Catalytic removal of acetaldehyde in saliva by a Gluconobacter strain

Haruhiko Yamaguchi,1 Miho Hosoya,1 Takefumi Shimoyama,1 Seiji Takahashi,1 Jian Feng Zhang,1 Eri Tsutsumi,2 Yukio Suzuki,3 Yoshihide Suwa,2 and Toru Nakayama1,*

Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, 6-6-11 Aza Aoba, Aramaki, Aoba-ku, Sendai, Miyagi 980-8579, Japan,1 Suntory Research Center, Suntory Holdings, 1-1-1 Wakayamadai, Shimamato-cho, Mishi-mama-gun, Osaka 618-8503, Japan,2 and SIFS Japan, Chuo-ku, Osaka 541-0047, Japan3

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Acetaldehyde (AA) accumulates in the oral cavity after alcohol intake and is responsible for an increased risk of alcohol-related upper aerodigestive tract (UADT) cancer among aldehyde dehydrogenase 2-inactive heterozygotes in particular. Thus, the removal of AA from the saliva to a level below its mutagenic concentration (50 μM) after drinking is a potentially straightforward method for reducing the risk of alcohol-related UADT cancer. Although microbial cells with AA-decomposing activity could potentially serve as a useful agent for the catalytic removal of AA from the saliva without the supplemental addition of cofactors, these cells generally exhibit strong AA-producing activity from ethanol, which is present in excess (50 mM) over AA (100 μM) in the saliva after drinking. In this study, we observed that Gluconobacter kondontii (GK) cells efficiently decomposed salivary AA (100–390 μM) without the supplemental addition of cofactors irrespective of the type of alcoholic beverages consumed, even in the presence of an excess of ethanol (63 mM). Hydrogen peroxide, which is carcinogenic in animal experiments, was not produced because of the AA removal. The GK cells incubated at 45°C and pH 3.5 for 15 h were killed, but they retained 80% of their original AA-decomposing activity. The treated cells were used as nontoxic microcapsules that harbor a membrane-bound AA-decomposing activity.

Key words: Acetaldehyde; Alcoholic beverages; Aldehyde dehydrogenase; Gluconobacter; Upper aerodigestive tract cancer

Chronic alcohol consumption is a significant risk factor for squamous cell carcinoma in the upper aerodigestive tract (UADT, consisting of the oral cavity, pharynx, larynx, and esophagus) (1–4). Recent biochemical and epidemiological data strongly suggest that acetaldehyde (AA), the first ethanol produced, plays an important role in alcohol-related carcinogenesis in the UADT (2,4). AA is toxic, mutagenic, and carcinogenic in animal experiments (5), and its minimum mutagenic concentration is estimated between 50 μM and 150 μM (4,6). AA occurs naturally in alcoholic beverages, and its concentration is dependent on the beverage type (7,8). The increased risk for UADT cancer due to chronic consumption of alcoholic beverages with a high AA content may be partly associated with the direct exposure of the UADT mucosa to the AA in these beverages (8,9). AA is generated from ethanol locally in the UADT by the alcohol dehydrogenases of oral microbes (such as α-hemolytic streptococci and yeasts), mucosal cells, and/or the salivary glands (1,10,11). Thus, the local in situ production and accumulation of AA may also play a role in carcinogenesis in the UADT.

