Inhibition of polysaccharide synthesis by the sinR orthologue PGN_0088 is indirectly associated with the penetration of Porphyromonas gingivalis biofilms by macrolide antibiotics

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Microbes commonly adhere to surfaces, aggregate in self-produced extracellular polymeric substances (EPS) and live in biofilms. Periodontitis is a serious oral infection that is initiated by the formation of biofilms by Porphyromonas gingivalis. EPS act as a barrier that protects biofilm-forming cells against sources of stress, including those induced by host immune cells and antimicrobial agents. Therefore, drugs intended to kill such micro-organisms cannot be used for the treatment of biofilm infections. Our previous studies revealed that subminimal inhibitory concentrations (subMIC) of two macrolide antibiotics (azithromycin, AZM and erythromycin, ERY) reduced P. gingivalis biofilms. Furthermore, we demonstrated that the Bacillus subtilis sinR orthologue (PGN_0088) inhibits the synthesis of carbohydrates that are components of EPS in P. gingivalis biofilms. Here, we constructed a novel sinR mutant from P. gingivalis ATCC 33277 and reveal that the increased abundance of carbohydrate in EPS of the mutant led to a reduced infiltration rate of AZM and ERY through EPS, and consequently elevated biofilm resistance to these macrolides. Detailed elucidation of the interaction between the product of the sinR gene and EPS will assist in the development of novel approaches that target EPS to prevent and inhibit the formation of biofilms.

INTRODUCTION

In nature, most micro-organisms adhere to any surface or to each other to form a biofilm, which is a structure comprising microbes and extracellular polymeric substances (EPS) (Costerton et al., 1981; O'Toole et al., 2000). Biofilms often form on medical devices such as catheters and artificial joints, causing chronic or refractory infections ('biofilm infections') (Costerton et al., 1987). Dental biofilms consist of multiple bacterial species and cause opportunistic infections such as dental caries and periodontal disease (Costerton et al., 1999; Donlan & Costerton, 2002). P. gingivalis, a Gram-negative oral anaerobic bacterium, is distributed throughout subgingival and extra-radicular biofilms and is one of the major pathogens that cause severe forms of marginal periodontitis and refractory peri-apical periodontitis (Noiri et al., 2004; Noguchi et al., 2005). Subgingival biofilms are located in the periodontal pocket (Noiri et al., 1997) and outside the root apex over the apical foramen (Noiri et al., 2002).

Biofilm-forming cells are generally resistant to certain antibiotics and biocides (Brown et al., 1988; Characklis, 1990; Costerton et al., 1999). Resistance may be imparted by slow or faulty penetration of antibiotics through EPS, expression of genes conferring antibiotic resistance, or slow growth of micro-organisms in the biofilm (Stewart & Costerton, 2001; Li et al., 2002). Similarly, P. gingivalis biofilms are resistant to high concentrations of minocycline and metronidazole that are effective against planktonic cells (Noiri et al., 2003). Furthermore, although chlorhexidine gluconate effectively reduces the viability of P. gingivalis in biofilms (Noiri et al., 2003), EPS remaining after chlorhexidine treatment serve as a scaffold for the attachment of bacterial cells to form a new biofilm (Yamaguchi et al., 2013). In contrast, subMIC levels of AZM and ERY inhibit P. gingivalis biofilms formed using static models, and AZM inhibits flow-cell biofilms (Maezono et al., 2011). Maezono et al. (2011) used confocal laser scanning microscopy (CLSM) to demonstrate that treatment of flow-cell biofilms with subMIC levels of AZM reduces the adherence of P.
Penetration of macrolides through *P. gingivalis* biofilm

*P. gingivalis*, although dead cells were undetected. Therefore, subMIC levels of AZM may not influence cell survival but may influence the maintenance of the biofilm structure. Unfortunately, the detailed mechanism of the effect of macrolides on biofilms is unknown.

