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Acid Stress Survival Mechanisms of the Cariogenic Bacterium *Streptococcus mutans*

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1. Introduction

Streptococcus mutans, the major etiological agent in human dental caries, is capable of forming a biofilm, or dental plaque, on the tooth surface (Loesche, 1986; Tanzer et al., 2001). *S. mutans* generates large amounts of acid within dental plaque from fermentable dietary carbohydrates. During meals, the ingestion of carbohydrates causes the pH of the dental plaque to fall below 4.0. Acid accumulation can eventually destroy the crystalline structure of teeth that is the hardest tissue in the human body, leading to the formation of a carious lesion (Quivey et al., 2001). The ability of *S. mutans* to survive in such a severe environment represents one of the most important virulence factors of this microorganism.

The mechanisms of acid tolerance that are most common among Gram-positive bacteria have been proposed to be: i) proton pumps; ii) protection and/or repair of macromolecules; iii) cell-membrane changes; iv) production of alkali; v) regulators; vi) cell density and biofilms; and vii) alteration of metabolic pathways (Fig. 1) (Cotter & Hill, 2003). Many researchers have sought to explain the mechanisms of acid tolerance in *S. mutans*, and various genes contributing to aciduricity in *S. mutans* have been identified. In this chapter, we review those genes that have been reported to be involved in *S. mutans* aciduricity, including those participating in two-component systems and others, especially targeting the *dgk* homolog.

2. Two-component system

Two-component systems (TCSs), prokaryote-specific signal transduction systems, are widespread in prokaryotes and play extensive roles in adaptation to environmental changes. The TCS operon (*tcs*) consists of *hk*, which encodes a sensory histidine kinase (HK), and *rr*, which encodes its cognate response regulator (RR). The HK undergoes autophosphorylation on a histidine residue in response to a specific environmental signal and relays this phosphate group to an aspartic acid residue on the cognate RR. The phosphorylated RR then binds target DNA elements with greater affinity, inducing or repressing the transcription of target genes (Hoch, 2000; Rampersaud et al., 1994). In this way, bacteria are able to adapt to the changes in external environment and to modulate gene expression. TCSs may be responsible for the acid tolerance of *S. mutans*.

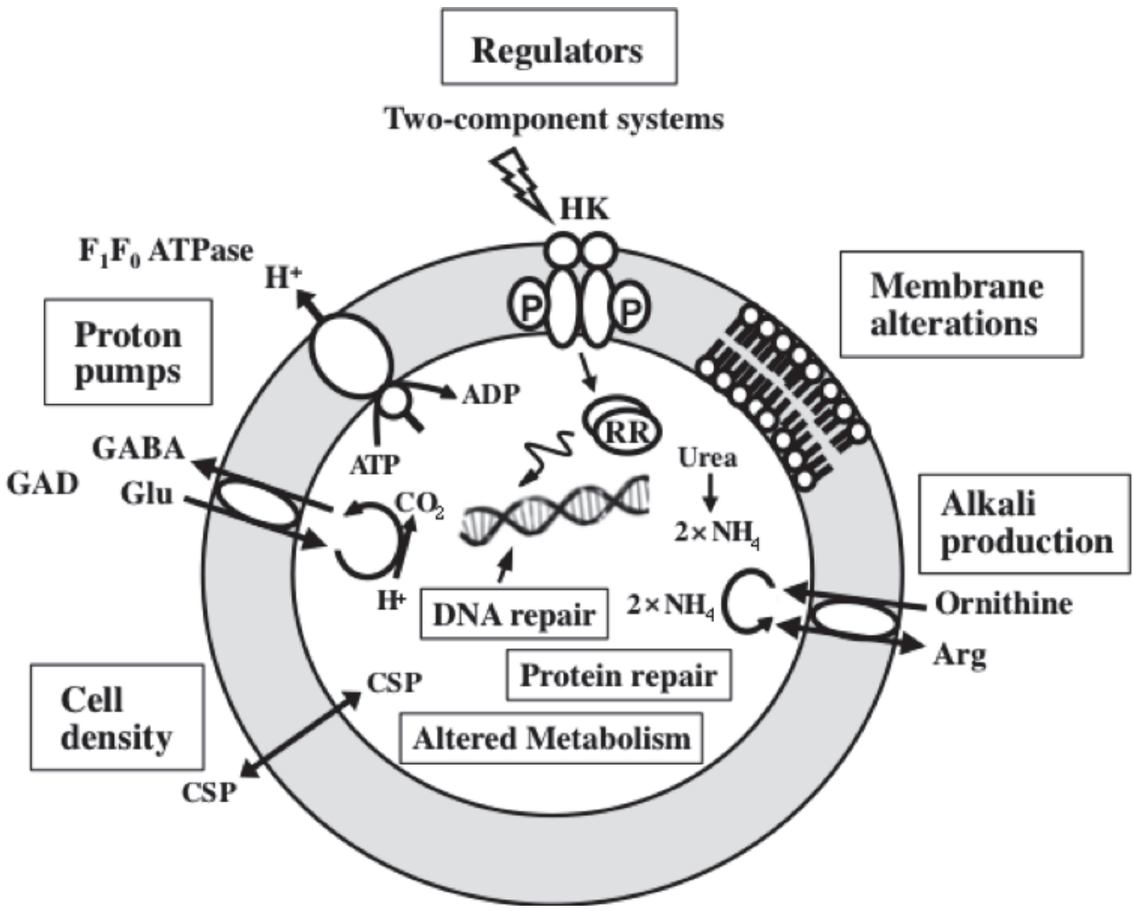

Fig. 1. Acid tolerance mechanisms proposed for gram-positive bacteria. The figure is taken from Cotter & Hill (2003) with some modification.

