Additive attenuation of virulence and cariogenic potential of *Streptococcus mutans* by simultaneous inactivation of the ComCDE quorum-sensing system and HK/RR11 two-component regulatory system

Yung-Hua Li,1,2 Xiao-Lin Tian,1 Gillian Layton,1 Chris Norgaard2 and Gary Sisson2

1Department of Applied Oral Sciences, Dalhousie University, Halifax, NS, Canada
2Department of Microbiology and Immunology, Dalhousie University, Halifax, NS, Canada

The genome of *Streptococcus mutans* harbours 13 two-component signal transduction systems (TCSTSSs). Of these, a peptide-mediated quorum-sensing system, ComCDE, and the HK/RR11 two-component system are well known to regulate several virulence-associated traits in *in vitro* experiments, including genetic competence, bacteriocin production, biofilm formation and stress responses. In this study, we investigated the hypothesis that inactivation of ComCDE, HK/RR11 or both systems would attenuate the virulence and cariogenicity of *S. mutans*. The results showed that simultaneous inactivation of both signal transduction systems additively attenuated *S. mutans* virulence and cariogenicity, since inactivation of either of these systems alone did not result in the same degree of effect. The double deletion mutant SMcde-hk11 was defective in genetic competence, had a reduced acid production, was unable to grow at pH 5.0 and formed an abnormal biofilm with reduced biomass. Animal studies showed that this mutant had reduced capabilities for oral colonization, succession and initiation of dental caries. A competitive index (CI) analysis using a mixed-infection animal model revealed that all the mutants, particularly SMcde-hk11, had reduced fitness in their ecological niches and were unable to compete with the wild-type strain for persistence in dental biofilms. The evidence from this study suggests that the ComCDE and HK/RR11 signal transduction systems can be considered to be novel targets for the development of strategies in the prevention and treatment of *S. mutans* infections.

**INTRODUCTION**

*Streptococcus mutans* is a Gram-positive bacterium that depends on a 'biofilm life-style' for survival and persistence in its natural ecosystem, dental plaque (Ajdic et al., 2002; Burne, 1998; Cvitkovitch et al., 2003). Under appropriate environmental conditions, this bacterium can rapidly produce acids from fermentable dietary carbohydrates and initiate demineralization of the tooth surface. *S. mutans* is therefore considered to be an important aetiological agent of dental caries (Kuramitsu, 2003). The ability of *S. mutans* to initiate dental caries depends on several significant virulence traits, including: (i) initiation of biofilm formation by adherence and accumulation on the tooth surface that is promoted by its synthesis of insoluble, extracellular polysaccharides; (ii) production of numerous bacteriocins that kill other species, favouring its competition in dental biofilms; (iii) high efficiency in catabolizing carbohydrates and producing acids; and (iv) the ability to tolerate low pH (Belli & Marquis, 1991; Li & Burne, 2001; Kuramitsu, 2003; Scheie & Petersen, 2004). These virulence-associated traits provide *S. mutans* with overwhelming ecological advantages in competition and succession in dental biofilms that cause caries (Marsh, 2000; Kuramitsu, 2003). *S. mutans* also causes corrosion of dental materials, leading to secondary caries around restorations (Marsh, 2000). Moreover, it can be a cause of subacute infective endocarditis (Ajdic et al., 2002; Mitchell, 2003).

To survive and initiate infections, bacteria must sense, and respond and adapt to their environments, a process that requires signal transduction across biological membranes.
(Barrett & Hoch, 1998). A major mechanism of signal transduction, widespread in bacteria, is represented by the so-called two-component signal transduction systems (TCSTSs), which enable bacteria to regulate their gene expression and coordinate activities in response to environmental stimuli (Barrett & Hoch, 1998; Beier & Gross, 2006; Hoch, 2000). A typical TCSTS consists of a membrane-associated histidine kinase (HK) protein, which senses a specific stimulus, and a cytoplasmic response regulator (RR) protein, which enables the cells to respond to the stimulus via regulation of gene expression (Hoch, 2000). Upon stimulation, the histidine kinase sensor protein interacts with a specific signal and activates autophosphorylation at a conserved histidine residue. The phosphoryl group is then transferred to the cognate regulator (RR) protein, which enables the cells to respond to the stimulus via regulation of gene expression (Hoch, 2000). Because of their importance in the environmental stimuli (Barrett & Hoch, 1998; Beier & Gross, 2006; Hoch, 2000). Many TCSTSs have been found to function as global regulators by initiating signalling cascades, in which large sets of genes are switched on and/or off. These systems provide the major means by which bacteria communicate with each other and the outside world. TCSTSs have been known to regulate diverse metabolic processes, the bacterial cell cycle, cell–cell communication and virulence factors in a wide range of bacterial species (Hoch, 2000). Because of their importance in the regulation of cellular physiology, adaptation to environments and virulence expression, TCSTSs have been considered to be important targets for the development of antimicrobial agents (Barrett & Hoch, 1998; Beier & Gross, 2006; Hoch, 2000).

