

## Identification of genes involved in fluoride resistance in oral streptococci

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# **Identification of genes involved in fluoride resistance in oral streptococci**

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Anion channels involved in fluoride resistance in oral streptococci

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## ABSTRACT

Recently, it has been reported that *eriC* and *crcB* are involved in bacterial fluoride resistance (Baker et al. 2012). However, the fluoride-resistance mechanism in oral streptococci remains unclear. BLAST studies showed that two types of *eriCs* (*eriC1* and *eriC2*) and two types of *crcBs* (*crcB1* and *crcB2*) are present across 18 oral streptococci, which were selected based on the following criteria: identification in  $\geq 10\%$  of 166 orally healthy subjects and  $\geq 0.01\%$  of the mean relative abundance. They were divided into three groups based on the distribution of these four genes: group I, only *eriC1*; group II, *eriC1* and *eriC2*; and group III, *eriC2*, *crcB1*, and *crcB2*. Group I consisted of *Streptococcus mutans*, in which one of the two *eriC1s* predominantly affected fluoride resistance. Group II consisted of eight species, in which *eriC1* was involved in fluoride resistance, but *eriC2* was not, in *Streptococcus anginosus* as a representative species. Group III consisted of nine species, in which both *crcB1* and *crcB2* were crucial for fluoride resistance, but *eriC2* was not, in *Streptococcus sanguinis* as a representative species. Based on these results, either EriC1 or CrcB play a role in fluoride resistance in oral streptococci. Complementation between *S. mutans* EriC1 and *S. sanguinis* CrcB1/B2 was confirmed in both *S. mutans* and *S. sanguinis*. However, neither transfer of *S. sanguinis* CrcB1/B2 into wild-type *S. mutans* nor *S. mutans* EriC1 into wild-type *S. sanguinis* increased the fluoride resistance of the wild-type strain. It is possible that EriC1 and CrcB1/B2 are responsible for fluoride resistance in oral streptococci by sharing specific pathways.

## INTRODUCTION

Fluoride is commonly used as an effective caries-preventive agent in many countries.

Organisms present in the oral cavity are frequently exposed to fluoride ions from drinking water or from the use of fluoride dentifrices and fluoride mouth rinses. Fluoride is known to interfere with metabolic processes in many organisms (Marquis et al. 2003), and high-concentration fluoride shows a bactericidal effect (Mayhew and Brown 1981) (Maltz and Emilson 1982). These anti-microbial effects contribute to the anti-caries effect of fluoride. However, the anti-microbial effects of fluoride may have an unexpected effect on the bacterial composition of oral microflora.

The widespread, long-term use of fluoride can result in the emergence of fluoride-resistant oral *Streptococcus* species, including *Streptococcus mutans*, a cariogenic bacterium (Streckfuss et al. 1980) (Hamilton 1969) (Bunick and Kashket 1981) (Brown et al. 1983) (Sheng and Liu 2000). These fluoride-resistant strains show clear phenotypic differences in growth, adherence, and metabolic activity compared to the fluoride-sensitive strains (Van Loveren et al. 1991) (Van Loveren et al. 1991) (Hoelscher and Hudson 1996). The emergence of fluoride-resistant oral *Streptococcus* species may not only decrease the anti-caries effects of fluoride, but also disrupt the composition of oral streptococci in the oral cavity, followed by the deterioration of oral health.

More than 700 bacterial species are present in the oral cavity, and oral streptococci are predominant. Oral streptococci account for approximately 20% of all bacteria in saliva (Moritani et al. 2015) and approximately 50% of those during the early stages of dental plaque formation (Takeshita et al. 2015). Oral streptococci pioneer early dental plaque

formation and have a specific temporal and spatial distribution that is crucial for the development of oral biofilms (Rickard et al. 2003).

Bacteria have evolved numerous strategies to alleviate the toxic effects of ions other than fluoride, and yet analogous systems for fluoride toxicity mitigation were notably absent (Silver 1996). Recently, it has been reported that the *eriC* gene of *Pseudomonas syringae* and the *crcB* gene of *Escherichia coli* are involved in bacterial fluoride resistance (Baker et al. 2012). The *eriC* gene is described as a ClC chloride channel in many bacteria, and the *crcB* gene has previously been implicated in resistance to camphor-induced chromosome decondensation. Furthermore, Liao et al. (2015) identified two single nucleotide polymorphisms (SNPs) in the gene cluster, including two *eriC* genes in the genome of the fluoride-resistant mutant *S. mutans* C180-2FR. These results suggested that *eriC* may be related to the response to fluoride in *S. mutans*. However, it remains unclear whether *eriC* and/or *crcB* play a role in fluoride resistance in oral streptococci. To maintain the appropriate bacterial composition of oral microflora, it is important to investigate the fluoride resistance mechanism in oral streptococci. In this study, we attempted to identify and characterize fluoride-resistance-related genes in oral streptococci.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

The following bacterial strains were used in this study: *Streptococcus mutans* UA159, *Streptococcus sobrinus* OMZ175, *Streptococcus mitis* ATCC 49456, *Streptococcus oralis* ATCC 10557, *Streptococcus gordonii* ATCC 10558, *Streptococcus sanguinis* SK36, *Streptococcus parasanguinis* ATCC 15912, *Streptococcus tigurinus* ATCC 15914, *Streptococcus australis* ATCC 700641, *Streptococcus infantis* ATCC 700779, *Streptococcus salivarius* HHT, *Streptococcus anginosus* NCTC 10707, *Streptococcus intermedius* ATCC 27335, and *Escherichia coli* DH5 $\alpha$ . *E. coli* strains were grown in 2 $\times$ YT Broth (Becton Dickinson, Franklin Lakes, NJ, USA). Oral streptococci strains were grown in brain heart infusion (BHI) broth (Becton Dickinson) at 37°C in 5% CO<sub>2</sub>. Antibiotics were used at the following concentrations: 300  $\mu$ g/mL erythromycin and 50  $\mu$ g/mL ampicillin for *E. coli*, 20  $\mu$ g/mL erythromycin for oral streptococci, 600  $\mu$ g/mL spectinomycin for *S. mutans* and *S. anginosus*, and 150  $\mu$ g/mL spectinomycin for *S. sanguinis*.

### Transformation

Transformation of oral streptococci was performed as described previously (Perry et al. 1983). Briefly, *Streptococcus* cells were grown for 18 h in BHI broth containing 10% heat-inactivated (56°C, 30 min) horse serum (BHI-HS), diluted 1:10 into fresh BHI-HS, and incubated at 37°C until an optical density of about 0.2 (at 550 nm) was attained. Competent cells were then exposed to DNA for 30 min, diluted 1:2 into fresh BHI-HS, and incubated for 2 h. An aliquot of cell suspensions was spread on BHI plates containing appropriate



antibiotics.

Transformation of *E. coli* was performed as described previously (Hanahan 1983). Briefly, *E. coli* cells were grown in SOB medium (Becton Dickinson) until the cell density reaches 0.3 (at 550 nm). Cell cultures (50 mL) were placed on ice for 10 min, pelleted at 5000 rpm for 10 min at 4°C, and resuspended in 17 mL of cool CCMB solution [80 mM CaCl<sub>2</sub>, 20 mM MnCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 10 mM potassium acetate, and 10% (v/v) glycerol]. Cell suspensions were again placed on ice for 20 min, harvested at 5000 rpm for 10 min at 4°C, and resuspended in 5mL of cool CCMB solution. Competent cells and DNA were mixed and placed on ice for 45 min. The mixture was heat-pulsed without agitation at 42°C for 60 sec and placed on ice for 2 min. After addition of 1 mL of fresh SOB medium, the cell suspension was incubated at 37°C for 1~4 h to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. The appropriate volume of transformed competent cells was spread onto 2×YT agar plate with appropriate antibiotic.