Analysis of the complete genome sequence of *S. mutans* UA159 suggested the presence of 13 *hk-rr* homologs and one orphan *rr* homolog (Table 1, *smtcs02-15*) (Ajdic et al., 2002). The roles of some specific *tcs* genes in acid tolerance have been evaluated. Li et al. showed that disruption of *smhk13* or *smrr13* resulted in a diminished log-phase acid-tolerance response in *S. mutans* BM71 (Li et al., 2001) and that only *smhk02* of *smtcs02* was involved in the acid-tolerance response of *S. mutans* NG8 (Li et al., 2002). Qi et al. (2004) and Ahn et al. (2006) reported that the *smhk08* mutant exhibited a significant growth defect, whereas the growth of both the *smrr08* mutant and *smhk-rr08* double mutant was similar to that of wild-type UA159 when grown at pH 6.4. Ahn et al. (2006) also showed that all *smhk08*, *smrr08*, or *smhk-rr08* mutants presented growth defects when grown at pH 5.5. Then, Lévesque et al. (2007) systematically inactivated each of the 13 *hk*, but not *rr*, genes in *S. mutans* UA159 and evaluated the roles of the *hk* genes in acid tolerance. They showed that *smhk09* and *smhk14* were involved in *S. mutans* acid tolerance. Furthermore, Biswas et al. (2007) found an additional *tcs* (Table 1, *smtcs01*) in the genome of *S. mutans* UA159 and examined the involvement of 14 *hk* genes in acid tolerance. They showed that only *smhk08* was involved in aciduricity. However, these studies focused only on the role of HKs and so did not provide a comprehensive overview of the role of TCSs in acid tolerance.

tcs genes	*hk gene*, *rr* gene	GenBank Locus Tag [a]	Gene order
smtcs01	*smhk01*	SMU.45	*hk-rr*
	smrr01	SMU.46	
smtcs02	*smhk02*	SMU.486	*hk-rr*
	smrr02	SMU.487	
smtcs03	*smhk03*	SMU.577c	*hk-rr*
	smrr03	SMU.576c	
smtcs04	*smhk04*	SMU.660	*rr-hk*
	smrr04	SMU.659	
smtcs05	*smhk05*	SMU.928	*rr-hk*
	smrr05	SMU.927	
smtcs06	*smhk06*	SMU.1009	*rr-hk*
	smrr06	SMU.1008	
smtcs07	*smhk07*	SMU.1037c	*rr-hk*
	smrr07	SMU.1038c	
smtcs08	*smhk08*	SMU.1128c	*rr-hk*
	smrr08	SMU.1129c	
smtcs09	*smhk09*	SMU.1145c	*rr-hk*
	smrr09	SMU1146c	
smtcs10	*smhk10*	SMU.1516c	*rr-hk*
	smrr10	SMU.1517c	
smtcs11	*smhk11*	SMU.1548c	*hk-rr*
	smrr11	SMU.1547c	
smtcs12	*smhk12*	SMU.1814c	*rr-hk*
	smrr12	SMU.1815c	
smtcs13	*smhk13*	SMU.1916c	*rr-hk*
	smrr13	SMU.1917c	
smtcs14	*smhk14*	SMU.1965c	*hk-rr*
	smrr14	SMU.1964c	
smtcs15	*smrr15*	SMU.1924c	*rr*

tcs, two-component system; *hk*, histidine kinase; *rr*, response regulator.
[a] GenBank locus tag was associated with the *S. mutans* genome at the Oral Pathogen Sequence Database site (http://www.stdgen.lanl.gov/oragen).

Table 1. The *tcs* genes identified in the *S. mutans* UA159 genome.

Therefore, we systematically constructed *rr* deletion mutants and *hk-rr* double mutants of *S. mutans* UA159 and examined the effect on acid tolerance (Kawada-Matsuo et al., 2009). Thirteen *rr* mutants and twelve *hk-rr* double mutants were obtained, the exceptions being *smrr10*, *smtcs10*, *smrr12*, and s*mtcs12*. The derivation of null mutations of these genes was unsuccessful, probably due to a loss of viability of these mutants. To examine the effects of these *rr* mutations on the acid tolerance of *S. mutans*, wild-type UA159 and the 25 mutants were grown in brain–heart infusion (BHI) broth adjusted to pH 7.2 or pH 5.5. Growth curves were generated, and the mid-log-phase doubling time was determined. All *rr* and *hk-rr* mutants grew similarly to wild-type UA159 at pH 7.2. However, as shown in Table 2, deletion of four *rr* genes (*smrr03, smrr05, smrr08*, and *smrr13*) caused significantly decreased

growth rates compared with that of wild-type UA159 when grown at pH 5.5. The growth rates of the *hk-rr* double mutants were similar to those of the corresponding *rr* mutants, and the differences in doubling time between them were not significant. The finding that *smrr08* and *smrr13* were involved in the acid tolerance of *S. mutans* is consistent with previous findings (Ahn et al., 2006; Li et al., 2001). On the other hand, *smrr03* and *smrr05* were, for the first time, demonstrated to be involved in *S. mutans* UA159 acid tolerance. However, Lévesque et al. (2007) and Biswas et al. (2007) showed that inactivation of their cognate *hk* genes did not affect acid tolerance. To confirm whether only the *rr* gene is involved in acid tolerance in the *smtcs03* and *smtcs0*5 mutants, the *hk* genes of *smtcs03* and *smtcs05* were individually inactivated, and the acid tolerance ability of these mutants compared. As shown in Table 3, the *smhk03* mutant exhibited a decreased growth rate compared with the wild type when grown at pH 5.5. This was not consistent with previous results. In contrast, the *smhk05* mutant grew similarly to wild-type UA159 at pH 5.5, as shown in previous studies, whereas the *smrr05* mutant and *smhk-rr05* double mutant exhibited reduced growth rates.

	Doublimg time (min) [a] in:	
UA159 [b]	123.8 ± 9.5	
tcs genes	$hk^{+}rr^{-}$	$hk^{-}rr^{-}$
smtcs01	116.8 ± 10.0	117.7 ± 10.3
smtcs02	131.5 ± 8.8	132.6 ± 5.9
smtcs03	146.4 ± 7.5*	148.5 ± 8.3*
smtcs04	127.4 ± 7.6	122.5 ± 3.7
smtcs05	160.1 ± 11.6**	155.7 ± 7.8*
smtcs06	132.8 ± 3.2	125.1 ± 10.2
smtcs07	131.0 ± 3.2	122.3 ± 7.2
smtcs08	146.3 ± 11.9*	145.2 ± 7.7*
smtcs09	126.3 ± 5.2	129.9 ± 5.0
smtcs11	126.4 ± 7.1	131.7 ± 11.0
smtcs13	141.5 ± 8.8*	140.9 ± 2.6*
smtcs14	132.5 ± 3.7	131.7 ± 12.1
smtcs15 [c]	127.6 ± 7.1	—

[a]Doubling time (Td) was calculated based on the formulas ln Z – ln Z0 = k (t – t0), where k is the growth rate, and g = 0.693/k, where g is the doubling time. Values are the mean ± standard deviation obtained from three independent experiments.
[b]Wild-type strain
[c]Orphan *rr*
* Significant increase from Td of wild-type UA159 by Tukey's HSD, $p < 0.05$
** Significant increase from Td of wild-type UA159 by Tukey's HSD, $p < 0.01$

Table 2. Doubling times of *rr* or *hk-rr* deletion mutants at pH 5.5.