There are 13 two-component systems that have been identified in the S. mutans genome (Adjic et al., 2002). Several TCSTSs have been characterized and recognized to regulate physiological activities and virulence-associated traits in S. mutans (Levesque et al., 2007; Biswas et al., 2008). A signal peptide-mediated quorum-sensing system encoded by comCDE has been found to play a central role in regulation of genetic competence, bacteriocin production, biofilm formation and stress response (Li et al., 2001a, b, 2002a; van der Ploeg, 2005). Another system, called HK/RR11, is involved in S. mutans survival at acidic pH (Li et al., 2002b). Since inactivation of either hk11 (SMu.486) or rr11 (SMu.487) results in an abnormal biofilm phenotype, which is similar to that formed by the comC mutant, HK11 is suspected to be the second receptor to the competence-stimulating peptide (CSP) (Li et al., 2002a). In S. mutans, the CiaHR system has also been characterized and found to play a coordinate role with the ComCDE quorum-sensing system in regulating genetic competence and stress response (Ahn et al., 2006). The VicRK system, which shares a high similarity to the CovSR of Streptococcus pyogenes, has been found to regulate sucrose-dependent biofilm formation in S. mutans (Lee et al., 2004; Senadheera et al., 2005). The ScnRK system in S. mutans has been found to regulate hydrogen resistance and macrophage killing (Chen et al., 2008). In addition, an orphan response regulator in the S. mutans genome has been found to play a role in sucrose-dependent adherence and cariogenicity (Idone et al., 2003). These studies have shown that the expression of virulence traits by S. mutans requires multiple signal transduction pathways and complex regulatory networks. The TCSTSs can be therefore considered to be an essential prerequisite for the virulence and cariogenicity of S. mutans (Levesque et al., 2007; Biswas et al., 2008). However, most of these studies are based on investigations of S. mutans in vivo experiments. Little is known of how these systems play roles in the virulence and pathogenesis of S. mutans in vivo. In this study, we used a specific-pathogen-free rat model to assess the effects of inactivation of the ComCDE, HK/RR11 or both signal transduction pathways on oral colonization, ecological fitness and the cariogenic potential of S. mutans. Here, we report the results of the experiments in which we address these questions.

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain, amplicon or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>S. mutans strains</strong></td>
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<tr>
<td>NG8</td>
<td>Wild-type, Erm&lt;sup&gt;+&lt;/sup&gt;, Spec&lt;sup&gt;+&lt;/sup&gt;, Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Li et al. (2001a)</td>
</tr>
<tr>
<td>NG8 [pDL276]</td>
<td>NG8 harbouring pDL276 to confer Kan&lt;sup&gt;+&lt;/sup&gt; marker</td>
<td>This study</td>
</tr>
<tr>
<td>SMhkk11</td>
<td>NG8 Δhk11::P&lt;sub&gt;C&lt;/sub&gt;Em, Erm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Li et al. (2002b)</td>
</tr>
<tr>
<td>SMcde-4</td>
<td>NG8 ΔcomCDE::Em, Erm&lt;sup&gt;+&lt;/sup&gt;, Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Li et al. (2002a)</td>
</tr>
<tr>
<td>SMcde-hk11</td>
<td>NG8 Δhk11::Em; ΔcomCDE::Spec, Erm&lt;sup&gt;+&lt;/sup&gt;, Spec&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>SMCE-1</td>
<td>NG8::pComE-KO; ComE, Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Li et al. (2001a)</td>
</tr>
<tr>
<td><strong>Amplicons</strong></td>
<td></td>
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<tr>
<td>Erm</td>
<td>Amplicon of ermAM cassette (860 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>Spec</td>
<td>Amplicon of spec cassette (1145 bp) from pDL278</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pDL276</td>
<td>Streptococcus–E. coli shuttle vector, Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Dunny et al. (1991)</td>
</tr>
<tr>
<td>pDL278</td>
<td>Streptococcus–E. coli shuttle vector, Spec&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Dunny et al. (1991)</td>
</tr>
<tr>
<td>pDL289</td>
<td>Streptococcus–E. coli shuttle vector, Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Li et al. (2001a)</td>
</tr>
</tbody>
</table>
METHODS