Aldehyde dehydrogenase (ALDH) catalyzes the oxidation of acetaldehyde to non-toxic acetic acid. In humans, there are multiple forms of ALDH, and the enzyme encoded by ALDH2 on chromosome 12 is thought to play a major role in the detoxification of AA produced during alcohol metabolism (12). In 40–50% of the East Asian population, ALDH2 exhibits a low activity due to an amino acid substitution in the enzyme arising from a single nucleotide polymorphism (13). The normal allele is termed ALDH2*1, whereas the inactive variant is designated ALDH2*2. People with low ALDH2 activity due to the heterozygous ALDH2 genotype (ALDH2*1/2) are termed “flushers”; however, they tolerate alcohol ingestion and may even become heavy drinkers or alcoholics. Epidemiological studies have shown that heavy drinkers with the heterozygous ALDH2 genotype constitute a high-risk group for alcohol-related cancer of the UADT (14). The relative risk for esophageal cancer in this high-risk group compared with ALDH2*1/1 homozygotes is reportedly 10–15-fold and the value for multiple esophageal carcinomas is as high as 54 (14–17). Analysis has shown that 30–180 min after drinking, the salivary AA concentrations in inactive ALDH2 heterozygotes are significantly higher than the concentrations in ALDH2 homozygotes (18). Moreover, in ALDH2-inactive heterozygotes, salivary AA levels are above the mutagenic concentration and are sustained for longer periods after drinking compared with ALDH2-active homozygotes. Thus, the mechanism underlying the ALDH2-associated increased risk of UADT cancer is likely related to the inability of the ALDH2-inactive heterozygotes to promptly eliminate AA from the UADT.

In an attempt to reduce the risk of alcohol-related UADT cancer, we investigated a microbial method to reduce the oral AA levels that are elevated after drinking. In the search for suitable AA-decomposing microorganisms, the following practical aspects must be considered: first, microorganisms with AA-decomposing
activity generally demonstrate a strong AA-producing activity from ethanol, which co-exists in excess (e.g., 63 mM [18]) of the AA in the saliva after the consumption of alcohol. A number of alcoholic beverages, such as beer, may contain unknown cofactors that activate the AA-producing activity of microorganisms. The cells used for this application should not accumulate AA, even after the consumption of alcohol, but should efficiently reduce the salivary AA concentration to a non-mutagenic level (less than 50 μM) irrespective of the type of beverage ingested. Second, the removal of AA should take place without the supplemental addition of cofactors. Third, no harmful byproducts shown to be carcinogenic in animal experiments, such as hydroxypropionaldehyde, should be produced because of the AA removal. Finally, the cells must be safe, and it is preferred that they are not genetically modified because the social acceptance of genetically modified microorganisms in foods varies from country to country.

In this study, we show that Gluconobacter kondonii strain NBRC3266 cells (termed GK cells) display an AA-decomposing activity that fulfills the requirements mentioned above. We characterized the activity of these bacterial cells in vitro to establish their catalytic properties, and we examined their potential application for the removal of the AA that accumulates in saliva after drinking.

MATERIALS AND METHODS

Chemicals and bacterial strains AA, ethanol, propanol, butanol, pentanal, hexanal, 2-nonanone, benzaldehyde, and vanillin were of analytical grade and were used as described previously. Chemicals in the GK medium were of edible grade. During cultivation of the bacteria, the temperature and pH of the medium were maintained at 28°C and pH 6.0 ± 0.1, respectively; the agitation rate was 200 rpm, the aeration rate was 1.0 v/v per min, and the inner pressure of the vessel was 0.7 kgf/cm². The GK cells were collected by centrifugation at 3000 × g for 15 min at 4°C and were subjected to lyophilization using a lyopholizer (model FDU-1200; Eyela, Tokyo, Japan). For large-scale cultivation, the GK cells were grown for 48 h in 1000 L of GK medium (see above) in a 2-kL fermentation tank. During cultivation, the temperature and pH of the medium were maintained at 28°C and pH 6.0 ± 0.1, respectively; the agitation rate was 140 rpm, the aeration rate was 1.0 v/v per min, and the inner pressure of the vessel was 0.7 kgf/cm². After cultivation, the bacterial cell culture was concentrated to 200 mL of water and re-concentrated by continuous centrifugation using a lyopholizer (model FDU-1200; Eyela, Tokyo, Japan).

