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# Oral Installation of Recombinant Methioninase-producing *Escherichia coli* into the Microbiome Inhibits Colon-cancer Growth in a Syngeneic Mouse Model

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**Abstract.** *Background/Aim: All cancer types so far tested are methionine-addicted. Targeting the methionine addiction of cancer with recombinant methioninase (rMETase) has shown great progress in vitro, in mouse models, and in the clinic. However, administration of rMETase requires multiple doses per day. In the present study, we determined if rMETase-producing Escherichia coli JM109 (E. coli JM109-rMETase) might be an effective anticancer agent when installed into the microbiome. Materials and Methods: E. coli JM109-rMETase was administered to a syngeneic model of MC38 colon cancer growing subcutaneously in C57BL/6 mice. JM109-rMETase was administered orally by gavage to the mice twice per day. Tumor size was measured with calipers. Results: The administration of E. coli JM109-rMETase twice a day significantly inhibited MC38 colon-cancer growth. E. coli JM109-rMETase was found in the stool of treated mice, indicating it had entered the microbiome. Conclusion: The present study indicates the potential of microbiome-based treatment of cancer targeting methionine addiction.*

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Cancer cells are generally addicted to methionine (1-6) due to excess transmethylation reactions occurring in cancer cells (7-10). Therefore, cancer cells require a greater amount of methionine than normal cells. Cancer cells cannot survive under conditions of methionine restriction (1-10). This specific feature of methionine addiction is a fundamental and general hallmark of cancer and is termed the Hoffman effect (11). Under the condition of methionine restriction, cancer cells are arrested in the late $S/G_2$ phase of the cell cycle (12-14).

Methionine restriction is effective against cancer *in vitro* and *in vivo* (1-10, 14-18). Methionine restriction of cancer cells sensitizes them to cytotoxic chemotherapy, possibly due to late $S/G_2$ cell-cycle arrest (16, 19-22). Recently, Gao *et al*. confirmed our findings that methionine restriction enhanced chemotherapy efficacy, using colorectal cancer and soft-tissue sarcoma in patient-derived xenograft models. The report of Gao *et al*. also showed that methionine restriction disrupts the flux of one-carbon metabolism and affects vulnerabilities involving redox and nucleotide metabolism in the patient-derived xenograft model and human studies (23). Dietary methionine restriction combined with cytotoxic agents showed efficacy over the cytotoxic agent alone in clinical trials of melanoma, glioma, colorectal cancer, and gastric cancer (24-27).

All protein sources contain methionine, and it is difficult for patients with cancer to restrict methionine strictly by diet alone. For this reason, we have developed recombinant methioninase (rMETase), which breaks down methionine (17, 28, 29). rMETase is produced by fermentation of recombinant *Escherichia coli* transformed with the methioninase gene from *Pseudomonas putida (P. putida)* (28, 29). Recently, we have reported the efficacy of rMETase for multiple cancer types using the patient-derived orthotopic xenograft model (18, 30-39). rMETase was initially

administered by intravenous or intraperitoneal injection. However, recently we showed that rMETase was highly effective when administered orally (40-54). However, oral administration of methioninase requires multiple doses each day. Therefore, in the present study, we investigated the efficacy of rMETase-producing *E. coli* JM109 (*E. coli* JM109-rMETase) when installed orally to the microbiome. We hypothesized that when *E. coli* JM109-rMETase is in the microbiome, rMETase can be delivered constantly to the intestine and inhibit tumor growth.

## Materials and Methods

*Culture of E. coli JM109-rMETase*. *E. coli* JM109 was used as the host strain for the expression of rMETase. The rMETase gene from *P. putida* was cloned into *E. coli* JM109 using plasmid pATG3131, which also includes the tetracycline (TC) resistance gene (28, 29). The resulting *E. coli* JM109-rMETase was pre-cultured in 5 ml of Luria-Bertani (LB) liquid medium with TC (32 μg/ml) for 8 hours at 37˚C. Preculture broth was transferred to a 400 ml culture medium with TC (32 μg/ml) and cultivated overnight. After culture, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.3 mM for 4 hours at 28˚C to induce expression of rMETase. The optical density at 600 nM was used to determine the amount of live *E. coli* JM109-rMETase in the medium. After manually counting colonies, we determined that an O.D. 600 of 0.7 comprises approximately $1.0\times10^9$ colony-forming units (CFU)/ml of *E. coli* JM109-rMETase. *E. coli* JM109-rMETase was harvested and diluted with phosphate-buffered saline (PBS), and 20% glycerin was added for storage at −80˚C until administration to mice.

