Antibacterial activity of disodium succinoyl glycyrrhetinate, a derivative of "glycyrrhetinic acid" against *Streptococcus mutans*

Short running title: Antibacterial agent to *S. mutans*

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Abstract

Streptococcus mutans is a cariogenic bacterium localized in the oral cavity. Glycyrrhetinic acid (GRA) is a major component of licorice extract. GRA and several derivatives, including disodium succinoyl glycyrrhetinate (GR-SU), are known to have anti-inflammatory effects in humans. We investigated the antimicrobial effect of GRA and its derivatives against the *S. mutans* UA159 strain. The minimum inhibitory concentrations (MICs) of GRA and GR-SU showed antibacterial activity against the *S. mutans* strain, while the other derivatives did not. Since GR-SU exhibited high solubility compared to GRA, we used GR-SU for further experiments. We evaluated the antibacterial activity of GR-SU against 100 *S. mutans* strains and found that all strains were susceptible to GR-SU, showing MIC values below 256 μg/ml. We performed a cell viability assay and found that GR-SU had a bacteriostatic effect on *S. mutans* cells. For growth kinetics, sub-MICs of GR-SU showed a growth inhibition effect. Then, we investigated the effect of GR-SU on *S. mutans* virulence. GR-SU at sub-MICs suppressed biofilm formation. Additionally, GR-SU greatly suppressed the pH drop caused by the addition of glucose. GR-SU suppressed the glucose-induced expression of the genes responsible for acid production (*ldh* and *pykF*) and tolerance

(*aguD* and *atpD*). Additionally, the expression of enolase, which is responsible for the carbohydrate phosphotransferase system (PTS), was not increased in the presence of GR-SU, indicating that GR-SU suppressed the incorporation of sugars in *S. mutans*. In conclusion, GR-SU showed not only antibacterial activity against *S. mutans* but also decreased *S. mutans* virulence.

Key Words:

Antibacterial agent, *Streptococcus mutans*

Introduction

Streptococcus mutans is an facultative anaerobic Gram-positive bacterium and is known to be a major cariogenic bacterium among oral bacteria (1, 2). *S. mutans* produces glucosyltransferases (GTFs), which mediate the formation of a sticky insoluble polysaccharide called glucan by using sucrose as a substrate (1, 2, 3). Glucan is necessary for the formation of dental plaques, especially on the smooth surface of the teeth. *S. mutans* produces acids, mainly lactic acid, by metabolizing sugars, including sucrose, to demineralize hydroxyapatite, which is a major component of the enamel of the teeth (3, 4). Therefore, *S. mutans* is strongly associated with tooth decay. Additionally, the maturation of dental plaques, especially subgingival plaques, is related to periodontitis.

Recently, many studies regarding the relationship between oral bacteria and systemic diseases have been published (5-7). Oral bacteria sometimes cause aspiration pneumonia, endocarditis and artificial joint replacement surgery infections (8, 9). Additionally, periodontitis is associated with lifestyle diseases, such as diabetes and atherosclerosis, as well as pregnancy and rheumatoid arthritis (10-15). Based on these reports, oral care is becoming more important to prevent not only oral diseases but also

systemic diseases. In oral care, inhibition/removal of dental plaque is important because dental plaque is a hotbed for dental caries and periodontitis (16). Mouthwashes sometimes been used for auxiliary oral care. Several types of mouthwash contain disinfectants, such as chlorhexidine gluconate, cetylpyridinium chloride and thymol, to kill bacteria (17-19). Additionally, natural products, which are extracts from several plants, including several flavonoids, are also added to mouthwash (20, 21).

Previously, we reported that glycyrrhetinic acid (GRA) and disodium succinoyl glycyrrhetinate (GR-SU) had antibacterial activity against *Staphylococcus aureus,* including methicillin-resistant *S. aureus* (MRSA) (22). GRA is a major component of licorice extract, which is a Leguminosae perennial found in the Mediterranean region, South Russia, central Asia, northern China and America. GR-SU is a derivative from GRA formed by the addition of a succinic acid moiety to increase water solubility (22). GRA has been reported to exhibit anti-inflammatory, antiallergic and antipeptic ulcer properties among others (23-26). In Japan, a preparation of monoammonium glycyrrhizinate (Stronger Neo-Minophagen C) is used to treat chronic hepatitis (27, 28). Additionally, several derivatives of glycyrrhetinic acid exhibit anti-inflammatory activities (29).

In this study, we evaluated the antibacterial activity of GRA and its derivatives against *S. mutans* strains. Since GR-SU showed strong antibacterial activity against *S. mutans*, we also investigated the effect of GR-SU on *S. mutans* virulence.

Materials and Methods

Bacterial strains and culture

S. mutans was grown in trypticase soy broth (TSB) (Becton Dickinson Microbiology Systems, Cockeysville, MD) at 37°C with 5% CO_2. Clinically isolated *S. mutans* strains were obtained from volunteers. *S. mutans* isolation was approved by the ethics committee of the Kagoshima University Graduate School of Medical and Dental Sciences (No. 701).

Glycyrrhetinic acid and its derivatives

Glycyrrhetinic acid (GRA) and its derivatives, including disodium succinoyl glycyrrhetinate, are shown in Fig. 1. These agents were obtained from Maruzen Pharmaceuticals Co., Ltd., Hiroshima, Japan. Dipotassium glycyrrhizate (GR-K) and

disodium succinoyl glycyrrhetinate (GR-SU) were solubilized in distilled water. Glycyrrhetinic acid (GRA) was solubilized in 100% dimethyl sulfoxide (DMSO). Stearyl glycyrrhetinate (GR-S) and glycyrrhetinyl stearate (GR-SA) were solubilized in 100% ethanol. Stock solutions of each reagent were prepared at a concentration of 20 mg/ml and were diluted in medium to the appropriate concentrations indicated in each experiment.

GR-SU antibacterial assay

The antibacterial activity of GRA and its derivatives was evaluated by the determination of MIC. The MICs were determined by using the microdilution method as previously described (30). Briefly, each agent was adjusted to 4,096 mg/L in TSB, and 2-fold serial dilutions were prepared in a 96-well microplate (Thermo Fisher Scientific, Roskilde, Denmark). Overnight bacterial cultures were adjusted to an OD_{660} of 1.0 (10^9 cells/ml) and diluted to 1:100 with TSB (10^7 cells/ml). Ten microliters of the bacterial culture (10^5 cells/well) was added to each well (100 μl total volume). The MICs of glycyrrhetinic acid and its derivatives were determined after the plate was incubated for 24 h at 37°C.

120

121 *S. mutans* growth curve

122 Two methods were used to determine the growth kinetics of *S. mutans* UA159. The
123 first method is as follows: First, TSB (5 ml) containing various concentrations of GR-
124 SU (4, 2, 1, 1/2, and 1/4 MIC) was prepared. Then, 5 x 10^5 cells were inoculated into
125 the prepared TSB. The optical density (OD) at 660 nm was monitored for 16 h.

126 The second method was as follows: An overnight culture of *S. mutans* was adjusted to
127 an OD_{660} of 1.0. Then, 100 μl of bacterial culture was inoculated into 5 ml of TSB and
128 incubated at 37°C with 5% CO_2. When the OD_{660} reached 0.2, various concentrations
129 of GR-SU were added to the medium, and the growth was monitored. To investigate
130 cell viability after the addition of GR-SU, appropriate dilutions of TSB were inoculated
131 onto a tryptic soy agar (TSA) plate. After overnight incubation at 37°C with 5% CO_2,
132 the colony forming units (CFUs) were counted. Three independent experiments were
133 performed, and the mean ± SD was calculated. The data were analyzed for statistically
134 significant differences compared to untreated controls at each timepoint by a two-way
135 ANOVA followed by Dunnett's post hoc test.

