Oral Microbiology Immunology 2009: 24: 173–176
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

Short communication

Role of two component signaling response regulators in acid tolerance of *Streptococcus mutans*

M. Kawada-Matsuo, Y. Shibata, Y. Yamashita
Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Fukuoka, Japan

Kawada-Matsuo M, Shibata Y, Yamashita Y. Role of two component signaling response regulators in acid tolerance of Streptococcus mutans*.*
Oral Microbiol Immunol 2009: 24: 173–176. © 2009 John Wiley & Sons A/S.

Introduction: In bacteria, two-component systems (TCS) involving the products of a histidine kinase gene (*hk*) and a response regulator gene (*rr*) play important roles in adaptation to environmental changes. Fourteen *hk-rr* homologs and one orphan *rr* homolog were identified in the *Streptococcus mutans* UA159 genome database. There have been no comprehensive evaluations of the roles of *rr* homologs in the acid tolerance of *S. mutans*.
Methods: The TCS genes (*tcs*) of *S. mutans* were designated *smtcs01–15*. Mutants of *S. mutans* UA159 with deletions of *rr* and *hk-rr* were constructed. Acid tolerance was evaluated by comparing the doubling times at pH 7.2 and pH 5.5 between the wild-type and mutant strains.
Results: Excluding *smtcs10* and *12*, for which viable mutants could not be obtained, a total of 13 *rr* deletion mutants were constructed. The *rr* deletions in *smtcs03*, *05*, *08*, and *13* resulted in diminished acid tolerance in comparison with UA159. The *hk-rr* double-mutants exhibited acid sensitivity levels similar to those of the corresponding *rr* mutants. The results of the present study reveal the involvement of the *rr* genes of *smtcs03* and *05* in acid tolerance. Deletion of *hk* and/or *rr* in *smtcs03* generated an acid-sensitive phenotype. In contrast, for *smtcs05*, while deletion of *rr* resulted in reduced acid tolerance, a single-deletion of *hk* had no effect on acid tolerance.
Conclusions: We implicated two *rr* genes in the acid tolerance of *S. mutans*. In particular, *smtcs05* is a novel *tcs*, the sole *rr* of which is involved in the acid tolerance of *S. mutans*.

Key words: acid tolerance; gene inactivation; response regulator; *Streptococcus mutans;* two-component system

Y. Yamashita, Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582 Japan
Tel.: + 81 92 642 6350; fax: + 81 92 642 6354; e-mail: yoshi@dent.kyushu-u.ac.jp
Accepted for publication September 17, 2008

Streptococcus mutans, which is a primary pathogen of human dental caries in the oral cavity (8), is capable of forming a biofilm, known as dental plaque, on the surfaces of teeth (17). Within the dental plaque, *S. mutans* produces large amounts of acid during carbohydrate metabolism. During meals, the ingestion of carbohydrates causes the pH of the dental plaque to fall below 4.0, initiating enamel demineralization (16). Induction of acid tolerance (AT) in *S. mutans* facilitates survival in the low pH environment of the plaque. Therefore, AT is an important property associated with virulence of *S. mutans*.

A two-component system (TCS) is a prokaryote-specific signal transduction system. The TCS genes (*tcs*) consist of a gene (*hk*) that encodes a sensory histidine kinase (HK) and a gene (*rr*) that encodes a cognate response regulator (RR). The sensor HK undergoes autophosphorylation on a histidine residue in response to a specific environmental signal and relays the phosphate group to an aspartic acid residue on the cognate RR. The phosphorylated RR then binds to target DNA elements with greater affinity, activating or repressing the transcription of target genes (4, 11). In this way, bacteria are able to adapt to the external environment and to regulate gene expression.

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

tcs genes	*hk* gene, *rr* gene	GenBank Locus Tag[1]	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	*smrr05*	SMU.928	*rr-hk*
	smhk05	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	SMU.1146c	
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.
[1]GenBank locus tag was associated with the *S. mutans* genome at the Oral Pathogen Sequence Database site (http://www.stdgen.lanl.gov/oragen).

