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***Streptococcus mutans* diacylglycerol kinase homolog: A potential target for anti-caries chemotherapy**

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Abbreviations: Dgk, diacylglycerol kinase; PSL, photostimulated luminescence; CFU, colony-forming unit.

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SUMMARY

Aciduricity is a major cariogenic characteristic of *Streptococcus mutans*, and various genes have been implicated in this ability of *S. mutans*. Sixteen *S. mutans* mutants, each defective in a different gene, were constructed and their aciduricity was assessed. Of the mutants, the diacylglycerol kinase (Dgk) homolog mutant and glucose-1-phosphate uridylyltransferase mutant displayed distinctly attenuated aciduricity when grown at pH 5.5. Considering the delayed growth rate of the latter at neutral pH, the *dgk* homolog appeared to be a gene responding specifically to pH reduction among the 16 genes tested. Two known eukaryotic Dgk inhibitors, R59949 and R59022, were selected as candidate inhibitors of the *S. mutans* Dgk homolog. R59949, but not R59022, significantly reduced the growth of *S. mutans* at pH<5.4. R59949 did not affect either the final pH of the medium or the internal pH of the organism. Furthermore, R59949 inhibited about 20% of Dgk kinase activity. Novel derivatives of R59949 may be useful for preventing the development of dental caries caused by *S. mutans*.

INTRODUCTION

Streptococcus mutans, the major etiological factor in human dental caries, is capable of forming the biofilm commonly known as ‘dental plaque’ on the surfaces of teeth (Loesche, 1986; Tanzer *et al.*, 2001). Within the dental plaque, *S. mutans* synthesizes large amounts of acids from fermentable dietary carbohydrates. Acid accumulation can eventually cause the teeth’s hard, crystalline structure to be dissolved, resulting in the formation of carious lesions (Quivey *et al.*, 2001). *S. mutans* has the ability to tolerate the typically low pH of dental plaque and continue to produce acids, contributing to its cariogenicity. The tolerance of low pH is considered to be one of this microorganism’s most important virulence factors.

Diacylglycerol kinase (Dgk) is an enzyme that phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA). In eukaryotic cells, both DG and PA are important lipid second messengers that regulate the expression of a number of enzymes. Therefore, Dgk plays an important role in regulating many intracellular signalling pathways (Kano *et al.*, 2002; Merida *et al.*, 2008; Topham & Prescott, 1999; van Blitterswijk & Houssa, 2000). In bacteria, Dgk functions in the recycling of DG produced during the turnover of membrane phospholipids (Hasin & Kennedy, 1982; Rotering & Raetz, 1983) and similar to eukaryotic Dgk, it also plays an important role in microbial physiology under conditions of environmental stress (Raetz & Newman, 1979; Walsh *et al.*, 1986; Yamashita *et al.*, 1993).

Many researchers have sought to explain the mechanisms of acid tolerance in *S. mutans*, and various genes contributing to this property have been identified. In a previous study (Shibata *et al.*, 2009), we reported that the C-terminal tail of a Dgk homolog was indispensable for tolerance to acid stress in *S. mutans* and that the Dgk homolog's kinase activity was closely related to the acid tolerance of this organism. Furthermore, a *dgk* deletion mutant exhibited markedly reduced levels of smooth-surface carious lesions in pathogen-free rats (Shibata *et al.*, 2009). Inhibitors of the Dgk homolog's enzymatic activity may therefore be useful in attenuating the virulence of *S. mutans*.

In this study, we generated mutations in genes that have been previously reported to be involved in acid tolerance in *S. mutans* and examined their contribution to acid tolerance, in order to determine the most desirable target for anti-caries agents. Additionally, we investigated the effects of inhibitors of the putative candidate on the acid tolerance of *S. mutans*, to assess their potential usefulness as anti-caries agents.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain RZDGK11 producing full-size *S. mutans* Dgk protein was constructed through transformation of the mutant strain RZ6, which lacks a functional *E. coli dgk* gene, as described previously (Shibata

et al., 2009). *E. coli* strains and *S. mutans* strains were maintained and grown routinely as described previously (Shibata *et al.*, 2002). Antibiotics were used at the following concentrations: 200 µg erythromycin ml⁻¹, or 50 µg ampicillin ml⁻¹ for *E. coli*; 10 µg erythromycin ml⁻¹ for *S. mutans*.