Generally, a signal sensed by a HK is thought to be transmitted to the cognate RR via transfer of phosphoryl groups, and deletion of either the *hk* or *rr* should generate a similar phenotype. However, we found that only the *rr* of *smtcs05* was involved in *S. mutans* acid tolerance. Furthermore, as mentioned above, Li et al. (2002) reported that the *hk*, but not the *rr*, of *smtcs02* was involved in the acid tolerance of *S. mutans* NG8. Qi et al. (2004) and Ahn et al. (2006) also reported that the *smhk08* mutant showed a significant growth defect, whereas the growth of both the *smrr08* mutant and *smhk-rr08* double mutant was similar to that of wild-type UA159. These results suggested involvement of several TCSs in *S. mutans* aciduricity via cross-talk between different TCS components. After all, among 15 TCSs, seven (Smtcs02, 03, 05, 08, 09, 13, and 14) appear to be involved in *S. mutans* aciduricity. Nevertheless, inactivation of no single TCS caused a complete loss of acid tolerance. Therefore, other TCSs that are definitively related to *S. mutans* acid tolerance may exist, but they cannot be identified by homology searching.

tcs genes	Strain	*hk*/*rr*	Doubling time (min) in:	
			BHI pH 7.2	BHI pH 5.5
smtcs03	SMHK03	–/+	53.6 ± 4.1	153.4 ± 7.9
	SMRR03	+/–	50.0 ± 2.7	146.4 ± 7.5
	SMTCS03	–/–	57.1 ± 8.6	148.5 ± 8.3
smtcs05	SMHK05	–/+	51.0 ± 3.4	128.8 ± 12.9 *
	SMRR05	+/–	49.7 ± 2.5	160.1 ± 11.6 * *
	SMTCS05	–/–	51.5 ± 4.6	155.7 ± 7.8

*p < 0.05 (Tukey's HSD)

Table 3. Doubling times of *hk*, *rr,* and *hk-rr* deletion mutants of *smtcs03* and *smtcs05* at pH 7.2 and pH 5.5.

3. Genes other than TCS involved in *S. mutans* acid tolerance

Many studies have implicated genes other than those involved in TCS in *S. mutans* aciduricity. Table 4 summarizes the characteristics of 14 such genes, of which the functions of the products of only nine have been experimentally verified.

These are *aguA*, encoding an agmatine deiminase that is involved in alkali production (Griswold et al., 2004); *dltC*, involved in the synthesis of D-alanyl-lipoteichoic acid, which is associated with alteration of membrane composition (Boyd et al., 2000); *gluA*, encoding a glucose-l-phosphate uridylyltransferase involved in the synthesis of UDP-D-glucose (Yamashita et al., 1998); *lgl*, encoding a lactoylglutathione lyase involved in the detoxification of methylglyoxal (Korithoski et al., 2007); *luxS*, encoding a S-ribosylhomocysteine lyase involved in autoinducer AI2 synthesis (Wen and Burne, 2004); and *uvrA*, encoding an excinuclease ABC subunit A that is involved in DNA repair (Hanna et al., 2001). Mutation of three genes (*ffh, ftsY, yidC2* genes) that are involved in the signal recognition particle pathway, significantly reduced H^+/ATPase specific activity compared with that of the wild type (Hasona et al., 2005). However, how these functions contribute to *S. mutans* aciduricity remains unclear. Furthermore, the functions of the five remaining gene products were predicted based on DNA sequence homology, and so estimation of their role in *S. mutans* aciduricity is much more difficult.

Gene	GenBank Locus Tag	Function	Evidence	Determination of the response against low pH	Reference
aguA	SMU.264	Agmatine deiminase	Testified experimentally	Not determined	Griswold et al., 2004
brpA	SMU.410	Transcriptional regulator	Putative	Acid tolerance & Acid killing	Wen et al., 2006
clpP	SMU.1672c	Clp protease (Serine protease)	Putative	Acid tolerance	Lemos & Burne, 2002
dltC	SMU.1689c	D-alanyl carrier protein	Testified experimentally	Acid tolerance & Acid killing & Acid tolerance response	Boyd et al., 2000
ffh	SMU.1060c	Signal recognition particle	Testified experimentally (partially)	Acid tolerance	Kremer et al., 2001
ftsY	SMU.744	Signal recognition particle receptor	Testified experimentally (partially)	Acid tolerance	Hasona et al., 2005
glrA	SMU.1035	ABC transporter ATP-binding-protein	Putative	Acid tolerance	Cvitkovitch et al., 2000
gluA	SMU.322c	Glucose-l-phosphate uridylyltransferase	Testified experimentally	Acid tolerance	Yamashita et al., 1998
htrA	SMU.2164	Serine protease	Putative	Acid tolerance (agar plate)	Biswas & Biswas, 2005
lgl	SMU.1603	Lactoylglutathione lyase	Testified experimentally	Acid tolerance & Acid tolerance response	Korithoski et al., 2007
luxS	SMU.474c	S-ribosylhomo-cysteine lyase	Testified experimentally	Acid tolerance & Acid killing & Acid tolerance response	Wen & Burne, 2004
ropA	SMU.91	Peptidyl-prolyl isomerase, trigger factor	Putative	Acid killing	Wen et al., 2005
uvrA	SMU.1851c	UV repair excinuclease	Testified experimentally	Acid tolerance & Acid tolerance response	Hanna et al., 2001
yidC2	SMU.1727	Oxa1(or A)-like protein	Testified experimentally (partially)	Acid tolerance	Dong et al., 2008

Table 4. Genes reported to be involved in *S. mutans* acid tolerance.