Biofilm-forming cells aggregate in the self-produced EPS (Costerton et al., 1995), which comprise proteins, carbohydrates and nucleic acids. EPS protect microbes from antibiotics and immune cells, and stabilize the biofilm structure (Flemming & Wingender, 2010). Therefore, traditional antimicrobial agents are less effective in suppressing biofilm infection, which emphasizes the importance of developing a novel strategy that targets EPS to control biofilm infections. In biofilms formed by the Gram-positive spore-former *Bacillus subtilis*, EPS consist of an exopolysaccharide (specified by the epsA–O operon) and TasA, a secreted protein encoded by the yqxm–sipW–tasA operon (Chu et al., 2008). The epsA–O and yqxm–sipW–tasA operons are controlled by the represor SinR (Chu et al., 2006; Lopez et al., 2009; Winkelman et al., 2009). We revealed previously that the number of genes that are expressed >1.5-fold was highest at the later stage of biofilm formation by *P. gingivalis* ATCC 33277 (Yamamoto et al., 2011). The gene encoding PGN_0088 (a sinR orthologue, which inhibits carbohydrate synthesis in *P. gingivalis* biofilms) was down-regulated by the greatest extent (Yamamoto et al., 2013) (Table S1, available in the online Supplementary Material).

The mutant strain constructed by insertion of the ERY resistance gene ermF (Yamamoto et al., 2013) cannot be used in studies of macrolides; therefore, it was necessary to construct new antibiotic-resistant sinR mutants with different mechanisms of resistance to macrolides. For this purpose, here we constructed a sinR mutant by inserting the chloramphenicol acetyltransferase gene (*cat*) into the parental strain (*P. gingivalis* ATCC 33277) to investigate the mechanism of action of AZM and ERY on biofilms, focusing on their effect on EPS.

**METHODS**

**Bacterial strains and culture conditions.** All bacterial strains used in this study are listed in Table 1. Similar to our previous studies (Yamaguchi et al., 2010; Yamamoto et al., 2011, 2013), *P. gingivalis* cells were grown anaerobically (10% CO2, 10% H2 and 80% N2) in Gifu anaerobic medium (GAM; Nissui) on Soybean–Casein–Digest agar (SCD agar; Nissui). For selection and maintenance of antibiotic-resistant strains, antibiotics were added to the medium at concentrations as follows: 100 μg ampicillin ml⁻¹; 20 μg chloramphenicol ml⁻¹; 200 μg gentamicin ml⁻¹; or 0.7 μg tetracycline ml⁻¹.

**Construction of bacterial strains.** We constructed strains ODP003 (sinR mutant strain; *ΔsinR::cat*) and ODP004 (*ΔsinR::complemented strain; *ΔsinR::cat/sinR*⁺) as previously described (Yamamoto et al., 2013). The former strain was constructed as follows: regions upstream and downstream of *sinR* were amplified by PCR using chromosomal DNA of *P. gingivalis* strain ATCC 33277 as a template and the primer-pairs SUF–SUR-Cm⁺ and SDF-Cm⁻–SDR to amplify the upstream and downstream regions, respectively. The DNA primers and plasmids used in this study are listed in Table 1. Amplicons were digested with *Kpn*I and *Hind*III, or *Hind*III and *Not*I, to generate the upstream or downstream regions, respectively, which were then inserted into *Kpn*I–*Not*I-digested pBluescript II SK(−) (Stratagene) to yield pOD005. The *Hind*III-digested *cat* DNA cassette was derived from a pCM7 plasmid (National BioResource Project (NBRP)) using *Hind*III digestion and inserted into the *Hind*III site of pOD005 to generate pOD006 (*ΔsinR::cat*). The *Bst*HII-linearized pOD006 DNA fragment was introduced into *P. gingivalis* ATCC 33277 using electroporation with a Gene-Pulser Xcell Microbial System (Bio-Rad Laboratories) set to 25 μF, 400 Ω, and 2.5 kV to yield strain ODP003 (*sinR* mutant; *ΔsinR::cat*). To construct the latter strain, the 0.7 kb DNA fragment containing the *sinR* region was amplified by PCR using *P. gingivalis* ATCC 33277 chromosomal DNA as a template. The amplified DNA fragment was cloned into a *Pst*I digested pGEM-T Easy vector (Promega). The *sinR* region obtained by NotI and *Bam*HI digestion was inserted into NotI–*Bam*HI-digested pTO/1 (Nagano et al., 2007) to yield pOD004 (*sinR*⁺). The pOD004 plasmid DNA was introduced into the *sinR* mutant by conjugation with *E. coli* S17-1 (Simon et al., 1983), harbouring pOD004, as a donor strain to generate strain ODP004 (*sinR*⁺–complemented strain; *ΔsinR::cat sinR*⁺). Ampicillin, ERY, gentamicin or tetracycline were used to select colonies that harboured these antibiotic-resistant gene cassettes.