Bacterial strains, media and growth conditions. Bacterial strains and their characteristics are listed in Table 1. The *S. mutans* wild-type strain was routinely grown on Todd–Hewitt medium plus 0.3 % yeast extract (THYE), whereas the mutants were maintained on THYE plus an appropriate antibiotic. Selective media, Mitis–Salivarius agar plus 0.2 U bacitracin ml⁻¹ (MSB) or THYE agar supplemented with appropriate antibiotics, were used to distinguish between the *S. mutans* strains and other bacteria in oral swab samples or dental plaques from animals. Blood agar plates were used to grow bacteria for enumeration of total viable cell counts of the oral samples.

Construction of the ComCDE-HK11 double deletion mutant. To determine the effect of simultaneous inactivation of both ComCDE and HK/R11 on the virulence and cariogenic potential of *S. mutans*, we used an existing *hk11* deletion mutant SMhk11 (Li et al., 2002b) to construct a double deletion mutant by deleting the internal region of the *comCDE* locus using a PCR-ligation mutagenesis strategy (Lau et al., 2002; Supplementary Fig. S1). Briefly, a 992 bp fragment (ComCDE-up) from the internal region of the *comC* start codon was amplified from *S. mutans* NG8 genomic DNA using primers CDE-P1 (5'-CAAAAGAAAAAACCCGAC-3') and CDE-P2 (5'-GGCCGGCCCACCAATGTTGTTCAAGAAGC-3'; AscI site underlined). Another fragment, ComCDE-dw (1408 bp), was amplified from an internal region of *comED* using primers CDE-P3 (5'-GGCCGGCCCGCAATGTTGTTCAAGAAGC-3'; FseI site underlined) and CDE-P4 (5'-GGCTTTTTGAGAAACAGAAGC-3'). A spectinomycin-resistance marker (1145 bp) from the Spectrofococcus–Escherichia coli shuttle vector pDL278 (Dunny et al., 1991) was amplified using primers Spec-P1 (5'-GGCCGGCCACTAATACGCTT- AAAGTGACTCGGC-3'; AscI site underlined) and Spec-P2 (5'-GGCCGGCCCTAATACGCTT- AAAGTGACTCGGC-3'; FseI site underlined). These amplicons were digested, cleaned and ligated to produce a ComCDE-up::Spec::ComCDE-dw fragment. The ligation product was then transformed into the SMhk11 mutant (Em') derived from *S. mutans* NG8. Following double-crossover homologous recombination, the internal region of the *comCDE* locus was completely replaced by the spectinomycin cassette (Supplementary Fig. S1). Transfornants that grew on THYE agar plates supplemented with both spectinomycin (500 μg ml⁻¹) and erythromycin (10 μg ml⁻¹) were selected for PCR confirmation. The PCR fragments of CDE-P1-Spec-P2 and Spec-P1-CDE-P4 from the mutant were sequenced to confirm the location and orientation of the insertion.

Growth rate and competence assays. Growth curves of all the strains were assayed by growing cells in 10 ml THYE broth in glass tubes for 20 h and OD₆₀₀ readings were obtained using a spectrophotometer. The mutant was also assayed for genetic competence to confirm the effect of gene deletion on genetic transformability using a protocol described previously (Syvitski et al., 2007). A *Streptococcus–Escherichia coli* shuttle vector pDL289 conferring kanamycin resistance was used as transforming DNA. The cultures were spread on THYE plates plus kanamycin (800 ng ml⁻¹), while an aliquot of the cell suspension was spread on THYE plates to determine the total numbers of recipient cells. The transformation frequency was calculated by the number of transformants divided by the total number of viable recipient cells (per millilitre cell suspension) and was expressed as a percentage.

Glycolytic pH drop assay. To determine acid production by the mutants, a glycolytic pH drop assay was performed using a method described elsewhere (Belli & Marquis, 1991). Briefly, stationary-phase cells (overnight culture) were harvested and resuspended in a salt solution (50 mM KCJ and 1 mM MgCl₂) to make a final cell density of OD₆₀₀ 1.0. The cell suspensions were adjusted to pH 7.4 and glucose was then added to a final concentration of 56 mM. Changes of the pH dropping profile were recorded for 2 h using a digital pH meter (Fisher) at room temperature.