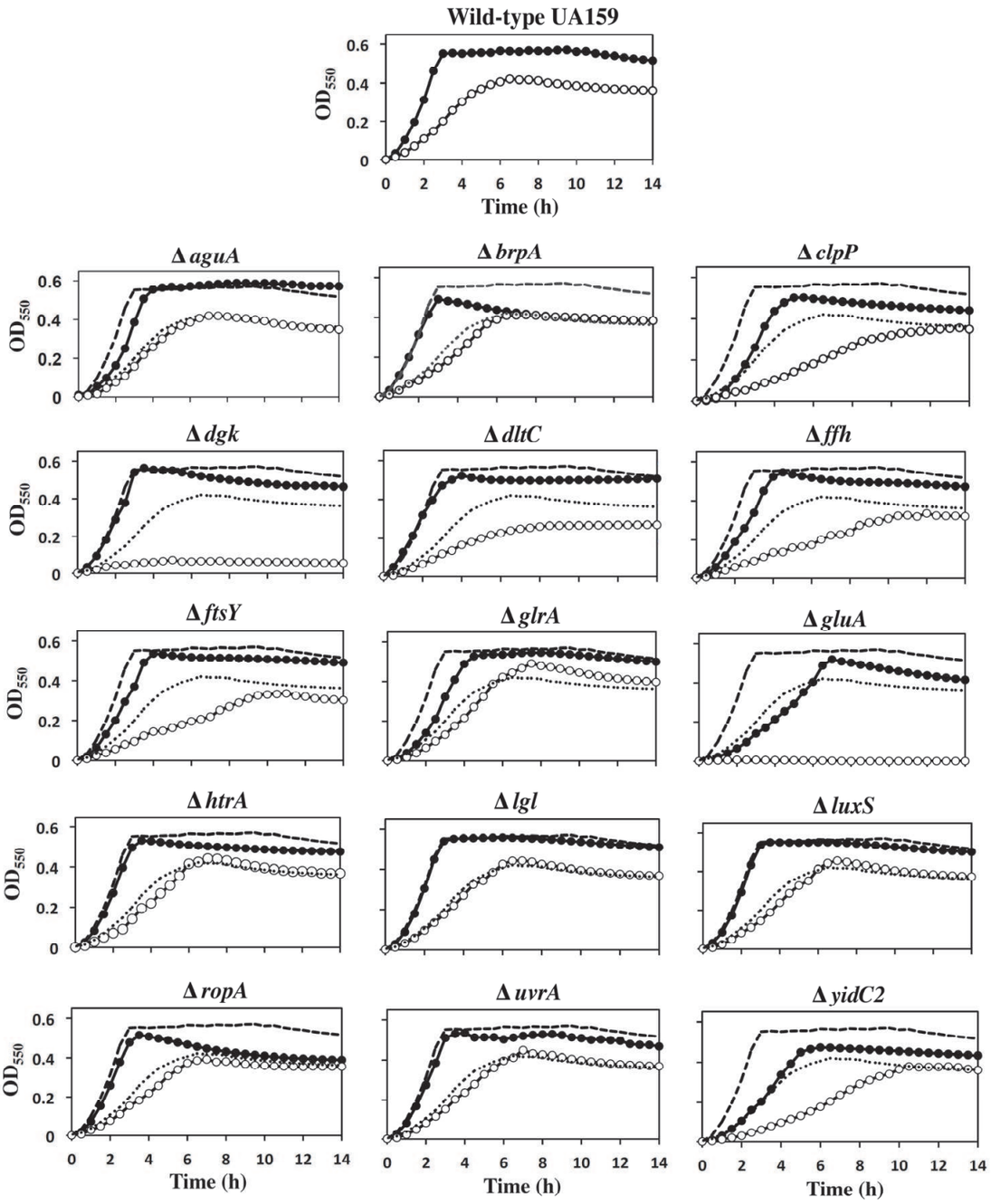

Fig. 2. Growth curves of *S. mutans* UA159 and mutant strains grown in BHI medium at pH 7.4 (●) or pH 5.5 (○). Growth was defined as the increase in OD_{550}, and was calculated by subtraction of OD_{550} at the initiation of growth from that at the times indicated. Data represent the means of three independent experiments. Graphs of mutant strains also represent the growth curves of wild-type UA159 at pH 7.4 (– – –) and pH 5.5 (·····) as controls.

To examine the extent to which a gene contributes to *S. mutans* acid tolerance, 14 mutants in which one of the genes listed in Table 4 was inactivated and the *dgk* mutant were constructed from *S. mutans* UA159, and their growth at pH 7.4 and at pH 5.5 was compared with that of the wild type (Fig. 2) (Shibata et al., 2011). Inactivation of *aguA*, *brpA*, *glrA*, *htrA*, *lgl*, *luxS*, *ropA*, or *uvrA* did not significantly affect the acid tolerance of *S. mutans* as compared with wild-type UA159 when grown at pH 5.5. Three types of method of assessing the acid tolerance of *S. mutans* are available. Simple acid tolerance is evaluated by a procedure in which over-night cultures or log-phase cells grown at neutral pH are subcultured in media or on plates at neutral and acidic pH. Another method is acid killing, in which log-phase cells grown at neutral pH are incubated at a lethally acidic pH, and then viability is determined by plate counts. The final method is determination of the acid tolerance response in which viability is estimated by plating after incubation at neutral or acidic pH of log-phase cells grown at neutral pH followed by incubation at killing pH. We used the first method, and so any discrepancy between this and other studies may derive from differences in the method used. However, comparing all of the mutants in terms of the most basic characteristics of acid tolerance is a worthwhile endeavor. Of course, a comparison using the other criteria is important, and will be performed as part of our next effort.

Notably, the *dgk* and the *gluA* mutants grew extremely slowly at pH 5.5, although the *clpP*, *dltC*, *ffh*, *ftsY,* and *yidC2* mutants also displayed significant reductions in growth rate at pH 5.5 compared with the wild-type UA159. However, only the *brpA*, *dgk*, *dltC*, *htrA*, *lgl*, *luxS*, *ropA*, and *uvrA* mutants showed growth rates comparable to the wild-type strain at pH 7.4. These findings suggest that the reduction in growth rates of the *clpP*, *ffh*, *ftsY, yidC2,* and *gluA* mutants at acidic pH values might be derived from reduced viability and not specifically related to acid tolerance. Therefore, *dltC* and *dgk* are likely specifically involved in acid tolerance. Of these two genes, the striking reduction in growth rate of the *dgk* mutant at acidic pH indicates that *dgk* is of great interest for elucidating the acid-tolerance mechanism of *S. mutans*.