136

137 Effects of GR-SU on cell viability

138 Overnight cultures of *S. mutans* UA159 cells were washed with 10 mM sodium
139 phosphate buffer (PB; pH 6.8) and suspended in PB. The bacterial suspension was
140 diluted to 10^7 cells/ml with PB, and 10 µl of the diluted suspension was inoculated into
141 500 µl of PB containing various concentrations of GR-SU (1x MIC: 128 mg/L) and
142 incubated for 1 h at 37°C with 5% CO_2. Dilutions of the reaction mixture (100 µl) were
143 plated onto TSA plates and incubated at 37°C overnight. The antibacterial effect was
144 calculated as the ratio of the number of surviving cells (survival rate %) to the total
145 number of bacteria incubated in PB. To verify the cell number of each strain inoculated
146 in PB, dilutions of the bacterial suspension prior to inoculation were plated onto TSA
147 and incubated at 37°C overnight. The data were analyzed for significant differences
148 compared to untreated controls using two-way ANOVA followed by Dunnett's post hoc
149 test.

150

151 Effect of GR-SU on biofilm formation

152 An overnight culture of *S. mutans* UA159 was adjusted to an OD_{660} of 1.0, and then,
153 the bacterial culture was diluted 100-fold (10^7 cells/ml). Aliquots (10 µl) of bacterial

154 culture were added to each well of a plastic 96-well plate. Each well contained 100 μl
155 of TSB containing 2% sucrose and various concentrations of GR-SU. GR-SU was
156 serially diluted 2-fold from 2048 μg/ml to 1 μg/ml. The plates were incubated at 37°C
157 with 5% CO_2 for 16 h. The medium was then removed, and the wells were washed with
158 distilled water three times. Finally, biofilm cells were stained with 0.1% safranin for 10
159 min. After washing with distilled water three times, biofilm quantification was
160 performed to evaluate the absorbance of each well at 530 nm. Three independent
161 experiments were performed.
162
163 ATP efflux from *S. mutans* planktonic cells.
164 ATP efflux of *S. mutans* cells exposed to GR-SU was evaluated by using BacTiter-Glo
165 reagent (Promega, Madison, WI) according to the manufacturer's protocol. For
166 planktonic cells, 5 ml of *S. mutans* overnight culture was centrifuged, and bacterial
167 cells were suspended in an equal volume of 10 mM phosphate buffer (pH 6.8). Then,
168 GR-SU was added to the bacterial suspension to reach final concentrations of GR-SU
169 of 4, 2, 1, 1/2 or 1/4 MIC. After 10 min and 60 min incubations, reaction solutions
170 (500 μl) were taken and centrifuged at 10,000 x g for 5 min. The supernatants (400 μl)

were taken and stored for ATP measurement. Equal volumes of stock solution and reagent were mixed. After 5 min of incubation at room temperature, GR-SU was removed and washed twice with PBS. The bioluminescence response in relative light units was detected with a TriStar^2 LB942 multimode plate reader (BERTHOLD). The ATP concentration of each sample was determined using standard ATP solutions (10 to 0.001 μM).

pH drop assay

The pH drop method was performed as described elsewhere (31). A small portion (100 μl) of overnight *S. mutans* UA159 culture was inoculated into 40 ml of fresh TSB, and the culture was incubated at 37°C with 5% CO_2. When the OD at 660 nm reached 0.8, bacterial cells were collected by centrifugation at 10,000 x g for 5 min. Then, bacterial cells were washed with PBS, and bacterial cells were suspended in 0.5 mM potassium phosphate buffer containing 37.5 mM KCl and 1.25 mM $MgCl_2$ (pH 6.5) (PB). After centrifugation, cells were suspended in PB containing various concentrations of GR-SU (1/4, 1/2, 1, and 2 MIC). Then, glucose (20% wt/vol) was added to the bacterial solution at a final concentration of 1% glucose. The pH was monitored at appropriate intervals.

188 We also investigated the expression of the genes associated with acid production and
189 tolerance. Lactate dehydrogenase (LDH) mediates the reaction of lactic acid generation
190 from pyruvic acid (32), and pyruvate kinase (PykF) mediates the reaction of pyruvic
191 acid generation from phosphoenolpyruvate (33). Enolase is responsible for the
192 production of phosphoenolpyruvate (PEP). PEP is responsible for not only lactic acid
193 production but is also a key component of the PEP: carbohydrate phosphotransferase
194 system (PTS) (34, 35). *atpD* is a part of the F_1F_o-ATPase operon responsible for the
195 proton pump (31, 36, 37), and *aguD is a* part of the agmatine deiminase system operon
196 for alkali production (38). *S. mutans* cells incubated in PB containing various
197 concentrations of GR-SU for 30 min were collected, and total RNA was extracted by a
198 FastRNA Pro Blue kit (MP Biomedicals, Solon, OH, USA) according to the
199 manufacturer's protocol. A 1 μg aliquot of total RNA was reverse-transcribed to cDNA
200 using a first-strand cDNA synthesis kit (Roche, Tokyo, Japan). Using the cDNA as a
201 template, quantitative PCR was performed using a LightCycler system (Roche, Tokyo,
202 Japan). Primers to amplify the gene were constructed, and *gyrB* was used as an internal
203 control. The primers used in this assay are listed in Table 1.
204

205 **Results**

206 Antibacterial activity of GRA and its derivatives

207 First, we evaluated the MIC of GRA and its derivatives against the *S. mutans* UA159
208 strain. The MIC for both GRA and GR-SU was 256 μg/ml, while the other three
209 derivatives showed MICs greater than 2048 μg/ml (Fig. 1). Since GR-SU showed the
210 lowest MIC and high solubility in water, we used GR-SU for further experiments. Then,
211 we determined the MIC of GR-SU in 100 *S. mutans* strains (Fig. 2). The MIC of GR-
212 SU showed variation with a range from 32 to 256 μg/ml. Among the 100 tested strains,
213 the number of strains showing MICs of 128 μg/ml and 256 μg/ml were 48 and 43 strains,
214 respectively.

215

216 Effect of GR-SU on growth

217 To investigate the effect of GR-SU on bacterial growth, various concentrations of GR-
218 SU were added to the medium prior to bacterial inoculation. By the addition of more
219 than 1/32 of the MIC of GR-SU, the growth of the bacteria was gradually suppressed in
220 a dose-dependent manner compared to the growth of bacteria in the absence of GR-SU
221 (Fig. 3A). After 24 h of incubation, the inhibition effect (% reduction compared to none)

of the 1/2, 1/4, 1/8, 1/16 and 1/32 MIC of GR-SU was 40.1, 34.9, 31.6, 25.9 and 22.0%, respectively.

We also investigated the effect of GR-SU on growing bacterial cells. When the optical density reached 0.18, various concentrations of GR-SU were added to the bacterial culture. Compared to the control (without GR-SU), the growth was highly suppressed by the addition of GR-SU. However, the growth was not completely inhibited by the addition of more than 1 MIC of GR-SU (Fig. 3B).

Effect of GR-SU on cell viability

To check whether GR-SU has bactericidal activity against *S. mutans* cells, we performed a cell viability assay. Fig. 4A shows that viable cell numbers were not affected by the addition of GR-SU compared with those without GR-SU addition. Additionally, we investigated ATP efflux by the addition of GR-SU and found no increase in ATP efflux, although 4 MIC of GR-SU slightly induced ATP efflux (Fig. 4B).

Effect of GR-SU on biofilm formation

To determine the effect of GR-SU on *S. mutans* virulence, we evaluated the biofilm

239 formation of *S. mutans* UA159 in the presence of various concentrations of GR-SU. In
240 Fig. 5, biofilm formation was inhibited by GR-SU in a dose-dependent manner.
241 Although the growth of *S. mutans* UA159 was suppressed by GR-SU (1/2 MIC to 1/32
242 MIC) (Fig. 3A), GR-SU at 1/64 and 1/128 MIC still reduced biofilm formation by
243 55.5% and 29.5%, respectively. Then, we evaluated the biofilm formation of 10 clinical
244 isolates in the presence of 1/4 and 1/16 MIC of GR-SU. Fig. 5B shows that sub-MICs
245 of GR-SU reduced the biofilm formation of all strains tested in this study. Since GTFs
246 are mainly involved in biofilm formation, we investigated the expression of *gtfs* grown
247 in the presence of GR-SU. However, *gtfB* and *gtfC* expression was not affected by the
248 addition of GR-SU (data not shown).
249
250 Effect of GR-SU on pH drop
251 By the addition of glucose (1% wt/vol), the pH of the *S. mutans* suspended solution
252 was lowered to 4.68 (after 15 min), 4.14 (30 min) and 3.52 (2 h) (Fig. 6). GR-SU
253 addition inhibited the pH drop in a dose-dependent manner. In the presence of 2 MIC
254 GR-SU, the pH drop was strongly suppressed, and the pH values were 6.32 (after 15
255 min), 6.16 (30 min) and 5.26 (2 h). Additionally, sub-MICs of GR-SU suppressed acid

256 production in a dose-dependent manner.

