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Expression of ssrA in non-pathogen-induced adaptation in the oral cavity through signal exchange with oral pathogens

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Abstract (J Korean Assoc Oral Maxillofac Surg 2012;38:14-9)

Introduction: This study was conducted to evaluate ssrA expression resulting from adaptation of *Escherichia coli* (*E. coli*) to oral pathogens through signal exchange.

Materials and Methods: Human cell lines Hep2 and HT29, wild-type *E. coli* (WT K-12), ssrA knock-out *E. coli* (Δ K-12), and *Scleropages aureus* (*S. aureus*) were used. A single culture consisting of Hep2, HT29, WT K-12, and Δ K-12, and mixed cultures consisting of Hep2 and WT K-12, Hep2 and Δ K-12, WT K-12 and *S. aureus*, Δ K-12 and *S. aureus*, and Hep2, WT K-12, and *S. aureus* were prepared. For HT29, a mixed culture was prepared with WT K-12 and with WT K-12 and *S. aureus*. Total RNA was extracted from each culture with the resulting expression of ssrA, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and p53 was evaluated by Reverse transcription polymerase chain reaction (RT-PCR).

Results: The expression of ssrA in a single culture of WT K-12 was lower than that observed in the mixed culture of WT K-12 with *S. aureus*. Greater ssrA expression was observed in the mixed culture of WT K-12 with Hep2 than in the single culture of WT K-12. The expression of NF-κB was higher in the mixed culture of Hep2 with Δ K-12 than that in the mixed culture of Hep2 with WT K-12, and was lowest in the single culture of Hep2. The expression of ssrA was higher in the mixed culture of WT K-12 with Hep2 and *S. aureus* than in the mixed culture of WT K-12 with Hep2.

Conclusion: These results suggest that ssrA plays an important role in the mechanism of *E. coli* adaptation to a new environment.

Key words: ssrA, *Escherichia coli*, Adaptation

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I. Introduction

Infection occurs when the balance of three elements- host, environment, and microbe- fails to maintain homeostasis[1]. Among them, a microbial element means dental infection by normal flora in the mucous membrane of the oral cavity or by bacteria carried from another place or surgical wound infection.

The neonatal oral cavity is known to be in aseptic condition at birth, but oral flora is formed as it begins to come into contact with the outside[2]. Likewise, over 300 kinds of microorganism are reportedly found in the various oral cavity environments of normal adults[3-5]. Most of infection occurs due to bacteria residing in a specific region, but is often caused by the microbes residing in another place such as *Escherichia coli* (*E. coli*) or *Clebsiella pneumonia*[6]. Normal flora in the oral cavity are not the cause of infection, the bacteria are carried to the oral cavity from another place through contact of a wound or the operator's hands[7].

An ssrA gene of *E. coli*, a small and stable mRNA molecule, has a various functions, such as destruction of abnormal protein, growth assistance for phage, and activation control of a protein combining with DNA[8-12]. An ssrA gene has information for tmRNA- which has two functions, tRNA

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and mRNA- and rescues the ribosome that was left alone unproductively[13,14]. As part of the trans-translation process added to the C-terminal of a protein with peptide tag secreted, it triggers proteolysis[15,16]. The existence of ssrA in the prokaryotic genome means the biological existence of a trans-translation system[13].

An ssrA is known as one of the genes necessary to adjust to stress in certain species. For example, *E. coli* ssrA mutant has variations in some control functions, and its growth speed declines in a temperature of 45°C. There are many variations in the phenotypes to phage as well[13,14,17,18].

ssrA also seems to work as the various controlling elements in building proteins related to some toxic elements in pathogenic organisms. For example, a lack of ssrA function is known to cause a mutant to lose the capability to survive as a macrophage, with considerable influence on the toxicity of *Salmonella* enteric or *Yersinia pseudotuberculosis*[19,20].

Nonetheless, the expression of ssrA is known to be necessary for *Bacillus subtilis* to grow effectively under strong stress[18]. Therefore, this gene is the one that plays a role in adjusting to stress. We need to know the role of ssrA in the adaptation of non-pathogen to regions except the normal residence area.