DNA manipulation. Standard DNA recombinant procedures, such as DNA isolation, endonuclease restriction, ligation, and agarose gel electrophoresis were carried out as described by Sambrook & Russell (2001). Transformation of *S. mutans* and *E. coli* was performed as described previously (Hanahan, 1983; Perry *et al.*, 1983).

Construction of the mutant forms of the genes that contribute to aciduricity in

***S. mutans*.** In this study, we created *S. mutans* UA159 mutants carrying mutated forms of the following 15 genes: *aguB* (Griswold *et al.*, 2004), *brpA* (Wen *et al.*, 2006), *ciaH* (Ahn *et al.*, 2006), *clpP* (Lemos & Burne, 2002), *dltC* (Boyd *et al.*, 2000), *ffh* (Kremer *et al.*, 2001), *ftsY* (Hasona *et al.*, 2005), *glrA* (Cvitkovitch *et al.*, 2000), *gluA* (Yamashita *et al.*, 1998), *htrA* (Biswas & Biswas, 2005), *lgl* (Korithoski *et al.*, 2007), *luxS* (Wen & Burne, 2004), *ropA* (Wen *et al.*, 2005), *uvrA* (Hanna *et al.*, 2001), and *yidC2* (Dong *et al.*, 2008). The *dltC* and *ciaH* genes were replaced by an erythromycin resistance gene using double cross-over homologous recombination, as described previously (Kawada-Matsuo *et al.*, 2009). The other mutants were generated through the interruption of linear target gene fragments by the same erythromycin resistance gene, inserted at appropriate restriction sites, using double cross-over homologous

recombination. Correct insertions or replacements in transformants were confirmed by PCR. The mutant UADGK1 (Shibata *et al.*, 2009), in which the C-terminal ten amino acid residues of Dgk were deleted, was used as a *dgk* mutant. The primers used to generate and confirm the identities of the mutants are listed in Supplementary Table S1.

Evaluation for acid sensitivity of strains. The 16 mutants and wild-type control UA159 cells were grown in brain heart infusion broth (BHI; Difco) overnight at 37 °C in 5% CO₂. The cultures were then diluted 1:10 into fresh BHI and grown to an OD₅₅₀ of ~0.5. Aliquots (20 µl) of the cell suspensions having the same turbidity were inoculated into wells that contained 200 µl of fresh BHI medium adjusted to either pH 7.4 or pH 5.5 with 50 mM sodium acetate buffer. Growth was monitored at 550 nm using a Spectramax 340PC³⁸⁴ microplate spectrophotometer (Molecular Devices). Wells containing only BHI were used as controls. Growth curves were generated from the data obtained from three independent experiments. In addition, each strain was grown at pH 7.4 or pH 5.5, as described above, and incubated for one additional hour after reaching the maximum OD₅₅₀. The cultures were then serially diluted, plated on BHI agar plates, and incubated at 37 °C in 5% CO₂ for 2 days before colonies were counted. The mutant/wild-type colony-forming unit (CFU) ratio was calculated. Three independent experiments were performed in triplicate.

Growth inhibition assay. The Dgk inhibitors,
6-{2-[4-[(*p*-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl}-7-methyl-5H-thiazolo

(3,2-a)pyrimidine-5-one (R59022) and 3-{2-{4[*bis*-(4-fluorophenyl)methylene]-1-piperidinyl}-2,3-dihydro-2-thioxo-4(1H)quinazolinone (R59949) were purchased from Merck. To examine the effects of R59022 and R59949 on the growth of *S. mutans*, wild-type UA159 cells were inoculated into microtitre plate wells, each containing 200 µl of fresh BHI medium (pH 7.4, 5.5, 5.4, 5.3, 5.2, 5.1, or 5.0) containing R59022 (100 µM), R59949 (25 µM), or dimethyl sulfoxide (control). After incubation at 37 °C in 5% CO₂ for 16 h, the OD₅₅₀ was measured. Three independent experiments were performed in triplicate.