257 Then, we investigated the expression of the genes responsible for acid production and
258 acid tolerance. The expression of *ldh*, *pykA*, *eno*, *aguD* and *atpD* was strongly induced
259 by the addition of glucose, while the expression of these genes was inhibited by the
260 addition of GR-SU, even at sub-MICs (Fig. 7).

261

262 **Discussion**

263 In this study, we investigated the antibacterial activity of GRA and its derivatives and
264 found that GRA and GR-SU had antibacterial activity against *S. mutans* strains, while
265 the other 3 GRA-derivatives had no antibacterial activity. Since our aim was to
266 determine whether GRA- or GRA-related agents have potential for clinical use in oral
267 care products such as mouthwash and toothpaste, GR-SU is a good candidate because
268 GR-SU is highly soluble in water compared to GRA. Although GR-K is also highly
269 soluble in water, GR-K did not show antibacterial activity against *S. mutans*. The loss
270 of antibacterial activity of GR-K is due to the addition of two glucuronic acids (Fig. 1)
271 causing structural alterations that influence the antibacterial activity.

272 Previously, we found that GRA and GR-SU had antibacterial activity against *S. aureus*

strains (22). The antibacterial activity of GRA and GR-SU against *S. aureus* was similar to that against *S. mutans*, showing an MIC range of 32-512 μg/ml for GRA and 16-256 μg/ml for GRA. Similar to *S. aureus*, GR-SU had a bacteriostatic effect on *S. mutans* cells (Fig. 4). Regarding the effect of GR-SU on bacterial growth, it is interesting to note that sub-MICs of GR-SU significantly affected bacterial growth. Even 1/32 MIC of GR-SU inhibited the maximum optical density (0.76 at 660 nm) at stationary phase compared to that without GR-SU (OD=0.98). This growth inhibition effect is dependent on the GR-SU concentration. In a biofilm assay (Fig. 5), we found that the addition of GR-SU at sub-MICs suppressed the biofilm formation of *S. mutans* strains. This suppression was mainly caused by the growth inhibitory effect of sub-MICs of GR-SU. However, in *S. mutans* UA159, biofilm formation was still inhibited by GR-SU at 1/128 MIC (Fig. 5A), while 1/128 MIC of GR-SU had a slight effect on bacterial growth (Fig. 3A). Since GtfB and GtfC are important for biofilm formation of *S. mutans* in the presence of sucrose, we investigated the expression of *gtfB* and *gtfC* in biofilm cells treated with GR-SU. As a result, we did not find significant alterations in gene expression (data not shown). Therefore, we think that additional factor(s) is involved in the inhibitory effect on biofilm formation by GR-SU together with the suppression of

290 bacterial growth.

291 For the pH drop experiments, GR-SU clearly inhibited acid production by in a dose-
292 dependent manner. *S. mutans* is known to produce mainly lactic acids by the
293 fermentation of sugars, including glucose and sucrose, under anaerobic and aerobic
294 conditions (1, 2). *S. mutans* takes up several sugars by PTS and non-PTS systems and
295 then metabolizes the sugars through the Embden-Meyerhof pathway (2, 4). When
296 excess sugars are in the environment, *S. mutans* produces many intermediate
297 metabolites, such as glucose 6-phosphate and fructose 1,6-bisphosphate, causing an
298 increase in the expression of pyruvate kinase (PK) and lactate dehydrogenase (LDH),
299 respectively (Fig. 8) (39-41). As a result, lactic acids are mainly produced by *S. mutans*
300 (39). We investigated the expression of the genes coding for LDH and PK and found
301 that GR-SU suppressed the expression of these genes. Additionally, we found that the
302 addition of GR-SU did not induce the expression of *atpD* and *aguD* responsible for acid
303 tolerance, indicating that the production of lactic acid was inhibited by GR-SU.
304 Therefore, we consider that GR-SU suppresses the uptake of sugars in the cytoplasm,
305 causing the suppression of sugar metabolism in *S. mutans*. This hypothesis is consistent
306 with our previous results in *S. aureus*. Comprehensive analysis of gene expression by

DNA microarray analysis in *S. aureus* showed that the expression of many genes related to sugar metabolism was altered by GR-SU treatment (22). Additionally, the minimum concentrations of glucose for the growth of *S. aureus* MW2 in chemically defined medium was increased by 4-fold in the presence of 1/2 MIC of GRA or GR-SU. Since acid production and acid tolerance in S. mutans are associated with cariogenic virulence (3, 31), the inhibitory effect of these factors by sub-MIC of GR-SU may be good advantage for the prevention of tooth decay.

In conclusion, we demonstrated the antibacterial activity of GR-SU against *S. mutans* strains. Although the precise mechanism of antibacterial activity by GR-SU was not defined, we hypothesize that GR-SU is associated with the inhibition of sugar uptake and metabolism, causing the inhibition of *S. mutans* growth. Taken together with previous reports of its anti-inflammatory effects, GR-SU may have potential for clinical use against *S. mutans* to prevent tooth decay and periodontitis.

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Disclosure

The authors declare no conflict of interest.

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Figure Legends

Figure 1. MIC of GRA and its derivatives in *S. mutans* UA159

Figure 2. MIC distribution of GR-SU in 100 *S. mutans* strains isolated from 100 subjects

Figure 3. Effect of GR-SU on the growth of *S. mutans* UA159

(A) Aliquots of bacterial cells were added to 5 ml of TSB containing various concentrations of GR-SU. The OD at 660 nm was measured over time. Three independent experiments were performed, and the mean ± SD was calculated. The data were analyzed for statistically significant differences compared to the control by a two-way ANOVA followed by Dunnett's post hoc test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$.

(B) Aliquots of bacterial cells were added to 5 ml of TSB. When the OD at 660 nm reached 0.15~0.2, various concentrations of GR-SU were added to individual bacterial cultures. The OD was measured over time.

457

458 Figure 4. Effect of GR-SU on the viability of *S. mutans*

459 (A) Viable cell numbers after treatment with various concentrations of GR-SU were
460 measured by the method described in the Materials and Methods. Three independent
461 experiments were performed, and the mean ± SD was calculated. The data were
462 analyzed for statistically significant differences compared to the control by a two-way
463 ANOVA followed by Dunnett's post hoc test.

464 (B) ATP efflux in response to treatment with various concentrations of GR-SU was
465 measured with the method described in the Materials and Methods. Three independent
466 experiments were performed, and the mean ± SD was calculated. The data were analyzed for
467 statistically significant differences compared to the control by a two-way ANOVA followed by
468 Dunnett's post hoc test. *$p < 0.05$ and **$p < 0.0001$.