Acid resistance assay. The mutant strains were assayed for the effect of acidic pH on their growth by growing bacterial cells on THYE plates at pH 7.0 and 5.0 using a protocol described previously (Li et al., 2002b). An aliquot (20 μl) of cell suspension diluted from overnight culture was inoculated onto THYE plates (pH 7.0 or 5.0) with additional buffer (10 mM potassium phosphate). The plates were then incubated at 37 °C for 48 h before assessment of their growth at low pH.

Biofilm formation assay. The mutants were also assayed for biofilm formation on a polystyrene surface by a method described previously (Li et al., 2002a). The growth of biofilms was initiated by inoculating 5 μl cell suspension into 300 μl 4 x diluted THYE broth in a 96-well microtitre plate or 25 μl into 2 ml broth in a 24-well microtitre plate (some wells contained coverslips). The plates were incubated at 37 °C for 16 h before removing planktonic cells. The 96-well microtitre plate was then stained by 0.1 % (w/v) safranin for 10 min, rinsed with water and air-dried for 3 h. Biofilms were quantified by reading OD₄₉₀ of stained biofilms using a multi-detection microplate reader (Synergy BioKet). Biofilms formed on cover slides were carefully removed for examination and photography by a phase-contrast microscope after staining with 0.1 % crystal violet for 5 min.

Rat model of oral colonization and cariogenic potential. To determine the effects of inactivation of *comCDE*, *hk11* or both on oral colonization, fitness and cariogenic potential, a total of 64 Sprague–Dawley female rats (19 days old) were purchased from the Charles River Breeding Laboratory. Upon arrival, the animals were divided into eight groups (n=8 per group). All the animals were fed with erythromycin water (100 μg ml⁻¹) and a regular diet for 3 days to lower the microbial load, and were tested to confirm the absence of *S. mutans* by swabbing and plating the samples on MSB. The animals then received a sucrose-containing diet (D12450B; Research Diet) throughout the entire experiment. On day 4, the animals were inoculated by pasting a bacteria–starch mix (10⁶ cells per millilitre of cooked starch) onto the surfaces of the animal’s molars once a day for five consecutive days to allow oral colonization. Swab samples were taken from the surfaces of animal molars on the first day and at the first, third, sixth, eighth and tenth weeks post-inoculation. The samples from each group were pooled in 2 ml 10 mM potassium phosphate buffer and sonicated for 30 s at a setting of 2 using a Fisher Sonic Dismembrator (Model 100). The samples were serially diluted and plated on MSB or THYE plates containing appropriate antibiotics and on blood agar plates for total cell counts. Samples from the wild-type group NG8 [pDL276] were plated on MSB plus kanamycin (500 μg ml⁻¹). The plates were incubated at 37 °C for 2 days before enumeration of colonies of *S. mutans* and total viable counts. The percentages of the *S. mutans* cells were calculated to determine oral colonization and succession profiles of individual strains in animals. At the end of the tenth week, all the animals were sacrificed in order to obtain dental plaque samples by a scaling and washing procedure. Samples from the same group were pooled, sonicated, serially diluted and inoculated on appropriate plates. The plates were incubated at 37 °C for 2 days before enumeration of colonies. Both jaws of the animals were then removed and suspended in 3.7 % formaldehyde until caries scoring. All molars of the animals were examined under a dissecting microscope (Fig. 6) and carious lesions were scored by a modification of the Keyes method (Keyes, 1958; Lee & Boran, 2003; Michalek et al., 1975; Yamashita et al., 1993). The results were analysed by Student’s t test, with P<0.05 considered statistically significant.
Competitive index (CI) analysis. To determine the ecological fitness of the mutants in the animals, we inoculated a 1:1 ratio of a mutant to the wild-type strain NG8 [pDL276] to establish mixed infections in the animals. A CI analysis was used to assess the ecological fitness of the mutants in terms of their ability to compete with the wild-type strain for oral colonization and succession. The CI was defined as the ratio of the mutant to the wild-type strain in the output samples (numbers of the organisms post-infection) divided by the ratio of the two strains in the input inoculum (Auerbuch et al., 2001). The CI values were determined by viable cell counts of the strains recovered from the selective media, while the resident flora was estimated from total viable counts on blood agar plates. The mean CI values were calculated from eight animals in each group. The data were analysed by Student’s t test, with $P<0.05$ considered statistically significant.