Measurement of intracellular pH. Intracellular pH measurements were performed as described previously (Sawatari & Yokota, 2007). Briefly, cells were cultured until mid-exponential phase, harvested, and washed twice with buffer A [150 mM potassium phosphate (pH 6.5), 1 mM MgSO₄]. The cells were resuspended to an OD₅₅₀ value of 0.5 in buffer A and incubated at 37 °C for 30 min in the presence of carboxyfluorescein diacetate succinimidyl ester (cFDASE, Molecular Probes), a fluorescent pH probe. To eliminate unbound probe, the cells were incubated with glucose for 1 h and then washed once in buffer A. The cells were subsequently resuspended in buffer B (buffer A supplemented with 100 mM 2-morpholinoethanesulfonic acid), and the intracellular pH was measured. Three independent experiments were performed.

Kinase assay. A kinase assay was conducted as described previously (Shibata *et al.*, 2009). Kinase activity in cell lysates was examined by an octyl glucoside mixed-micelle

assay (Preiss *et al.*, 1986), using undecaprenol (Larodan Fine Chemicals) as a substrate. The synthesized undecaprenyl phosphate, radiolabelled by [γ - 32 P]-ATP, was separated on silica gel 60 TLC plates (Merck), detected by BAS2500 (Fujix), and finally represented as photostimulated luminescence (PSL). For the kinase inhibition assay, the Dgk inhibitors were used at a concentration of 100 μ M. Three independent experiments were performed.

RESULTS AND DISCUSSION

Mutant acid sensitivity

To examine the effects of the mutations on *S. mutans* acid tolerance, the 16 mutants and wild-type control UA159 cells were grown in BHI broth adjusted to pH 7.4 or 5.5 (Fig. 1 and Table 1). At pH 7.4, the *brpA*, *ciaH*, *dgk*, *dltC*, *htrA*, *lgl*, *luxS*, *ropA*, and *uvrA* mutants grew similarly to the wild-type UA159 cells, whereas the other mutants, particularly the *gluA* and *yidC2* mutants, grew more slowly than the wild-type strain (Fig. 1a). Inactivation of the *aguB*, *brpA*, *glrA*, *htrA*, *lgl*, *luxS*, *ropA*, or *uvrA* did not significantly affect the acid tolerance of *S. mutans*, as assessed by growth at pH 5.5 (Fig. 1b). In contrast, the *ciaH*, *clpP*, *dgk*, *dltC*, *ffh*, *ftsY*, *gluA*, and *yidC2* mutants displayed significantly reduced growth rates. Notably, the *dgk* and the *gluA* mutants grew extremely slowly at pH 5.5. Not all mutants grew at rates similar to that of

wild-type UA159 cells, even at pH 7.4. Calculation of the mutant/wild-type CFU ratio at pH 5.5 revealed that the inactivation of the *dgk* or *gluA* gene markedly attenuated the aciduricity of *S. mutans* (Table 1). The growth data of all strains tested are summarized in Table 1. The *gluA* mutant exhibited a reduced growth rate at both neutral and acidic pH, and thus we focused on *dgk* as a gene responding specifically to pH reduction in *S. mutans*.

Effects of Dgk inhibitors on the growth of *S. mutans*

Mammalian Dgks have been extensively studied, and several compounds that inhibit their activity have been identified. R59022 and R59949 are the most commonly used inhibitors, and both interact directly with Dgk. R59022 inhibits Dgk without affecting phosphodiesterase or phosphatidylinositol kinase (de Chaffoy de Courcelles *et al.*, 1985). R59949 acts on the enzyme's catalytic domain (Jiang *et al.*, 2000). In contrast, the inhibition of prokaryotic Dgk enzymes has not been studied. We therefore evaluated the effects of R59022 and R59949 on the growth of *S. mutans*. Both inhibitors can normally be used at a concentration of 100 μ M in the hydrophobic conditions of enzyme assays. However, in the growth assay, R59949 did not fully dissolve in BHI medium (hydrophilic) at a concentration of 100 μ M, although R59022 did. Thus, R59022 and R59949 were used at concentrations of 100 and 25 μ M, respectively, in the growth inhibition assay. We confirmed that the addition of the inhibitors did not affect the pH of the buffered BHI medium. While neither R59022 nor R59949 influenced the

growth of *S. mutans* at pH 7.4, R59949 significantly inhibited the growth of *S. mutans* at acidic pH (Fig. 2a). R59022 had no effect on *S. mutans* growth rate (Fig. 2b). The inhibition ratio of R59949 increased with decreasing pH, with ratios of 13, 29, 58, 68, and 78% at pH 5.4, 5.3, 5.2, 5.1, and 5.0, respectively.