469

470 Figure 5. Effect of GR-SU on the biofilm formation of *S. mutans*

471 (A) *S. mutans* UA159 was grown in TSB containing 2% sucrose and various
472 concentrations of GR-SU. (B) Ten *S. mutans* strains were grown in TSB containing 2%
473 sucrose and 1/4 or 1/16 MIC of GR-SU. Biofilm assays were performed by the method

474 described in the Materials and Methods. Three independent experiments were
475 performed, and the mean ± SD was calculated. The data were analyzed for statistically
476 significant differences compared to the control by a two-way ANOVA followed by
477 Dunnett's post hoc test. *$p < 0.05$ and **$p < 0.0001$.
478
479 Figure 6. Effect of GR-SU on pH change in *S. mutans* UA159
480 The pH value of the *S. mutans* suspension in 0.5 mM potassium phosphate buffer (PB)
481 containing 37.5 mM KCl, 1.25 mM $MgCl_2$ and various concentrations of GR-SU was
482 monitored for up to 2 h of incubation at 37ºC. The detailed methods are described in
483 the Materials and Methods. Three independent experiments were performed, and the
484 mean ± SD was calculated. The data were analyzed for statistically significant
485 differences compared to the control by a two-way ANOVA followed by Dunnett's post
486 hoc test. *$p < 0.05$ and **$p < 0.0001$.
487
488 Figure 7. Effect of GR-SU on the expression of acid production-related genes
489 *S. mutans* cells incubated in 0.5 mM potassium phosphate buffer containing 37.5 mM
490 KCl, 1.25 mM $MgCl_2$ and various concentrations of GR-SU for 30 min were collected.
491 RNA extraction, cDNA synthesis and quantitative PCR were performed by the methods

492 described in the Materials and Methods. Three independent experiments were
493 performed, and the mean ± SE was calculated. The data were analyzed for statistically
494 significant increases compared to control by a two-way ANOVA followed by Dunnett's
495 post hoc tests. *, p <0.05; **, p < 0.0001

496

497 Fig. 8. Pathway of lactic acid production in *S. mutans* UA159

498 EI, enzyme I; EII, enzyme II

499

500

501 **List of abbreviations**

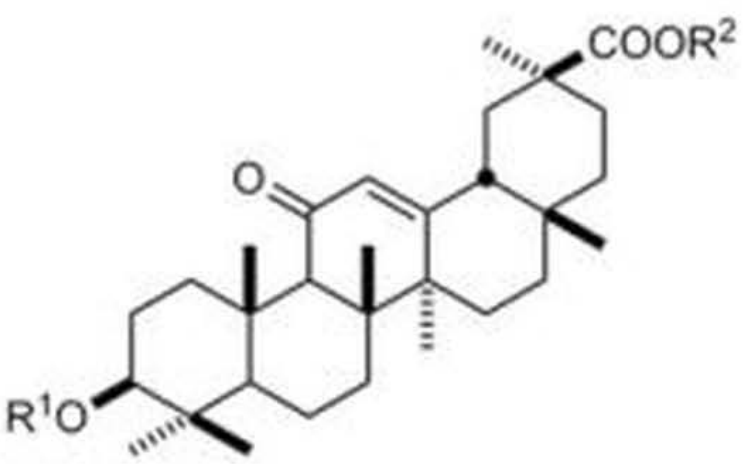
502 CFU, colony forming unit; GRA, glycyrrhetinic acid; GR-K, dipotassium glycyrrhizate;
503 GR-SU, disodium succinoyl glycyrrhetinate; GR-S, stearyl glycyrrhetinate; GR-SA,
504 glycyrrhetinyl stearate; GTF, glucosyltransferase; LDH, lactate dehydrogenase; MIC,
505 minimum inhibitory concentration; PEP, phosphoenolpyruvate; PK, pyruvate kinase;
506 PTS, phosphotransferase system; TSA, tryptic soy agar; TSB, tryptic soy broth

507

508

Table 1. primers used in this study

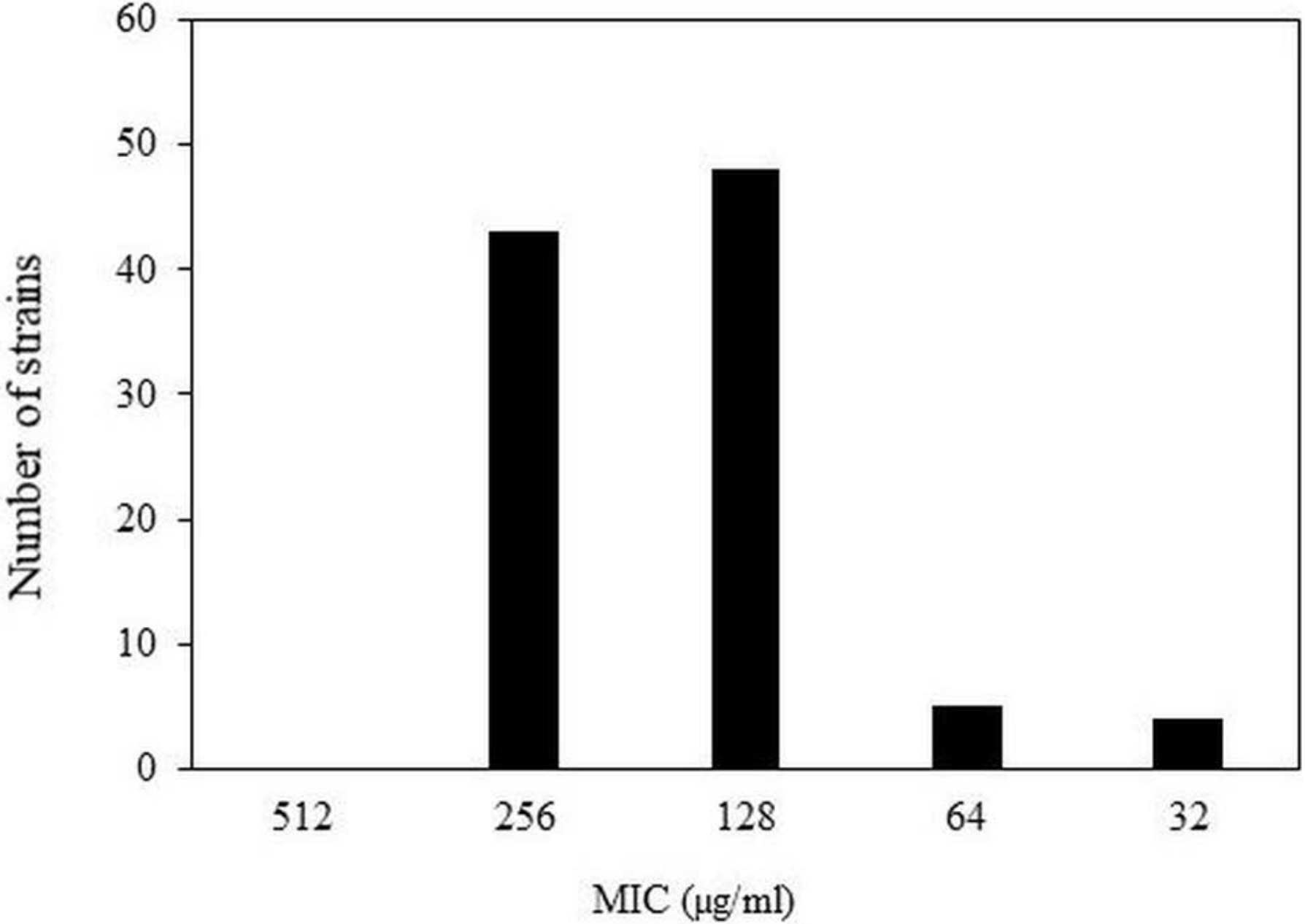
target gene ID	primer-forward	primer-reverse
Primers for quantitative PCR		
ldh	5’- TAT GAA GAC TGT GCG GAT GC -3’	5’- GGT TAG CAG CAA CGA GGA AG -3’
pykF	5’- GAA TTG GCA CGT CAA AAG GT -3’	5’- ATA TCA AGA CCG CCT GCA AC -3’
aguD	5’- TGG TGC TGC TCT TGC TAA TG -3’	5’- TAA AAG GAC GCG GTG TAT CC -3’
atpD	5’- TGT TGA TGG TCT GGG TGA AA -3’	5’- TTT GAC GGT CTC CGA TAA CC -3’
eno	5’- CAG CGT CTT CAG TTC CAT CA -3’	5’- TCA CTC AGA TGC TCC AAT CG -3’

