Development of SNAP-tag-mediated live cell labeling as an alternative to GFP in Porphyromonas gingivalis

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Porphyromonas gingivalis; SNAP-tag; biofilm; confocal laser scanning microscopy (CLSM); green fluorescent protein (GFP); Streptococcus gordonii.

Abstract
Porphyromonas gingivalis is an anaerobic periodontal pathogen that resides in the complex multispecies microbial biofilm known as dental plaque. Effective reporter tools are increasingly needed to facilitate physiological and pathogenetic studies of dental biofilm. Fluorescent proteins are ideal reporters for conveniently monitoring biofilm growth, but are restricted by several environmental factors, such as a requirement of oxygen to emit fluorescence. We developed a fluorescent reporter plasmid, known as the SNAP-tag, for labeling P. gingivalis cells, which encode an engineered version of the human DNA repair enzyme O6-alkylguanine-DNA alkyltransferase. Fluorescent substrates containing O6-benzylguanine covalently and specifically bind to the enzyme via stable thioether bonds. For the present study, we constructed a replicative plasmid carrying SNAP26h under the control of the P. gingivalis endogenous trxB promoter. The P. gingivalis-expressing SNAP26 protein was successfully labeled with specific fluorophores under anaerobic conditions. Porphyromonas gingivalis biofilm formation was investigated using flow cells and confocal laser scanning microscopy. A specific distribution of a strong fluorescence signal was demonstrated in P. gingivalis-SNAP26 monospecies and bispecies biofilms with Streptococcus gordonii-GFPmut3*. These findings show that the SNAP-tag can be applied to studies of anaerobic bacteria in biofilm models and is a useful and advantageous alternative to existing labeling strategies.

Introduction
Periodontal diseases are multifactorial infections initiated by multispecies bacterial communities organized in the complex and dynamic structure called a biofilm (Rosan & Lamont, 2000). Early colonizers of the salivary pellicle on the tooth surface, mainly commensal oral streptococci such as Streptococcus gordonii, initiate biofilm formation by favoring adherence and colonization by late pathogenic colonizers, including Porphyromonas gingivalis. Porphyromonas gingivalis, a Gram-negative, black-pigmented anaerobic rod, is widely recognized as an important etiological agent of periodontal disease (Lamont & Jenkinson, 1998). Porphyromonas gingivalis possesses several virulence factors, including proteases, adhesins, and endotoxins. Although organisms such as P. gingivalis are considered to be responsible for the destruction of periodontal tissues, in the oral cavity, P. gingivalis accumulates into mixed species biofilms (Kuboniwa et al., 2006); thus, it is important to develop a multispecies biofilm model under anaerobic and flowing conditions. We chose to study biofilm formation and the role of P. gingivalis in the pathogenesis of periodontal disease, through the development and analysis of the bispecies S. gordonii/P. gingivalis biofilm model in a flow cell that mimics the natural conditions in the oral cavity.

The analysis of a biofilm structure is usually performed by confocal laser scanning microscopy (CLSM). This technology has led to a better understanding of the architecture of biofilms and their spatiotemporal development. CLSM, combined with suitable fluorophores, allows the real-time investigation of intact hydrated microbial biofilms and the examination of the three-dimensional architecture of complex multispecies microbial biofilm known as dental plaque.
(Lawrence & Neu, 1999). However, these analyses require labeling bacterial cells with compatible fluorescent markers that enable time-resolved in situ observation. Visualizing specific bacteria and following their position and evolution inside a complex structure requires the use of specific labels. In the last decade, the green fluorescent protein gene (gfp) has emerged as the most useful reporter gene and live cell marker in both bacteria and higher organisms due to its bright clear fluorescence, which is detectable even in single cells (Chalfie et al., 1994; Errampalli et al., 1999). The use of the GFP protein and its variants in combination with CLSM has led to major insights into biofilm architecture and organization (Tolker-Nielsen et al., 2000; Drenkard & Ausubel, 2002; Hentzer et al., 2002; Klausen et al., 2003). A major problem with GFP as a reporter molecule is the requirement of oxygen for the proper maturation of the protein, making it inappropriate for anaerobic environments (Tsien, 1998).