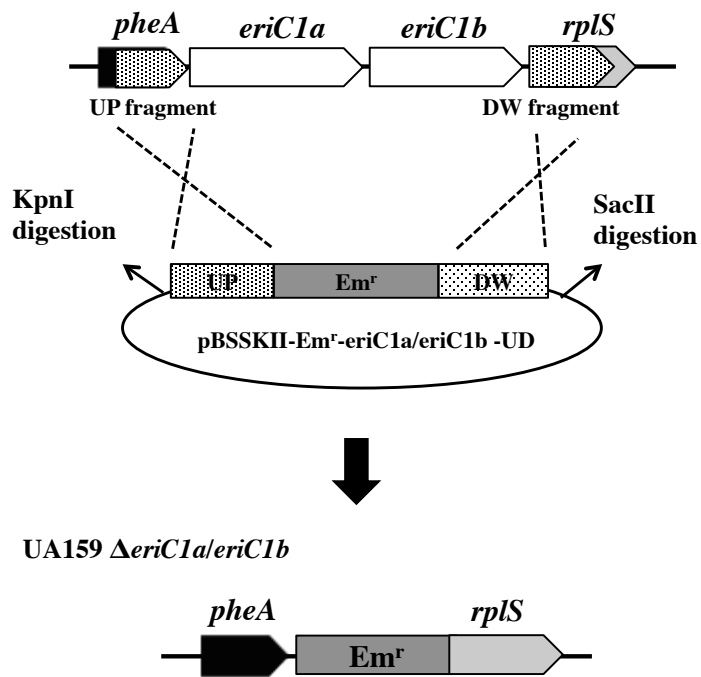
### **Construction of mutant forms of the genes in *Streptococcus* species**

Various deletion mutants were constructed by replacing the target gene with an erythromycin resistance or spectinomycin resistance gene using double cross-over homologous recombination, as described previously (Li et al. 2012). As an example, we describe a strategy for construction of the *eriC1a/eriC1b* double mutant (Fig. 1). An 869-bp fragment upstream of *eriC1a* and a 767-bp fragment downstream of *eriC1b* were amplified from *S. mutans* UA159 genomic DNA and inserted upstream and downstream, respectively, of the Em<sup>r</sup> gene in pBSSKII-Em<sup>r</sup> (Kawada-Matsuo et al. 2009), in which the Em<sup>r</sup> fragment was cloned into HindIII- and EcoRV-digested pBluescript SK II (+). The resultant plasmid

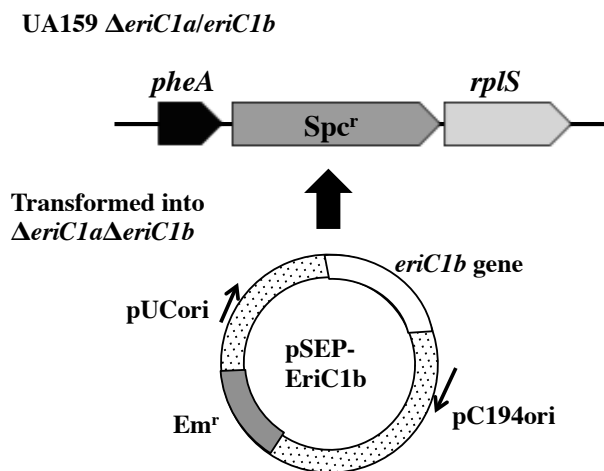
(pBSSKII-Em<sup>r</sup>-*eriC1a/eriC1b* -UD) was digested with KpnI and SacII, and the assembled fragment was transformed into *S. mutans* UA159. Correct insertions or replacements of transformants were confirmed by PCR.

Complementation was performed using the shuttle vector, pSEP, which consisted of an erythromycin resistance gene, pC194ori for replication in Gram-positive organisms, pUCori for replication in *E. coli*, and the promoter region of the erythromycin resistance gene derived from Gram-positive organisms. Fig. 2 shows a strategy for complementation of *eriC1a/eriC1b* by *eriC1b* in the *eriC1a/eriC1b* double mutant of *S. mutans*.

*S. mutans* UA159



**Fig. 1.** Construction of the *eriC1a/eriC1b* double mutant



**Fig. 2.** Construction of the mutant complemented by *EriC1b*

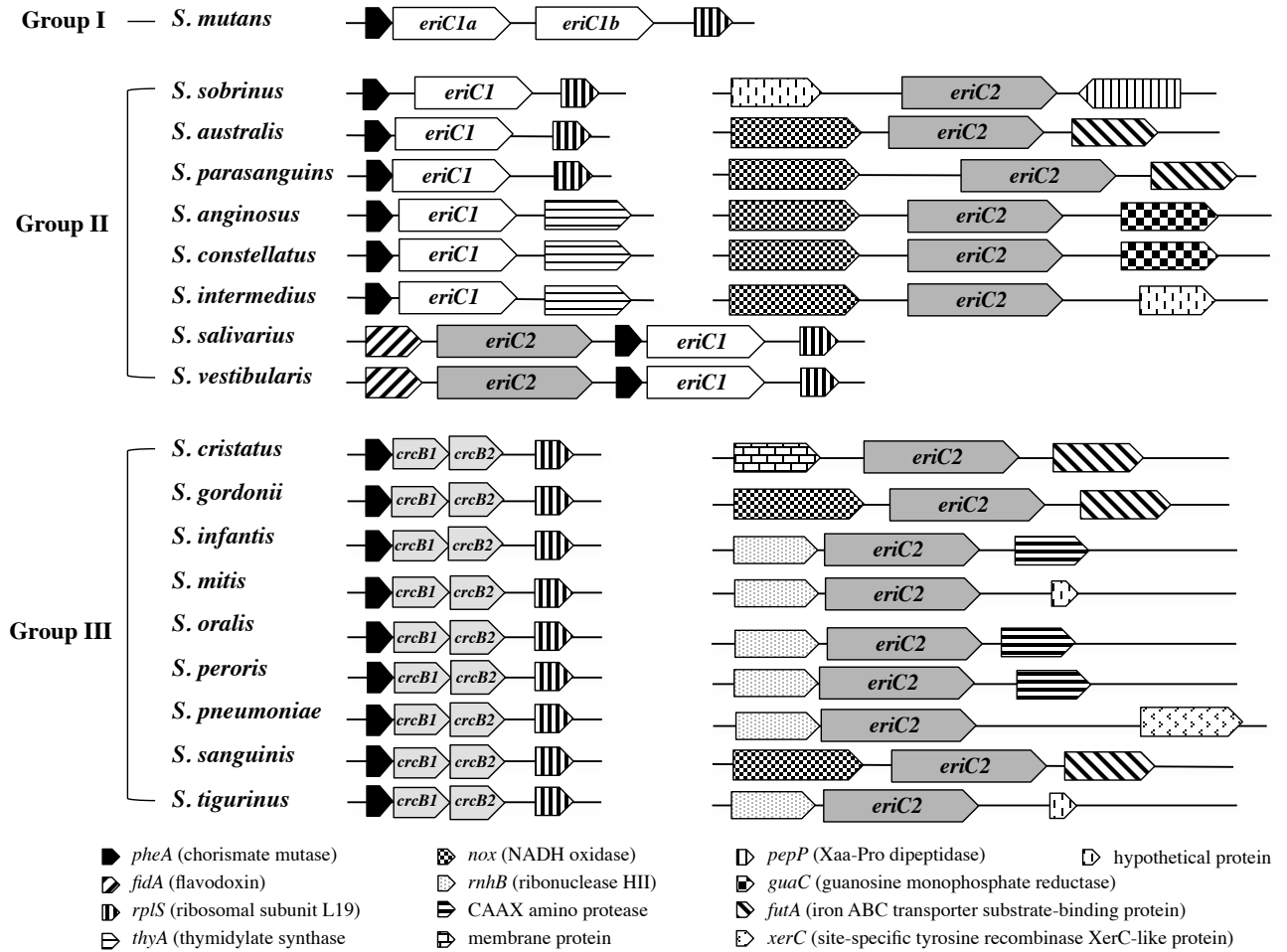
### **Evaluation of the fluoride sensitivity of strains**