Effect of the Dgk inhibitors on the enzymatic activity of *S. mutans* Dgk

The effects of R59022 and R59949 on the kinase activity of *S. mutans* Dgk were assayed. Cell lysates obtained from *E. coli* RZDGK11 expressing full-length *S. mutans* Dgk protein were used as crude *S. mutans* Dgk samples in a kinase activity assay. It has been shown that cell lysates from *E. coli* RZ6, the parent strain of RZDGK11, do not exhibit any kinase activity when provided with undecaprenol as a substrate (Shibata *et al.*, 2009). We first confirmed that the addition of the inhibitors did not affect the pH of the reaction mixture for kinase activity assay. Neither R59022 nor R59949 affected Dgk kinase activity at pH 7.4 (data not shown), consistent with the above observations that the inhibitors did not affect the growth rate of *S. mutans* at neutral pH.

As intracellular pH is not necessarily equal to that of the local environment, we determined the intracellular pH of *S. mutans* at various external pH values. The intracellular pH values of *S. mutans* were 6.57 ± 0.07 , 6.51 ± 0.07 , 6.45 ± 0.07 , 6.38 ± 0.07 , 6.32 ± 0.07 , and 6.26 ± 0.07 at external pH values of 5.5, 5.4, 5.3, 5.2, 5.1, and 5.0, respectively. Moreover, the addition of R59949 did not affect the intracellular pH.

Considering that R59949 showed greater than 50% inhibition of *S. mutans* growth at pH 5.2, the kinase inhibition experiment was performed at pH 6.4. The kinase activities in *E. coli* RZDGK11 cell lysates using undecaprenol as a substrate were 37.3 ± 2.9 PSL/mm² and 29.4 ± 4.3 PSL/mm², respectively, in the absence and presence of R59949, reflecting an inhibition of about 20% ($p < 0.05$, Student's *t*-test) by R59949. In contrast, R59022 did not inhibit kinase activity under these conditions (data not shown).

Collectively, these results support the previous conclusion that Dgk kinase activity is closely related to *S. mutans* acid tolerance. However, the inhibitory effect of R59949 on Dgk kinase activity was not as strong as expected. In our previous study (Shibata *et al.*, 2009), *S. mutans* UADGK6 expressing Dgk6, a truncated form of Dgk lacking five C-terminal amino acids, exhibited 40% and 60% reduced growth at pH 5.5 and 5.4, respectively, compared with *S. mutans* expressing full-length Dgk. However, R59949 had no effect on the growth of *S. mutans* at pH 5.5 and caused significant growth inhibition (>50%) at pH 5.2. This indicates that the effect of R59949 was weaker than that of the C-terminal truncation of Dgk. Thus, given that Dgk6 displayed 60% reduced kinase activity with undecaprenol as a substrate, compared with full-length Dgk, the calculated 20% reduction in Dgk kinase activity caused by R59949 seems reasonable.

Unlike eukaryotic Dgks, bacterial Dgks, including that of *S. mutans*, are small proteins exhibiting minimal amino acid sequence similarity with their eukaryotic homologs. Moreover, the principal substrate for *S. mutans* Dgk is undecaprenol, not diacylglycerol

(Lis & Kuramitsu, 2003). Nevertheless, R59949, an inhibitor of mammalian Dgks, inhibited the Dgk activity and acid tolerance of, *S. mutans*. A second inhibitor of mammalian Dgks, R59022, produced no such effect. Both R59022 and R59949 are known to selectively inhibit Type-I Dgks (de Chaffoy de Courcelles *et al.*, 1985; Jiang *et al.*, 2000), although their inhibitory effects on different Dgk isoforms have not been comprehensively evaluated. Thus, a few (minor) differences in isoform selectivity between R59949 and R59022 may exist. Moreover, differences in the molecular structures of R59949 and R59022 may contribute to the difference in their effectiveness against prokaryotic Dgks. It is not clear why an inhibitor of mammalian Dgks also inhibited Dgk activity in *S. mutans*. However, our study clearly shows that the addition of R59949 caused a reduction in aciduricity, one of the main virulence factors of *S. mutans*. Unfortunately, the direct use of R59949 in patients is not realistic. The development of more potent Dgk inhibitors specific for prokaryotic enzymes, through specific modifications based on comparisons of the molecular structures of R59949 and R59022, may lead to the discovery of new anti-caries agents.