RESULTS

Characteristics of the double deletion mutant SMcde-hk11

Previous studies have shown that the comCDE knockout mutant SMcde-4 is significantly defective in its genetic transformability (Li et al., 2001a). Introduction of a second genetic construct into this mutant through transformation was, if not impossible, very difficult. However, deletion of hk11 resulted in inactivation of the entire signal transduction pathway, but did not affect the natural transformability of S. mutans (Li et al., 2002b). We took advantage of this by using an existing hk11 deletion mutant, SMhk11, to construct a double deletion mutant by transforming a construct of comCDE-up::Spec::comCDE-dw into SMhk11. The internal region (2192 bp) of the comCDE locus was completely replaced by a spectinomycin cassette through allelic exchange during double-crossover recombination. The transformants that grew on THYE plates plus both spectinomycin (500 $\mu$g ml$^{-1}$) and erythromycin (10 $\mu$g ml$^{-1}$) were selected for PCR and sequencing confirmation. The result confirmed that the Spec cassette was inserted into the correct location and completely replaced the internal region of the comCDE locus (Supplementary Fig. S1). The newly constructed mutant was named SMcde-hk11 ($\Delta$comCDE::Spec, $\Delta$hk11::Em, Spec$^c$, Erm$^\gamma$). The double mutant had a slower growth rate [doubling time ($T_d$)=1.72 h] than the parent ($T_d$=1.28 h) when grown in THYE broth, and was significantly defective in genetic competence (data not shown). In addition, the mutant cells grown in THYE broth appeared to aggregate and deposit on the bottom of test tubes. Interestingly, mutant SMcde-4 had the highest cell density (OD$_{600}$ 1.45) compared with the parent (OD$_{600}$ 1.20) when grown in THYE broth.

Effects on glycolytic pH drop

To determine the effects of inactivation of these systems on acid production, all the mutants along with their parent strain NG8 were assayed for glycolytic pH reduction. As shown in Fig. 1, strain NG8 could rapidly generate acids from glycolysis, reducing the pH to 4.75 in just 10 min. The lowest pH value for NG8 to carry out glycolysis was about pH 3.7. If the acid was neutralized glycolysis took place again, suggesting that glycolytic activity ceased due to inhibition by the lower pH but not by glucose depletion. In comparison with the parental strain, the double deletion mutant SMcde-hk11 had a slower acid production rate, since the mutant took about 20 min to reach pH 4.76. This was almost two times slower than the parental strain in glycolytic pH drop. In addition, the lowest pH value that allowed SMcde-hk11 to carry out glycolysis was pH 4.5, which was 0.8 units higher than the value for the parent strain (pH 3.7). Clearly, the double deletion mutant was less tolerant of lower pH. Interestingly, it was observed that similar pH values stopped the glycolysis of single deletion mutants SMcde-4 and SMhk11, suggesting that both mutants were less tolerant of acidic pH. Nevertheless, these mutants appeared to have similar glycolytic pH values in the first 10 min, when pH was not a major factor in inhibiting their growth.

Effects on growth at low pH

To further determine how these mutants tolerated low pH, we examined the ability of all three mutants, SMcde-4, SMhk11 and SMcde-hk11, to grow at pH 5.0. As shown in Fig. 2, inactivation of comCDE (SMcde-4) alone did not appear to affect the growth of this mutant at pH 5.0, since the numbers and size of the colonies of this mutant were similar to those of the parental strain (Fig. 2a). At the same pH, however, the growth of the SMhk11 mutant was diminished and the double deletion mutant SMcde-hk11 completely stopped growing (Fig. 2b). We checked the viability of the overnight culture of these strains and found
no significant viability loss in the overnight culture (pH 4.4). This was also supported by the fact that the same strain from the same overnight culture grew reasonably well on control plates (pH 7.0), although the size of colonies was smaller than that of the parental strain. The data suggest that simultaneous inactivation of both signal transduction systems additively attenuated the ability of *S. mutans* to grow at acidic pH.

**Effects on biofilm formation**

To determine the effect of inactivation of these systems on biofilm formation, the double deletion mutant SMcde-hk11 was assayed for biofilm formation on a polystyrene surface. The results showed that SMcde-hk11 formed a biofilm with reduced biomass and exhibited a sponge-like architecture (Fig. 3a). Phase-contrast microscopy revealed that the biofilm formed by SMcde-hk11 was composed of cells in very long chains that appeared to favour cell aggregation (Fig. 3b). We also found that the biofilm of this mutant was loosely attached to the surface and could be easily removed with a gentle wash or by a mechanical shear force.