The mutant strains and wild-type control strain cells were grown in BHI broth overnight at 37°C in 5% CO<sub>2</sub>. The cultures were then diluted 1:10 into fresh BHI and grown to an OD<sub>550</sub> of ~0.5. Aliquots (20 μL) of cell suspensions with the same turbidity were inoculated into wells containing 200 μL of fresh BHI medium with several different sodium fluoride (NaF) concentrations. Growth was monitored by measuring OD<sub>550</sub> using a SpectraMax 340PC384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Wells containing only BHI were used as controls. Growth yields were estimated based on the means of the data obtained from three independent experiments.

## RESULTS

### Distribution of *eriC* and *crcB* genes in oral streptococci

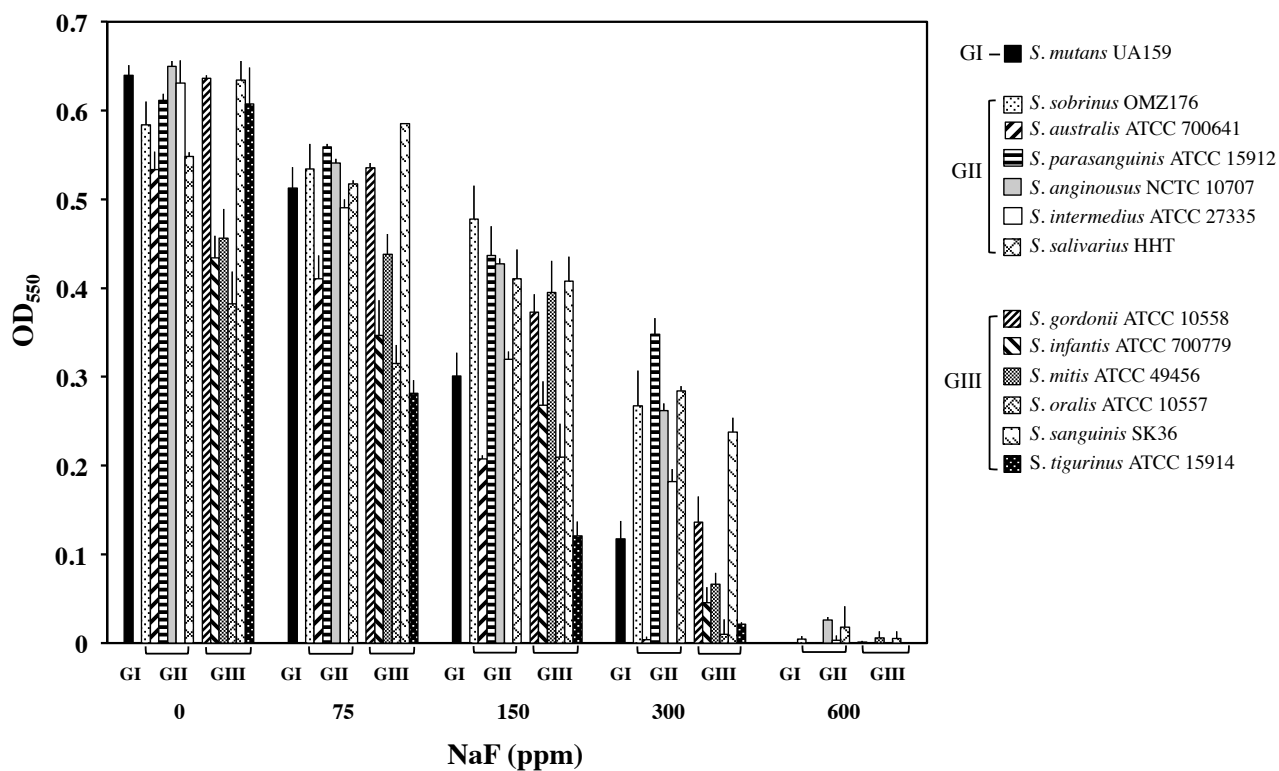
To examine the distribution of *eriC* and *crcB* genes in oral streptococci, we performed sequence homology analysis for the complete genome sequences of 18 types of oral streptococci with the BLAST program via the National Center for Biotechnology Information server. These species were selected based on the following criteria: identification in  $\geq 10\%$  of 166 orally healthy subjects and  $\geq 0.01\%$  of the mean relative abundance. There were two types of *eriC* genes (*eriC1* and *eriC2*) and two types of *crcB* genes (*crcB1* and *crcB2*) in oral streptococci (Fig. 3). The *eriC1* gene product showed about 50% similarity with EriC of *P. syringae* DC3000, which was identified as a fluoride channel protein in a previous report (Baker et al. 2012). On the other hand, another EriC2 had no similarity with *P. syringae* EriC. Both *crcB* products showed about 50% similarity with CrcB of *E. coli* K-12, which was involved in fluoride resistance in a previous report (Baker et al. 2012). As shown in Fig. 3, these oral streptococci were divided into three groups based on the distribution of *eriC* and *crcB* genes: group I with only *eriC1* (*S. mutans*), group II with *eriC1* and *eriC2* (*S. sobrinus*, *S. salivarius*, *S. vestibularis*, *S. australis*, *S. parasanguinis*, *S. anginosus*, *S. constellatus*, and *S. intermedius*), and group III with *eriC2*, *crcB1*, and *crcB2* (*S. cristatus*, *S. gordonii*, *S. infantis*, *S. mitis*, *S. oralis*, *S. peroris*, *S. pneumonia*, *S. sanguinis*, and *S. tigurinus*). The *eriC1* and *crcB* genes were flanked by highly similar gene arrangements.



**Fig. 3.** Locations of *eriC* and *crcB* genes in 18 oral streptococci. These species were selected based on the following criteria: identification in  $\geq 10\%$  of 166 orally healthy subjects and  $\geq 0.01\%$  of the mean relative abundance.

## Fluoride resistance of oral streptococci

To examine the fluoride resistance of oral streptococci, 13 available species among the above 18 oral *Streptococcus* species were grown in BHI broth with various concentrations of NaF. As shown in Fig. 4, these oral streptococci showed various resistances to fluoride. *S. parasanguinis*, *S. sanguinis*, *S. sobrinus*, *S. salivarius*, and *S. anginosus* grew fairly well, even in the presence of 300 ppm NaF. *S. australis*, *S. oralis*, *S. infantis*, *S. mitis*, and *S. tigurinus* grew poorly under the same conditions. In the presence of 600 ppm NaF, all 13 oral streptococci did not grow. Among species with the higher fluoride-resistance, *S. parasanguinis*, *S. sobrinus*, *S. salivarius*, and *S. anginosus* belong to group II and *S. sanguinis* belongs to group III. On the other hand, among species with the lower fluoride-resistance, *S. oralis*, *S. infantis*, *S. mitis*, and *S. tigurinus* belonged to group III and *S. australis* belonged to group II. *Streptococcus mutans*, which belonged to group I, showed medium fluoride-resistance. No strong relationship was observed between the distribution of *eriC* and *crcB* genes and fluoride resistance. Next, we explored the related gene(s) for fluoride resistance in *S. mutans*, *S. anginosus*, and *S. sanguinis*, which possess a fair fluoride resistance, as representative species of the three groups, respectively.