*E. coli* JM109 competent cells were prepared the same as *E. coli* JM109-rMETase but without TC in the LB medium, and IPTG was not added to the culture.

*E. coli JM109-rMETase tolerability study in C57BL/6 mice*. Firstly, we conducted a preliminary study to evaluate the tolerability of the C57BL/6 mouse to *E. coli* JM109-rMETase. Three different doses of *E. coli* JM109-rMETase ($10^6$, $10^8$ and $10^{10}$ CFU/100 μl) were prepared and fed by oral gavage to healthy C57BL/6 mice in the morning and evening, for 1 week, and the weight of each mouse was assessed.

*rMETase activity assay*. rMETase activity was determined from α-ketobutyrate produced from L-methionine, as previously reported (55). We examined the rMETase activity produced by different amounts *E. coli* JM109-rMETase. Assuming an intestinal environment, 20 mM deoxycholic acid was added to *E. coli* JM109-rMETase treated with IPTG, as described above, in order to release the rMETase from the bacteria (56). After the addition of 0.5 mM pyridoxal 5'-phosphate and 0.5 mg/ml dithiothreitol for 3 hours, rMETase activity was evaluated.

We examined rMETase activity in *E. coli,* cultured with IPTG, from mouse stool. We adjusted the bacterial density and sonicated the bacteria for 30 seconds after adding, pyridoxal 5'-phosphate and dithiothreitol and then evaluated rMTEase activity.

*Animals and diets*. C57BL/6 mice (AntiCancer Inc., San Diego, CA, USA) aged 4-6 weeks old were used in the present study. The mice were housed in a barrier facility with a HEPA-filtered rack under typical light/dark cycles of 12 hours. An autoclaved laboratory rodent diet was fed to mice from 9 a.m. to 5 p.m. during this study. Approval was received from the AntiCancer Institutional Animal Care and Use Committee's ethical committee under National Institutes of Health Guide Assurance Number 3873-1. All experiments were performed in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 criteria (57).

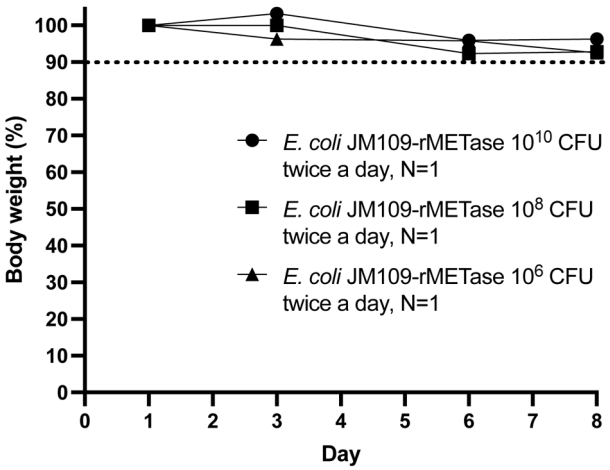


Figure 1. *Tolerability of C57BL/6 mice to recombinant methioninase (rMETase)-producing Escherichia coli JM109 (E. coli JM109-rMETase) as shown by body weight. CFU: Colony-forming units.*

*Cell culture*. The MC38 mouse colon cancer cell line (58) was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 IU/ml penicillin/streptomycin at 37˚C in a humidified environment containing 5% carbon dioxide.

*Establishment of subcutaneous tumors*. Eighteen C57BL/6 male mice were injected subcutaneously with $10^6$ MC38 cells in the right flank. One week after injection, subcutaneous tumors were established.

*Treatment study design*. Mice implanted with MC38 subcutaneously were randomized into three groups of six mice each: Group 1: Untreated control given 100 μl PBS orally twice daily (9 a.m. and 5 p.m.) for 14 days; Group 2: *E. coli* JM109 competent cells ($10^{10}$/100 μl) orally by gavage twice daily (9 a.m. and 5 p.m.) for 14 days: Group 3: *E. coli* JM109-rMETase cells ($10^{10}$/100 μl) orally by gavage twice daily (9 a.m. and 5 p.m.) for 14 days. TC (0.5 g/l) was added to the mouse's drinking water to prevent plasmid shedding only in group 3 (29). Tumor volume and body weight were measured every 2 days during the treatment. Tumor volume ($mm^3$) was calculated with the following formula: Tumor volume ($mm^3$)=length (mm) × width (mm) × width (mm) × 1/2.