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FIGURE LEGENDS

Fig. 1. Growth curves of *S. mutans* UA159 and mutant strains grown in BHI medium at pH 7.4 (a) or 5.5 (b). Growth was defined in terms of increased OD₅₅₀, and was calculated by subtracting the initial OD₅₅₀ value from those recorded after the indicated periods of growth. Data represent the means of three independent experiments.

Fig. 2. Effect of R59949 (a) and R59022 (b) on the growth of *S. mutans*. The y-axis represents the OD₅₅₀ after 16 h of incubation. Data are the means \pm standard deviations of three independent experiments. Differences in growth rate between cells cultured in the presence and absence of Dgk inhibitor were analysed using Student's *t*-test. *, $p < 0.05$; **, $p < 0.0001$.

Table 1. Growth data of *S. mutans* UA159 and sixteen mutants at pH 7.4 and 5.5

Doubling time was calculated based on the formulas $\ln Z - \ln Z_0 = k(t - t_0)$, where k is the growth rate, and $g = 0.693/k$, where g is the doubling time.

CFU ratio was calculated between the mutant (MT) and wild-type UA159 (WT).

Values are the mean (\pm standard deviation) of three independent experiments.

Strain	pH 7.4				pH 5.5			
	Doubling time (min)	Maximum OD ₅₅₀	Final OD ₅₅₀	CFU ratio (% MT/WT)	Doubling time (min)	Maximum OD ₅₅₀	Final OD ₅₅₀	CFU ratio (% MT/WT)
UA159	55.9 (3.3)	0.59 (0.08)	0.52 (0.08)	100	109.3 (7.2)	0.43 (0.06)	0.36 (0.03)	100
$\Delta aguB$	55.7 (4.9)	0.57 (0.03)	0.52 (0.05)	84.2 (10.1)	110.8 (22.9)	0.49 (0.03)	0.39 (0.01)	196.3 (31.7)
$\Delta brpA$	50.0 (4.5)	0.49 (0.07)	0.38 (0.06)	72.9 (8.3)	127.1 (12.4)	0.43 (0.03)	0.38 (0.02)	172.8 (7.6)
$\Delta ciaH$	45.3 (2.7)	0.53 (0.07)	0.49 (0.07)	100.9 (19.0)	166.7 (11.5)	0.34 (0.04)	0.29 (0.03)	60.7 (16.5)
$\Delta clpP$	62.1 (0.7)	0.51 (0.05)	0.44 (0.05)	52.4 (11.0)	189.3 (18.5)	0.38 (0.02)	0.35 (0.01)	85.3 (10.1)
Δdgk	46.8 (4.0)	0.57 (0.07)	0.43 (0.04)	89.9 (13.4)	>1000	0.08 (0.02)	0.06 (0.02)	8×10^{-2} (10.9×10^{-2})
$\Delta dltC$	56.6 (2.4)	0.52 (0.05)	0.51 (0.04)	92.1 (12.2)	171.4 (4.9)	0.27 (0.04)	0.27 (0.04)	41.2 (12.4)
$\Delta fflh$	62.5 (1.1)	0.55 (0.04)	0.47 (0.02)	78.1 (9.1)	178.5 (4.0)	0.34 (0.03)	0.32 (0.02)	53.5 (6.8)
$\Delta fisY$	68.3 (5.5)	0.54 (0.04)	0.49 (0.04)	73.2 (8.9)	169.0 (11.5)	0.34 (0.02)	0.30 (0.02)	63.5 (9.2)
$\Delta glrA$	60.2 (7.4)	0.56 (0.06)	0.50 (0.07)	81.9 (5.5)	129.6 (13.8)	0.49 (0.08)	0.40 (0.03)	213.6 (22.1)
$\Delta gluA$	116.0 (13.8)	0.52 (0.03)	0.41 (0.02)	52.1 (9.6)	>1000	0.01 (0.00)	0.00 (0.01)	15.0×10^{-1} (2.1×10^{-1})
$\Delta htrA$	50.6 (4.4)	0.53 (0.03)	0.48 (0.07)	71.0 (3.9)	115.0 (10.1)	0.46 (0.04)	0.37 (0.03)	178.9 (20.9)
Δlgl	46.3 (3.9)	0.56 (0.04)	0.51 (0.03)	83.5 (7.6)	121.0 (11.9)	0.45 (0.04)	0.37 (0.00)	170.4 (26.8)
$\Delta luxS$	46.6 (3.7)	0.56 (0.04)	0.50 (0.07)	81.2 (11.4)	121.8 (18.1)	0.47 (0.02)	0.37 (0.00)	185.8 (25.1)
$\Delta ropA$	51.5 (0.5)	0.52 (0.04)	0.39 (0.04)	61.4 (6.0)	120.3 (0.8)	0.40 (0.04)	0.36 (0.04)	85.3 (7.0)
$\Delta uvrA$	49.5 (8.6)	0.55 (0.06)	0.47 (0.08)	83.7 (5.1)	116.8 (4.4)	0.45 (0.00)	0.37 (0.02)	82.1 (8.0)
$\Delta yidC2$	81.1 (9.6)	0.47 (0.07)	0.43 (0.06)	49.9 (8.5)	176.3 (5.6)	0.40 (0.03)	0.36 (0.03)	118.0 (24.5)