**Characterization of *P. gingivalis ΔsinR::cat* biofilms.** To confirm that there were no differences in the characteristics of biofilms formed by strains *ΔsinR::cat* (this study) and *ΔsinR::ermF* (Yamamoto et al., 2013), we performed quantitative analysis, CLSM observations, scanning electron microscopy (SEM) observations and sonic disruption assays of *P. gingivalis* biofilms described previously (Yamamoto et al., 2013).

**Measurement of MICs and minimum biofilm inhibitory concentrations (MBICs) of macrolides.** We determined the MICs and MBICs for AZM (Tocris Bioscience) and ERY (Wako Pure Chemical Industries) as previously reported (Maezono et al., 2009). We briefly, to measure macrolide MICs for planktonic *P. gingivalis*, we dispensed 100 μl of culture into 96-well U-bottom microplates (Becton Dickinson). Subsequently, 100 μl of macrolides (dissolved in DMSO and diluted with GAM) were immediately added at a concentration range of 0–1 μg ml⁻¹. The MIC was defined as the lowest concentration of AZM or ERY that visibly inhibited *P. gingivalis* growth after anaerobic incubation (37 °C for 2 days). For determination of the MBICs for adherent *P. gingivalis*, 200 μl of culture was dispensed into 96-well flat-bottom microplates (Becton Dickinson) and incubated under anaerobic conditions for 3 days at 37 °C. Macrolides were added at a final concentration range of 0–1 μg ml⁻¹, and the cells were incubated for 3 days. Quantitative analysis of adherent cells was performed using a published method (Maezono et al., 2011). DMSO without macrolides was added as a control for MIC and MBIC assays, which were performed in triplicate.

**Biofilm penetration assay.** This penetration assay was conducted using the methods described by Anderl et al. (2000) and Sammanyake et al. (2005) with some modifications. Each isopore membrane filter (diameter, 25 mm; pore size, 0.22 μm; Millipore) was placed on an SCD agar plate and then inoculated with 2.0 × 10⁸ cfu ml⁻¹ *P. gingivalis* in GAM broth (50 μl). Sterile GAM broth was used as a control. The inoculated agar plates were incubated anaerobically at 37 °C to dry the culture medium, and the plate was inverted after 1 h and incubated anaerobically at 37 °C for 9 days (Fig. 1a). Every day during this incubation period, each filter with the growing *P. gingivalis* biofilm was transferred onto a plate containing fresh SCD agar.

After 9 days, the biofilms on the membrane filters were placed on SCD agar plates containing 0, 30 or 250 μg ml⁻¹ of AZM or ERY. Isopore membrane filters (diameter, 13 mm; pore size, 0.22 μm;
Millipore) were then placed on top of 9 day-old *P. gingivalis* biofilms. A blank antibiotic disk (diameter, 6.35 mm; thickness, 0.8–0.9 mm; Difco) moistened with 50 μl of sterile 0.9 % saline solution (Otsuka Pharmaceutical) was then placed on top of the first membrane; wetting the disk prevents passive capillary perfusion of the antibiotic through the biofilm. The whole assembly was then incubated anaerobically at 37 °C for 4 h (Fig. 1b).

After 4 h, the disk was removed and placed on a fresh SCD agar plate with 100 μl of *P. gingivalis* ATCC 33277. The plates were then incubated anaerobically at 37 °C for 3 days, after which we used calipers to measure the diameters of the zones of inhibition. Before use, filters and disks were sterilized by exposure to UV light (wavelength, 253.7 nm) for 15 min per side and manipulated with sterile disposable tweezers (Tokyo Glass Kikai). This assay was conducted in duplicate on three separate occasions for each *P. gingivalis* strain.

**Statistical analysis.** The significance of intergroup differences of all data was analysed using one-way ANOVA and Scheffe’s tests with the statistical software SPSS Statistics, Version 22 (IBM). *P* < 0.001 was considered statistically significant.