*Stool and tissue collection*. Mouse stool was collected before treatment (day 0) and on the evening of day 14. The mice were sacrificed on day 15, and the tumor was removed.

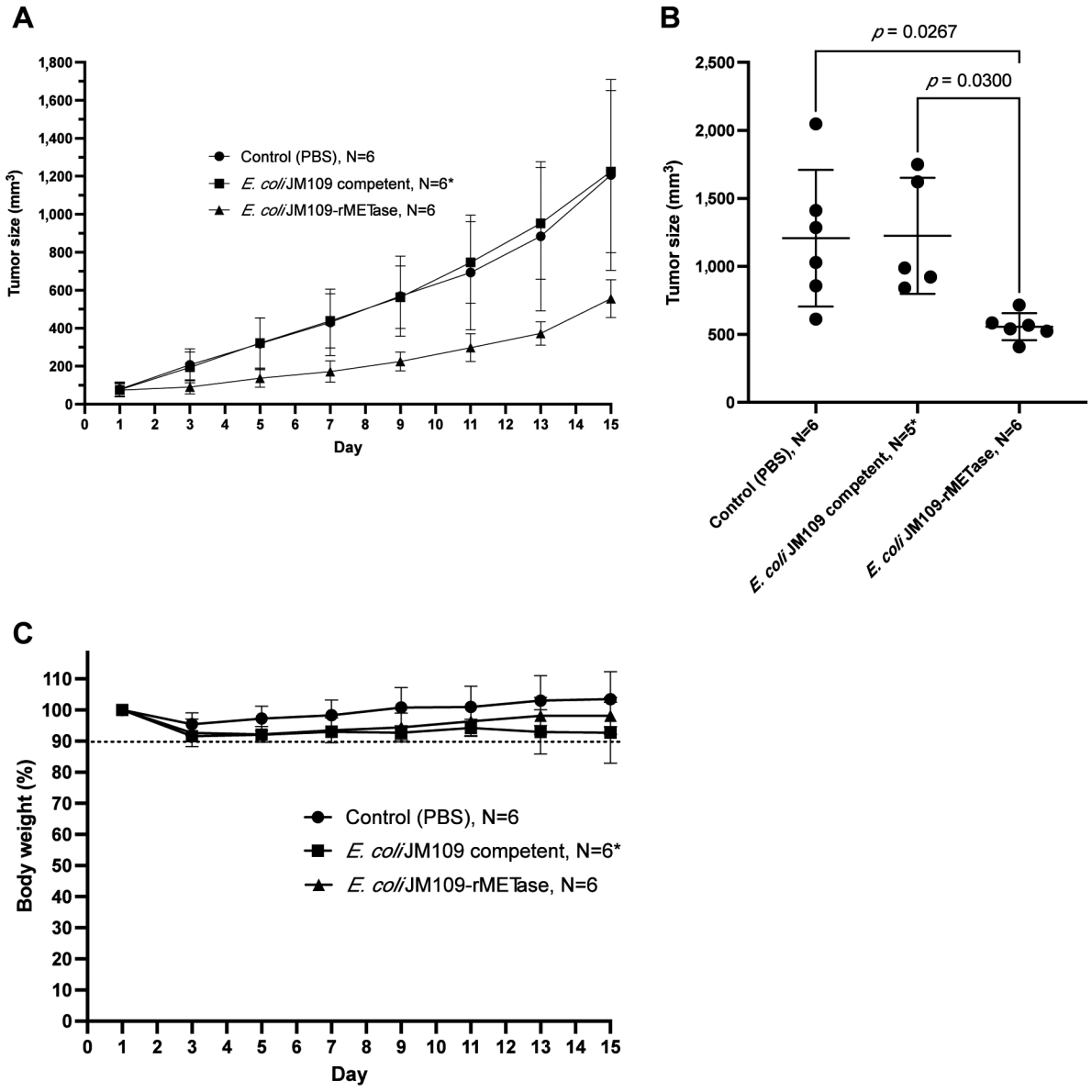


Figure 2. *Efficacy of recombinant methioninase (rMETase)-producing Escherichia coli JM109 (E. coli JM109-rMETase) on the syngeneic MC38 colon-cancer in the C57BL/6 model. A: Tumor growth curve. B: Tumor size on day 15. C: Body weight change. Data are shown as the mean±standard deviation. *One mouse was dead on day 11.*