**Effects on oral colonization and succession**

A specific-pathogen-free rat model with mono-infection was used to determine the effects of inactivation of ComCDE, HK11 or both on oral colonization and succession of *S. mutans*. At the first week post-inoculation all the strains successfully colonized the animals (Fig. 4), although the numbers of colonized cells varied with the strains. The swab samples taken from the animals during the first week predominantly contained the target organisms, accounting for 40–68% of the total viable cells. Among all the strains, SMcde-hk11 had the lowest percentage of recovered cells post-inoculation, suggesting that this mutant was less efficient in establishing initial colonization. However, SMcde-4 was very similar to the parental strain in the percentage of recovered cells. We also
took swab samples at different time points throughout the experiment to monitor succession of these strains in the animals. Although the numbers of recovered cells varied considerably, all the target organisms could be identified from the viable cell counts at any given time, suggesting that all the strains succeeded and persisted in the mono-infection animals. However, the percentages of the target organisms decreased, suggesting that the resident organisms slowly extended their populations. At the end of the 10-week experiment, the percentages of target organisms in samples taken from dental plaque were 29% for NG8 [pDL276], 27% for SMcde-4, 18% for SMhk11 and 9% for the double deletion mutant SMcde-hk11 (Fig. 4).

**CI analysis for ecological fitness**

To determine the ecological fitness of the mutants in the animals, we used a mixed-infection model for CI analysis by inoculating a 1:1 ratio of a mutant to the wild-type strain NG8 [pDL276]. CI analysis is considered to be a sensitive measure of the virulence attenuation of bacterial pathogens. It can distinguish between mutant strains whose attenuation is too subtle to be detected in mono-infections. By testing the virulence level of a mutant versus the parental strain within the same animal, sample variations from time to time or from animal to animal are usually decreased. Our results revealed that at the first week post-inoculation all the strains successfully colonized and initiated a mixed infection in the animals. The mean CI values at the first week post-inoculation were 0.69 ± 0.32 for SMcde-4/NG8 [pDL276], 0.54 ± 0.29 for SMhk11/NG8 [pDL276] and 0.28 ± 0.12 for SMcde-hk11/NG8 [pDL276]. Interestingly, the percentages of recovered *S. mutans* cells from all the groups were similar to that of NG8-kan in the mono-infections (data not shown), suggesting that the inoculated *S. mutans* strains dominated in the flora during the first week post-inoculation. However, the succession of the individual mutants in the mixed infections differed dramatically from that in the mono-infections in that the CI values in all the groups were greatly decreased at the tenth weeks post-inoculation (Fig. 5). The CI values of the groups of SMcde-4/NG8 [pDL276] and SMhk11/NG8 [pDL276] were about 1.5–2 orders of magnitude lower than those at the first week. The CI value of group SMcde-hk11/NG8 [pDL276] was almost four orders of magnitude lower than that at the first week. Interestingly, the recovered *S. mutans* cells from the mixed-infection animals at week 10 were not significantly different from the values for NG8 [pDL276] from the mono-infected animals. The percentages of the recovered cells were 21% for SMcde-4/NG8 [pDL276], 17% for SMhk11/NG8 [pDL276] and 19% for SMcde-hk11/NG8 [pDL276]. Clearly, SMcde-hk11 was almost completely overtaken by the population of NG8 [pDL276] by the end of the experiment, indicating that this mutant was much less competitive in dental biofilms than the parental strain. The results suggest that simultaneous inactivation of both signal transduction pathways significantly reduces the ecological fitness of *S. mutans* in dental biofilms.

![Fig. 4. Oral colonization by *S. mutans* NG8 [pDL276] and mutants SMcde-4, SMhk11 and SMcde-hk11 in mono-infected animals during the first week and the last (tenth) week post-inoculation.](image)