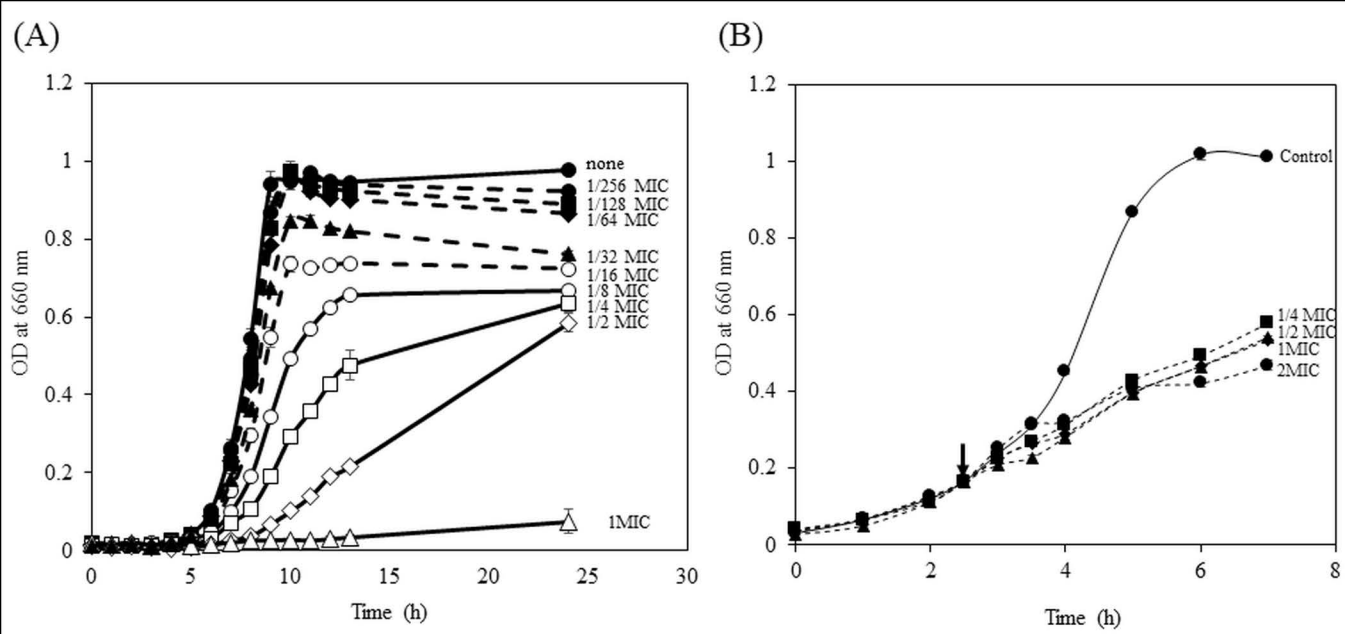


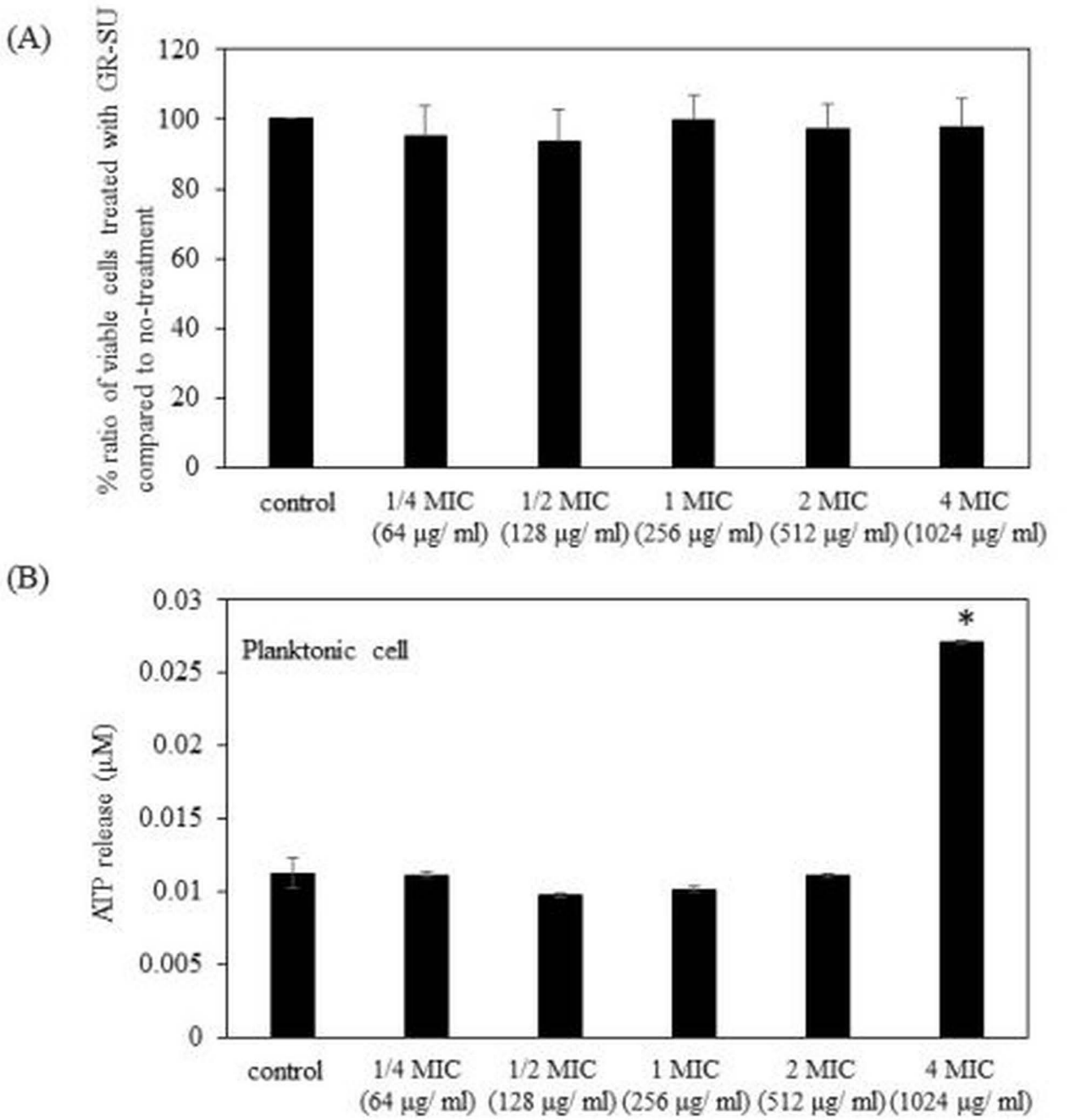
	R^1	R^2
1	-$GlcA^{2}$-$^{1}GlcA$	-H
2	-H	-H
3	$-CO(CH_2)_2COOH$	-H
4	-H	$-(CH_2)_{17}CH_3$
5	$-CO(CH_2)_{16}CH_3$	-H