AA decomposition assays The standard assay system consisted of 0.114 mM of AA, 25 μM of potassium phosphate buffer (pH 6.0), and 0.1 mg (dry weight) of the GK cells, in a final volume of 500 μL. The assay mixture, without the GK cells, was pre-incubated at 30°C for 5 min. The reaction was initiated by the addition of the GK cells, and the mixture was incubated at 30°C for 5 min. The reaction was stopped by the addition of 30 μL of 6 M of perchloric acid. The AA concentration in the resultant mixture was determined by head-space gas chromatography on an INNOWax 19091N–23318 column (0.25 mm × 30 m) using a Varian CP-3800/Tekmar 7000 apparatus (Varian, Inc., Tokyo, Japan). A unit (U) of AA-decomposing activity is defined as the amount of activity that results in the decomposition of 1 nmol of AA per min. To determine kinetic parameters, the initial velocity of AA decomposition was determined by using a standard assay system (see above) with varying AA concentrations. Km and Vmax values for AA by the GK cells were calculated using the curve fitting module of a SigmaPlot 11 and the Michaelis–Menten equation:

\[
V = \frac{V_{\text{max}} [\text{AA}]}{K_{\text{m}} + [\text{AA}]}
\]

For stoichiometric studies, the acetic acid concentration was determined using an F-kit (acetic acid, J.K. International). The rate of oxygen consumption was measured using an Oxygenigraph system (Hansatech Instruments, Ltd., Norfolk, UK).

For large-scale cultivations, the GK cells (20 mg dry cells/mL) were incubated in 0.104 M sodium citrate, pH 3.5, or 0.05 M potassium phosphate, pH 7.0, at 30°C, 40°C, 50°C, 60°C, and 70°C for 1 h. After incubation, the mixture was cooled on ice and assayed for residual activity, as previously described.

The GK cells (20 mg dry cells/mL) were incubated in 0.104 M sodium citrate, pH 3.5, at 45°C for 24 h with gentle stirring. At appropriate time intervals, an aliquot of the mixture was removed and was assayed for residual activity, as previously described. The viability of the GK cells was determined by plating the cells on a GK agar medium (agar concentration, 1.5% w/v) after the appropriate dilution of the cells with sterilized water.

Preparation of dried citrate-treated preparation of GK cells (DCTP-GK) For large-scale cultivations (500 g dry cells, corresponding to 83 g dry weight) were suspended in 0.104 M sodium citrate (final concentration), pH 3.5, at a final cell concentration of 20 mg dry cells/mL and incubated in a 10-L jar fermentor (model BMS-C, Able) at 45°C for 15 h at an agitation rate of 100 rpm. The cells were collected at 4°C by centrifugation using a Hitachi centrifuge (model CR20E) equipped with a R10C rotor and were freeze-dried using a lyopholizer (model FD-108M; Nikon Techno Service, Ibaraki, Japan). This GK cell preparation was termed the dried citrate-treated preparation of GK cells (DCTP-GK).

The viabilities of strain NBRC3266, total bacteria, staphylococci, and coliform bacteria in the DCTP-GK were examined using EIA agar medium (see above), nutrient agar (standard method agar “Nissui,” Nissui Pharmaceutical, Tokyo, Japan), Staphylococcus agar no. 110 (Nissui Pharmaceutical), and XM-g agar (Nissui Pharmaceutical), respectively.

Removal of AA in saliva by DCTP-GK Method I: Saliva (10 mL each) was collected from 6 healthy individuals, mixed, and centrifuged at 12,000 rpm for 15 min. Ethanol and AA were added to the supernatant to a final concentration of 63 mM and 100 μM, respectively, and 500 μL samples of the mixture were incubated at 37°C for 5 min in stoppered Eppendorf tubes. DCTP-GK (5.5, 16, 82, or 164 units) were added and the mixture was incubated at 37°C for 0.5, 10, 3.0, 5.0, 10, or 30 min. The reaction was stopped by the addition of 50 μL of 6 M perchloric acid. The amount of AA remaining in the mixture was determined by head-space gas chromatography, as described above.