This study was conducted to determine ssrA's role in the interaction between *E. coli* and *Scleropages aureus* (*S. aureus*) as host cell and pathogen, respectively, in the non-residence regions.

II. Materials and Methods

1. Cell culture

The Hep2 cell line and HT29 cell line obtained from Korean Cell Line Bank were cultured under conditions of 37°C, 5% CO_2 using Dulbeco's modified Eagle's medium (DMEM) with 10% fetal bovine serum added. Wild-type *E. coli* (WT K-12) and ssrA knockout *E. coli* (Δ K-12) were obtained from Hirosaki University in Himeno, Japan.

2. RNA isolation

To obtain total RNA for reverse transcription polymerase chain reaction (RT-PCR) and microarray, WT K-12, Δ K-12, and *S. aureus* were inoculated with a toothpick from a stock culture (one-night culture of colony in LB agar plate) and cultured respectively for 18 hours in a 15 mL falcon tube including 3 mL LB broth. A single culture of WT K-12 and Δ K-12 and a pre-culture of *S. aureus* were mixed at 30 μL each, and such mixed culture was done for 4 hours. To extract a human RNA, 3×10^6 Hep2 cells were cultured for 18 hours in a 60 mm culture plate using DMEM with 10% FBS added.

As a control group, Hep2 and WT K-12 were used. On the other hand, the experiment groups included a mixed culture of *S. aureus* and WT K-12 (inoculation of *S. aureus* and WT K-12 with concentration of 30 μL/3 mL), a mixed culture of *S. aureus* and Δ K-12 (inoculation of *S. aureus* and Δ K-12 with concentration of 30 μL/3 mL), a mixed culture of Hep2 and WT K-12 (inoculation of WT K-12 into the Hep2 culture with concentration of 30 μL/3 mL), a mixed culture of Hep2 and Δ K-12 (inoculation of Δ K-12 into the Hep2 culture with concentration of 30 μL/3 mL), and a mixed culture of Hep2, *S. aureus*, and WT K-12 (inoculation of *S. aureus* and Δ K-12 into the Hep2 culture with concentration of 30 μL /3 mL). The positive control group included a single culture of HT29 or a mixed culture of HT29, WT K-12 (inoculation of WT K-12 into the HT29 culture with concentration of 30 μL /3 mL), or Δ K-12 (inoculation of Δ K-12 into the HT29 culture with concentration of 30 μL/3 mL). After cell collection, the total RNAs were identified using Easy blue (iNtRON Biotechnology, Seongnam, Korea). The ratios of 28S and 18S to the total RNA samples were measured in 260/280 nm and 260/230 nm. Purity for microarray was identified using agarose gel electrophoresis.

3. RT-PCR

The RT-PCR of total RNAs was done using the RT PreMix Kit. One mg of RNA and 100 pmol of primer were put in a mixing bowl after pre-inoculation for 5 minutes at temperature of 70°C. The reacting dose was 20 μL cDNA synthesis was performed for 60 minutes at temperature of 45°C.

Afterward, a reverse transcriptase was inactivated for 5

Table 1. Sequence of primers used in RT-PCR for bacterial K-12

Name	Sequence of primers
ssrA	F 5'-attctggattcgacgggatt-3'
	R 5'-gcgatctcttttgggtttg-3'
GAPDH	F 5'-aaggcgctaacttcgacaaa-3'
	R 5'-ggaacgccataccagtcagt-3'

(RT-PCR: reverse transcription polymerase chain reaction, GAPDH: glyceraldehyde 3-phosphate dehydrogenase)

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minutes at temperature of 94°C. After that, 2-5 μL of RT-generated cDNA was amplified using a polymerase chain reaction (PCR) PreMix Kit. The primers used for cDNA amplification are listed in Tables 1, 2.

PCR was performed through 30 cycles of denaturation step for 30 seconds at temperature of 94°C, annealing step for 30 seconds at temperature of 55°C, and extension step for 30 seconds at temperature of 72°C. As an expected PCR output, human NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) 1 was 299 bp, p53 was 251 bp, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was 326 bp. On the other hand, 194 bp for ssrA and 300 bp for GAPDH were the expected PCR output from K-12.