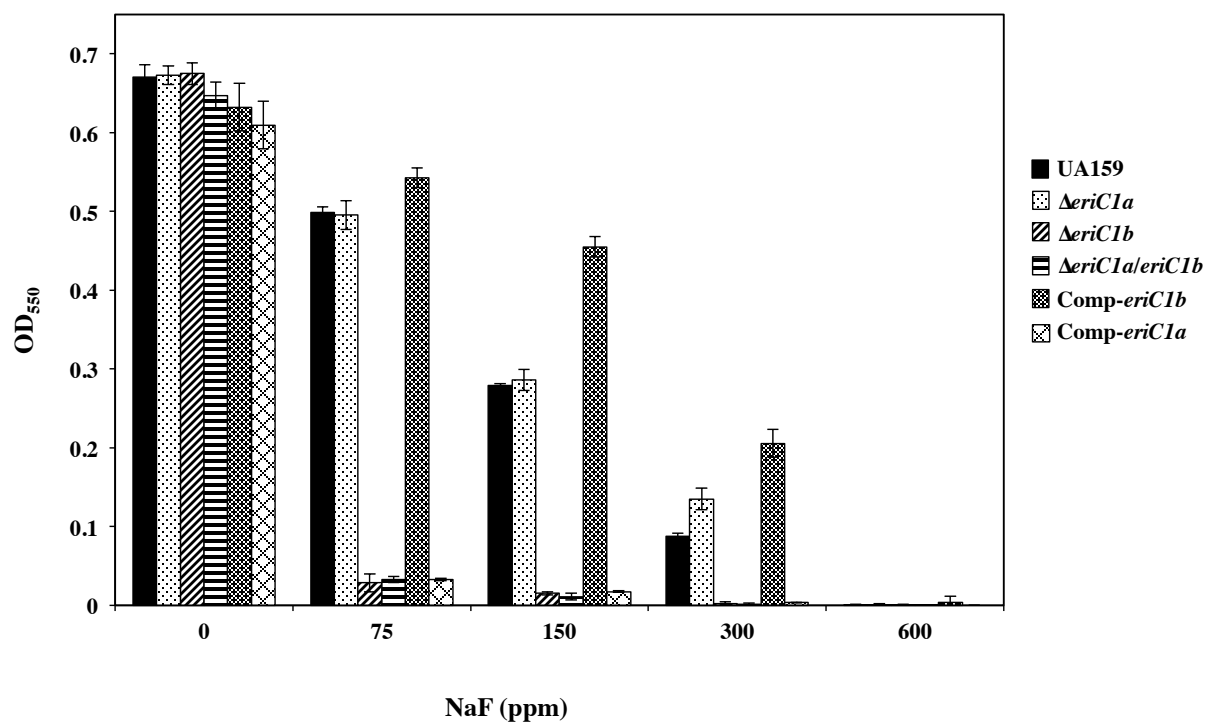


**Fig. 4.** Growth yields of 13 oral streptococci at various NaF concentrations. The y-axis represents the  $OD_{550}$  after incubation for 16 h. The data represent the mean standard deviations of three independent experiments.



### **Identification of fluoride-resistance-related genes in *S. mutans***

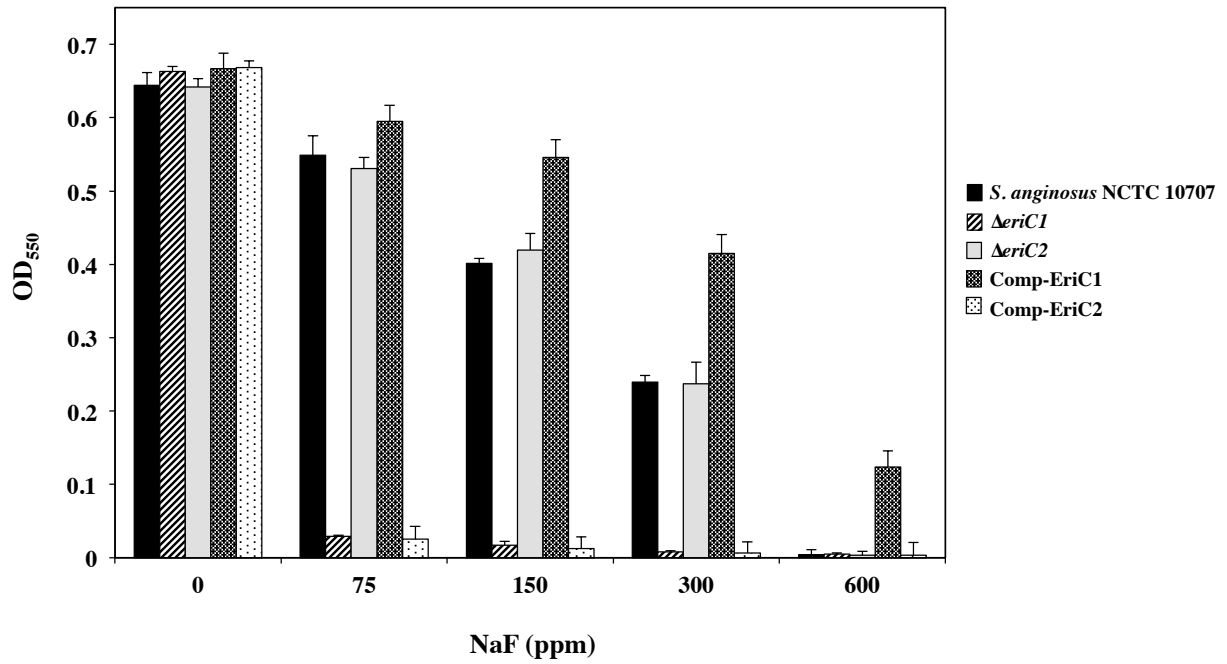
Sequence homology analysis revealed two *eriC1* genes [SMU\_1290 (*eriC1a*) and SMU\_1289 (*eriC1b*)] in *S. mutans* UA159. *eriC1a* and *eriC1b* exist in tandem and in the same orientation. Both *eriC1a* and *eriC1b* encode a protein of 406 amino acids, named the chloride channel permease. The amino acid sequences deduced from *eriC1a* and *eriC1b* showed 52.6 and 52.5% similarity, respectively, to *P. syringae* EriC. The amino acid sequence similarity between EriC1a and EriC1b was 74.4%. To examine whether inactivation of *eriC1a* or/and *eriC1b* resulted in the loss of fluoride resistance, *eriC1a* and *eriC1b* single mutants, and the *eriC1a/eriC1b* double mutant were constructed using double-crossover homologous recombination, as described in the Materials and Methods. As shown in Fig. 5, the *eriC1b* mutant showed extremely limited growth in the presence of 75 ppm NaF, while it grew similarly to the wild-type UA159 in the absence of NaF. In contrast, the *eriC1a* mutant showed a growth rate similar to wild-type UA159 in both the presence and absence of NaF, and inactivation of *eriC1a* did not result in a loss of fluoride resistance. Furthermore, the growth rates of the *eriC1a/eriC1b* double mutant and the *eriC1b* single mutant were similar in the presence of NaF. Complementation of *eriC1b* was performed using the shuttle vector, pSEP, in the *eriC1a/eriC1b* double mutant. The complemented strain showed rescued fluoride resistance (Fig. 5). On the other hand, introduction of *eriC1a* into the *eriC1a/eriC1b* double mutant did not restore fluoride resistance. These results demonstrated that EriC1b was responsible for the fluoride resistance in *S. mutans*.