Method II: Saliva (10 mL each) was collected from 6 healthy individuals, mixed, and centrifuged at 12,000 rpm for 3 min. To the supernatant, one of the following alcohol beverages was added: white wine, red wine, rice wine, beer, Shaoxing wine, shochu, whisky, and carvados. The final concentration of ethanol in the saliva was 63 mM. AA was added to the saliva/alcohol mixtures to a final concentration of 100 ± 20 μM, and 500 μL samples of each mixture were incubated at 37°C for 5 min in stoppered Eppendorf tubes. DCTP-GK (82 units) were added, and the mixture was incubated at 37°C for 5 min. The reaction was stopped by the addition of 50 μL of 6 M perchloric acid. The amount of AA remaining in the mixture was determined by head-space gas chromatography, as described above.

RESULTS

Microbial screening Microorganisms associated with food manufacturing were obtained from type culture collections. In addition, microorganisms that could grow on an agar medium containing 1% (w/v) acetaldehyde were isolated from natural environments. The microorganisms were examined for their ability to decompose 250 μM AA in the presence of 0.6 M ethanol. The strains that were capable of decomposing AA under these conditions included Gluconobacter, Pseudomonas, and Lactobacillus. These strains were then examined for their ability to decompose 200 μM AA in a 1:1 mixture of beer (ethanol, 4% v/v) and 0.05 M potassium phosphate buffer, pH 6.0, containing 400 μM AA. The only strain that decomposed AA efficiently was G. kondonii NBRC3226, which had been isolated previously from strawberry fruits (19); the other strains examined accumulated AA transiently under the same conditions (Fig. S1 in supplementary data). When AA decomposition by the G. kondonii NBRC3226 cells (termed the GK cells) was assayed...
using the standard assay system, the addition of supplemental cofactors, such as NAD, NADP, FAD, FMN, or ADP + Pi, was not required for the activity. Moreover, no detectable amounts of hydrogen peroxide were produced under the assay conditions. Thus, the AA decomposition catalyzed by this bacterium appeared to satisfy the requirements outlined in the Introduction section of this paper, and a detailed characterization of the bacterium was performed in vitro.

Characterization of the AA-decomposing activity of the GK cells

During the cultivation of the GK cells at pH 6.0 and 28°C, the AA-decomposing activity of the cells reached the highest level when the cells reached the stationary phase (Fig. 1). The cells harvested at stationary phase exhibited a specific activity of 159 ± 25 U/mg of dry cells under the standard assay conditions (at pH 7.0 and 37°C). When the cellular localization of the AA-decomposing activity in the GK cells was examined by differential centrifugation (Table 1), the majority of the activity was observed in the precipitate fractions, strongly suggesting that the activity was membrane-bound.

The AA decomposition did not occur when assayed using the N2-substituted assay system, suggesting that oxygen is required for the activity. Moreover, no detectable amounts of hydrogen peroxide were produced under the assay conditions. Thus, the AA decomposition catalyzed by this bacterium appeared to satisfy the requirements outlined in the Introduction section of this paper, and a detailed characterization of the bacterium was performed in vitro.

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TABLE 1. Differential centrifugation analyses of the cellular localization of the AA-decomposing activity of GK cells.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Recovery of AA-decomposing activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract and debrisb</td>
<td>100</td>
</tr>
<tr>
<td>Centrifugation (at 20,000 g x 10 min)</td>
<td>65.1</td>
</tr>
<tr>
<td>Precipitate</td>
<td>34.1</td>
</tr>
<tr>
<td>Supernatantc</td>
<td>34.1</td>
</tr>
<tr>
<td>Ultracentrifugation (at 125,000 g x 1 h)</td>
<td>28.0</td>
</tr>
<tr>
<td>Precipitate</td>
<td>3.2</td>
</tr>
</tbody>
</table>

a Total activity of cell extract and debris was taken as 100%.
b Cell extract and debris were subjected to centrifugation at 20,000 g x 10 min.
c This supernatant fraction was subjected to ultracentrifugation at 125,000 g x 1 h.