Table 2. Bacterial strains used in this study

Strain	Relevant properties	Source or reference
E. coli DH5α	*supE44* ΔlacU169 (080lacZΔM15) HSDR17 *recA1 endA1 gyrA96 thi-1 relA1*	(13)
UA159	serotype c wild-type strain	(9)
SMRR01	*smrr01* deletion mutant of UA159; Em^r	This study
SMRR02	*smrr02* deletion mutant of UA159; Em^r	This study
SMRR03	*smrr03* deletion mutant of UA159; Em^r	This study
SMRR04	*smrr04* deletion mutant of UA159; Em^r	This study
SMRR05	*smrr05* deletion mutant of UA159; Em^r	This study
SMRR06	*smrr06* deletion mutant of UA159; Em^r	This study
SMRR07	*smrr07* deletion mutant of UA159; Em^r	This study
SMRR08	*smrr08* deletion mutant of UA159; Em^r	This study
SMRR09	*smrr09* deletion mutant of UA159; Em^r	This study
SMRR11	*smrr11* deletion mutant of UA159; Em^r	This study
SMRR13	*smrr13* deletion mutant of UA159; Em^r	This study
SMRR14	*smrr14* deletion mutant of UA159; Em^r	This study
SMRR15	*smrr15* deletion mutant of UA159; Em^r	This study
SMT01	*smtcs01* deletion mutant of UA159; Em^r	This study
SMT02	*smtcs02* deletion mutant of UA159; Em^r	This study
SMT03	*smtcs03* deletion mutant of UA159; Em^r	This study
SMT04	*smtcs04* deletion mutant of UA159; Em^r	This study
SMT05	*smtcs05* deletion mutant of UA159; Em^r	This study
SMT06	*smtcs06* deletion mutant of UA159; Em^r	This study
SMT07	*smtcs07* deletion mutant of UA159; Em^r	This study
SMT08	*smtcs08* deletion mutant of UA159; Em^r	This study
SMT09	*smtcs09* deletion mutant of UA159; Em^r	This study
SMT11	*smtcs11* deletion mutant of UA159; Em^r	This study
SMT13	*smtcs13* deletion mutant of UA159; Em^r	This study
SMT14	*smtcs14* deletion mutant of UA159; Em^r	This study
SMHK03	*smhk03* deletion mutant of UA159; Em^r	This study
SMHK05	*smhk05* deletion mutant of UA159; Em^r	This study

Analysis of the complete genome sequence of *S. mutans* UA159 suggested the presence of 13 *hk-rr* homologs and one orphan *rr* homolog (2). Lévesque et al. (5) systematically inactivated each of the 13 *hk* genes, but not the *rr* genes, in *S. mutans*, and evaluated the roles of the *hk* genes in the AT of this organism. Furthermore, Biswas et al. (3) found an additional *tcs* in the genome of *S. mutans*, and examined the involvement of 14 *hk* genes in AT. The roles of some specific *tcs* genes in AT have been evaluated in other studies (1, 6, 7, 10, 14). However, these studies failed to include all of the *rr* genes, and did not provide a comprehensive overview of the role of TCS in AT. In the present study, *rr* deletion mutants were systematically constructed in *S. mutans* UA159 and examined for effects on AT. Surprisingly, Ahn et al. (1) found that an *hk* single-mutant of *smtcs08* reduced AT to a greater extent than did the *hk-rr* double-mutant. Therefore, we compared the AT levels of the *rr* mutants with those of the corresponding *hk-rr* double-mutants.

