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研究題目：Recombinant Sox enzymes of *Paracoccus pantotrophus* degrade volatile sulfur compounds

目 的：

Oral malodor, the presence of unpleasant foul smelling breath, is a common problem throughout the world, and significantly impacts on social interactions. Studies have shown that nearly 90% of oral malodor cases are the result of intra-oral causes. Oral malodor is derived from the action of anaerobic bacteria that produce malodorous molecular species including volatile sulfur compounds（VSCs). Hydrogen sulfide（H_2S）and methyl mercaptan（CH_3SH）are the major malodorous components of VSCs. Many trials have been shown to develop new products including oral rinse solutions to reduce VSCs. However, the effect was limited and there have been no useful means to prevent oral malodor. In industrial area, several chemotrophs including *Paracoccus pantotrophus* are used for the removal of H_2S based on biological process. *P. pantotrophus* is a lithoautotrophic, aerobic, Gram-negative bacterium, and able to use sulfide as electron donors. In this study, we cloned sulfur-oxidizing（Sox）enzymes of *P. pantotrophus* and evaluated the activity to degrade H_2S and CH_3SH in view of oral malodor prevention.

材料および方法：

Paracoccus pantotrophus GB17 was used throughout this study. The strain was cultured aerobically in BHI broth supplemented with 4 mM $MgSO_4 \cdot H_2O$ at 37°C. Four genes coding SoxXA, SoxB, SoxCD and SoxYZ, were respectively amplified from *P. pantotrophus* GB17. Each fragment was cloned into the vector for the expression of 6 x His-tagged fusion protein in *Escherichia coli* XL II. *E. coli* XL II harboring Sox plasmid was cultured aerobically in Luria-Bertani medium supplemented with ampicillin（100 μg/ml）at 37°C. After incubation, *E. coli* cells were collected and suspended in phosphate buffer containing 8 M urea, then sonicated. The supernatant was collected and applied to Ni-NTA resin affinity chromatography. Eluted proteins were refolded in sequential dialysis against urea-decreasing phosphate buffer. Purification of recombinant proteins was verified by 12% SDS-PAGE and western blot using antibody against 6 x His-tag. Sox enzymes activity was analyzed in 1 ml of PBS（pH 7.0）containing whole cells of *P. pantotrophus* GB17 or recombinant Sox enzymes（consisting of SoxXA, SoxB, SoxCD, and SoxYZ), and 0.01 mM NaHS. Periodontopathic bacteria, *Fusobacterium nucleatum* ATCC10953 and *Porphyromonas gingivalis* 381, were also used to generate H_2S and CH_3SH. To determine H_2S and CH_3SH degrading activity, the reaction tube was sealed and incubated at 37°C for appropriate time. After incubation, 2.5 ml of headspace air in the reaction tube was taken and used to determine the concentration of H_2S and CH_3SH by gas chromatography.

結果および考察：

1. Cloning of Sox genes

The gene cluster of *P. pantotrophus* GB17 coding for Sox enzymes comprises at least two transcriptional units with 16 genes（Fig. 1). Seven genes, *soxXYZABCD*, code for proteins essential for sulfur oxidation *in vitro*. Essential Sox genes were rearranged and positioned in the sequence of *SoxXA*, *SoxB*, *SoxCD*, and *SoxYZ*. The genes were amplified by PCR and shown in order of *SoxXA*（2.2 kbp), *SoxB*（1.7 kbp), *SoxCD*（2.4 kbp), and *SoxYZ*（0.66 kbp）（Fig. 2).

Fig. 1. Schematic map of the sox gene cluster of *P. pantotrophus* GB17.

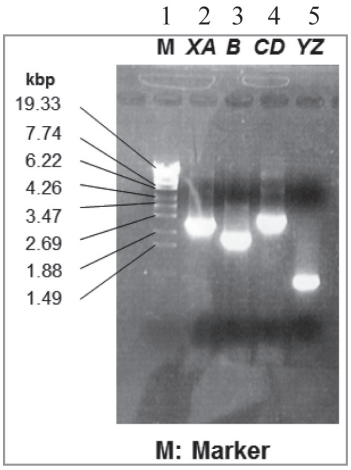

Fig. 2. Cloning of Sox genes amplified by PCR. Lane 1, marker; lane 2, *soxXA*; lane 3, *soxB*; lane 4, *soxCD*; and lane 5, *soxYZ*.