For the facultative anaerobic bacterium S. gordonii, Hansen and colleagues reported the potential use of a variant of the GFP protein called GFPmut3* to visualize S. gordonii biofilms, even under anaerobic conditions. Hansen et al. (2001) demonstrated that, when S. gordonii DL1 expresses GFPmut3* in biofilms grown in flow cells under strict anaerobic conditions, fluorescence was quickly detectable by CLSM only after oxygen was supplied. These results suggest that GFPmut3* maturation in S. gordonii is not affected during biofilm development under anaerobic conditions and could be considered a useful tool for studying S. gordonii biofilms under anaerobic conditions. Nevertheless, the GFP protein has already been expressed in P. gingivalis, and Liu et al. (2000) were unable to detect under a microscope even after 4 h of oxygenation. The authors suspected that GFP was rapidly degraded in P. gingivalis. However, most laboratories working with anaerobic bacteria use fluorochromes such as the general nucleic acid stain SYTO, which can label, without the fixation step, the DNA of most living bacteria (Neu et al., 2000). The use of the SNAP-tag is especially appealing because the SNAP-tag, an oxygen-independent enzyme, becomes specifically and covalently labeled when exposed to synthetic fluorophores presented in the suitable form of a benzyguanine substrate (Regoes & Hehl, 2005). These SNAP-tag substrates are cell-permeable and allow live-cell labeling and imaging. Until recently, the SNAP-tag system was developed only for the specific labeling of fusion proteins (Keppler et al., 2006), and no studies exist on the use of the SNAP-tag for the unique objective of staining bacteria.

The aims of the present study were to develop fluorescently labeled P. gingivalis expressing an SNAP-tag without any fusion protein and to test the strain in mono- and multispecies biofilms with S. gordonii-GFPmut3* after anaerobic and flowing conditions. This tool will be useful for in vitro and in situ studies of P. gingivalis biofilm formation and progression in a complex multispecies community like that of the oral flora.

Materials and methods

Bacterial strains and growth conditions

Porphyromonas gingivalis ATCC 33277 and S. gordonii DL1 pCM18 (GFPmut3*/gfpΔmut3*), a gift from S. Molin (Molecular Microbial Ecology Group, Technical University of Denmark), were grown on blood Columbia agar plates and/or in a brain–heart infusion broth (BHI) (AES Chemunex, Combourg, France) supplemented with menadione (10−2 g L−1) and hemin (5 × 10−2 g L−1) (Sigma, Saint Quentin Fallavier, France). Porphyromonas gingivalis was incubated at 37 °C in an anaerobic chamber (MAC 500B) with a 10% H2, 10% CO2, and 80% N2 atmosphere. Streptococcus gordonii was cultivated at 37 °C under aerobic conditions. Erythromycin (5 μg mL−1) (Sigma) was added to experiments in which a selection pressure was applied. Escherichia coli JM109 and HB101 competent cells (Promega, Charbonnières, France) were grown at 37 °C in Luria–Bertani broth.

Construction of P. gingivalis strains expressing the SNAP26 protein

For SNAP26 protein expression, SNAP26b was cloned under the control of the P. gingivalis endogenous trxB promoter. The SNAP-Cell Starter Kit (Ozyme, Saint Quentin Yvelines, France) contains the bacterial expression plasmid pSNAP-tag® (T7) encoding SNAP26b.

A vector expressing the SNAP26 protein was constructed in E. coli by cloning SNAP26b (Covalys Biosciences AG) in a shuttle vector that replicates in both E. coli and P. gingivalis. The resulting plasmid, pYPK028ErmF-SNAP26b, was then introduced into P. gingivalis.

The tetracycline resistance cassette of the pYPK028 plasmid (Kumagai et al., 2003) was replaced by an erythromycin resistance cassette (ErmF) in order to construct the pYPK028ErmF plasmid. The tetB gene was removed from the pYPK028 plasmid using restriction enzymes SmaI and Sphl. The ErmF cassette was amplified from the pYHF1 plasmid (Takahashi et al., 1999) by PCR using primers containing the SmaI and Sphl restriction sites (Table 1). The PCR product was then digested with SmaI/Sphl and cloned into...
the SmaI/SphI-digested pYPK028 plasmid to obtain the pYPK028ErmF plasmid (Fig. 1).