4. Diacylglycerol kinase

Diacylglycerol kinase (Dgk) catalyses the ATP-dependent phosphorylation of *sn*-1,2-diacylglycerols, resulting in production of phosphatidic acid. In eukaryotic cells, diacylglycerol and phosphatidic acid are immediate second cellular messengers responding to extracellular signals, suggesting that Dgk is a key enzyme in cellular signal transduction (Moolenaar et al., 1986; Murayama & Ui, 1987; Nishizuka, 1984; Topham & Prescott, 1999). Among bacterial Dgk, only that of *Escherichia coli* has been well-characterized, and it is a small integral membrane protein with a molecular mass of 13.2 kDa. This enzyme functions in the recycling of the diacylglycerol produced during turnover of membrane phospholipids (Hasin & Kennedy, 1982; Rotering & Raetz, 1983) and plays an important physiological role in responding to environmental stress as well as its role in eukaryotic cells (Raetz & Newman, 1979; Walsh et al., 1986). On the other hand, the *S. mutans* Dgk homolog has a molecular mass of 15.3 kDa and comprises 137 amino acids. It is interesting that insertion of the transposon Tn*916* into the codon for the tenth amino acid from the C terminus of the Dgk homolog resulted in defective growth of the mutant (GS5Tn1) at acidic pH values

(Yamashita et al., 1993). In addition to attenuation of aciduricity, this mutant possessed reduced resistance to high osmolarity and temperature (Yamashita et al., 1993). The C terminus of the Dgk homolog may thus play an important role in signal transduction during environment stress.

To evaluate how the C terminus of Dgk contributes to *S. mutans* acid tolerance, we sequentially truncated amino acids from the C terminus of Dgk and finally constructed 11 mutants termed UADGK0–10, expressing Dgk0–10 (Fig. 3) (Shibata et al., 2009). The mutants showed no significant difference in growth rate at neutral pH (doubling times: 53.8 to 61.6 min; Table 5). Most, with the exception of UADGK0 to UADGK2, showed a reduction in growth rate at pH 5.5 compared with the wild type (Table 5 and Fig. 4). UADGK3, in which three amino acid residues had been deleted from the C-terminus of Dgk, showed a slight reduction in growth rate. Subsequent deletion of amino acids from the C-terminus resulted in further reductions in growth rate at acidic pH. Indeed UADGK4, UADGK5, and UADGK6 had significantly increased doubling times ($p < 0.05$, $p < 0.001$, and $p < 0.0001$, respectively) compared with UADGK0. UADGK7, in which seven amino acid residues had been deleted, showed extremely limited growth in the first 9 hours. Further truncation of the C-terminus of Dgk (UADGK8 to UADGK10) resulted in no growth at pH 5.5. These results suggest that the C-terminal of the Dgk homolog is indispensable for its function in aciduricity of *S. mutans*. We further constructed two additional UA159 *dgk* mutants, UADGK11 and UADGK12 (Fig. 3) to evaluate the function of truncated Dgk. There were only negligible differences in the growth rates of these two mutants at pH 5.5, 5.8, or 6.3, compared with that of UADGK10.

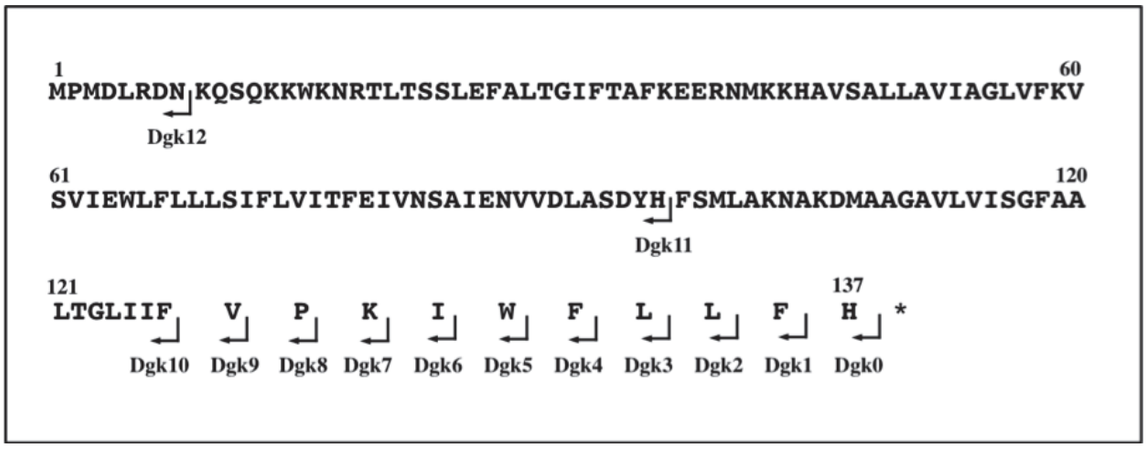

Fig. 3. Representation of the truncated Dgk proteins used. The deduced amino acid sequence of the *dgk* gene from *S. mutans* UA159 is presented. The terminal amino acid of the truncated Dgk expressed in each UA159 *dgk* mutant and each *E. coli* RZ transformant is indicated along the sequences by a curved arrow, which indicates that the sequence is deleted from the right up to this site. All the truncated Dgk proteins names were changed from those of previous paper (Shibata et al., 2009) to help readers understand.