2. Purification of rSox proteins and western blot analysis

By SDS-PAGE analysis, protein bands were observed after staining the gel with Coomassie brilliant blue（Fig. 3). The molecular mass of each protein is SoxX, 23 kDa；SoxA, 29 kDa；SoxB, 60 kDa；SoxC, 47 kDa；SoxD, 50 kDa；SoxY, 18 kDa；and SoxZ, 36 kDa. Western blot analysis revealed all bands observed are fused with 6 x His-tag（Fig. 4).

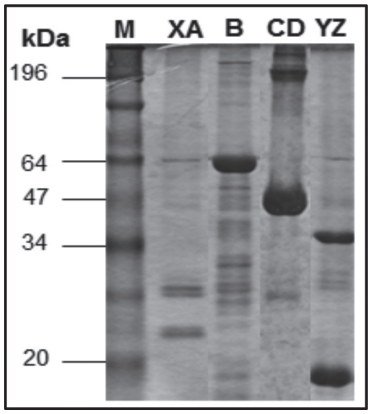

Fig. 3. SDS-PAGE analysis of purified rSox proteins on a 12% polyacrylamide gel. Lane 1, Molecular standards; lane 2, SoxXA; lane 3, SoxB; lane 4, SoxCD, and lane 5, SoxYZ.

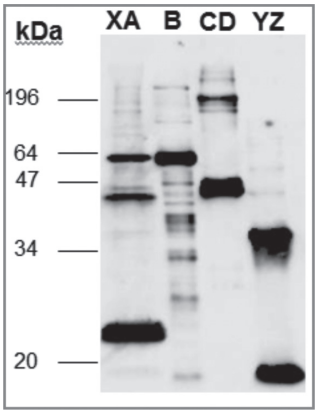

Fig. 4. Western blot analysis. rSox poteins were applied to 12% SDS-PAGE gel and were transferred to a nitrocellulose membrane. rSox proteins were detected using an antibody against 6 x His-tag.

3. *P. pantotrophus* GB17 activity to degrade H2S generated from NaHS

The ability of *P. pantotrophus* GB17 cells to degrade H_2S generated from NaHS was examined. As shown in Fig. 5, *P. pantotrophus* GB17 degraded H_2S in a cell number-dependent manner.

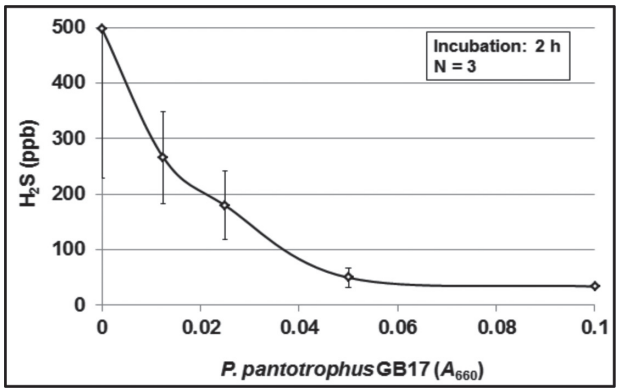

Fig. 5. H_2S degrading activity by whole cells of *P. pantotrophus* GB17. Bacterial cells were suspended in PBS with 0.01 mM NaHS and incubated for 2 hours at 37°C.

4. rSox enzymes activity to degrade H_2S generated from NaHS

The assay mixture contained various concentrations of rSox enzymes (SoxXA, SoxB, SoxCD, and SoxYZ). NaHS was used to generate H_2S. rSox enzymes degraded H_2S in dose-dependent and time-dependent manners (Fig. 6, 7).

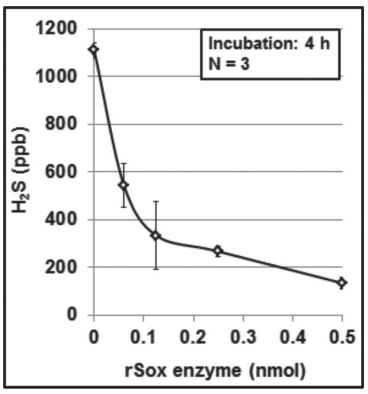

Fig. 6. H_2S degrading activity by rSox enzymes. The assay mixture containing various concentrations of rSox enzymes and 0.01 mM NaHS was incubated for 4 hours at 37°C.