**Fig. 5.** Growth yields of the parent strain, *eriC1a* mutant, *eriC1b* mutant, *eriC1a/eriC1b* double mutant and complemented strains in *S. mutans* at various NaF concentrations. The y-axis represents the OD<sub>550</sub> after incubation for 16 h. Data represent the mean standard deviations of three independent experiments.

### **Identification of fluoride-resistance-related genes in *S. anginosus***

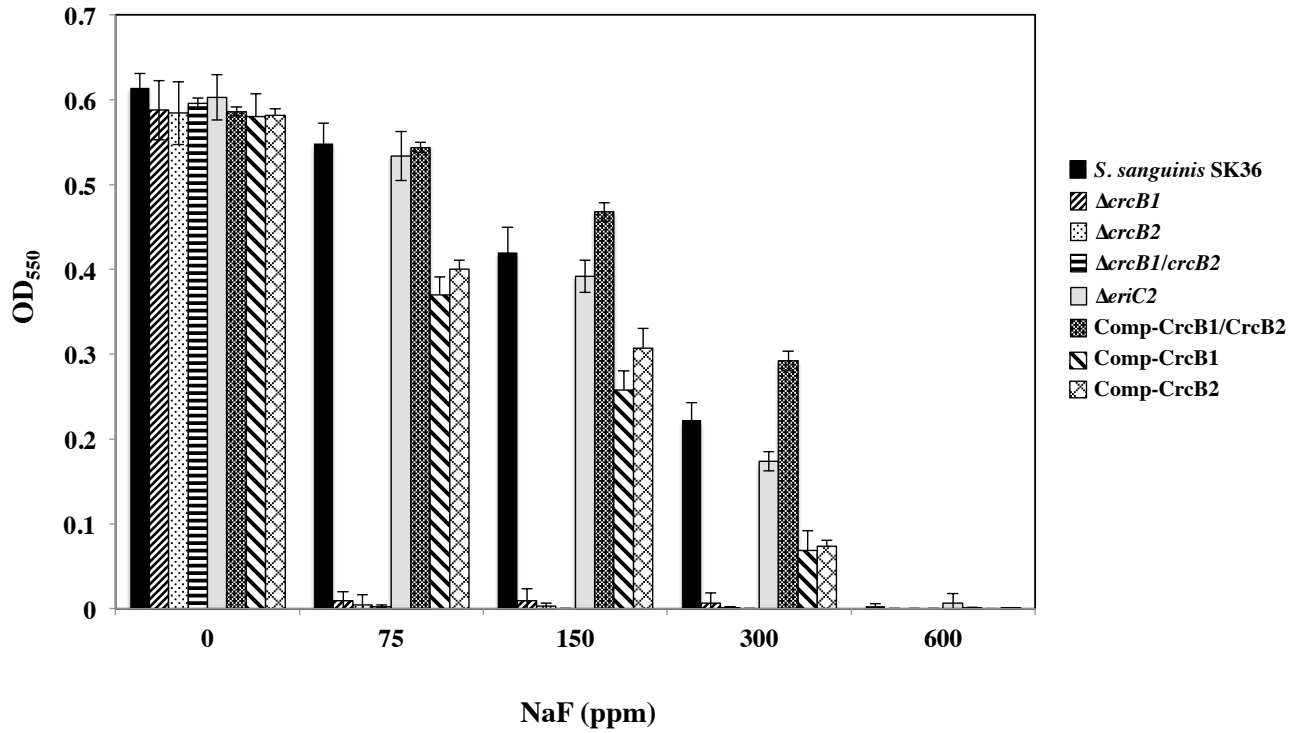
*S. anginosus* possesses two types of *eriC* genes. *eriC1* encodes a protein of 405 amino acids with amino acid sequence similarity of 52% to the *P. syringae* EriC, and is named the voltage-gated chloride channel protein. *eriC2* encodes a protein of 518 amino acids with no amino acid sequence similarity to the *P. syringae* EriC. To examine whether the inactivation of *eriC1* or *eriC2* leads to the loss of fluoride resistance, the *eriC1* and *eriC2* single mutants were constructed in *S. anginosus* NCTC 10707 in the same manner as the *eriC1a/eriC1b* double mutant. The *eriC1* mutant hardly grew in the presence of 75 ppm NaF, while it grew similarly to wild-type NCTC 10707 in the absence of NaF (Fig. 6). On the other hand, the *eriC2* mutant showed a growth rate similar to that of wild-type NCTC 10707 in both the absence and presence of NaF. Furthermore, as shown in Fig. 6, complementation of *eriC1* rescued fluoride resistance, while the introduction of *eriC2* did not. These results demonstrated that EriC1 was responsible for the fluoride resistance in *S. anginosus*.



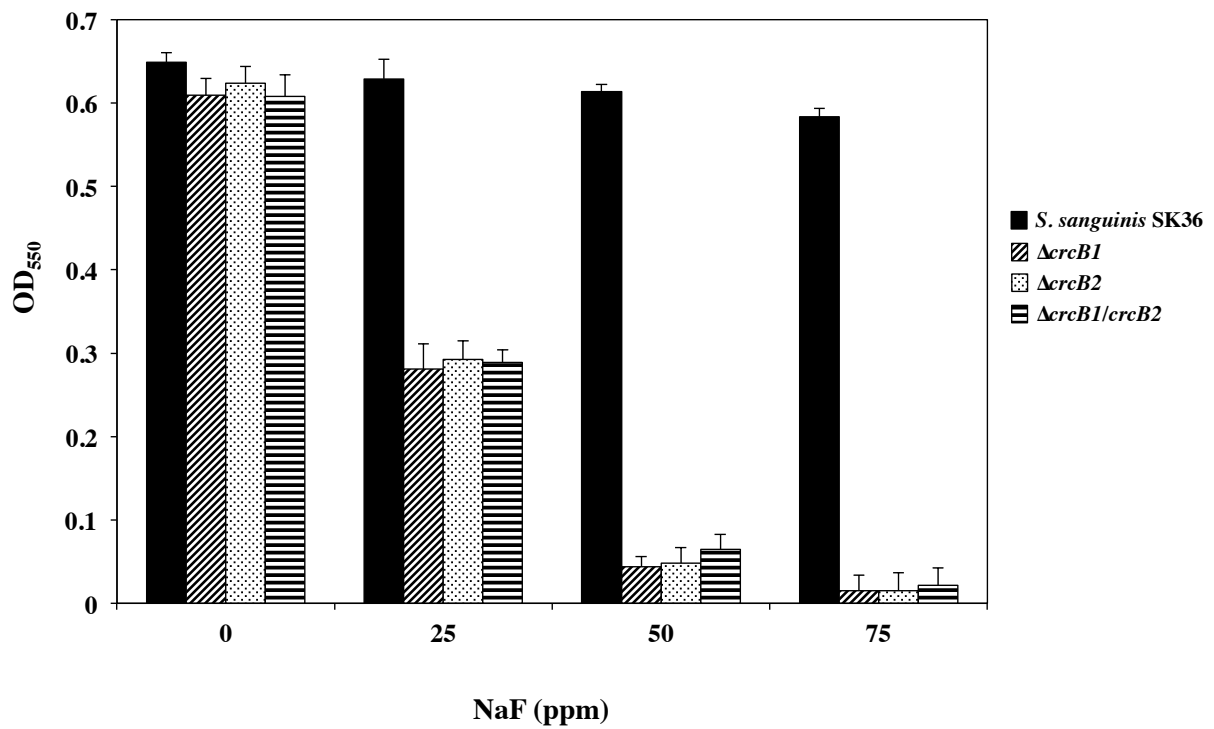
**Fig. 6.** Growth yields of the parent strain, *eriC1* mutant, *eriC2* mutant, and complemented strains in *S. anginosus* at various NaF concentrations. The y-axis represents the OD<sub>550</sub> after incubation for 16 h. Data represent the mean standard deviations of three independent experiments.