SNAP26b (Ozyme) was placed under the control of the trxB promoter of P. gingivalis (C0 to C1) in the E. coli plasmid pBlueScript s II Phagemid Vector (pSK) (Stratagene, Lyon, France).

The trxB gene corresponds to PGN_1232 (GeneID 6330860 (location: 1376369–1377310). The promoter region of trxB (location: 1375869–1376368) was amplified using the oligos 5protrxB-XhoI and 3protrxB-EcoRI, and the terminator region of trxB (location: 1377311–1377811) was amplified using the oligos 5termtrxB-BamHI and 3termtrxB-XbaI. SNAP26b was amplified using the oligos 5SNAP26-EcoRI and 3SNAP26-BamHI. The pSK-protrxB_SNAP26 termtrxB construction was then amplified using the oligos 5protrxB-SphI and 3termtrxB-SphI, digested with SphI, and cloned into the SphI-digested and dephosphorylated pYPK028ErmF plasmid. The resulting plasmid, pYPK028ErmF-protrxB_SNAP26_termtrxB, was constructed as shown in Fig. 1, prepared in large quantities using the Qiagen plasmid midi kit (Qiagen, Courtaboeuf, France), and used for transforming P. gingivalis.

SNAP-tag labeling
SNAP-Cell 505 (green BG-505) and SNAP-Cell TMR Star (red TMR-Star) substrates (Ozyme) were dissolved in dimethyl sulfoxide according to the manufacturer’s instructions and diluted in a growth medium to a final working concentration of 5 and 3 μM, respectively. Porphyromonas gingivalis cells were incubated with SNAP-Cell 505 or SNAP-Cell TMR Star solutions at 37°C for 30–60 min. After labeling, cells were washed and suspended in BHI for confocal microscopy.

A nonfluorescent-negative control blocking agent (10 μM SNAP-Cell Block), which interacts with the SNAP-tag protein, was included in the experiments.

Biofilm formation in the flow-cell system
A mounted flow-cell chamber was assembled with glass cover-slips (Ludin Chambers Life Imaging Services, Switzerland) (750-μL volume) and was connected to a peristaltic pump (flow rate 7 mL h⁻¹) that pulled fresh medium through the system and evacuated liquid to a waste container through a silicone tubing. The flow-cell system was placed at 37°C under anaerobic conditions and coated with 10 mL of sterile human saliva. The flow cell was inoculated by flowing the system with a SNAP-Cell TMR Star-labeled P. gingivalis-SNAP26 culture (OD₆₀₀nm 0.1) for 4 h and

<table>
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<th>Genes</th>
<th>Primer names</th>
<th>Primer sequences (5‘–3‘)*</th>
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<tr>
<td>ermF</td>
<td>5ermF-SphI</td>
<td>GGGGGCATATGACATAAGAAATTCCT¹</td>
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<td></td>
<td>3ermF-SmaI</td>
<td>GGGGGGCCC GGGCTAGGAAGGATGAAATTTTC¹</td>
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<td></td>
<td>5protrxB-XhoI</td>
<td>GCCCTCGAGAAAGACCAATTGTACGCC¹</td>
</tr>
<tr>
<td></td>
<td>3protrxB-EcoRI</td>
<td>GGGCCAAATCACTGCTGTTTTGTTTTCG¹</td>
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<tr>
<td>termtrxB</td>
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<td></td>
<td>3termtrxB-XbaI</td>
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<tr>
<td>SNAP26</td>
<td>5SNAP26-EcoRI</td>
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<td>3SNAP26-BamHI</td>
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<td>protrxB-SNAP26-termtrxB</td>
<td>5protrxB-SphI</td>
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<td></td>
<td>3termtrxB-SphI</td>
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*Underlined sequences represent restriction enzyme sites.
¹SphI.
²SmaI.
²²XhoI.
²²²EcoRI.
²²²²BamHI.
²²²²²²XbaI.