AA decomposition catalyzed by the GK cells displayed Michaelis–Menten kinetics (see Fig. S2 in the supplementary materials), with Vmax and K_m values of 805 ± 123 nmol/mg dry cells/min and 0.596 ± 0.148 mM, respectively, at pH 6.0 and 30°C.

Specificity

The ability of the GK cells to decompose aldehydes and alcohols was examined under the conditions described in Materials and methods (Table 2). The GK cells decomposed propanal, butanal, pentanal, hexanal, 2-nonenal, and benzaldehyde at rates that were comparable to the AA decomposition rate. Vanillin was a poor substrate for the GK cells. The GK cells decomposed ethanol and 1-propanol slowly (the relative activity was 12% and 14%, respectively, of the rate of AA decomposition), which is consistent with the observation that the cells did not accumulate AA, even in the presence of excess ethanol over AA in the reaction system (see above).

Stability of GK cells and preparation of DCTP-GK

The AA-decomposing activity of the GK cells was not lost when the cells were incubated at 20°C, 30°C, 40°C, 50°C, and 60°C for 1 h in 0.05 M potassium phosphate buffer, pH 7.0, or 0.104 M sodium citrate buffer, pH 3.5. However, the activity was lost at 70°C. Because G. kondoni is a mesophilic microorganism, these observations led us to examine the temperatures that killed GK cells effectively without the loss of their AA-decomposing activity (Fig. 3). GK cells (a final cell concentration, 20 dry cell mg/mL) were incubated at 45°C in 0.104 M sodium citrate buffer, pH 3.5, with gentle stirring. At timed intervals, the cells were harvested by centrifugation, and the precipitate was subjected to lyophilization. The dried cells were assayed for their AA-decomposing activity and their viability. The results demonstrate that following incubation for
15 h or longer, the cell preparation (per 10 mg dry cells) contained no detectable viable GK cells (Fig. 3B) but retained 60–80% of its original AA-decomposing activity (Fig. 3A). When the substrate specificity of the resultant GK cells, termed the dried citrate-treated preparation of GK cells or DCTP-GK, was examined, the ethanol-decomposing activity of the cells was completely lost (Table 2). Moreover, the same preparation contained no detectable amount of total bacteria, staphylococci, or coliform bacteria. Therefore, the DCTP-GK (see Materials and methods) were used to further examine the removal of AA in saliva (see below).

### Removal of AA in saliva using DCTP-GK

The ability of the DCTP-GK to eliminate AA in saliva was examined in vitro. To mimic oral conditions after drinking, the initial concentrations of AA and ethanol in the reaction mixture were set based on the respective salivary concentrations observed after drinking in the previous analyses (18). Fig. 4A shows the course of AA removal (initial concentration, 100 mM) by varying the amounts (6, 16, 82, or 164 U) of DCTP-GK in the saliva (500 μL) in the presence of 63 mM ethanol at 37°C. Following the addition of 82 and 164 U of DCTP-GK to the saliva, the AA concentrations diminished almost immediately (i.e., within 15 s) to levels that were significantly lower than the minimum mutagenic AA concentration (50 mM) and subsequently reached a level below the detection limit. With smaller amounts (6 and 16 units) of DCTP-GK, the removal of AA occurred more slowly, taking 6 and 22 min to reduce AA to the level below the mutagenic concentration, respectively. A higher concentration of AA (391 mM) (7,8) was also decomposed to a level below mutagenic concentrations within 3 min after the addition of 164 U of DCTP-GK to 500 μL of saliva (Fig. 4B).

We examined further the ability of DCTP-GK to eliminate AA from the saliva in the presence of unknown components of alcoholic beverages that are expected to remain in the oral cavity after drinking. A variety of alcoholic beverages (see Fig. 5) were diluted with saliva to a final ethanol concentration of 63 mM and were supplemented with AA to an approximate final concentration of 100 μM. DCTP-GK (82 units) was added to these mixtures (500 μL each), followed by incubation for 5 min. The results showed that irrespective of the beverage types examined, DCTP-GK reduced the AA concentration to a level significantly lower than the mutagenic concentration within 1 min.