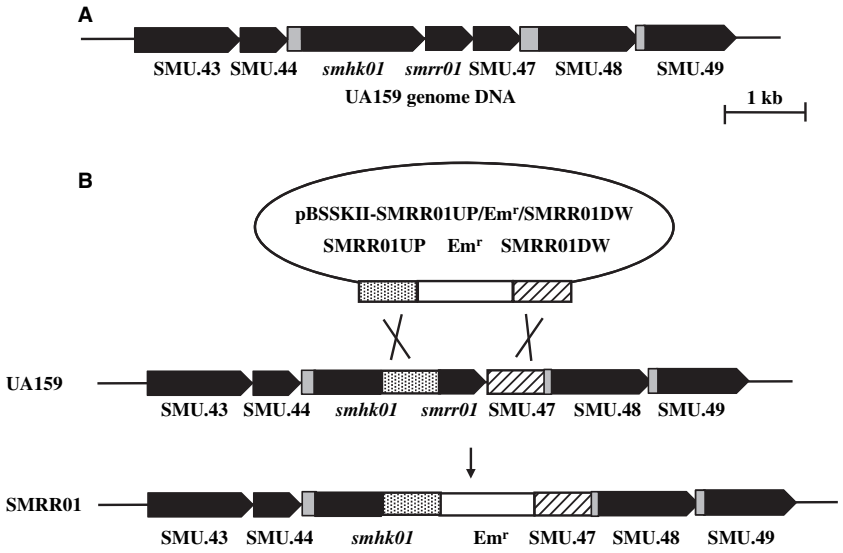

Fig. 1. (A) A map of *smrr01* and flanking regions in *Streptococcus mutans* UA159. The black arrows indicate the open reading frames. The gray box denotes the intergenic regions. (B) The strategy for constructing SMRR01 by double-crossover homologous recombination. The details are given in the text.

Construction of *rr* single-deletion mutants and *hk-rr* double-deletion mutants by double-crossover recombination

Fourteen *hk-rr* homologs and one orphan *rr* homolog identified in the *S. mutans* UA159 genome (2, 3) were designated *smtcs01–15*, with *hk* and *rr* designated as *smhk01–14* and *smrr01–15*, respectively (Table 1).

We constructed 15 *rr* deletion mutants in *S. mutans* UA159 using double-crossover homologous recombination. Given that the *hk* and *rr* genes are located in tandem in the genome, we planned to construct 14 *hk-rr* double-deletion

mutants. The primers used to construct and confirm the gene deletions are listed in Table S1 (see Supporting information). Figure 1 shows the strategy used to construct the *smrr01* deletion mutant, as an example. First, the Em^r fragment that contains the erythromycin resistance gene from the plasmid pResEmNot (15) was amplified using the Em-F and Em-R primers with *Hin*dIII and *Pvu*II sites, respectively, engineered into their 5′-ends. The Em^r fragment was cloned into pBluescript SK II (+) (Stratagene, La Jolla, CA) digested with *Hin*dIII and *Eco*RV, yielding pBSSKII-Em^r. Next, a 574-base-pair fragment upstream of the *smrr01* gene (SMRR01UP) was amplified from *S. mutans* UA159 genomic DNA using the primers SMRR01UP-F (with a *Kpn*I site at the 5′-end) and SMRR01UP-R (with a *Xho*I site at the 5′-end). This fragment was digested with *Kpn*I and *Xho*I, and then inserted into the corresponding sites of pBSSKII-Em^r, to yield pBSSKII-SMRR01UP/Em^r. Finally, a 507-base-pair fragment downstream of the *smrr01* gene (SMRR01DW) was amplified with the primers SMRR01DW-F (blunt end) and SMRR01DW-R (with a *Sac*I site at the 5′-end). The SMRR01DW fragment was digested with *Sac*I and then cloned into *Sma*I/*Sac*I-digested pBSSKII-SMRR01UP/Em^r. The resulting plasmid, designated pBSSKII-SMRR01UP/Em^r/SMRR01DW, was digested with *Kpn*I and *Sac*I, and the assembled fragment was then transformed into *S. mutans* UA159. The resulting mutant was designated SMRR01. Correct replacement in the transformant was confirmed by polymerase chain reaction (data not shown). A similar strategy was used to construct the other mutants. The derivation of null mutations of *smrr10*, *smtcs10*, *smrr12*, and *smtcs12* was unsuccessful, probably because of the loss of viability of these mutants. Consequently, we constructed 13 *rr* deletion mutants (SMRR01–SMRR09, SMRR11, and SMRR13–SMRR15) and 12 *hk-rr* double-deletion mutants (SMT01–SMT09, SMT11, SMT13, and SMT14) (Table 2).

Table 3. Doubling times of *rr* or *hk-rr* deletion mutants at pH 5.5

Doubling time (min)[1]		
UA159[2]	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[3]	127.6 ± 7.1	—

[1]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. Values are the mean ± standard deviation obtained from three independent experiments.
[2]Wild-type strain.
[3]Orphan *rr*.
*Significant increase from T_d of wild-type UA159 by Tukey's HSD, $P < 0.05$.
**Significant increase from T_d of wild-type UA159 by Tukey's HSD, $P < 0.01$.