### **Identification of fluoride-resistance-related genes in *S. sanguinis***

The *eriC2*, *crcB1*, and *crcB2* genes are present in *S. sanguinis*. The *eriC2* gene product is a protein of 518 amino acids with no similarity to *P. syringae* EriC, but with a similarity of 71% to *S. anginosus* EriC2, which is not involved in fluoride resistance in *S. anginosus*. The *crcB1* gene encodes a protein of 124 amino acids with amino acid sequence similarity of 51% to *E. coli* CrcB (127-aa protein), which is involved in fluoride resistance in *E. coli*, and the *crcB2* gene product encodes a protein of 116 amino acids with a similarity of 62.2% to *E. coli* CrcB. Amino acid sequence similarity between CrcB1 and CrcB2 was 58.4%. The *crcB1* and *crcB2* genes exist in tandem and in the same orientation. To examine whether the inactivation of *eriC2*, *crcB1*, or *crcB2* leads to the loss of fluoride resistance, *eriC2*, *crcB1*, and *crcB2* single mutants were constructed in *S. sanguinis* SK36 in the same manner as the *eriC1a/eriC1b* double mutant. As shown in Fig. 7, inactivation of *eriC2* did not affect fluoride resistance of wild-type SK36. On the other hand, both *crcB1* and *crcB2* single mutants showed extremely limited growth in the presence of 75 ppm NaF, while they grew similarly to wild-type SK36 in the absence of NaF. The *crcB1/crcB2* double mutant showed a growth rate similar to that of the *crcB1* or *crcB2* single mutants in both the absence and presence of NaF. At a lower concentration of 25 and 50 ppm NaF, the growth rates of these three mutants were similar (Fig. 8). Furthermore, as shown in Fig. 7, complementation of *crcB1/crcB2* by *crcB1/crcB2* restored fluoride resistance to wild-type levels. However, the fluoride resistance of the *crcB1/crcB2* mutant complemented by either *crcB1* or *crcB2* did not reach the level of the mutant complemented by *crcB1/crcB2*. These results demonstrated that both *crcB1* and *crcB2* genes were critical for fluoride resistance in *S. sanguinis*.



**Fig. 7.** Growth yields of the parent strain, *crcB1* mutant, *crcB2* mutant, *crcB1/crcB2* double mutant, *eriC2* mutant, and complemented strains in *S. sanguinis* at various NaF concentrations. The y-axis represents the OD<sub>550</sub> after incubation for 16 h. Data represent the mean standard deviations of three independent experiments.

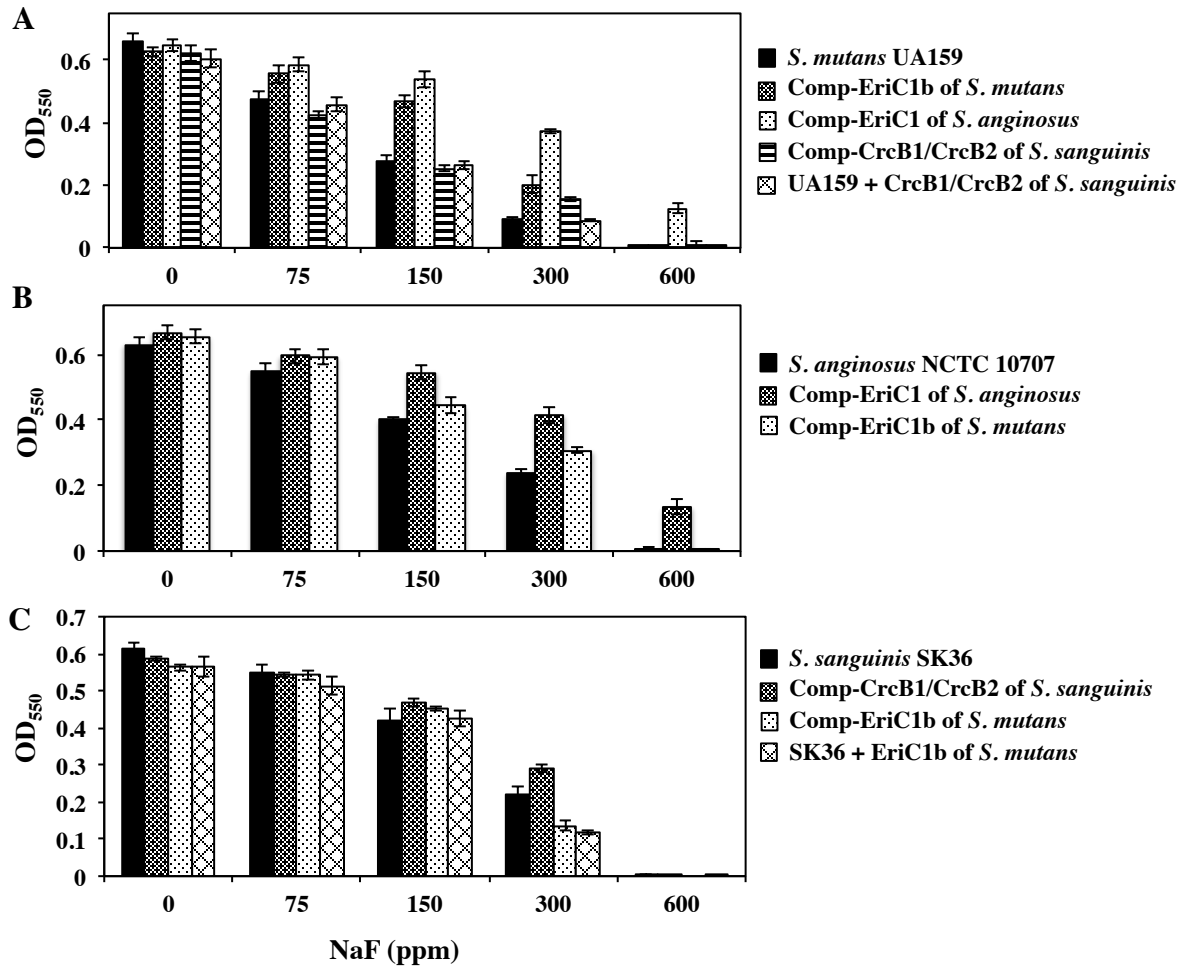


**Fig. 8.** Growth yields of the parent strains, *crcB1* mutant, *crcB2* mutant, and *crcB1/crcB2* double mutant at a lower concentration of NaF (25 and 50 ppm). The y-axis represents the OD<sub>550</sub> after incubation for 16 h. Data represent the mean standard deviations of three independent experiments.