Electroporation of P. gingivalis
The electroporation of P. gingivalis cells was performed as described previously (Belanger et al., 2007). Briefly, P. gingivalis cells were made competent by washing them with cold electroporation buffer (10% glycerol, 1 mM MgCl₂) and then concentrated 100 times. The electroporation was carried out with 5 mg of plasmid DNA for 100 μL of competent cells. The cells were pulsed using an electroporator at 2.5 kV/201 Ω/5 ms/25 μF (easyject®, Equibio), added to 450 μL of BHI, and incubated for approximately 12 h. The cells were plated on a blood Columbia solid medium containing 5 μg mL⁻¹ erythromycin and incubated anaerobically at 37°C. Erythromycin-resistant colonies were detected after a 7-day incubation period. Approximately 1–5 clones were obtained per microgram of DNA.

Table 1. Primer sequences

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pulling fresh diluted BHI containing erythromycin for an additional 20 h to allow a biofilm to form.

The bispecies S. gordonii/P. gingivalis biofilm was generated using a flow-cell system as described above by simultaneously inoculating the Ludin chamber with S. gordonii-GFPmut3/C3 (OD600 nm 0.02) and SNAP-Cell TMR Star-labeled P. gingivalis-SNAP26 (OD600 nm 0.1). Biofilm development was monitored at 4 and 24 h using CLSM under aerobic conditions.

CLSM observation was carried out using a CLSM (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany) equipped with an inverted microscope (Fluorescence Microscopy Platform, IFR 140 GFAS, Université de Rennes 1). An HC PL Apo 63X, 1.4 NA, oil immersion objective lens was used for image capture and a ×2 numerical zoom was applied. Microscope piloting and image acquisition were carried out using LEICA software (Confocal Software 3D).

SNAP-Cell 505 (green BG-505) has an excitation maximum at 504 nm (blue laser Ar, 488 nm) and an emission maximum at 532 nm. SNAP-Cell TMR Star (red TMR-Star) has an excitation maximum at 554 nm (diode laser, 561 nm) and an emission maximum at 580 nm. GFPmut3 has an excitation maximum at 490 nm (blue laser Ar, 488 nm) and an emission maximum at 511 nm.

Biofilm stacks with an area of $119 \times 119 \mu m^2$ were scanned. Images were acquired at 0.4-μm z-intervals from the bottom to the top of the biofilm and image averaging was carried out. The number of images in each stack varied according to the thickness of the biofilm. A series of fluorescent optical x–y sections were collected to create digitally reconstructed images ($z$-projection of $x$–$y$ sections) of the communities using IMAGEJ V1.34s (National Institutes of Health).

Results

The SNAP26b gene was cloned under the control of a constitutive promoter of P. gingivalis and placed in a vector able to replicate in P. gingivalis. Thus, we investigated the level of SNAP26 protein expression and visualized P. gingivalis in a biofilm in the presence of an SNAP-Cell substrate using CLSM.

Expression of SNAP26b in P. gingivalis

The pYPK028ErmF-protrxB_SNAP26b vector was stably maintained in P. gingivalis.

To test the expression of SNAP26b and its functional gene product in P. gingivalis, we observed grown P. gingivalis cells carrying an SNAP26b-tagged plasmid and labeled with green BG-505 or red TMR-Star. The microscopic observations revealed green or red fluorescent cells when excited with the 488-nm Ar or the 561-nm diode laser, respectively. The SNAP26 protein was distributed in a uniform and stable form throughout the bacterial cell. Porphyromonas gingivalis-SNAP26 fluoresced brightly (Fig. 2a and b), whereas wild-type bacteria (nontransfected control cells) stained with BG-505 or TMR-Star exhibited no signal (Fig. 2c). The results demonstrated a specific labeling of P. gingivalis and the absence of endogenous hAGTm in these bacterial cells. The viability and morphology of cells treated with BG-505 or TMR-Star did not change compared with wild-type cells (data not shown). The specificity of SNAP-tag labeling in living cells was shown by introducing a nonfluorescent...
SNAP-tag substrate (SNAP-Cell Block) that inhibited the labeling of *P. gingivalis*-SNAP26 with TMR-Star (Fig. 2d).