### Results

**Effect of exchanging antibiotic-resistance gene cassettes from *ermF* to *cat* on the characteristics of *sinR* mutants**

The surface structure of the *sinR* mutant biofilm (*sinR*) formed a mesh, unlike those of the WT and *sinR*+ complemented strains (*sinR*-C) (Fig. 2a–c). Further, only
*sinR* formed a mesh-like structure revealed by exopolysaccharide staining (Fig. 3 a, b). Colorimetry revealed no significant difference in protein content among the three strains (Fig. 2d). However, the amount of carbohydrate produced by *sinR* was significantly higher compared with that of the WT and *sinR*-C (Fig. 2e). Further, CLSM quantification indicated that the volume of adherent cells was not changed; however, the volume of the exopolysaccharide was increased significantly in the *sinR* biofilm compared with that of the other two strains (Fig. 2d, e). Further, application of a shear force to biofilms of the three strains revealed that the mechanical strength of the *sinR* biofilm was significantly higher compared with that of the WT and *sinR*-C (Fig. 4). These results show that replacing *ermF* with *cat* did not influence the high carbohydrate content of the biofilm formed by the *sinR* mutant.

**DISCUSSION**

Micro-organisms that exist in biofilms synthesize EPS (Costerton *et al.*, 1995), which protect the cells against bactericidal agents and host immune defences (Flemming & Wingender, 2010). Lopez *et al.* (2009) reported that *sinR*, a transcriptional regulator of *B. subtilis*, controls the production of EPS. We used an *ermF*-insertion mutant to reveal that PGN_0088 (*sinR*), a *B. subtilis* orthologue, suppresses the synthesis of polysaccharides present in the EPS of *P. gingivalis* biofilms (Yamamoto *et al.*, 2013). In the present study, we mutated *sinR* by inserting *cat* and showed that the increase in carbohydrate observed in the mutant strain biofilm was similar between methods. Micro-morphological and 3D observations, quantification and sonic disruption assays showed that replacing *ermF* with *cat* did not influence the high carbohydrate content of the biofilm formed by the *sinR* mutant.

Although the MICs for AZM or ERY of the *P. gingivalis* strains tested here were equal, the MBICs of *sinR* were higher compared with those of the WT or *sinR*-C strains. These results show that deleting *sinR* may not affect macrolide resistance of the cell but may contribute to the resistance of biofilms to macrolides. SubMIC levels of AZM inhibit biofilm formation by *Pseudomonas aeruginosa* (Ichimiya *et al.*, 1996), *Haemophilus influenzae* (Starner *et al.*, 2008) and *P. gingivalis* (Maezono *et al.*, 2011). Furthermore, *P. gingivalis* biofilm formation, determined using a static
model, was repressed by subMIC levels of ERY (Maezono et al., 2011). Therefore, AZM and ERY may have anti-biofilm effects that differ from their antimicrobial effects. The largest and most important difference between planktonic cell and biofilms is the presence of EPS that cover the micro-organisms. The expression of sinR and the carbohydrates present in EPS may influence resistance of P. gingivalis to AZM and ERY.

One reason that accounts for the antimicrobial resistance of biofilm-forming microbes is the slow penetration of these drugs through EPS in biofilms (Mah & O’Toole, 2001; Stewart & Costerton, 2001). It is possible that if the proportion of each component of EPS changes, the resistance of biofilm-forming cells against antimicrobials will shift because of a decreased rate of penetration through the EPS. For example, under all macrolide conditions used here, the radius of the inhibition zone of sinR was significantly smaller compared with that of the WT and sinR-C (Fig. 5). We conclude that the increased carbohydrate content of EPS of the sinR mutant delays access of AZM or ERY to the blank antibiotic disk, and reduces the content of these macrolides on the disk.

Slow penetration of antibiotics through EPS of the biofilm is one reason why biofilm-forming cells resist antibiotics. Reduced metabolic activity and changes in gene expression in biofilms may represent other mechanisms (Mah & O’Toole 2001; Stewart & Costerton, 2001). Although the foregoing mechanisms were considered to explain the difference in
antibiotic sensitivity between planktonic cells and biofilm-forming cells, these concepts should be applied to explain the disparities in antibiotic sensitivity among bacterial strains that form biofilms. Thus, slow penetration, reduced metabolic activity, changes in gene expression or any or all of these may explain why the MBICs of mutant strains were significantly higher compared with those of the WT and complementary strains.