*Mouse stool culture*. We performed stool culture to examine whether *E. coli* JM109-rMETase was incorporated into the mouse microbiome or not. The stool was diluted 1:10 by weight into PBS and then homogenized by mechanical disruption. Large debris was pelletized with short centrifugation at 200 × *g*. Then 100 μl of supernatant was plated onto LB agar with 32 μg/ml TC and incubated overnight at 37˚C.

*Screening for E. coli JM109-rMETase in mouse stool culture*. We performed Gram staining of colonies formed from stool culture. The Gram-negative bacterial colonies were plated on a modified M9 medium with TC (32 μg/ml) and IPTG (0.3 mM) to determine whether these bacteria produced rMETase or not. The modified M9 medium contained 6 g/l disodium hydrogen phosphate, 3 g/l potassium dihydrogen phosphate, 0.5 g/l sodium chloride, 5 g/l L-methionine, 0.24 g/l magnesium sulfate, 0.011 g/l calcium chloride, 2 g/l glucose, 32 μg/ml TC, and 0.3 mM IPTG. Before pouring the plates, phenol red was added to the medium as an indicator for methioninase at a final concentration of 0.007% (w/v) at a pH of 7.0 (59, 60). We determined whether colonies produced rMETase based on the pink color of the colonies because ammonium, produced from methionine by rMETase, makes the colony pink due to phenol red in the modified M9 medium.

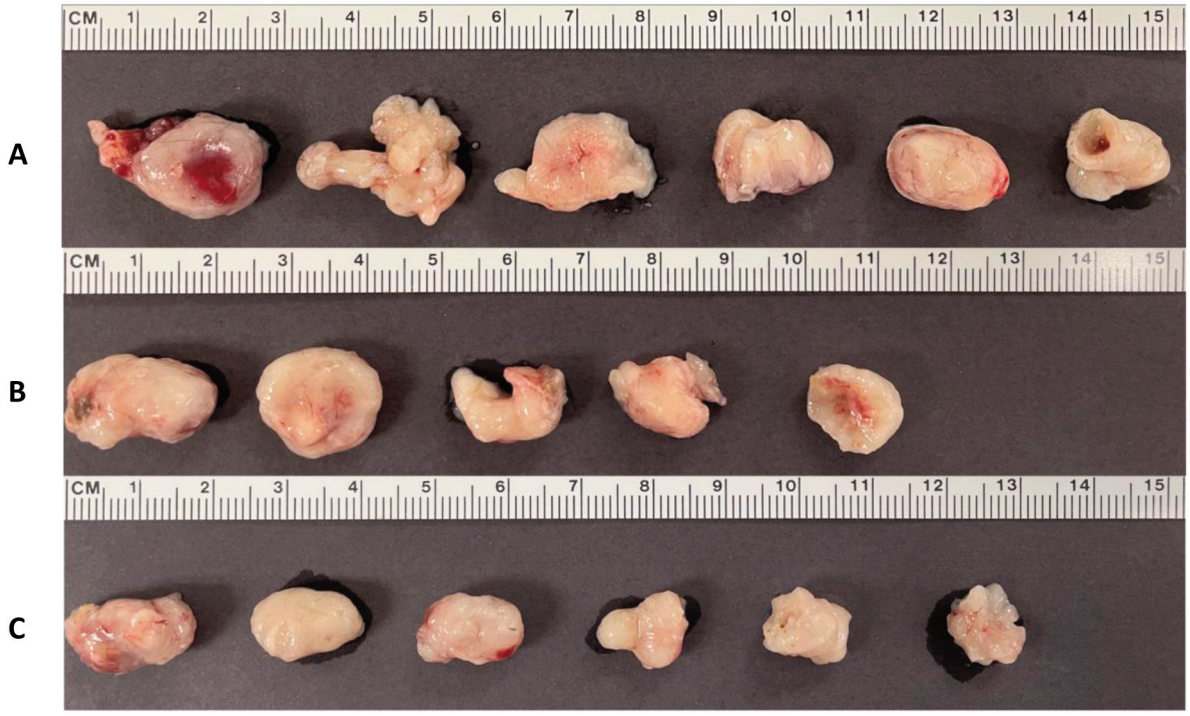


Figure 3. *Photographs of tumors excised from mice treated with phosphate-buffered saline (control) (A); Escherichia coli JM109 competent cells (B); or recombinant methioninase-expressing E. coli JM109 cells (C).*