Supplementary Table S1. Primers used in this study

Primer	Nucleotide sequence (5'-3')*	Restriction site	Amplicon (bp)
AguB-F	AGAGGATCCAAGGCATTTTCCGTTT	BamHI	1,734
AguB -R	CAGCAACAAAGACGACAC		
BrpA-F	AGAGGATCCTGATTCTATTGATCTG	BamHI	1,650
BrpA -R	GGAGCCATAAGAAGAACT		
CiaHUP-F	CGCGGTACCCATGCAGGTTTTTGATGGTG	KpnI	620
CiaHUP-R	CGCCTCGAGCTCATCCTGCTTTTGTTT	XhoI	
CiaHDW-F	CAGATTCGGTTAAGTATG		285
CiaHDW-R	CGCGCGGCCCGCGTGCTGAACCTAACGTT	NotI	
ClpP-F	AGAGGATCCGGTAGAATATCTGGTT	BamHI	1,765
ClpP -R	TCGCCTCAATTGCTTTAC		
DltCUP-F	CGCCTCGAGCTTCATGTCTTCATGTCT	XhoI	985
DltCUP-R	CGCAAGCTTTGAGGGTATAACGGAGTT	HindIII	
DltCDW-F	ACTCCTTTATTTCTTTGAT		880
DltCDW-R	CGCTCTAGATGGAAGCCTTTGAGTCTT	XbaI	
Ffh-F	AGAGGATCCCTAAGAAGGTTGCATCT	BamHI	1,636
Ffh-R	TCTTCCATTGATCCCATG		
FtsY-F	AGAGGATCCTCTTAAAGCTATCGAA	BamHI	1,411
FtsY-R	CTGAACACCGACATCAGA		
GlrA-F	GAAAGAGCGCGTTTAGAA		1,445
GlrA-R	AGAGGTACCGAACACATGAATTTCA	KpnI	
GluA-F	TTATCACACGCGGTCTAG		1,617
GluA-R	AGAGGTACCACTTCTTTGTTTGGGCTT	KpnI	
HtrA-F	AGAGGATCCGCATTAAGTATGAGGA	BamHI	1,690
HtrA-R	CTACCTTCTTCCCATCAA		
Lgl-F	AGAGGATCCGTCAGGCTTTTGATCA	BamHI	1,801
Lgl-R	TGGTATGATCTATCCTAC		
LuxS-F	AGAGGATCCGTTTATCCGTTCTAT	BamHI	1,582
LuxS-R	TTTCACAACAACGTGGTT		
RopA-F	AGAGGATCCAATCGCTCTTAGCTAA	BamHI	1,693
RopA-R	GCTGCATATTACCCATGA		
UvrA-F	AGAGGATCCTTGAGTGTTATCAACT	BamHI	1,795
UvrA-R	TCACCTCCTGATAGCGTT		
YidC2-F	TTACTAGTCTCTGGTCGT		1,747
YidC2-R	AGAGGTACCAAGCGGAAGTTATCAA	KpnI	
Em ^r -F	GAAGCAAACCTTAAGAGTG		846
Em ^r -R	TTATTTCTCTCCCGTTAAA		

*Nucleotides that are underlined in each primer sequence show the position of the restriction endonuclease site incorporated to facilitate cloning.

