food such as *E. coli* and *S. aureus* (*19*−*21*). Regarding the mechanisms of the antimicrobial activity of melanoidins, a possibility that melanoidins killed *E. coli* by irreversible changing in both the inner and outer membranes was shown (*21*). Melanoidins possessed the magnesium-chelating properties which cause a disruption and destabilization of the membrane of *E. coli*, leading to cell death (*22*). In addition, melanoidins exerted a bacteriostatic activity and decreased the virulence of pathogenic bacteria by iron-chelating properties (*22*). Most of those studies focused on the antimicrobial activities of melanoidin, the advanced Maillard reaction products. Little is known about the relationship between the Maillard reaction products and its anti-*H. pylori* activity (*13*), and no study regarding the antimicrobial activity of AR to *H. pylori* was reported. This is a first report that AR, an intermediate during Maillard reaction, but not its related melanoidins, showed the growth inhibition of *H. pylori*, leading to the conclusion that AR will be a good candidate for preventing the threat caused by *H. pylori* infection.

3.2. Killing Ability of AR in *H. pylori*. The growth inhibition of AR against all 24 strains was recognized. Next, to determine whether the inhibitory effect of AR is bactericidal and/or bacteriostatic, the killing assay with 24 strains was performed in the presence of 1, 2, or 5 × MIC of AR. The killing assay has been proposed as the most reliable method for determining the susceptibility of microorganisms to compounds and antibiotics (*37*). As shown in **Table 2**, it should be noted that AR had an ability of bactericidal effect for all 24 strains tested at the concentration of 5 × MIC or lower within 1 h after incubation. The most potent bactericidal activity was exhibited for SS1 at the concentration of 1 × MIC (0.5 mM). For SS1, NY11, PYI, and KMT127, the bactericidal concentrations were the same with MIC values, whereas it was observed in the other strains that the bactericidal activity of AR was reached at the concentration higher than the bacteriostatic concentration (2× MIC; 5 × MIC). The bactericidal effects for the other 3 isolates (NY11, PYI, and KMT127), 3 isolates (NY31, NY33, TK1402), and remaining 12 isolates were observed at the concentrations of 1, 2, and ≤5 × each MIC, respectively. These results indicated that the killing effect of AR differs among individual clinical isolates and is not associated with the properties of *H. pylori*, such as drug susceptibility, clinical outcomes, and geographical districts. In addition, the killing activity was no different between wild-type HPK5 and its five isogenic mutants, such as HPK5SA4, HPK5BA4, HPT207, HPT208, and HPKT510 strains. On the basis of the MIC value and multiple of MIC required for bactericidal effect, the highest dose of AR required for bactericidal effect was calculated, resulting in 25 mM for KMT 114 (5 × MIC (5 mM)). Because the concentration of AR in UHT-milk was from 0.1 to 0.625 mM, the estimation indicated that at most 25 mM AR in 400 μL broth culture is sufficient for effective bactericidal activity in all strains examined in vitro, and this amount of AR corresponds to 16−100 mL of UHT-milk (*26*). It is difficult to exactly

Table 2. Killing Ability of Aminoreductone to *H. pylori* Strains[a]

strain/AR concentration	MIC	2 × MIC	5 × MIC	bactericidal concentration (mM)	degradation products of 5 × MIC
NY11	+	+	+	3	+
NY31	-	+	+	2	-
NY33	-	+	+	4	-
PYI	+	+	+	1	-
TK1402	-	+	+	2	-
KMT86	-	-	+	15	-
KMT89	-	-	+	20	-
KMT92	-	-	+	15	+
KMT93	-	-	+	20	+
KMT114	-	-	+	25	+
KMT117	-	-	+	15	-
KMT127	+	+	+	4	+
KMT129	-	-	+	12.5	-
KMT130	-	-	+	22.5	-
NCTC11637	-	-	+	17.5	-
26695	-	-	+	20	-
SS1	+	+	+	0.5	-
J99	-	-	+	20	-
HPK5	-	-	+	20	-
HPKT510	-	-	+	20	-
HPK5SA4	-	-	+	20	-
HPK5BA4	-	-	+	20	-
HPT208	-	-	+	20	-
HPT209	-	-	+	20	-

[a] Bactericidal (+) and no bactericidal (-) effects observed were shown.

estimate the *H. pylori* population colonized in the human stomach due to individual host immunity, lifestyle, age, and diet. However, these results strongly suggest that the uptake of 100 mL of UHT-milk could kill at least 10^8~10^{10} *H. pylori* organisms.