**Table 2. Caries scores for *S. mutans* NG8 [pDL276] and its mutants in a rat caries model**

<table>
<thead>
<tr>
<th>Group*</th>
<th>Mean caries score ± SD</th>
<th>Percentage of caries on smooth surface†</th>
<th>P value‡</th>
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<tbody>
<tr>
<td><strong>Mono-infection</strong></td>
<td></td>
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</tr>
<tr>
<td>NG8 [pDL276]</td>
<td>56.4 ± 8.5</td>
<td>55</td>
<td>–</td>
</tr>
<tr>
<td>SMcde-4</td>
<td>36.7 ± 6.4</td>
<td>35</td>
<td>0.012</td>
</tr>
<tr>
<td>SMhk11</td>
<td>35.6 ± 7.2</td>
<td>24</td>
<td>0.015</td>
</tr>
<tr>
<td>SMcde-hk11</td>
<td>28.6 ± 5.8</td>
<td>13</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Mixed infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMcde-4/NG8 [pDL276]</td>
<td>53.5 ± 6.8</td>
<td>56</td>
<td>0.48</td>
</tr>
<tr>
<td>SMhk11/NG8 [pDL276]</td>
<td>52.4 ± 7.4</td>
<td>54</td>
<td>0.55</td>
</tr>
<tr>
<td>SMcde-hk11/NG8 [pDL276]</td>
<td>54.7 ± 6.5</td>
<td>55</td>
<td>0.42</td>
</tr>
</tbody>
</table>

* n=8 animals per group.
† (Number of smooth surface caries/total number of caries scored) × 100.
‡ A comparison with the wild-type group and significant by Student’s *t* test (*P*<0.05).
Effects on cariogenic potential

The effects of inactivation of ComCDE, HK11 or both on the cariogenic potential of *S. mutans* were evaluated in the rat caries models (Fig. 6). All the animals were fed with a sucrose-containing diet throughout the entire experiment. The results revealed that all the mutants were compromised in their ability to initiate dental caries, resulting in lower caries scores than for the parental strain (Table 2). Interestingly, the total caries score in the group of the double deletion mutant was about twofold lower than that for the wild-type NG8 [pDL276] (*P*<0.01), while those for the other two mutants, SMcde-4 and SMhk11, were both 1.5-fold lower than the score for NG8 [pDL276] (*P*<0.05). Again, the results suggest that inactivation of both signal transduction systems additively attenuated the cariogenic potential of *S. mutans*. We also examined the incidence of caries in animals with a mixed infection of a 1:1 ratio of a mutant and NG8 [pDL276]. The results showed very similar numbers of caries lesions in all the groups to that of control group NG8 [pDL276], suggesting that the wild-type strain played the major role in caries development in these animals.

**DISCUSSION**

The human oral cavity is a highly dynamic environment that undergoes rapid and often substantial changes in nutrient availability, nutrient type, pH, oxygen tension and inter-bacterial interactions involving either competition or cooperation (Burne, 1998; Marsh, 2000). Bacteria such as *S. mutans* living in dental biofilms are frequently exposed to cycles of such environmental challenges (Marsh, 2000). To colonize and succeed in dental biofilms, *S. mutans* has evolved multiple signal transduction systems to cope with rapid and unexpected environmental fluctuations. Two-component signal transduction is one of the most common mechanisms by which *S. mutans* regulates large sets of genes in response to changing environments (Levesque *et al.*, 2007; Biswas *et al.*, 2008). In this study, we have
investigated the effects of the inactivation of the signal transduction systems ComCDE, HK/RR11 or both on oral colonization, ecological fitness and cariogenic potential of S. mutans in a specific-pathogen-free rat caries model. One of the interesting findings from this study was that simultaneous inactivation of both signal transduction pathways additively attenuated the virulence and cariogenic potential of S. mutans, since inactivation of either of these systems alone did not result in effects of the same degree or extent. The double deletion mutant was much less able than the parent to succeed and persist in dental biofilms. Clearly, inactivation of both signal transduction systems greatly reduced the ecological fitness of S. mutans in its natural ecosystem. The fact that inactivation of both systems additively attenuated the virulence and cariogenic potential of S. mutans suggests that the ComCDE and HK/RR11 signal transduction systems function independently to regulate the physiological activities, ecological fitness and virulence of S. mutans. However, which gene products are directly responsible for these traits and how these signal transduction systems regulate these gene products remain to be studied. Genome-wide analysis of individual pathways using microarray technology may provide clues to answer these questions.