*Statistics*. All statistical analyses were performed with GraphPad Prism 9.4.0 (GraphPad Software, Inc., San Diego). Tukey-Kramer was performed for the parametric test of comparison between groups. All data are displayed as the mean±standard deviation. *p*-Values ≤0.05 were regarded as significant.

## Results

*Tolerability of E. coli JM109-rMETase in C57BL/6 mice*. Different amounts of *E. coli* JM109-rMETase administered orally in C57BL/6 mice for 1 week allowed the body weight of mice to be maintained at all amounts tested, indicating *E. coli* JM109-rMETase was therefore not toxic (Figure 1).

*rMETase activity at different E. coli JM109-rMETase cell densities*. The rMETase activity of $10^{11}$ CFU/ml *E. coli* JM109-rMETase was 39.7 U/ml compared to 7.9 U/ml for $10^{10}$ CFU/ml *E. coli* JM109-rMETase. Based on these results, the dose of *E. coli* JM109-rMETase to be used in subsequent experiments was determined to be $10^{10}$ CFU/100 μl every morning and evening for 2 weeks.

*Antitumor efficacy of oral E. coli JM109-rMETase*. *E. coli* JM109-rMETase significantly inhibited MC-38 tumor growth in C57BL6 mice compared to the untreated control or non-rMETase-producing *E. coli* JM109 competent cells (control *vs*. *E. coli* JM109-rMETase: $p$=0.0267; *E. coli* JM109 competent cells *vs*. *E. coli* JM109-rMETase: $p$=0.03) (Figure 2A and B, and Figure 3).

*Body weight change*. Figure 2C shows no significant change in mouse body weight for each group, confirming the tolerability of $10^{10}$ CFU/100 μl *E. coli*.

*Stool culture to determine the presence of E. coli JM109-rMETase*. Culture of stool bacteria on modified M9 agar resulted in pink colonies of Gram-negative bacilli, consistent with the presence of *E. coli* JM109-rMETase (Figure 4). Furthermore, after the isolation of these colonies, they were grown to $10^{11}$ CFU/ml and examined for rMETase activity. All of the pink colonies had rMETase activity (data not shown).

## Discussion

Although we have previously reported the efficacy of oral rMETase in many types of cancer *in vivo* (40-54, 61-62), this is the first report that shows oral administration of *E. coli* JM109-rMETase inhibits tumor growth. When *E. coli* is administered orally, its toxicity to mice should be considered. A tolerability study demonstrated that up to $2\times10^{10}$ CFU/day