### **Complementation of the fluoride-resistance-related genes between *S. mutans* and *S. anginosus* or *S. mutans* and *S. sanguinis***

We explored whether fluoride-resistance-related genes were able to complement each other between *S. mutans* and *S. anginosus* or *S. mutans* and *S. sanguinis*. As shown in Fig. 9A, complementation of *eriC1a/eriC1b* by *S. anginosus eriC1* in the *eriC1a/eriC1b* double mutant rescued fluoride resistance, and the fluoride resistance ability of this complemented strain was higher than that complemented by *eriC1b*. Complementation of *eriC1* by *eriC1b* in *S. anginosus* also restored fluoride resistance, and the fluoride resistance ability of this transformant was lower than that complemented by *eriC1* (Fig. 9B). These results may reflect the fact that the fluoride resistance ability of *S. anginosus* wild-type NCTC 10707 tended to be higher than that of *S. mutans* wild-type UA159. Next, we performed complementation of *eriC* and *crcB* between *S. mutans* and *S. sanguinis*. Both the *eriC1a/eriC1b* mutant complemented by *crcB1/crcB2* and the *crcB1/crcB2* mutant complemented by *eriC1b* showed restored fluoride resistance (Fig 9A, C), as well as that *E. coli crcB* knock-out mutant rescued fluoride resistance by introduction of the fluoride-resistance- related EriCs of other bacteria (Stockbridge et al. 2012). The fluoride resistance ability of the *eriC1a/eriC1b* mutant complemented by *crcB1/crcB2* was lower than that complemented by its own *eriC1b*, while the fluoride resistance ability of *S. sanguinis* wild-type SK36 tended to be higher than that of *S. mutans* wild-type UA159. Furthermore, we examined whether introductions of *eriC1b* and *crcB1/crcB2* into wild-type *S. sanguinis* and *S. mutans*, respectively, affects fluoride resistance in the wild-type strains. Neither introduction of *eriC1b* nor *crcB1/crcB2* increased the fluoride resistance of the wild-type strain (Fig. 9 A, C).

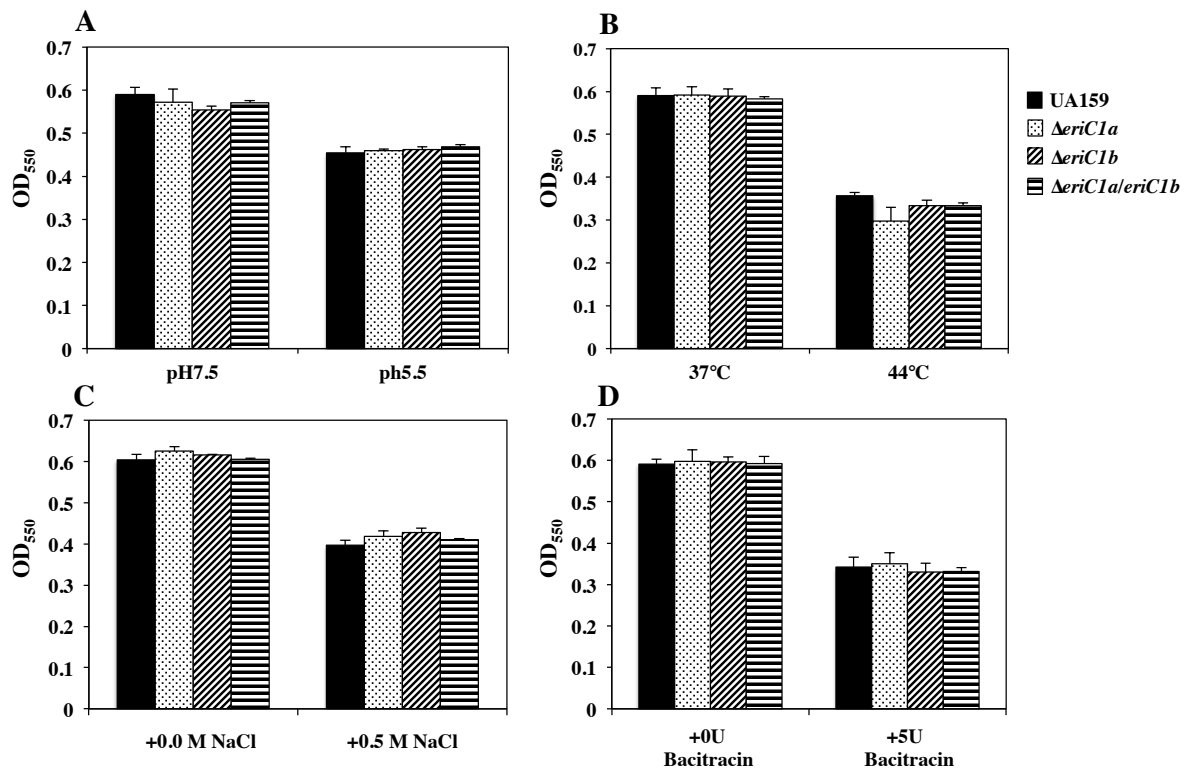




**Fig. 9.** Growth yields of the strains obtained by complementation between *S. mutans* EriC1b and *S. anginosus* EriC1 and between *S. mutans* EriC1b and *S. sanguinis* CrcB1/B2. The y-axis represents the OD<sub>550</sub> after incubation for 16 h. The data represent the mean standard deviations of three independent experiments.

**Responses of the *eriC1a* mutant, the *eriC1b* mutant, and the *eriC1a / eriC1b* double mutant to bacitracin stress and environmental stressors other than fluoride**

The *eriC1a* mutant, the *eriC1b* mutant, and the *eriC1a / eriC1b* double mutant of *S. mutans* were tested for its ability to grow at a low pH (5.5), an elevated temperature (44°C), and high osmotic pressure (NaCl at a final concentration of 0.585 M). As shown in Fig. 10, these mutants showed growth rates similar to that of wild-type UA159 under the above stress conditions.



**Fig. 10.** Growth yields of the parent strain, UA159, the *eriC1a* mutant, the *eriC1b* mutant, and the *eriC1a/eriC1b* double mutant at pH 7.5 or 5.5 (A), at 37°C and 44°C (B), in BHI broth supplemented with 0.5 M NaCl (C), and in BHI broth supplemented with 5U/mL bacitracin (D). The y-axis represents the OD<sub>550</sub> after incubation for 16 h. The data represent the mean standard deviations of three independent experiments.

## DISCUSSION

ClC-type anion-selective channels are widespread throughout eukaryotic organisms and play a crucial role in controlling the ionic composition of the cytoplasm and the volume of cells, as well as the regulation of membrane electrical excitability. Although these channels may conduct other anions (e.g.,  $\Gamma$  or  $\text{NO}_3^-$ ) better than  $\text{Cl}^-$ , they are often called chloride channels because  $\text{Cl}^-$  is the most abundant anion in organisms (Jentsch et al. 2002). BLAST homology searches revealed that many microbial genomes contain members of the ClC family, and the prokaryotic ClC channel was designated an *E. coli*-derived ClC chloride channel homolog (EriC) (Maduke et al. 1999). *E. coli* EriC has been confirmed experimentally to function as a ClC  $\text{Cl}^-$  channel. On the other hand, *P. syringae* EriC and *Clostridium difficile* EriC have been reported to be associated with fluoride resistance (Baker et al. 2012). The *crcB* (confers resistance to camphor B) gene has previously been implicated in resistance to camphor-induced chromosome decondensation. However, it has recently been reported that an *E. coli* *crcB* knock-out mutant became sensitive to fluoride, and *crcB* is predicted to code for membrane proteins belonging to a superfamily composed predominantly of transporters (Baker et al. 2012). Furthermore, it was shown that the eukaryotic *crcB* homolog [renamed *FEX* (fluoride exporter)] encodes a previously unrecognized class of fluoride exporters required for survival under standard environmental conditions (Li et al. 2013).