### DISCUSSION

#### Removal of AA from saliva

Several lines of evidence show that oral bacteria serve as a main source of AA in saliva (18), for example, the salivary AA level after drinking in active ALDH2...
homozygotes could be reduced using a mouth rinse containing antiseptics (20,21). Therefore, tooth brushing (i.e., removal of dental plaques) or the use of aseptic mouthwash before or after drinking may partly inhibit the AA production in the oral cavity. However, AA production was not necessarily fully inhibited using these methods (20), and the oral AA level in inactive ALDH2 heterozygotes could potentially exceed the mutagenic stoichiometry (L-cysteine: AA, mol/mol), and an excess of pure L-cysteine above the amount of AA (e.g., 5 mg) (22) is required for efficient AA removal. The repeated ingestion of L-cysteine can potentially cause an amino-acid imbalance. Moreover, the reaction of L-cysteine with AA is a reversible process that can potentially regenerate AA. In contrast, AA removal by GK cells is an irreversible and catalytic process, which avoids the potential problems caused by the buccal L-cysteine tablets.

**Mechanistic consideration of AA removal by GK cells** Acetic acid bacteria, including the genus *Gluconobacter*, are characterized by their ability to oxidize ethanol to acetic acid (24,25). This process, which is referred to as acetic acid fermentation, involves the conversion of ethanol to AA and subsequently to acetate catalyzed by membrane-bound alcohol dehydrogenases and ALDHs (26–30), respectively. Supposedly, in *Gluconobacter* strains, these enzymes are located in the bacterial inner membranes (31). Electrons produced by the enzymatic oxidation of ethanol and AA are transferred to redox mediators (e.g., ubiquinones) that are also present in the inner membrane, followed by transfer to oxygen catalyzed by membrane-bound ubiquinol oxidases, either with or without the production of energy (32). The membrane-bound ALDHs of acetic acid bacteria generally exhibit their catalytic activities without supplementation with redox cofactors, such as NAD, FAD, FMN, and PQQ. However, enzyme structures and the chemical nature of the prosthetic groups associated with these ALDHs remain to be established (33). The biochemical and genetic characterization of the ALDH complex from *Acetobacter europaeus* suggested that this multi-subunit enzyme complex might contain heme, a [2Fe-2S] cluster, hemeb, and molybdopterin cofactors (34). Recent biochemical and spectroscopic characterization of a membrane-bound ALDH complex of *Gluconacetobacter diazotrophicus* suggested that this heterodimeric ALDH complex should be a quinoheme protein containing pyrroloquinoline quinone, cytochrome b, and cytochrome c, where cytochromes b and c constitute an intramolecular redox sequence that delivers electrons to the membrane ubiquinones (35).

The AA-decomposing activity of the GK cells was membrane-bound and did not require the supplemental addition of cofactors. These results, along with the stoichiometry of the AA decomposition catalyzed by the GK cells, suggested that the catalytic removal of AA by the GK cells was similar to the AA oxidation catalyzed by the membrane-bound ALDHs of acetic acid bacteria, as follows:

\[
\begin{align*}
2\text{CH}_3\text{CHO} + 2\text{H}_2\text{O} + 2X &\rightarrow 2\text{CH}_3\text{COOH} + 2\text{XH}_2 \\
2\text{XH}_2 + 2\text{Ubq} &\rightarrow 2\text{X} + 2\text{UbqH}_2 \\
2\text{UbqH}_2 + \text{O}_2 &\rightarrow 2\text{Ubq} + 2\text{H}_2\text{O} \\
2\text{CH}_3\text{CHO} + \text{O}_2 &\rightarrow 2\text{CH}_3\text{COOH}
\end{align*}
\]

where XH2 and X denote the oxidized and reduced forms of an ALDH cofactor, respectively, and Ubq and UbqH2 denote ubiquinone and ubiquinol, respectively. Reactions 1 and 2 are catalyzed by ALDH and reaction 3 is catalyzed by ubiquinol oxidases without coupling to energy production.