The killing assay using the degradation products of AR (melanoidin), the browning compounds obtained by auto-oxidative process of AR at room temperature, also was carried out. Interestingly, in only 5 isolates of 24 strains, the killing activities were observed at the concentration equal to the degradation products of 5 × each MIC. No killing activity of the degradation products of AR has been found for the remaining 19 strains even in 5 × each MIC (**Table 2**). Of the five isolates, three isolates represented 2 × MIC for bactericidal effect of AR, while the two isolates (KMT92 and KMT93) showed 5 × MIC. These suggest that the bactericidal concentration of AR for the two isolates might be less than 5 × MIC since the killing assay was performed with 1, 2, and 5 × MIC. However, these results revealed that melanoidin has a slight ability of bactericidal effect and that the killing activity of AR to *H. pylori* is considerably higher than that of its derived melanoidin. Hiramoto et al. (*13*) showed that a variety of melanoidins derived from casein-lactose inhibited the adherence of *H. pylori* urease to gastric mucin, which suppressed the colonization of *H. pylori* in the stomach. Therapeutic efficacy of melanoidin was also confirmed in an animal model and human subjects infected with *H. pylori*. However, to our knowledge, no report concerned with the direct antimicrobial effects and its mechanism of AR as well as melanoidin against *H. pylori* was found. In this study, AR definitely possesses the direct antimicrobial effect to all *H. pylori* strains examined, such as growth inhibition with bactericidal activity.

The bactericidal effects of *H. pylori* after exposure to AR at the concentration of 5 × MIC in the acidic PBS (pH 3) supplemented with urea (10 mM) were tested for SS1 (the lowest MIC (0.5 mM)), KMT114 (the highest MIC (5 mM)), KMT127 (MNZ-resistant), KMT117 (CMA-resistant), HPK5, and its derivative HPKT510 strains. Urea is essential for keeping *H. pylori* alive in the acidic conditions (*43*). The killing ability of AR for all six strains in the acidic PBS with or without the presence of urea was examined. All strains did not survive in the acidic PBS without urea but completely survived with urea, which is consistent with the results from using PBS (pH 7) as control. However, in the acidic PBS with the presence of urea and AR, no bacteria survived, indicating that AR has a steadily active bactericidal effect on *H. pylori* even in the acidic conditions including at pH 1 (data not shown).

3.3. Cluster Analysis. On the basis of MIC values and the multiple of MIC required for bactericidal activity, cluster analysis was performed, demonstrating that all strains were definitely divided into two groups (**Figure 1**). Group 1 (95% similarity) contains 17 strains including all isogenic mutants, which have relative high MIC values and a high level of multiples of MIC required for killing. The dose of AR required for bactericidal activity was calculated as more than 12.5 mM in group 1. On the contrary, relatively low MIC values and low levels of multiples of MIC were observed in seven isolates of group 2 (80% similarity). The dose of AR required for bactericidal activity in group 2 was less than 4 mM. These results indicated that AR with low dose has a more effective bactericidal activity in *H. pylori* strains belonging to group 2. No common clinical characteristics related with the strains in each group were discovered; however, the group 1 strain has a major prevalence, suggesting that *H. pylori* might alter the tolerant ability of AR during persistent infection in the human stomach due to individual lifestyle and adapt to the microenvironment as principal component clines. Thus, in the near future, to investigate mechanisms by which AR possesses the anti-*H. pylori* activity is of interest, and will probably allow us to understand the ancestral property of *H. pylori*. In addition to the antibiotic-resistant strains that were observed in both groups, HPK5 and its isogenic mutants including the strains became MNZ-resistant and showed the same susceptibility to AR, suggesting that anti-*H. pylori* activity of AR would be an independent mechanism by the antibiotics. Furthermore, except KMT127 and NY11 isolates, a significant positive correlation at the 0.01 level ($r = 0.90$; $n = 22$) was observed between MIC values and the required multiple of MIC for bactericidal activity. This result implicated that the strains requiring a high value in MIC have also a propensity to require a high multiple value of the MIC for killing. However, at most, 25 mM AR is sufficient to exhibit the bactericidal activity in all 24 strains examined.