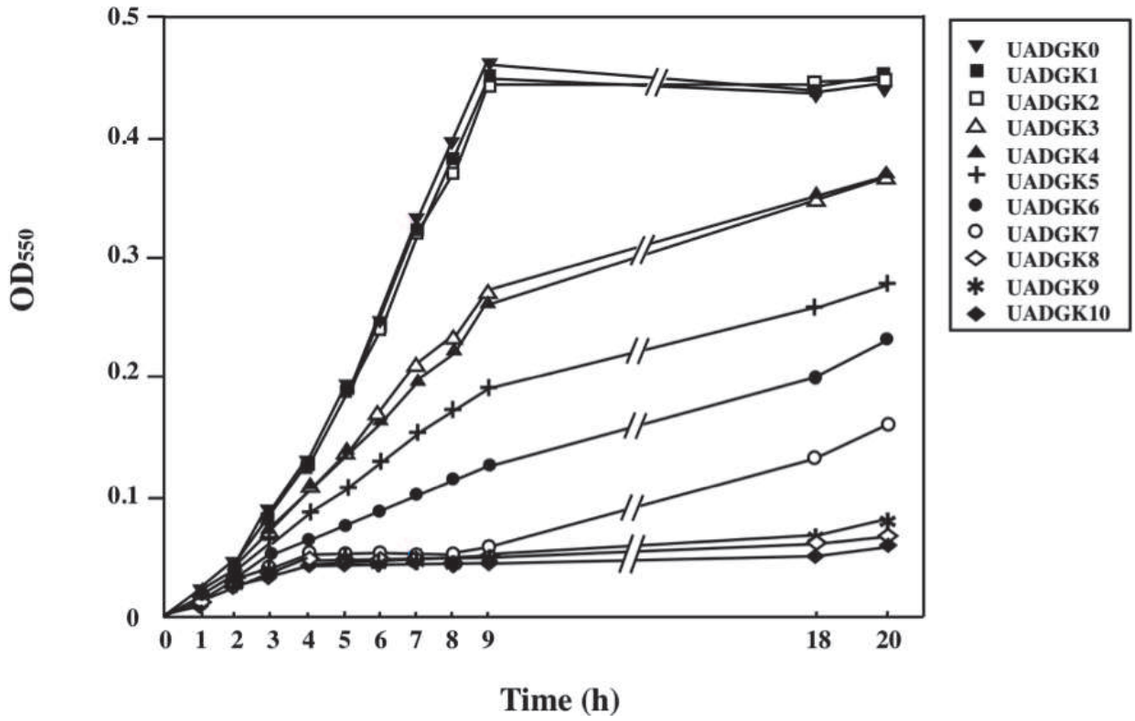

Fig. 4. Growth curves of *S. mutans* UA159 *dgk* mutants grown in BHI medium at pH 5.5. Growth was defined as the increase in OD_{550}, and was calculated by subtraction of OD_{550} at the initiation of growth from that at the times indicated. Data represent the means of three independent experiments. All the mutants names were changed from those of previous paper (Shibata et al., 2009) to help readers understand.

Strain	Doubling time (min) in:	
	BHI pH 7.45	BHI pH 5.5
UADGK0	57.8 ± 4.4	132.3 ± 12.9
UADGK1	58.2 ± 6.4	131.2 ± 11.7
UADGK2	61.2 ± 5.8	133.6 ± 11.7
UADGK3	61.6 ± 1.8	177.6 ± 19.0
UADGK4	55.5 ± 3.4	202.5 ± 24.4*
UADGK5	57.5 ± 2.5	248.9 ± 32.2**
UADGK6	55.3 ± 5.4	271.3 ± 34.6 ***
UADGK7	55.1 ± 6.0	> 1000 [a]
UADGK8	53.8 ± 3.7	> 1000 [a]
UADGK9	58.4 ± 6.7	> 1000 [a]
UADGK10	57.0 ± 6.1	> 1000 [a]

Differences in the doubling time between UADGK0 and UADGK1-10 were analyzed by Bonferroni multiple comparison test (*, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$).
[a]Statistical analyses were not carried out because of too slow growth rates.
All the mutants names were changed from those of previous paper (Shibata et al., 2009) to help readers understand.

Table 5. Effect of low pH on growth of *S. mutans* UA159 *dgk* mutants.

We next constructed recombinant Dgk proteins corresponding to *S. mutans* strains UADGK10, UADGK7, UADGK5, and UADGK0 utilizing *E. coli* strains RZDGK10, RZDGK7, RZDGK5, and RZDGK0, respectively. The kinase activity in cell lysates of *E. coli* transformants was examined by an octyl glucoside mixed-micelle assay (Preiss et al., 1986), using undecaprenol as a substrate because of it has a higher substrate specificity for the *S. mutans* Dgk homolog compared with diacylglycerol (Lis & Kuramitsu, 2003).

As shown in Fig. 5A, whereas the full-size *S. mutans* Dgk protein expressed in RZDGK0 catalyzed a high level of phosphorylation of undecaprenol, the Dgk missing five amino acid residues from the C terminus expressed in RZDGK5 exhibited markedly reduced kinase activity. Furthermore, RZDGK7 (seven amino acids missing from the C terminus) exhibited much weaker kinase activity than did RZDGK5. The deletion of 10 C-terminal amino acid residues of Dgk in RZDGK10 resulted in a total lack of kinase activity. These differences were confirmed by quantitative analysis (Fig. 5B). These data indicate that the C-terminus of the *S. mutans* Dgk homolog plays an important role in kinase activity and may harbor residues required for catalysis. Alternatively, incorrect folding of the protein due to the missing C-terminal residues may cause loss of kinase activity. Therefore, its catalysis of undecaprenol phosphorylation is closely related to *S. mutans* acid tolerance.