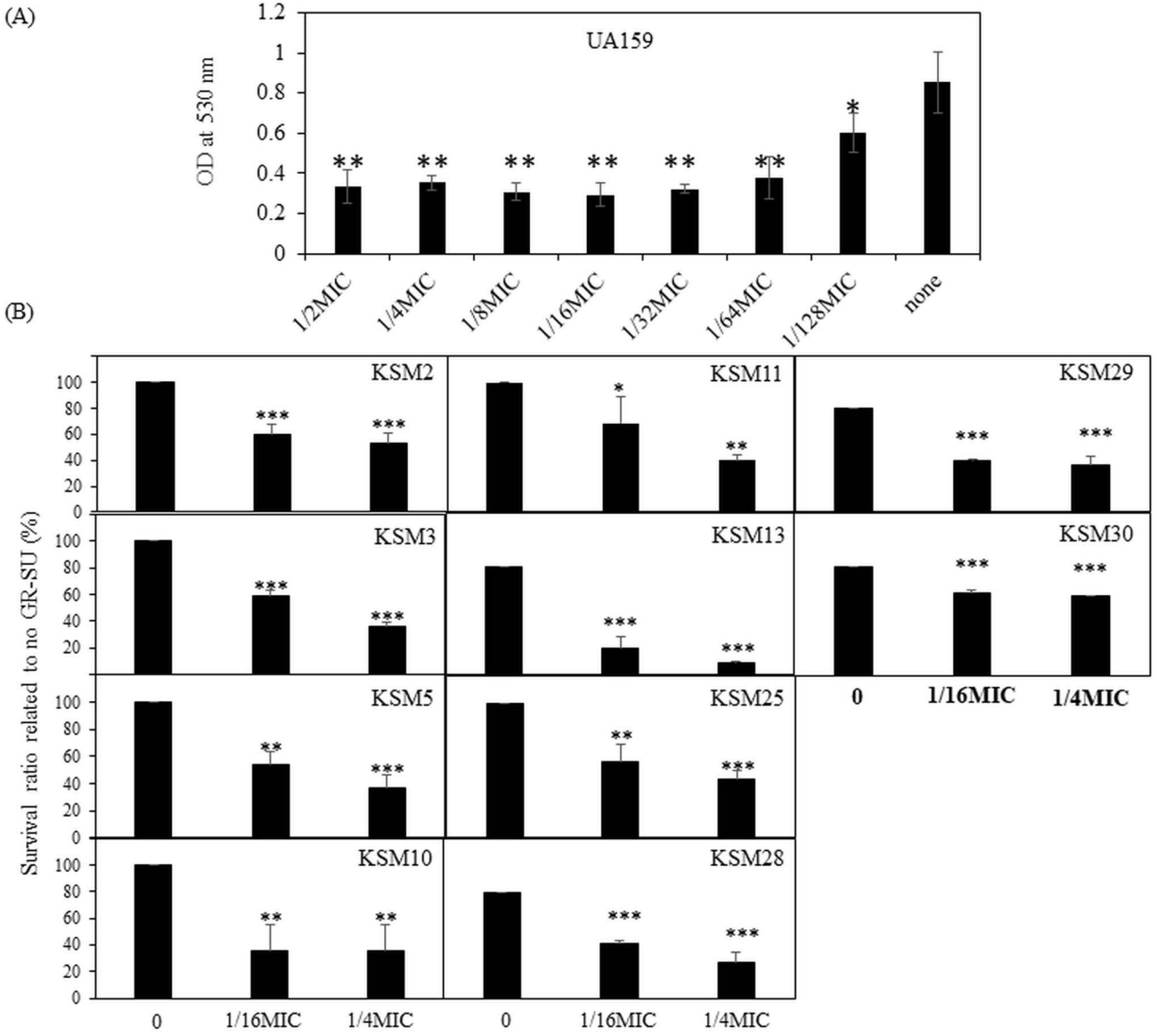
$GlcA^{2}$-$^{1}GlcA$: optical isomer of glucuronic acid

		MIC of Nisin A (μg/ml)
1	Dipotassium glycyrrhizate (GR-K)	>2048
2	Glycyrrhetinic acid (GRA)	256
3	disodium 3-succinyloxy beta-glycyrrhetinate (GR-SU)	256
4	glycyrrhetinyl stearate (GR-SA)	>2048
5	stearyl glycyrrhetinate (GR-S)	>2048

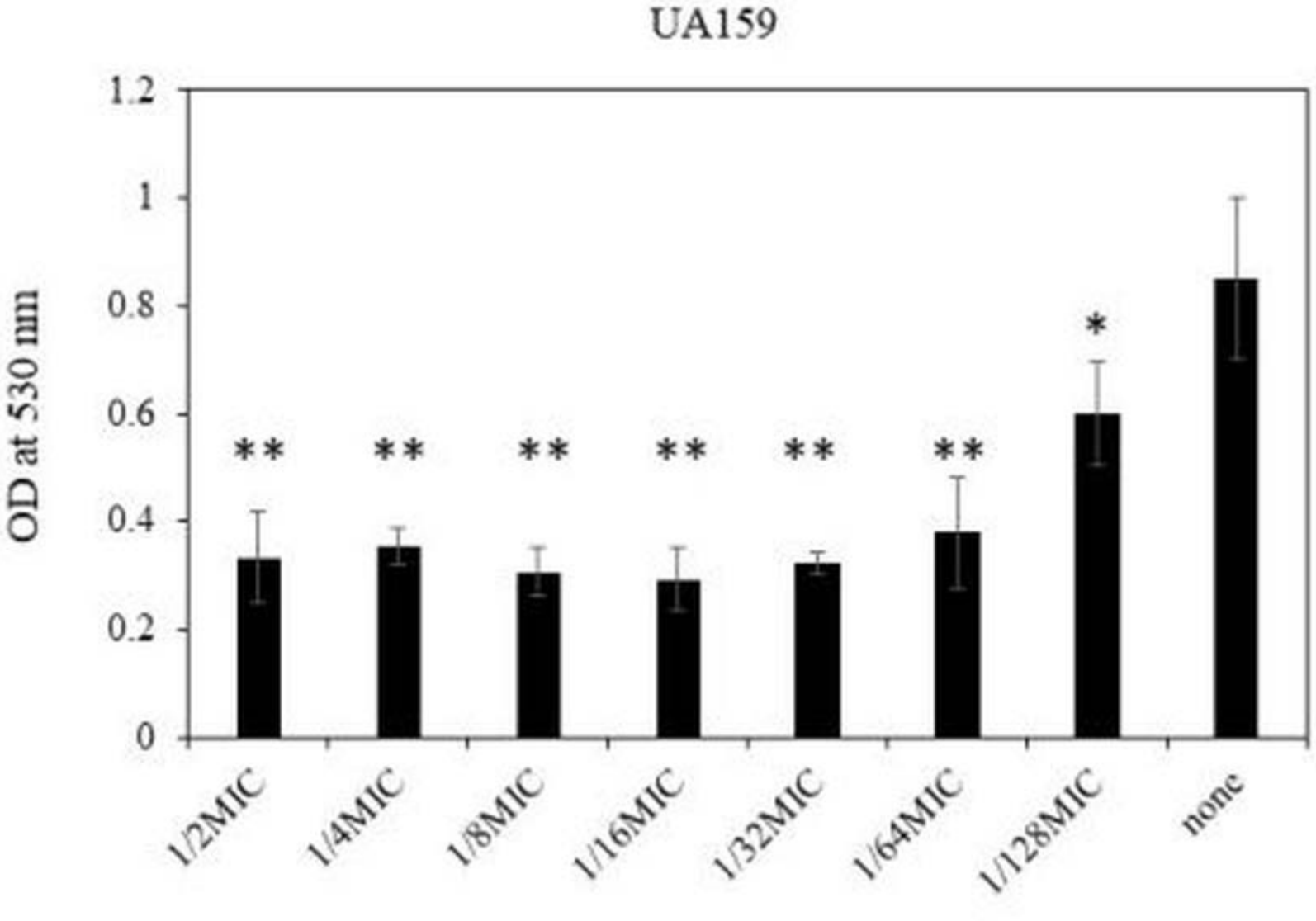

60
50
40
30
20
10
0
Number of strains
512
256
128
64
32
MIC (μg/ml)