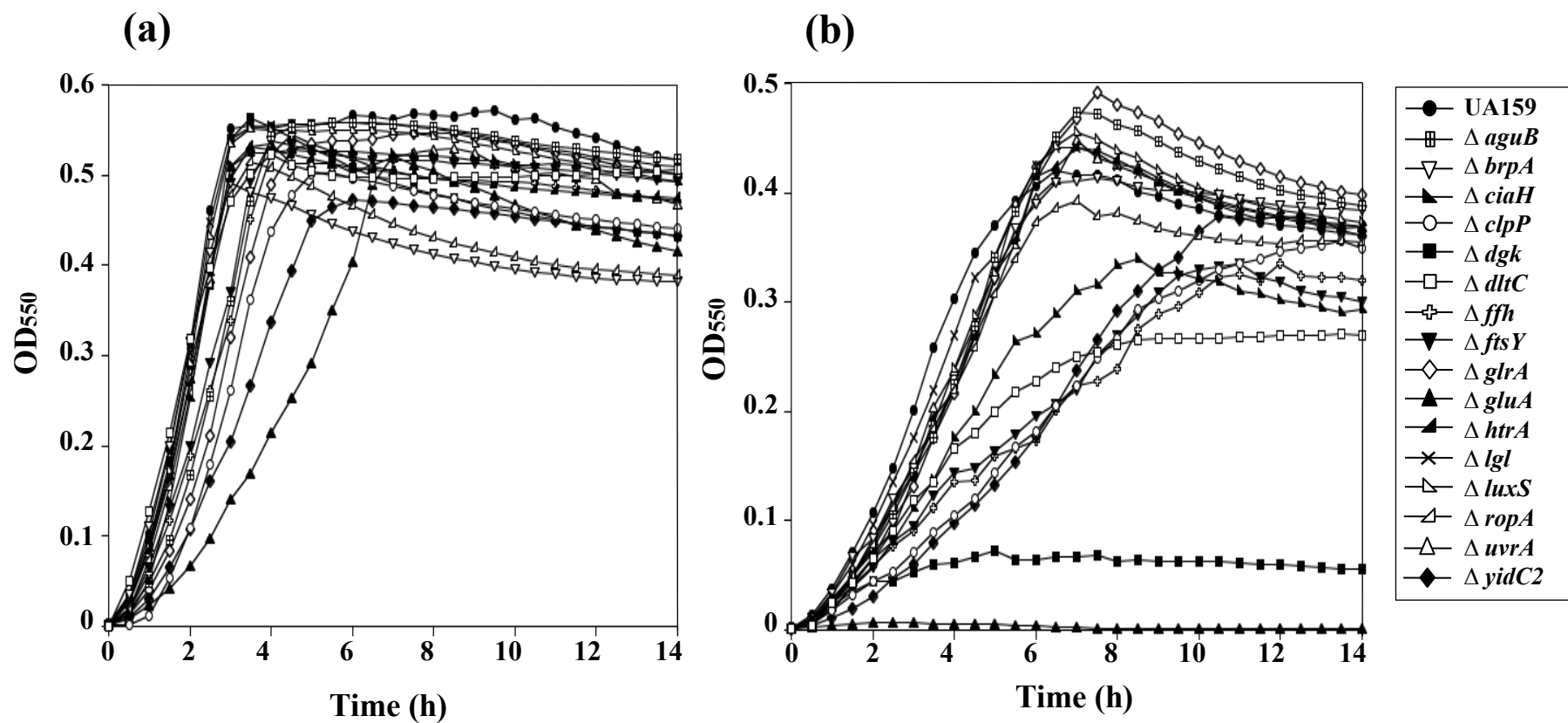


Fig. 1