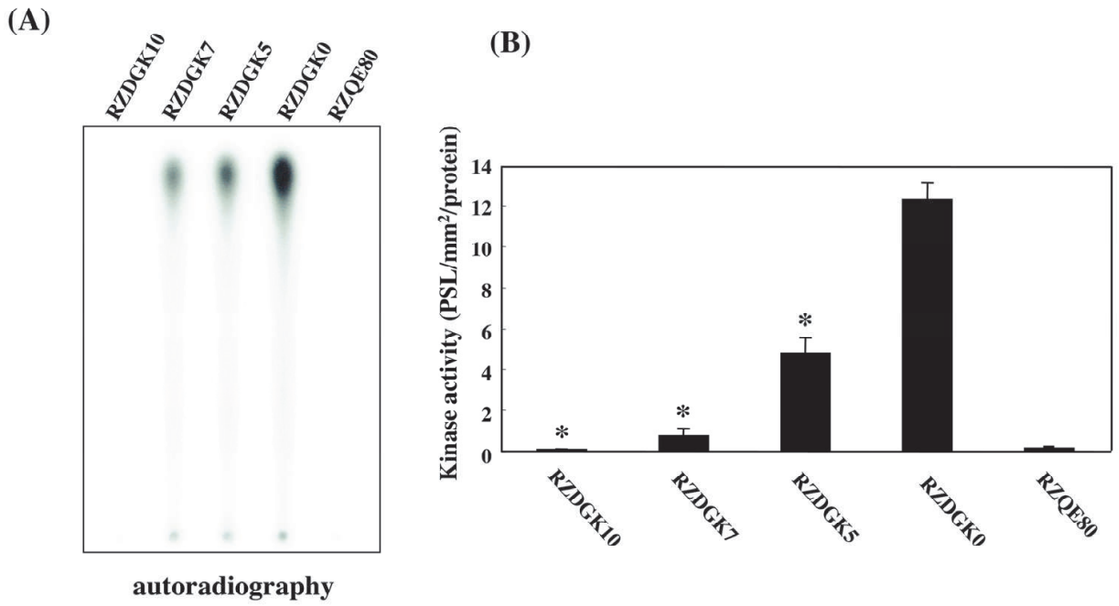

Fig. 5. Effect of deletion of the C-terminal tail of Dgk on undecaprenol kinase activity. (A) Comparison of undecaprenol kinase activity of the full-size Dgk and various C-terminally truncated forms of Dgk. The undecaprenol kinase activity in the lysates from *E. coli* RZ cells was determined using an octyl glucoside mixed-micelle assay. (B) Quantification analysis of the kinase assay. Quantification was carried out by normalization of radioactive bands in the kinase assay using the protein level. Vertical bars represent standard deviation. Differences in kinase activity between RZDGK0 and RZDGK5, RZDGK7, or RZDGK10 were analyzed by Student's *t* test (*, $p < 0.0001$). All the *E.coli* strains names were changed from those of previous paper (Shibata et al., 2009) to help readers understand.

Moreover, the importance of the C-terminal end of Dgk in *S. mutans* acid tolerance was examined in a specific pathogen-free animal model (Table 6). The *dgk* mutant strain clearly displayed a significant reduction in smooth-surface caries compared with the wild type ($p < 0.005$). In contrast, no significant difference in plaque extent was observed between the wild-type and *dgk* mutant strains. These results suggest that aciduricity regulated by the *dgk* gene product might play a critical role in *S. mutans* virulence.

Treatment	Plaque extent (Δ)	Initial dentinal fissures (ΔΔ)	Advanced dentinal fissures (ΔΔ)	Smooth-surface caries (ΔΔΔ)	Total bacteria CFU (10^7)	Total streptococci CFU (10^7)	Total *S. mutans* CFU (10^7)
Water control	$2.8 \pm 0.63^{a\,b}$	9.5 ± 1.72^{a}	$6.6 \pm 2.80^{a\,b}$	0.5 ± 0.97^{a}	4.4 ± 2.36^{a}	2.7 ± 2.26^{a}	ND $^{a\,b}$
UA159 (wt)	1.1 ± 0.32^{a}	11.5 ± 1.27^{a}	10.8 ± 1.62^{a}	$9.5 \pm 6.55^{a\,c}$	7.9 ± 3.21^{a}	$7.4 \pm 2.79^{a\,c}$	$4.0 \pm 2.11^{a\,c}$
UADGK10 (*dgk*)	1.5 ± 0.53^{b}	10.6 ± 0.84	9.3 ± 1.2^{b}	2.5 ± 2.68^{c}	6.4 ± 2.40	4.4 ± 2.53^{c}	$2.3 \pm 1.20^{b\,c}$

ND, Not determined. Δ, 4 units at risk; ΔΔ, 12 fissures at risk; ΔΔΔ, 20 units at risk.
[a]Significant difference between water control and UA159 (wt), $p < 0.05$.
[b]Significant difference between UADGK1 (*dgk*) and water control, $p < 0.05$.
[c]Significant difference between UA159 (wt) and UADGK1 (*dgk*), $p < 0.05$.
The mutant name was changed from those of previous paper (Shibata et al., 2009) to help readers understand.

Table 6. Influence of *dgk* deletion on smooth-surface plaque extent, initial and advanced dentinal fissure lesions, smooth-surface caries, and colonization properties.

5. A potential target for anti-caries chemotherapy

Development of an effective anti-caries agent is the ultimate goal of our work. Considering the characteristics of known mutants, the Dgk homolog seems to be the most promising target for anti-caries agents. Dgks have been extensively studied in mammals, and several inhibitory compounds, e.g., R59022 and R59949 (Fig. 6), have been reported. In contrast, inhibitors of prokaryotic Dgk have not yet been elucidated.

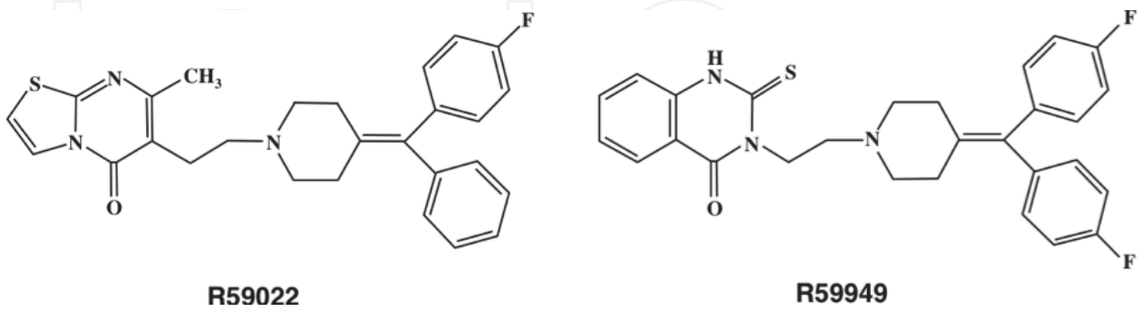

Fig. 6. Structures of R59022 and R59949.

When first attempting to discover inhibitors of prokaryotic Dgk, we tested the effects of R59022 and R59949 on the growth of *S. mutans* (Shibata et al., 2011). Although neither R59022 nor R59949 influenced growth at pH 7.4, R59949, but not R59022, showed a significant inhibitory effect at acidic pH (Fig. 7). Inhibition by R59949 increased by 13, 29, 58,

68, and 78% at pH 5.4, 5.3, 5.2, 5.1, and 5.0, respectively (Fig. 7A). These findings were particularly interesting because R59022 and R59949 were used at concentrations of 100 µM and 25 µM, respectively, due to the limited solubility of R59949.