Table 2. Sequence of primers used in RT-PCR for human

Name	Sequence of primers
NF-κB1-F	F 5'-ggtctctgggggtacagtca-3'
	R 5'-gtccttcctgcccataatca-3'
p53	F 5'-ccaacaacaccagctcctct-3'
	R 5'-tgagtcaggcccttctgtct-3'
GAPDH	F 5'-cgagatccctccaaaatcaa-3'
	R 5'-acagtcttctgggtggcagt-3'

(RT-PCR: reverse transcription polymerase chain reaction, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, GAPDH: glyceraldehyde 3-phosphate dehydrogenase)

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Table 3. Amount of total RNA extracts of K-12 strains stimulated by *S. aureus* (A)

	Total RNA		
	Conc (ng/uL)	260/280	260/230
W	1,042.28	2.07	2.26
Δ	1,129.40	2.10	2.31
A+W	1,421.40	2.06	2.18
A+Δ	1,714.60	2.09	2.40

(Conc: concentration, W: wild-type *Escherichia coli* [WT K-12], Δ: ssrA knock-out *E. coli* [Δ K-12])

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III. Results

1. Total RNA preparation from K–12 strains following the stimulation of *S. aureus* for microarray

After a single culture of K-12 and a mixed culture of K-12 and *S. aureus*, refined RNA was detected as shown in Fig. 1 and Table 3. High-purity RNAs were extracted in all the groups.

2. Expression of ssrA and GAPDH in K–12 strains stimulated by *S. aureus*

After a single culture of K-12 and a mixed culture with *S. aureus*, ssrA in the mixed culture was slightly more expressed than that in the single culture but was not expressed in K12 with ssrA knock out.(Fig. 2) Meanwhile, GAPDH was

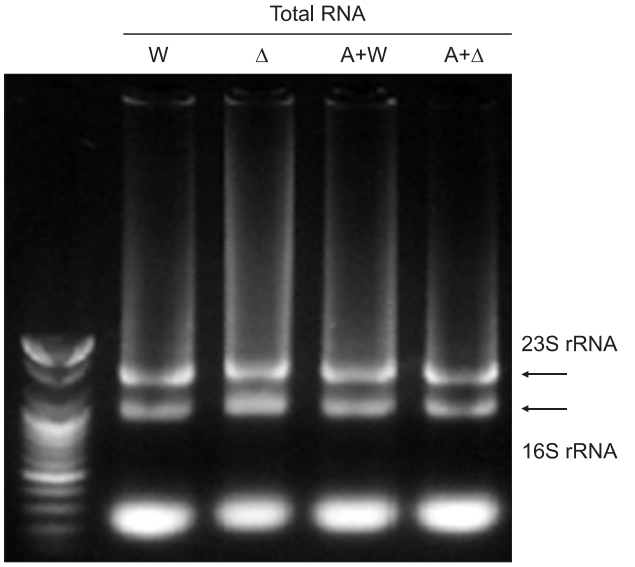

Fig. 1. Agarose gel fraction of total RNA of K-12 strains stimulated by *Scleropages aureus* (A). (W: wild-type *Escherichia coli* [WT K-12], Δ: ssrA knock-out *Escherichia coli* [Δ K-12], GAPDH: glyceraldehyde 3-phosphate dehydrogenase)

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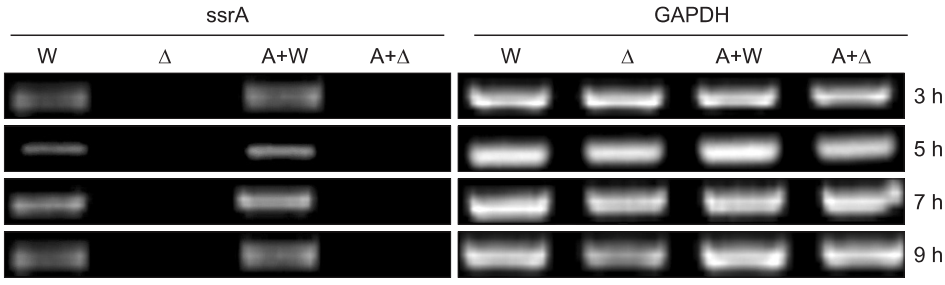