Another interesting finding from this study was that inactivation of the ComCDE quorum-sensing system alone did not affect oral colonization and succession of S. mutans in mono-infected animals. However, the caries score in this group was significantly lower than that of the parental strain (P<0.05), indicating that inactivation of the ComCDE quorum-sensing system still attenuated the cariogenic potential of S. mutans. The results suggest that colonization of S. mutans in dental biofilms may not be sufficient to explain its virulence and cariogenic activity, although it is a prerequisite for infection. The mechanism behind this is not very clear. One possibility is that this mutant is less tolerant the lower pH, so that it has less potential to initiate caries. Another possibility is that SMcde-4 in mono-infected animals had less inter-species competition because of reduced numbers of the resident flora due to the use of antibiotic water. In these animals, SMcde-4 dominated until the sixth week post-inoculation, and the mean viable cell count of this mutant at the tenth week remained the same as that of the parental strain (Fig. 4). Recent studies have shown that the same quorum-sensing system ComCDE that regulates genetic competence in S. mutans also controls the production of several bacteriocins and bacteriocin immunity proteins (Kreth et al., 2005a, b; van der Ploeg, 2005; Matsumoto-Nakano & Kuramitsu, 2006). These compounds can kill other related species and favour S. mutans for competition in multi-species dental biofilms (Kreth et al., 2005a, b). These quorum sensing-controlled compounds and activity are believed to act as a ‘two-edged sword’ to kill other species and release DNA, which can be used by S. mutans for genetic exchange (Kreth et al., 2005b). In contrast, many other species of streptococci, including Streptococcus pneumoniae, need two independent quorum-sensing systems, the ComCDE and BlpRH systems, to regulate these phenotypes (Martin et al., 2006). Thus, the ComCDE quorum-sensing system in S. mutans forms a unique regulatory mechanism that may provide S. mutans with an ecological advantage to cope with competing species in its natural ecosystem.

The attenuation of the cariogenic potential of S. mutans by inactivating the ComCDE quorum-sensing mechanism and the activities that it controls may hold promise for developing anti-quorum-sensing compounds. These compounds could function as inhibitors to block the quorum sensing-controlled activities and reduce the cariogenic potential of S. mutans, even if this organism is still present in dental biofilms. Several recent studies have described such a strategy and the application of quorum-sensing antagonists to achieve the inhibition of quorum-sensing-controlled activities and to prevent opportunistic infections caused by Pseudomonas aeruginosa and Staphylococcus aureus (Hentzer & Givskov, 2003; Wright et al., 2005). Our recent study has also identified several signalling peptide antagonists that show some degree of inhibition of the quorum-sensing activity in S. mutans (Svyitski et al., 2007).

One of the major advantages of using this strategy is that such anti-quorum-sensing compounds that specifically block or override bacterial signalling pathways may control unwanted pathogenic activities without significant effects on bacterial viability (Hentzer & Givskov, 2003; Wright et al., 2005). As bacterial viability is not affected, there is much less selection pressure to create resistant microbes with the use of these novel anti-microbial compounds.

Unlike the ComCDE mutant, the HK11 deletion mutant consistently showed noticeable levels of attenuation in oral colonization, succession and cariogenic potential in both single- and dual-infection models, although the degree of attenuation of these phenotypes was less severe than that for SMcde-hk11. In the in vitro experiments, we also found that SMcde-hk11 exhibited some phenotypes that looked similar to mutant SMhk11 in terms of their growth in broth, acid-resistance profile (Fig. 2) and formation of biofilms (Fig. 3). The HK/RR11 two-component system has been previously suspected to act as the second pathway to CSP (Li et al., 2002a), since deletion of either hk11 or rr11 results in a mutant that forms a biofilm with a sponge-like architecture composed of cells in very long chains, a feature that was also observed with the biofilm formed by the comC mutant (Li et al., 2002b). More interestingly, the HK11 histidine kinase protein has been found to cross-talk with an unknown response regulator in response to an acidic pH shift (Li et al., 2001b). However, there is no additional evidence so far to determine whether or not HK11 is the second receptor to CSP and how HK11 cross-talks with a response regulator. Although the evidence from this study is insufficient to answer these questions, the additive effects on physiological activities, virulence and the cariogenic potential of S. mutans of simultaneous inactivation of both signal transduction systems appear to
favour the possibility that the two signal transduction systems function independently to regulate different sets of genes, although the resulting phenotypes are similar. Clearly, further study is necessary to answer these questions.

In summary, this study has shown that simultaneous inactivation of the ComCDE quorum-sensing system and HK/RR11 two-component regulatory system additively attenuates the virulence and cariogenic potential of *S. mutans*. The evidence from this study suggests that the ComCDE and HK/RR11 signal transduction systems can be considered as novel targets for the development of strategies for the prevention and treatment of *S. mutans* infections.

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