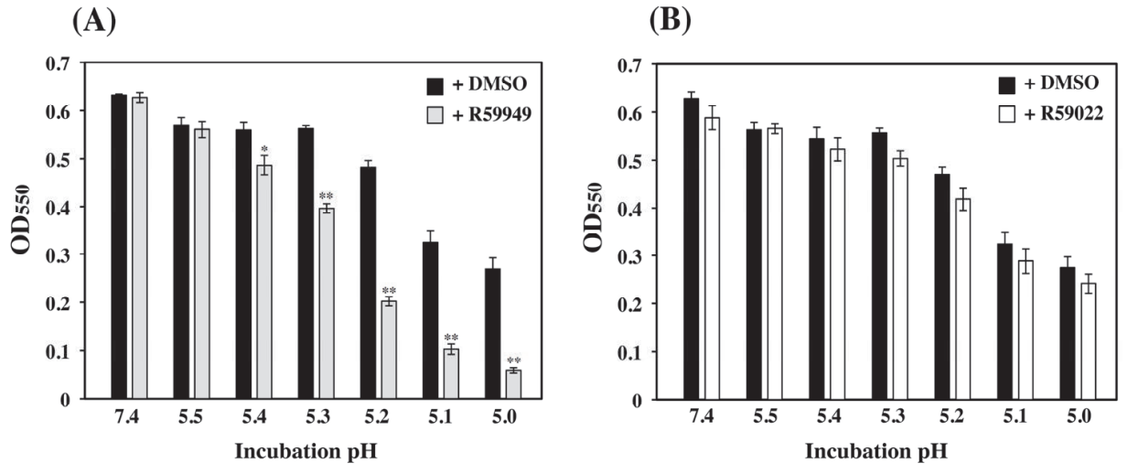

Fig. 7. Effect of R59949 (A) and R59022 (B) on the growth of *S. mutans.* Data represent the mean ± standard deviation. Differences in growth rate between cells cultured in the presence and absence of Dgk inhibitor were analyzed using Student's *t* test. *, $p < 0.05$; **, $p < 0.0001$.

Furthermore, we examined the inhibitory effects of R59022 and R59949 on the kinase activity of *S. mutans* Dgk. Neither R59022 nor R59949 inhibited kinase activity at pH 7.4; this is in agreement with their lack of effect on *S. mutans* growth at neutral pH. As mentioned above, R59949 significantly inhibited the growth of *S. mutans* at acidic pH values (below 5.4). When evaluating the effect of R59949 on enzyme activity at acidic pH, it is important to know the intracellular pH of *S. mutans* cells; the intracellular pH of *S. mutans* cells was 6.4 when cultured in broth at pH 5.2. Therefore, we determined the inhibitory effect of R59949 and R59022 on *S. mutans* Dgk kinase activity at pH 6.4. R59949, but not R59022, inhibited kinase activity with undecaprenol as a substrate by around 20% (Fig. 8).

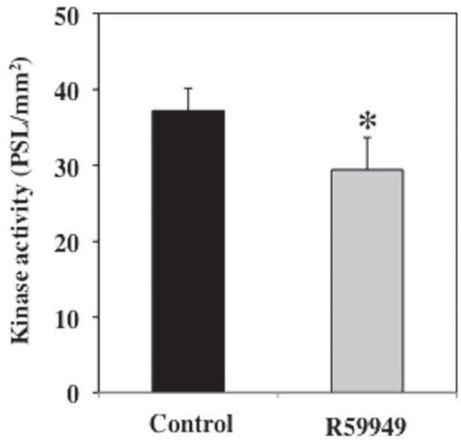

Fig. 8. Effect of R59949 on kinase activity with undecaprenol as a substrate. Data represent the mean ± standard deviation. Differences in kinase activity between cells cultured in the presence and absence of R59949 were analyzed by Student's *t* test. *, $p < 0.05$.

S. mutans Dgk is inherently different from mammalian Dgk in terms of its molecular size, molecular structure, and substrate specificity. However, it is interesting that R59949 inhibits the enzymatic activity of *S. mutans* Dgk even with undecaprenol as the substrate. Additionally, the difference in inhibitory activity between R59949 and R59022 means that a comparison of their molecular structure may lead to discovery of further potent Dgk inhibitors specific for prokaryotic enzymes, that is, new anti-caries agents.

6. Conclusion

The reduction of environmental pH in dental plaque by the cariogenic microorganisms is important step in the development of dental caries. The cariogenic microorganisms should survive in a relentless environment produced by themselves in order to exhibit their maximum virulence. In this chapter, we described the acid tolerance characteristics of the cariogenic microorganism, *S. mutans*. TCSs seem to be the most suitable system for adaptation to environmental conditions. However, no TCS seems to be definitively responsible for *S. mutans* acid tolerance. At present, identification of TCS depends on gene homology searching, which may not identify all genes encoding TCS that contribute to *S. mutans* acid tolerance.

We focus on *dgk* because it is the most promising contributor to *S. mutans* acid tolerance when assessed using a simple acid tolerance assay. Although the precise mechanism by which the gene product is involved in acid tolerance has not yet been elucidated, *dgk* is the only gene whose product has been definitively implicated in cariogenicity in an animal model. Furthermore, potential specific inhibitors of the gene product have been introduced. This fact may aid in development of next-generation anti-caries therapies based on the ability of this microorganism to adapt to environmental conditions.

However, much detail of the acid tolerance mechanisms of *S. mutans* remains unknown, and so further study is required.

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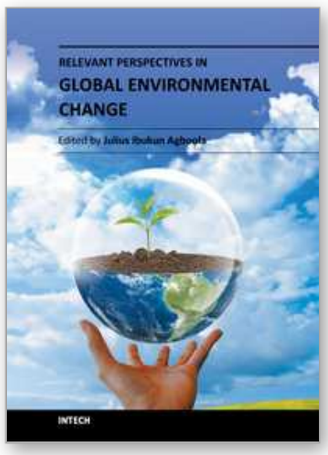

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