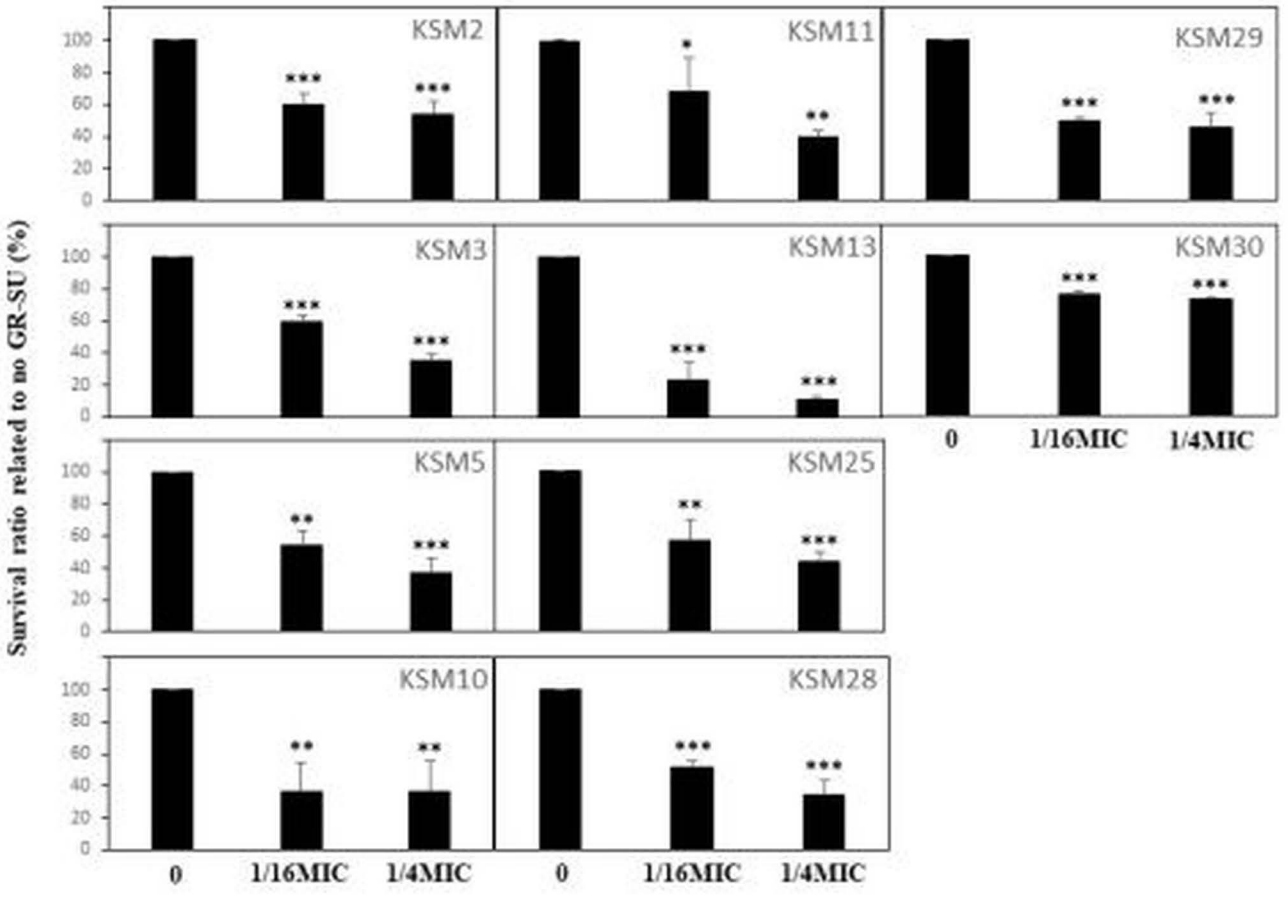

(A)
OD at 660 nm
Time (h)
none
1/256 MIC
1/128 MIC
1/64 MIC
1/32 MIC
1/16 MIC
1/8 MIC
1/4 MIC
1/2 MIC
1MIC
(B)
OD at 660 nm
Time (h)
Control
1/4 MIC
1/2 MIC
1MIC
2MIC

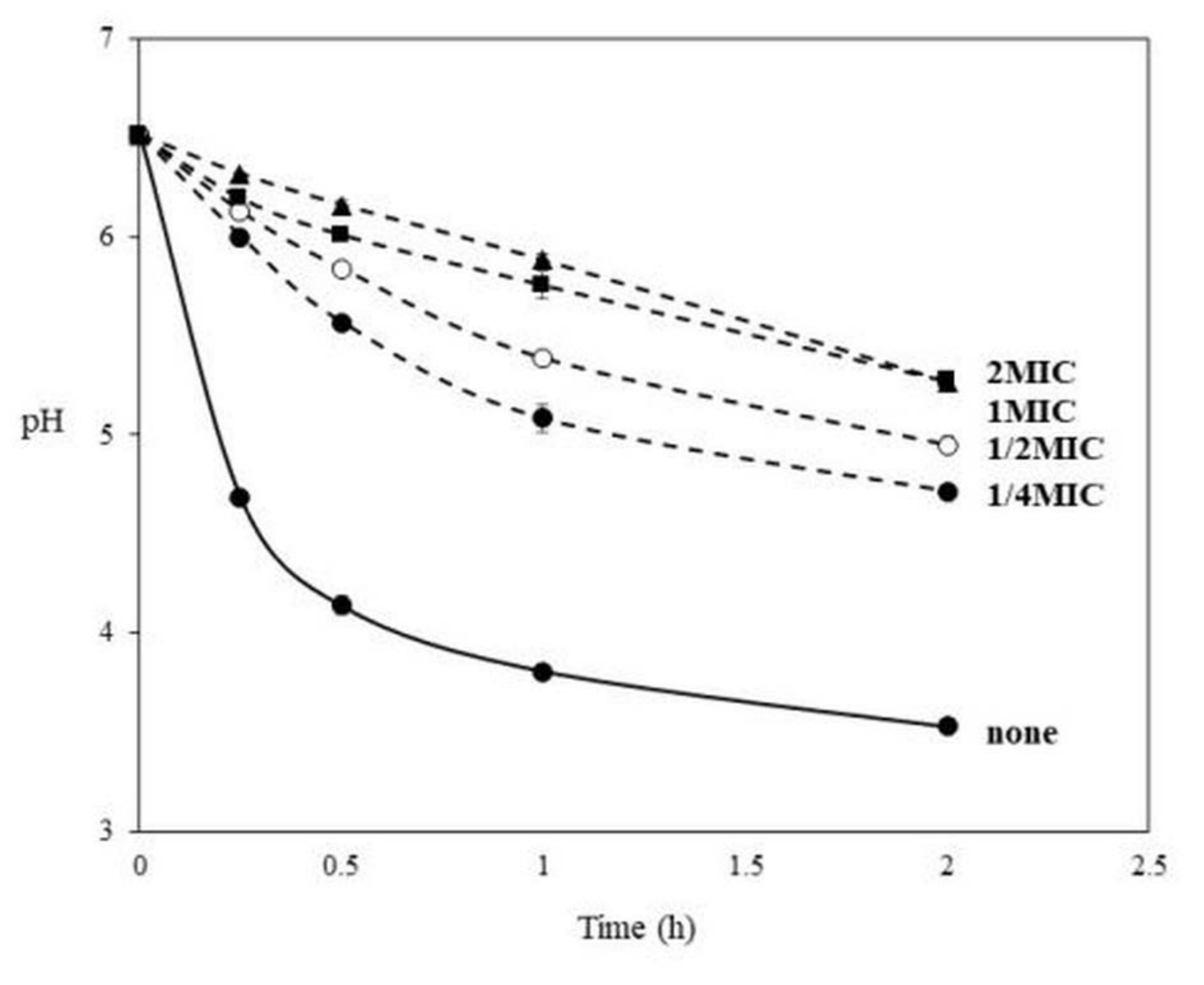

(A)
% ratio of viable cells treated with GR-SU compared to no-treatment
120
100
80
60
40
20
0
control
1/4 MIC (64 μg/ ml)
1/2 MIC (128 μg/ ml)
1 MIC (256 μg/ ml)
2 MIC (512 μg/ ml)
4 MIC (1024 μg/ ml)
(B)
ATP release (μM)
0.03
0.025
0.02
0.015
0.01
0.005
0
Planktonic cell
*
control
1/4 MIC (64 μg/ ml)
1/2 MIC (128 μg/ ml)
1 MIC (256 μg/ ml)
2 MIC (512 μg/ ml)
4 MIC (1024 μg/ ml)