Roles of the *rr* genes in AT

To examine the effects of the *rr* mutations on the AT of *S. mutans*, the bacteria were grown in brain–heart infusion broth (BHI; Difco Laboratories, Detroit, MI) at 37°C in 5% CO_2 overnight. The cultures were then diluted 1 : 10 into fresh BHI and grown to an optical density of approximately 0.5 at 550 nm (OD_{550}). Aliquots (20 μl) of the cell suspension having the same turbidity were inoculated into wells that contained 200 μl fresh BHI medium adjusted to pH 7.2 or pH 5.5 with 50 mM phosphate–acetate buffer. To maintain anaerobic conditions, sterile mineral oil (50 μl per well) was overlaid on the cultures. The absorbance was monitored every 30 min for 12 h at 37°C using a SPECTRA max 340PC^{384} (Molecular Devices, Sunnyvale, CA). Wells that contained uncultured BHI were used as controls. From the data obtained, growth curves were generated and the mid-log phase doubling time (T_d) was determined.

All of the *rr* and *hk-rr* mutants showed growth patterns similar to that of wild-type UA159 at pH 7.2 (T_d values: for UA159, 52.1 ± 0.7; for the mutants, 48.1 ± 3.3 to 57.1 ± 8.6). However, as shown in Table 3, deletion of four *rr* genes (*smrr03*, *smrr05*, *smrr08*, and *smrr13*) resulted in decreased growth rates compared to that of wild-type UA159 when grown at pH 5.5. The differences in T_d between the wild-type and each mutant were statistically significant. The *hk-rr* double-mutants showed acid sensitivities similar to those of the corresponding *rr* mutants, and there were no significant differences in their growth rates. The involvement of *smrr08* and *smrr13* in AT has been reported previously (1, 6), and our results are consistent with these previous findings. On the other hand, in the present study, *smrr03* and *smrr05* are newly demonstrated to be involved in the AT of *S. mutans* UA159.

Roles of *hk* and *rr* of *smtcs03* and *smtcs05* in AT

Lévesque et al. (5) and Biswas et al. (3) showed that inactivation of *smhk03* and *smhk05* did not affect the AT of *S. mutans*. Considering these results, *smtcs03* and *smtcs05* seem to be novel *tcs* genes, only the *rr* of which is involved in the AT of *S. mutans*. To confirm this, the *hk* genes of *smtcs03* and *smtcs05* were individually inactivated and the AT values of these mutants were compared as described above. As shown in Table 4, SMHK03 exhibited a decrease in growth rate compared to the wild-type when grown at pH 5.5. Unexpectedly, this result was not consistent with the previous results, even when we repeated the trial. In contrast, the growth of the SMHK05 mutant was similar to that of wild-type UA159 at pH 5.5, as shown in previous studies, while SMRR05 and SMT05 had reduced AT values. Although the involvement of *smhk03* in the AT of *S. mutans* remains uncertain, because of discrepancies between the results of the present study and previous studies, *smtcs05* is clearly a novel *tcs*, only the *rr* of which is involved in the AT of *S. mutans*.

Generally, a signal that is sensed by HK is thought to be transmitted to the cognate RR via transfer of phosphoryl groups, and

Table 4. Doubling times of *hk*, *rr*, and *hk-rr* deletion mutants of *smtcs03* and *smtcs05* at pH 7.2 and pH 5.5

			Doubling time (min)		
tcs genes	Strain	*hk*/*rr*	pH 7.2 BHI	pH 5.5 BHI	
smtcs03	SMHK03	−/+	53.6 ± 4.1	153.4 ± 7.9	
	SMRR03	+/−	50.0 ± 2.7	146.4 ± 7.5	
	SMT03	−/−	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	
	SMT05	−/−	51.5 ± 4.6	155.7 ± 7.8	

*$P < 0.05$ (Tukey's HSD).

deletion of either the *hk* or *rr* should generate a similar phenotype. However, in the present study, we found that only the *rr* of *smtcs05* was involved in the AT of *S. mutans*. Previously, Li et al. (7) reported that the *hk*, but not the *rr*, of *smtcs02* was involved in the AT of *S. mutans* NG8. Similar phenomena have been reported for *Escherichia coli* (18) and *Mycobacterium tuberculosis* (12). Yamamoto et al. (18) confirmed *trans*-phosphorylation between non-cognate HK-RR pairs in *E. coli*. The results of our AT tests suggest that the Smrr05 protein receives the pH signal from the other HK by cross-talk. It will be of interest to identify the actual HK involved in the cross-talk that occurs via the Smrr05 protein in SMHK05.