Oral streptococci are classified into four groups; namely, the mutans, the salivarius, the mitis, and the anginosus groups, according to their 16S rRNA sequences (Kawamura et al. 1995). The present study suggests that oral streptococci possess either *eriC1* or *crcB*, which are associated with fluoride resistance. Among oral streptococci examined in this study, all

*Streptococcus* species belonging to the mutans and the salivarius groups contained EriC1. Nine of 11 *Streptococcus* species belonging to the mitis group possessed CrcB and the other 2 species possessed EriC1. Although a distinct relationship between the type of genes (*eriC1* or *crcB*) and the fluoride-resistance level was not observed, oral streptococci with *eriC1* were prone to higher fluoride resistance than those with *crcB*. The difference in the fluoride-resistance level related to the gene types suggests that the mechanisms through EriC1 and CrcB are not identical.

Baker et al. (2012) showed that *P. syringae* EriC and *C. difficile* EriC associated with fluoride resistance commonly carry a specific set of amino acids in the conserved anion selectivity filter region (Dutzler, *et al.*, 2002), which are distinct from validated chloride-specific EriC proteins (Table). In this study, two novel genes encoding EriC1b of *S. mutans* and EriC1 of *S. anginosus* were shown to be involved in fluoride-resistance, and subsequently seven EriC1s in other oral streptococci were predicted to be responsible for fluoride resistance of these organisms based on amino acid sequence homology. Our table shows amino acid sequences of the conserved anion selectivity filter region of the 18 EriC homologs; 3 EriCs are Cl<sup>-</sup> channels and 15 EriCs are predicted to be F<sup>-</sup> channels. All EriC1s of oral streptococci possessed a similar set of amino acid sequences to those of the F<sup>-</sup> channel EriCs, but they were distinct from those of Cl<sup>-</sup> channel EriCs. Although it is possible that EriC1s in oral streptococci may be generally responsible for the fluoride resistance, the role of EriC1s from oral streptococci other than *S. mutans* and *S. anginosus* in fluoride resistance should be examined. The amino acid sequences of the region in the streptococci were more similar to those of *C. difficile*, *Eubacterium ventriosum*, and *Lactococcus lactis* EriCs than those of *P. syringae*, *Pirellula staleyi*, and *Ralstonia picketti* EriCs, possibly due to the

difference between Gram-positive and Gram-negative bacteria. On the other hand, EriC2s identified in all oral streptococci examined in this study, except for *S. mutans*, possessed amino acid sequences with conserved Cl<sup>-</sup> channels (data not shown), suggesting that EriC2 may be related to Cl<sup>-</sup> channels.

Complementation between *S. mutans* EriC1b and *S. anginosus* EriC1 and that between *S. mutans* EriC1b and *S. sanguinus* CrcB1/B2 were confirmed in this study. Introduction of *S. anginosus* EriC1 into an EriC1s-knockout mutant of *S. mutans* increased fluoride resistance when compared with the same complementation experiment with EriC1b. On the other hand, when EriC1b was transferred into the EriC1-knockout mutant of *S. anginosus*, fluoride resistance of the complemented strain was lower than that complemented with its own EriC1. The fluoride resistance level of *S. anginosus* NCTC10707 was higher than that of *S. mutans* UA159, and the differences in fluoride resistance levels between the above species may be attributed to differences in the kinetics of ion transport between EriC1s. Moreover, the EriC1s-knockout mutant of *S. mutans* complemented by *S. sanguinis* CrcB1/B2 and the CrcB1/CrcB2-knockout mutant complemented by *S. mutans* EriC1b showed restored fluoride resistance. However, neither introduction of *S. sanguinis* CrcB1/B2 into wild-type *S. mutans* nor *S. mutans* EriC1b into wild-type *S. sanguinis* increased the fluoride resistance level of the wild-type strain. It is possible that EriC1 and CrcB1/B2 may be responsible for fluoride resistance in oral streptococci by sharing pathways.

Fluoride application is highly effective in preventing dental caries. On the other hand, we must consider that fluoride has anti-bacterial effects. The frequent application of fluoride results in the emergence of fluoride resistant strains, leading to the dysbiosis of oral microflora. This disturbance may affect not only oral health conditions, but also general

health conditions. Exploring the mechanism of fluoride resistance may contribute to countermeasures against the risk of emergence of the fluoride-resistant strains in oral microflora.

Liao et al. (Liao, *et al.*, 2015) reported that two SNPs related to fluoride resistance were identified in the genome of the fluoride resistant strain *S. mutans* C180-2FR. These were located in the region of the gene cluster composed of SMU\_1291, *eriC1a*, and *eriC1b*; one in its promoter region and the other in *eriC1b*. Expression of the cluster was approximately 10-fold higher in C180-2FR than in the parent strain C180-2. However, we demonstrated that *eriC1b* is involved in fluoride resistance of *S. mutans*, while *eriC1a* is not. It remains unknown why *S. mutans* possesses two EriC1s, unlike other oral streptococci, and why *eriC1a* is not involved in fluoride resistance in *S. mutans*, even though the two EriC1s showed amino acid sequence similarities of 74%.

Inactivation of *eriC1a* or/and *eriC1b* did not affect the resistance to other stress conditions, such as low pH and high osmotic pressure, in *S. mutans*. On the other hand, the function of EriC1a is not clear, while EriC1b is obviously involved in fluoride resistance. EriC1a might be responsible for the transport of other molecules. Anyhow, it is interesting to investigate, including the additional role of EriC1b, what kind of molecules EriC1a transports in order to respond to environmental stressors other than those examined in the present study.

**Table.** Amino acid sequences of the conserved anion selectivity filter region of several EriCs. Eight EriCs (shadowed) were shown to be involved in fluoride resistance (Baker et al. 2012) (Stockbridge et al. 2012). Boxes show the amino acid sequences associated with fluoride resistance. Related EriCs commonly contain a specific set of amino acids, which are distinct from validated chloride-specific EriCs.

Substrate	Organism	Cl <sup>-</sup> selectivity filter residues
Cl <sup>-</sup>	human (ClC-1)	<b>G S G I P ... G K E G P ... G G F M P ... Y</b>
Cl <sup>-</sup>	<i>Escherichia coli</i>	<b>G S G I P ... G R E G P ... G I F A P ... Y</b>
Cl <sup>-</sup>	<i>Salmonella typhimurium</i>	<b>G S G I P ... G R E G P ... G I F A P ... Y</b>
F <sup>-</sup>	<i>Pseudomonas syringae</i>	<b>G N N L I ... G R E G T ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Pirellula staleyi</i>	<b>G N N L L ... G R E G T ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Ralstonia picketti</i>	<b>G N N L L ... G R E G T ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Clostridium difficile</i>	<b>G M N L I ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Eubacterium ventriosum</i>	<b>G M N L V ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Lactococcus lactis</i>	<b>G M G L I ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus mutans</i>	<b>G M G L I ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus anginosus</i>	<b>G M T L I ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus sobrinus</i>	<b>G M G L V ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus australis</i>	<b>G M E L L ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus parasanguinis</i>	<b>G M G L I ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus salivarius</i>	<b>G M G L I ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus vestibularis</i>	<b>G M G L I ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus constellatus</i>	<b>G M T L I ... G R E G V ... G E V T P ... Y</b>
F <sup>-</sup>	<i>Streptococcus intermedius</i>	<b>G M T L I ... G R E G V ... G E V T P ... Y</b>



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