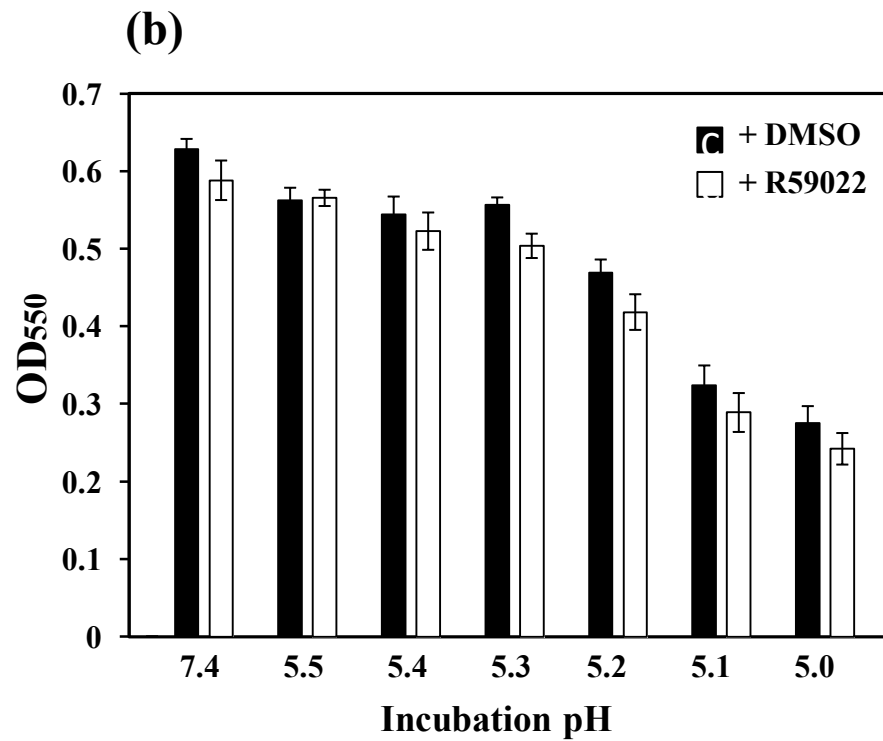
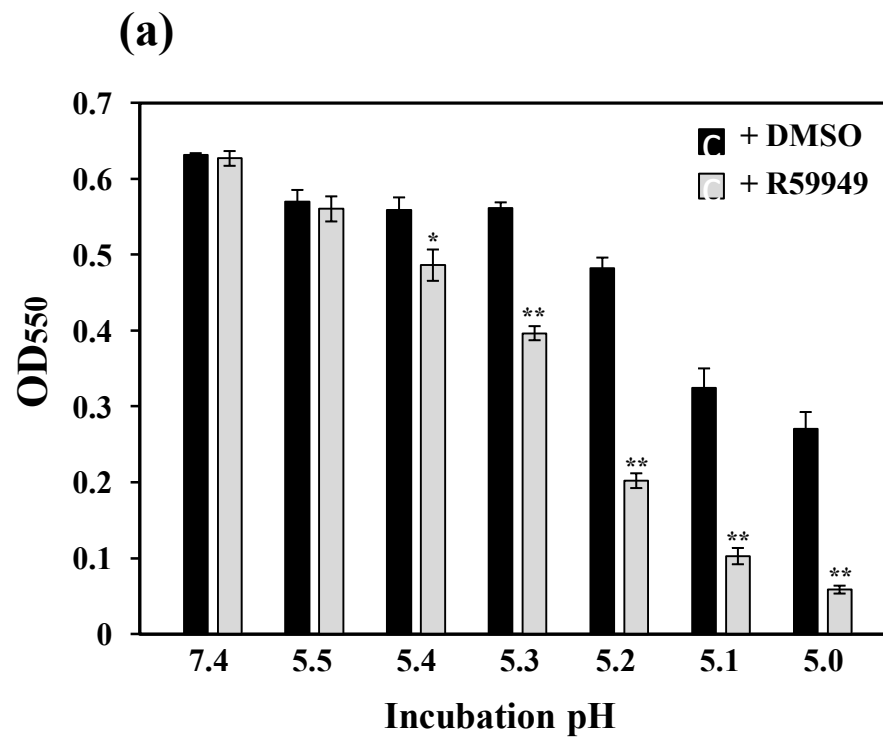


Fig. 2