Reduced metabolic activity may explain the significant difference in MBICs among the mutant strains, because the growth curve of the \( \text{sinR} \) mutant was identical to that of the WT and complementary strains (data not shown). In \( \text{Escherichia coli} \), the expression of \( \text{yhcQ} \), encoding a putative multidrug-resistance pump, and \( \text{yeeZ} \), putatively involved with extracellular wall functions, was inhibited in the biofilm formed by a strain harbouring a mutation in \( \text{rapA} \) that encodes an RNA polymerase-associated protein, and resistance to penicillin G was decreased compared with that of the WT (Lynch et al., 2007). Because the genes involved in the resistance of \( \text{P. gingivalis} \) to macrolides are unknown, it is difficult to determine whether the change in gene expression in the \( \text{sinR} \) mutant biofilm influences resistance

![Fig. 4. Tenacity of biofilms formed by \( \text{P. gingivalis} \) strains. Relative numbers of \( \text{P. gingivalis} \) present in biofilms before and after sonication were calculated according to the number of WT cells without sonication, which was defined as 1.0. Percentages indicate the amount of the remaining biofilm after sonication. The error bars indicate SEM. Different letters above the error bars indicate significant differences of the means (\( P<0.001 \) using one-way ANOVA and Scheffe’s tests).](image)

![Table 2. MICs of AZM and ERY for planktonic \( \text{P. gingivalis} \) strains ATCC 33277 (WT), ODP003 (\( \text{sinR} \)) and ODP004 (\( \text{sinR-C} \))](image)

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<th>( \text{P. gingivalis} ) strain</th>
<th>MIC (( \mu \text{g ml}^{-1} ))</th>
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<tr>
<td></td>
<td>AZM</td>
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<tr>
<td>WT</td>
<td>0.5</td>
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<tr>
<td>( \text{sinR} )</td>
<td>0.5</td>
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<tr>
<td>( \text{sinR-C} )</td>
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![Table 3. MBICs of AZM and ERY for adherent \( \text{P. gingivalis} \) strains ATCC 33277 (WT), ODP003 (\( \text{sinR} \)) and ODP004 (\( \text{sinR-C} \))](image)

<table>
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<th>( \text{P. gingivalis} ) strain</th>
<th>MBIC (( \mu \text{g ml}^{-1} ))</th>
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<tbody>
<tr>
<td>AZM</td>
<td>ERY</td>
</tr>
<tr>
<td>WT</td>
<td>0.125</td>
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<tr>
<td>( \text{sinR} )</td>
<td>0.25</td>
</tr>
<tr>
<td>( \text{sinR-C} )</td>
<td>0.125</td>
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![Fig. 5. Penetration of macrolides through biofilms formed by \( \text{P. gingivalis} \) strains. The radii of the growth-inhibition zones formed by AZM (a) or ERY (b) contained in the antibiotic disk that penetrated through biofilms of \( \text{P. gingivalis} \) strains were measured. Two values (30 and 250) displayed as the labels of the x-axis indicate the final concentrations of macrolides added to the SCD agar plates used in the two-membrane system. Control indicates that GAM broth was contained in the two-membrane system instead of the biofilms of the \( \text{P. gingivalis} \) strains. The error bars indicate SEM. The letters above the error bars indicate significant differences of the means (\( P<0.001 \), one-way ANOVA and Scheffe’s tests).](image)
to macrolides. However, it may be possible that any resistance-associated gene is either up- or down-regulated by the deletion of sinR, which encodes a putative transcription factor. Thus, the change in gene expression caused by deletion of sinR may account for the difference between the MBICs for AZM and ERY.

In conclusion, our study shows that the transcriptional regulator sinR contributes to resistance to AZM and ERY by inhibiting carbohydrate synthesis in the EPS of the P. gingivalis biofilm. In B. subtilis, sinR is a component of a complex cascade of reactions, which is controlled by spo0A- P (sporulation transcription regulator) and sinI (antagonist of SinR) and regulates the expression of the epsA−O and yqxA−mipW−tasA operons (Lopez et al., 2009). Identifying the cascade involving sinR in P. gingivalis may contribute to the development of an inhibitor of the synthesis of EPS, leading to the destruction of biofilms. Further, PGN_0088 (sinR) is a highly conserved gene among Gram-negative bacteria such as Porphyromonas, Bacteroides, Tannerella, Prevotella and Pseudomonas, as well as Gram-positive bacteria such as Bacillus, Clostridium, Staphylococcus and Streptococcus (http://www.kegg.jp/). This study was supported by the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (24390425, 24390424, 24890118 and 26861593).

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