Acetic acid bacteria are frequently isolated from fresh fruits, are widely used in the industrial processing of foods, such as vinegar.
and tropical fermented products, and are generally regarded as safe (GRAS). Although the membrane-bound ALDH activities of acetic acid bacteria, along with electron transport systems to oxygen, are generally applicable to the removal of AA from saliva after drinking, it must be stressed that the acetic acid bacteria can potentially produce AA during drinking due to their alcohol dehydrogenase activities. Indeed, in most cases examined, the cells transiently accumulated AA above its mutagenic level when the cells were incubated with 200 \( \mu \text{M} \) AA ata 1:1 mixture of buffer and beer (ethanol, 4% v/v) (see Fig. S1 in the supplementary materials), which might have contained unknown activators of alcohol dehydrogenases. However, the GK cells exhibited low alcohol-decomposing activities (see Table 2) and effectively decomposed AA even under these conditions.

The in vitro studies show that GK cells are useful for the removal of the AA that accumulates in the oral cavity after drinking alcohol. The GK cells reduced the salivary AA concentration to non-mutagenic levels (less than 50 \( \mu \text{M} \)) after drinking alcohol. The GK cells reduced the salivary AA concentrations after drinking exhibit multi-phase absorption to non-mutagenic levels (less than 50 \( \mu \text{M} \)), without the addition of cofactors. The AA-decomposing activity was at a maximum at pH 4–6, which corresponds to the post-prandial pH of saliva (pH 4–5). No harmful byproducts, such as hydrogen peroxide, were produced because of the AA removal. The GK cells were killed by treatment with 0.104 M citric acid, pH 3.5, at 45°C for 15 h without the loss of AA-decomposing activity. The resultant DCTP-GK were useful as noniviable microcapsules that harbor a membrane-bound AA-decomposing system, which likely consists of ALDHs, ubiquinol oxidases, and redox mediators (e.g., ubiquinones).

**Practical aspects** Previous analyses suggest that changes in salivary AA concentrations after drinking exhibit multi-phase kinetics. For example, after drinking, the salivary AA concentration increases to an average level of 353 \( \mu \text{M} \) within 30 s (termed the burst phase), followed by an immediate decrease to 70–100 \( \mu \text{M} \) (7). The analyses also show that in inactive ALDH2 heterozygotes, salivary AA concentrations 30–180 min after drinking were maintained above or near the mutagenic level, irrespective of the type of alcoholic beverage ingested (18). In this phase (termed the steady-state phase), the rates of AA production from oral ethanol and the AA decomposition catalyzed by the enzymes of oral microbes, mucosal cells, and/or salivary glands should be almost balanced. The buccal use of GK cells greatly enhances the AA-decomposing activity in the oral cavity and would be particularly effective for the rapid reduction of the maximum salivary concentrations of AA to non-mutagenic levels in both burst and steady-state phases. It can be estimated that 820 units of AA-decomposing activity (corresponding to 7–15 mg DCTP-GK, depending on the specific activity) for each buccal use would be sufficient for the removal of a steady-state concentration of AA (approximately 100 \( \mu \text{M} \)) in the saliva to a non-mutagenic level within 1 min (assuming the amount of saliva is 5 mL). Because the oral amount of GK cells will decrease due to swallowing, pharmaceutical studies are needed to determine the most effective dosage forms (e.g., slow-release tablets, troches, or chewing gum) of the GK cells to retain the cells in the oral cavity for as long as possible. For example, combining GK cells with food thickeners (36), such as guar gum and xanthan gum, would render the GK cells cohesive to the oral mucosa and allow the cells to remain in the fine structures of the oral cavity for prolonged periods. Moreover, after the rapid removal of salivary AA, the salivary AA level is expected to be maintained below the mutagenic concentration by the small amount of the GK cells that remain in the oral cavity. These properties will be confirmed in future studies using human subjects, for which the bio-safety evaluation of DCTP-GK will be required.

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