Fig. 2. Expression of *ssrA* and GAPDH in single and mixed cultures after stimulation by *Scleropages aureus* (A). (W: wild-type *Escherichia coli* [WT K-12], Δ: ssrA knock-out *Escherichia coli* [Δ K-12], GAPDH: glyceraldehyde 3-phosphate dehydrogenase)

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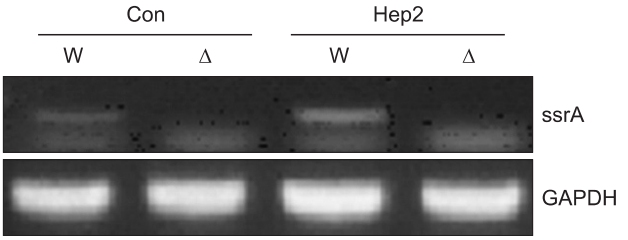

Fig. 3. Expression of *ssrA* in the mixed culture of wild-type *Escherichia coli* (WT K-12) with Hep2. (Con: control, W: WT K-12, Δ: ssrA knock-out *E. coli* [Δ K-12], GAPDH: glyceraldehyde 3-phosphate dehydrogenase)
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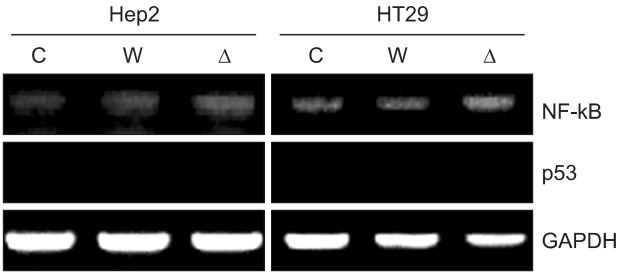

Fig. 4. Expression level of NF-κB and p53 from Hep2 and HT29 following K-12 treatment. (C: control, W: wild-type *Escherichia coli* [WT K-12], Δ: ssrA knock-out *E. coli* [Δ K-12], GAPDH: glyceraldehyde 3-phosphate dehydrogenase)
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expressed in all groups, showing an almost similar tendency to 5 hours after culture.(Fig. 2)

3. ssrA expression in WT K-12 strain stimulated by Host

The expression of ssrA in the K-12 culture mixed with Hep2 was analyzed using RT-PCR.

The expression of ssrA in the mixed culture with Hep2 showed greater increase than the one in a single culture of WT K-12.(Fig. 3)

4. NF-κB and p53 expression test in Hep2

The expression of NF-κB- as a factor related to inflammation- in a mixed culture with WT-K12 showed greater increase compared to the control group. Note, however, that the expression of NF-κB in a mixed culture with Δ K-12 showed much more increase. Expression of p53 was not observed in both single culture of Hep2 and mixed culture with K-12.(Fig. 4) In the case of HT29, the expression of

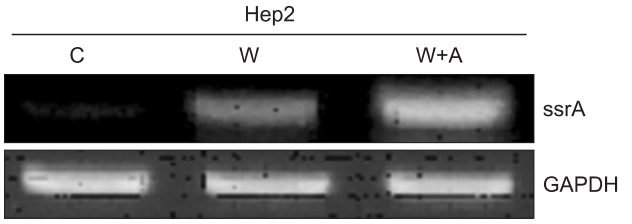

Fig. 5. Expression of *ssrA* in wild-type *Escherichia coli* (WT K-12) from a mixed culture with *Scleropages aureus* (*S. aureus*). (C: control, W: WT K-12, A: *S. aureus,* GAPDH: glyceraldehyde 3-phosphate dehydrogenase)
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NF-κB showed an increase in a single culture or a mixed culture with Δ K-12, not WT K-12.(Fig. 4)

5. ssrA expression in K-12 strains stimulated by the host and *S. aureus*

The expression of ssrA in a mixed culture of WT K-12, *S. aureus*, and Hep2 showed greater increase than that in a mixed culture of WT-12 and Hep2.(Fig. 5)

IV. Discussion

Human is born in aseptic condition including internal organs, and normal flora adapted to the environment of each part settles according to contact with the external environment. Normal flora in the oral cavity is known to consist of over 300 kinds[2-5], which is not pathogenic in normal state but causing infection through a wound in the oral cavity or an operated part. Bacteria from other regions may travel through various route. This study was conducted to determine how such a non-normal flora adapts to normal flora and the cells with different environment. Hep2 originating from the occipital region was used as a cell, *S. aureus*, as a normal flora, and K-12 cell line, *E. coli*, as non-normal flora.