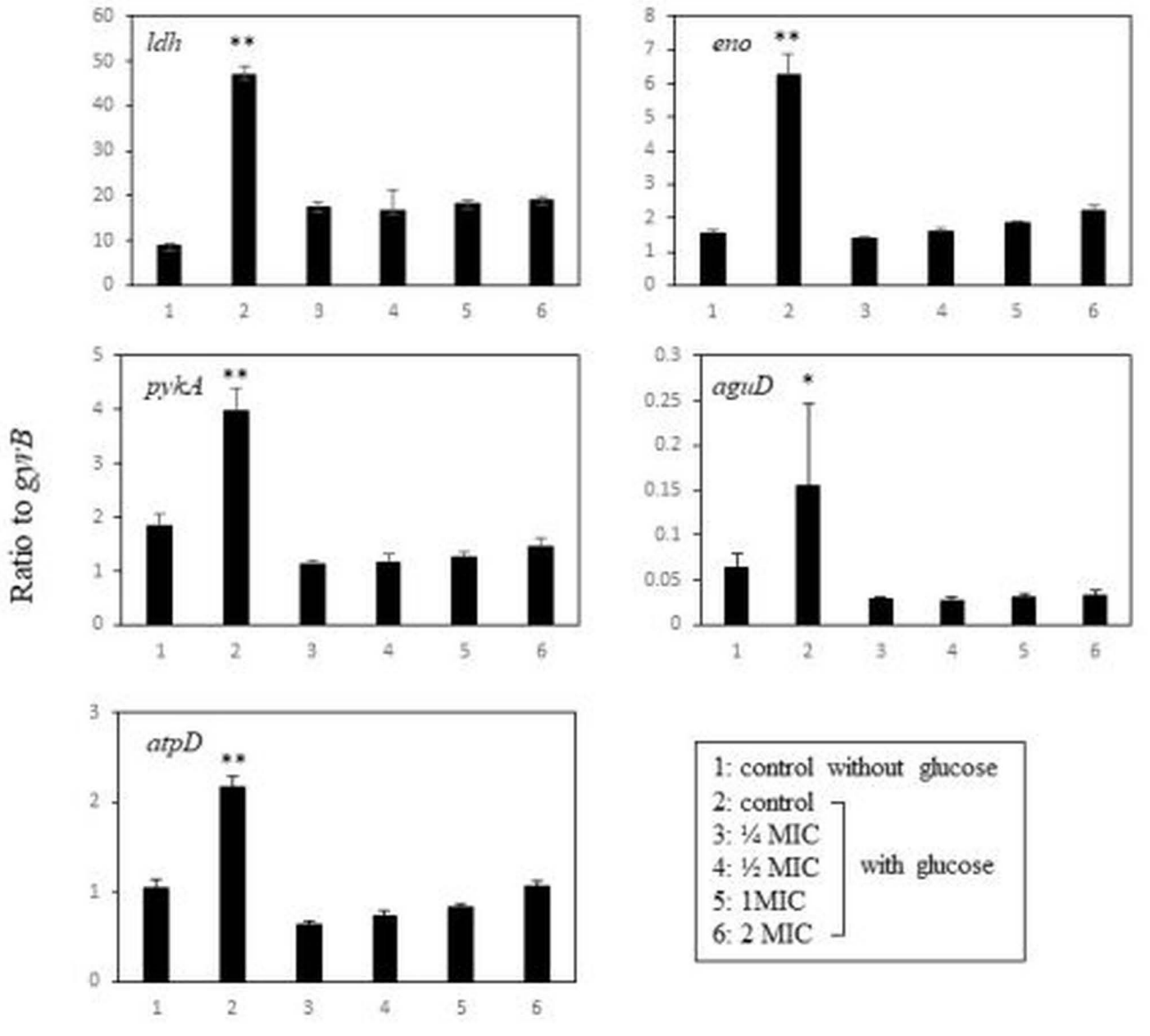

(A)
UA159
OD at 530 nm
1.2
1
0.8
0.6
0.4
0.2
0
1/2MIC
1/4MIC
1/8MIC
1/16MIC
1/32MIC
1/64MIC
1/128MIC
none
(B)
Survival ratio related to no GR-SU (%)
KSM2
KSM11
KSM29
KSM3
KSM13
KSM30
KSM5
KSM25
KSM10
KSM28
0
1/16MIC
1/4MIC

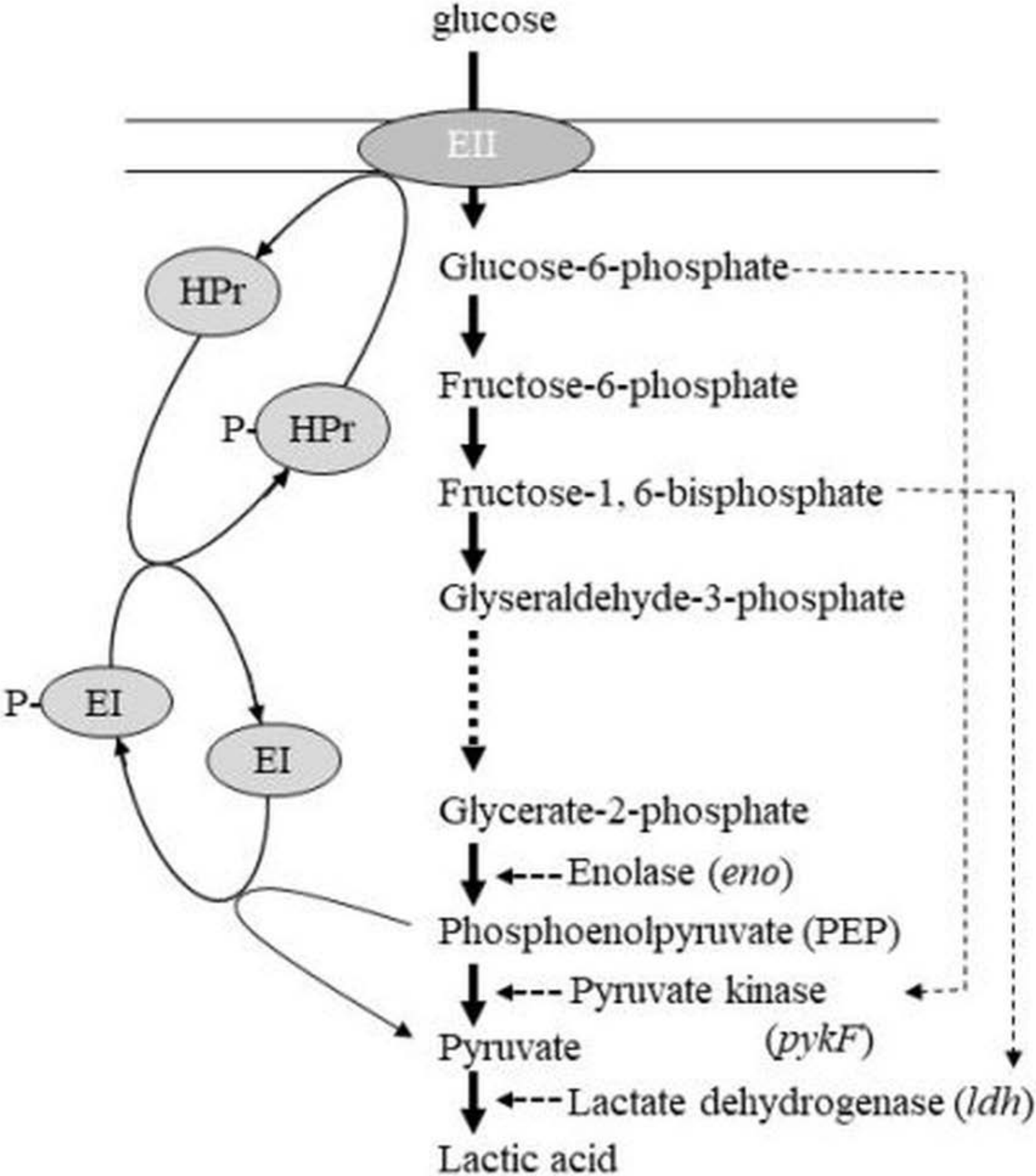
(A)


UA159
OD at 530 nm
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
**
**
**
*
1/2MIC
1/4MIC
1/8MIC
1/16MIC
1/32MIC
1/64MIC
1/128MIC
none


KSM2
KSM11
KSM29
KSM3
KSM13
KSM30
KSM5
KSM25
KSM10
KSM28
Survival ratio related to no GR-SU (%)
0
1/16MIC
1/4MIC


7
6
5
4
3
pH
0
0.5
1
1.5
2
2.5
Time (h)
2MIC
1MIC
1/2MIC
1/4MIC
none


ldh
**
eno
**
pykA
**
aguD
*
atpD
**
Ratio to gyrB
1: control without glucose
2: control
3: ¼ MIC
4: ½ MIC
5: 1MIC
6: 2 MIC
with glucose


glucose
EII
HPr
P-HPr
P-EI
EI
Glucose-6-phosphate
Fructose-6-phosphate
Fructose-1, 6-bisphosphate
Glyseraldehyde-3-phosphate
Glycerate-2-phosphate
Enolase (eno)
Phosphoenolpyruvate (PEP)
Pyruvate kinase
(pykF)
Pyruvate
Lactate dehydrogenase (ldh)
Lactic acid