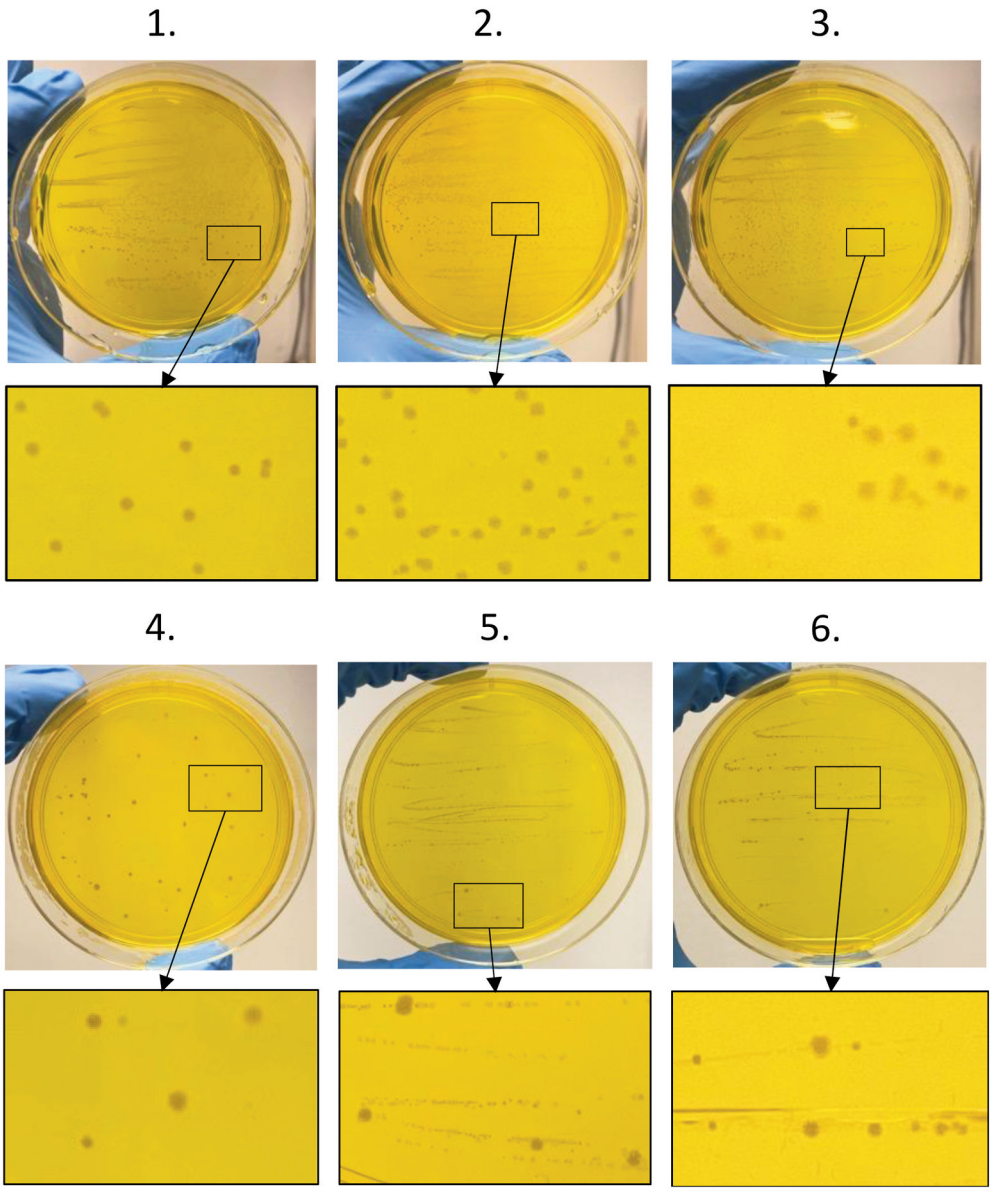


Figure 4. *Stool culture on day 15 from six mice treated with recombinant-methioninase-producing Escherichia coli JM109 cells (modified M9 medium with tetracycline).*

of *E. coli* JM109-rMETase is tolerable. *E. coli* JM109-rMETase ($10^{11}$ CFU/ml), after exposure to IPTG, had rMETase activity of 39.7 U/ml, which is about one-tenth of the rMETase we usually use for mice (500 U/ml) (62). The present results indicate that $2\times10^{10}$ CFU/day of *E. coli* JM109-rMETase inhibited MC-38 tumor growth in C57BL6 mice.

In the present study, we also found *E. coli* JM109-rMETase in mouse stool after 2 weeks of oral administration of *E. coli* JM109-rMETase, indicating it was incorporated into the microbiome.

In conclusion, bacterial therapy using rMETase-producing *E. coli* JM109 showed growth-inhibitory efficacy against syngeneic MC38 colon cancer in C57BL/6 mice. A previous study showed the anticancer efficacy of asparaginase-producing *Salmonella typhimurium* (63). Oral methioninase has been shown to be active clinically for prostate cancer (64-66), pancreatic cancer (67), and rectal cancer (68). Future studies will test microbiome-resident rMETase-producing *E. coli* for multiple cancer types. Recent advances in the manipulation of the gut microbiome indicate the future

potential of treating disease as shown in the present report with E. coli JM109-rMETase (69, 70).

## Conflicts of Interest

The Authors declare no competing interests regarding this work.

## Authors' Contributions

YK and RMH created the concept and design and wrote the article. YK performed acquisition, analysis, and interpretation of data. QH provided the recombinant methioninase-producing *E. coli*. KH, YA, NM, KO, AB, MB, and TT reviewed the article.

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