In conclusion, we have identified two novel *rr* genes (*smrr03* and *smrr05*) that are involved in the AT of *S. mutans* UA159, and we have shown that *smtcs05* is a novel *tcs*, only the *rr* gene of which is involved in the AT of *S. mutans*.

Acknowledgments

This work was supported in part by Grants-in Aid for Scientific Research 19592408 (Y. S.) and 20659329 (Y. Y.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Ahn SJ, Wen ZT, Burne RA. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect Immun 2006: **74**: 1631–1642.
2. Ajdic D, McShan WM, McLaughlin RE et al. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc Natl Acad Sci U S A 2002: **99**: 14434–14439.
3. Biswas I, Drake L, Erkina D, Biswas S. Involvement of sensor kinases in the stress tolerance response of *Streptococcus mutans*. J Bacteriol 2008: **190**: 68–77.
4. Hoch JA. Two-component and phosphorelay signal transduction. Curr Opin Microbiol 2000: **3**: 165–170.
5. Lévesque CM, Mair RW, Perry JA, Lau PCY, Li YH, Cvitkovitch DG. Systemic inactivation and phenotypic characterization of two-component systems in expression of *Streptococcus mutans* virulence properties. Lett Appl Microbiol 2007: **45**: 398–404.
6. Li YH, Hanna MN, Svensäter G, Ellen RP, Cvitkovitch DG. Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. J Bacteriol 2001: **183**: 6875–6884.
7. Li YH, Lau PCY, Tang N, Svensäter G, Ellen RP, Cvitkovitch DG. Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. J Bacteriol 2002: **184**: 6333–6342.
8. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986: **50**: 353–380.
9. Murchison HH, Barrett JF, Cardineau GA, Curtiss R III. Transformation of *Streptococcus mutans* with chromosomal and shuttle plasmid (pYA629) DNAs. Infect Immun 1986: **54**: 273–282.
10. Qi F, Merritt J, Lux R, Shi W. Inactivation of the *ciaH* gene in *Streptococcus mutans* diminishes mutacin production and competence development, alters sucrose-dependent biofilm formation, and reduces stress tolerance. Infect Immun 2004: **72**: 4895–4899.
11. Rampersaud A, Harlocker SL, Inouye M. The OmpR protein of *Escherichia coli* binds to sites in the *ompF* promoter region in a hierarchical manner determined by its degree of phosphorylation. J Biol Chem 1994: **269**: 12559–12566.
12. Saini DK, Malhotra V, Tyagi JS. Cross talk between DevS sensor kinase homologue, Rv2027c, and DevR response regulator of *Mycobacterium tuberculosis*. FEBS Lett 2004: **565**: 75–80.
13. Sambrook J, Russell DW. Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2001.
14. Senadheera MD, Lee AWC, Hung DCI, Spatafora GA, Goodman SD, Cvitkovitch DG. The *Streptococcus mutans vicX* gene product modulates *gtfB/C* expression, biofilm formation, genetic competence, and oxidative stress tolerance. J Bacteriol 2007: **189**: 1451–1458.
15. Shiroza T, Kuramitsu HK. Construction of a model secretion system for oral streptococci. Infect Immun 1993: **61**: 3745–3755.
16. Svensäter G, Larsson UB, Greif ECG, Cvitkovitch DG, Hamilton IR. Acid tolerance response and survival by oral bacteria. Oral Microbiol Immunol 1997: **12**: 266–273.
17. Tanzer JM, Livingston J, Thompson AM. The microbiology of primary dental caries in humans. J Dent Educ 2001: **65**: 1028–1037.
18. Yamamoto K, Hirao K, Oshima T, Aiba H, Utsumi R, Ishihama A. Functional characterization *in vitro* of all two-component signal transduction systems from *Escherichia coli*. J Biol Chem 2005: **280**: 1448–1456.

Supporting information

Additional Supporting information may be found in the online Version of this article:

Table S1. Primers used in this study (Word document)

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.