3.4. Comparison for Wild-Type HPK5 and Its Isogenic Mutants. The five isogenic mutants were utilized in this study. HPK5SA4 lacking SabA protein and HPK5BA4 lacking BabA protein strains can not bind to Le^b and sialic acid expressed on the epithelial cells in the stomach, respectively. These bacterial adhesion proteins were involved in persistent infection. HPT208 and HPT209 mutants do not possess UreA and UreB proteins, respectively, subunits of *H. pylori* urease. These four proteins localized on the surface of bacteria were also involved in direct interaction with microenvironmental materials. Therefore, at least these bacterial proteins were unnecessary for AR function. Furthermore, HPKT510 loses the *cdrA* protein which suppresses the cell division and gains the ability of tolerance to β-lactum antibiotics and more severe microenvironments than HPK5 (*31*). Deletion of the *cdrA* in *H. pylori* genome was observed during persistent infection due to adaptation to the harsh microenvironment in the stomach (*35*). On the basis of the results with the HPKT510 strain, AR might be applicable to such mutant strain possessing high capability for surviving under severe conditions. Taken together, our study with these five mutants indicated that the antimicrobial activities of AR operate independently on the pathway relevant to these bacterial proteins and that other bacterial molecules might be involved in AR function for

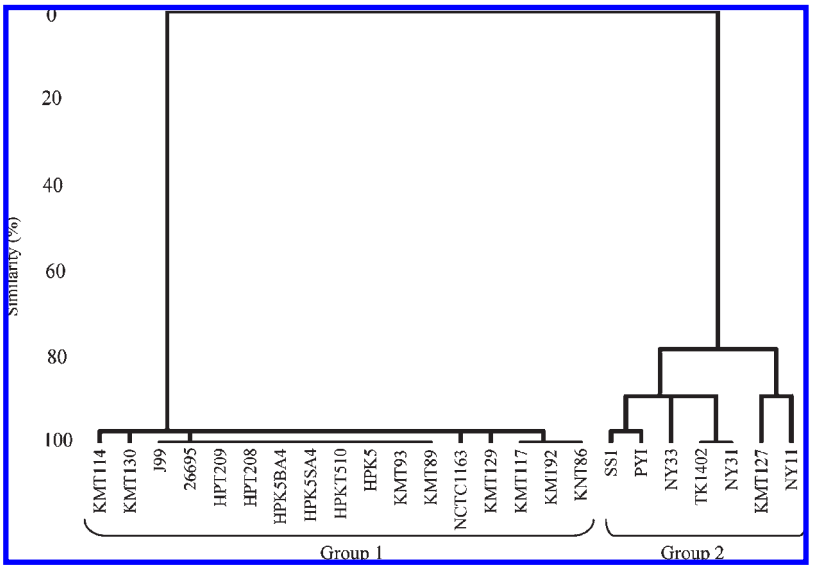

Figure 1. Dendogram produced by cluster analysis of *H. pylori* strains using the MIC value and multiples of MIC required for killing ability. Group 1 and 2 strains are 95% and 80% similarities, respectively.

H. pylori. Microscopic observation of *H. pylori* treated with AR demonstrated that no particular morphological changes such as coccoid formation, filamentation, and lysis were observed (data not shown), suggesting that such bactericidal effects differ from the mechanisms induced by antibiotics.

However, the exact antimicrobial mechanism is still unknown. The high molecular weight structure ($M > 1000$ Da) of the Maillard reaction product was required for antimicrobial activity (*13*, *23*, *41*). These high molecular weight products exert their antimicrobial activity by chelating important metals (such as iron or magnesium) which are necessary for the bacterial survival (*22*). On the contrary, AR possessing a low molecular weight structure ($M = 217$ Da) showed a higher microbial activity than its derivative products with high molecular weight such as melanoidins. The low molecular weight product (like AR) has no chelating property. So, it should have a different mechanism of action with melanoidin. We need more investigation to determine the antimicrobial mechanism of AR, an early Maillard reaction product, to understand the application with these products for therapeutic efficacy. The present study suggests that foods containing AR such as milk and dairy products (*16*, *17*) may be a promising and effective source for decreasing the risk associated with colonization of *H. pylori* in the stomach.

This is a first report that AR, but not its derived melanoidins, effectively inhibits the growth and viability of *H. pylori* irrespective of susceptibility to antibiotics and clinical manifestation. Furthermore, AR exhibited bactericidal activity with different effective doses, and at most 25 mM AR was sufficient for all strains examined. These effectives doses were confirmed in the acidic conditions, arising to the possibility that foods containing AR are valuable sources as anti-*H. pylori* ingredients. Investigating the mechanisms of AR function and anti-*H. pylori* effectives in vivo could open up a new approach to food technology that combines good quality with health benefits.

ABBREVIATIONS USED

H. pylori, *Helicobacter pylori*; AR, aminoreductone; MIC, minimum inhibitory concentration.

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