As a germ that normal flora of the large intestine of human and animal, *E. coli* becomes the cause of various diseases by leading to many infections in the other regions as well as the large intestine[21]. ssrA is known to be an essential element in some kinds of germs such as Coliform bacillus. *E. coli* with variation of ssrA shows the reduction of growth speed and abnormal control function due to non-adaptation to temperature and stress[13,17,18]. Since *E. coli*'s ssrA has information on tmRNA, a small and stable RNA molecule,

it reportedly takes a role of various functions such as tagging for degrading abnormal proteins, growth assistance of the bacteria, and activation control of proteins combined with DNA[8-12]. According to Muto et al.[18], an ssrA gene is involved in maintaining the homeostasis of the bacteria, increasing when exposed to heat or chemical stimuli. This study sought to investigate the reaction in the host and pathogen using *E. coli* with ssrA (in charge of adjusting to stress) removed and normal *E. coli*.

In K-12 with ssrA knocked out, ssrA was not expressed in single culture and mixed culture with *S. aureus*. In normal *E. coli*, the expression of ssrA could be identified beginning 3 hours after culture. The expression of ssrA in a mixed culture of *E. coli* and *S. aureus* showed greater increase than that in a single culture of *E. coli*. Since ssrA was not detected in a single culture of *E. coli* with ssrA knocked out or in a mixed culture with *S. aureus*, ssrA could be said to be not expressed in *S. aureus*. Meanwhile, GAPDH was expressed in all the groups. This finding was consistent with the result of Muto et al.[18]. The expression of ssrA increases to adapt to stress for maintaining the homeostasis of a cell. As the result of Karzai et al.[13], ssrA contributes to signal exchange between the bacteria.

The expression of ssrA in a mixed culture of Hep2 and K-12 showed greater increase than that in a single culture of K-12; no expression of ssrA was found in a single culture or a mixed culture in case of K-12 with ssrA knocked out. However, such result was again consistent with that of Muto et al.[18] and Karzai et al.[13], signal exchange between bacteria and the host. This suggested that K-12 received a certain signal from Hep2. Nonetheless, further study on such signal system will be needed in the future.

The expression of NF-κB related to inflammations in a mixed culture with WT K-12 showed greater increase than that in a single culture of Hep2, a cell line originating with the occipital region. The greatest expression was shown in a mixed culture with K-12 with ssrA knocked out. No expression of p53 was found in all the cases. This implies that ssrA is also related to the toxicity of bacteria, and that, in a variant with ssrA mutated, a non-toxic germ can be changed to a toxic one. Meanwhile, since the mixed culture with HT29, a cell line originating from the large intestine, was deemed similar to the K-12 residence region, the expression of NF-κB was similar to that in a single culture of HT29 or a mixed culture with WT K-12. In a mixed culture with K-12 with ssrA knocked out, however, the expression of NF-κB increased. Such result indirectly implies that K-12 receives more stimuli in the non-residence region rather than in the residence region, and that, without ssrA, toxicity can occur in both regions.

The expression of ssrA in a mixed culture of K-12, Hep2, and *S. aureus* simultaneously showed greater increase than that in a mixed culture of K-12 and Hep2. K-12's receipt of simultaneous stimuli from Hep2 and *S. aureus* was believed to have led to the increase of ssrA expression.

These results suggested that ssrA gene is involved in *E. coli's* adaptation to a new environment in case of mixed culture by combining *E. coli* with *S. aureus* and Hep2, contributing to changing non-pathogenic bacteria in the residence region to pathogenic bacteria in the other regions. Detailed study on the mechanism related to ssrA will be necessary in the future, however.

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