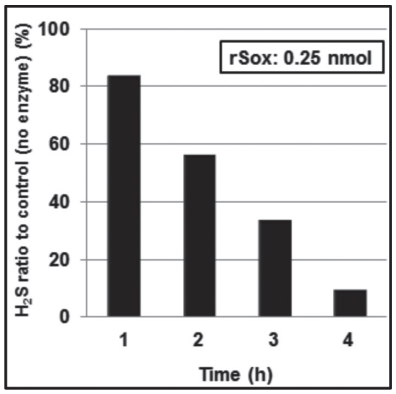

Fig. 7. H_2S degrading activity by rSox enzymes. The assay mixture containing 0.25 nmol of rSox enzymes and 0.01 mM NaHS was incubated up to 4 hours at 37°C.

5. rSox enzymes activity to degrade H_2S and CH_3SH produced by bacteria

Fusobacterium nucleatum ATCC10953 and *Porphyromonas gingivalis* 381, as periodontopathic bacteria, were used to produce H_2S and CH_3SH from L-cysteine and L-methionine. After incubation, the ratio of H_2S to control (no enzyme) decreased in a time-dependent manner (Fig. 8). rSox enzymes also degraded CH3SH produce by *P. gingivalis* (Fig. 9).

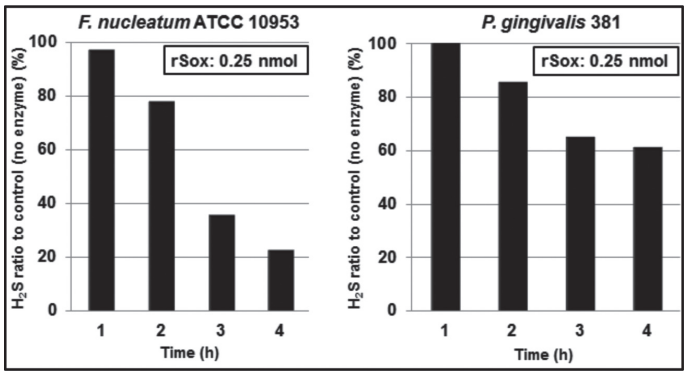

Fig. 8. rSox enzyme activity to degrade H_2S produced by periodontopathic bacteria. The assay mixture containing rSox enzymes, bacterial cells and L-cysteine or L-methionine was incubated up to 4 hours at 37°C.

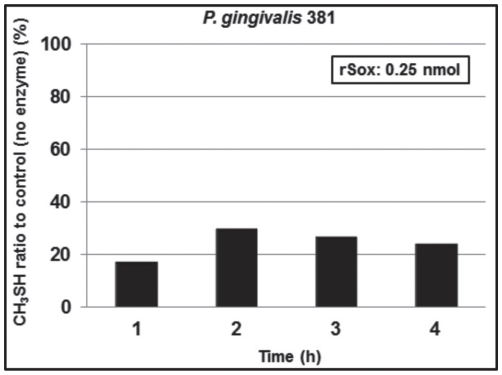

Fig. 9. rSox enzyme activity to degrade CH_3SH produced by *P. gingivalis*. The assay mixture containing rSox enzymes, bacterial cells and L-methionine was incubated up to 4 hours at 37°C.

In conclusion, combination of seven recombinant Sox enzymes of *P. pantotrophus* GB17 has an activity reducing H_2S and CH_3SH. The results suggest that combination of seven recombinant Sox enzymes from *P. pantotrophus* GB17 could be useful for the prevention of oral malodor.

成果発表：

1. Ramadhani A., Matsuo M., Komatsuzawa H., and Oho T.：Recombinant Sox enzymes of *Paracoccus pantotrophus* degrade hydrogen sulfide. 62nd Annual Meeting of Japanese Association for Dental Research, Osaka, December 2014.

2. Ramadhani A., and Oho T.：Recombinant Sox enzymes of *Paracoccus pantotrophus* degrade volatile sulfur compounds. 第 64 回日本口腔衛生学会総会，つくば，2015 年 5 月．（発表予定）