Our observations also revealed that the bacteria tolerated extended excitation without photobleaching, demonstrating the photostability of these new fluorophores.

Biofilm analysis using fluorescent labels

A *P. gingivalis* biofilm expressing SNAP26 labeled with TMR-Star in continuous-culture flow cells was observed by CLSM. During the two phases of biofilm development (4–24 h), only single cells and small bacterial aggregates were observed by CLSM. The red fluorescence of *P. gingivalis*-SNAP26 in the biofilm was homogeneously distributed throughout the monospecies biofilm and provided excellent CLSM fluorescence recordings throughout the course of biofilm development, up to 24 h of incubation.

The surface coverage and microcolony distribution of *P. gingivalis*-SNAP26 and *S. gordonii*-GFPmut3* in the entire biofilm at 4 and 24 h are shown in Fig. 3. In the bispecies biofilm of *P. gingivalis*-SNAP26 and *S. gordonii*-GFPmut3*, bacterial cells emitted their specific fluorescence, but not both, when the TMR-Star and GFP images were merged. Thus, the use of GFPmut3* and SNAP-tag markers allows for the specific visualization of two distinct bacterial species forming the biofilm constructed under anaerobic conditions (Fig. 3).

Discussion

In biofilm research, the ability to monitor biofilms in real time is quite advantageous compared with other characterization techniques, which are often destructive and time consuming. In a laboratory setup, this can be conveniently achieved using flow cells and CLSM. In fact, a combination of flow cells and CLSM has allowed for the acquisition of new insights into the development of the three-dimensional architecture of biofilms (Palmer & Sternberg, 1999; Heydorn *et al.*, 2002). Flow cells are simple and inexpensive tools to study biofilm growth under varying conditions, such as flow, substrate loading, and species interactions (Palmer & Sternberg, 1999; Christensen *et al.*, 2002; Purevdorj *et al.*, 2002). Flow cells observed using CLSM allow the monitoring of biofilm growth if used in conjunction with a reporter molecule, such as GFP, for bacterial labeling. Nevertheless, the requirement of oxygen for fluorescence excludes the use of GFP in strict anaerobic bacteria and in *P. gingivalis*, where it has been shown that GFP was not detectable (Liu *et al.*, 2000). Thus, our study aimed to test the functionality of the SNAP-tag protein adapted for anaerobic conditions (Regoes & Hehl, 2005) in this bacterium.

To demonstrate the feasibility of labeling strict anaerobic periodontopathogens with the SNAP-tag, we constructed a replicative plasmid encoding the SNAP26 protein under the control of the trxB promoter of *P. gingivalis*. We observed that the expression of this vector does not deteriorate the growth of *P. gingivalis*. In the presence of a permeable substrate (TMR-Star or BG-505 fluorophores), the SNAP-tag enzyme clearly revealed *P. gingivalis* cells carrying the plasmid and stained specifically red or green. The advantage of this tool is that a single construct can be used with different dye substrates to label with multiple colors without the need for new manipulations. In contrast, proteins from the GFP family require separate cloning and expression for each color. The SNAP-tag forms a highly stable, covalent thioether bond with fluorophores or other substituted groups on the benzylguanine substrate (Tirat *et al.*, 2006). This reaction is highly specific in live cells without significant cell toxicity. Moreover, the trxB promoter was also proven to be sufficiently strong in allowing specific labeling of *P. gingivalis* through a significant level of SNAP26 expression.

The combination of both tags, the GFPmut3*/SNAP-tag in a bispecies *S. gordonii/P. gingivalis* biofilm, allowed the specific visualization of these two bacterial species in the same biofilm. The results showed bacteria carrying GFP easily distinguished from those bearing the SNAP-tag. The signals were easily discriminated and, therefore, their
combination is suitable for double-fluorescence labeling experiments. The present study found that GFP conditions are compatible with SNAP-tag labeling in vitro. Although the SNAP-tag technology is complementary to the GFP technique, there are several applications in which the SNAP-self-labeling technology is advantageous. In contrast to GFP, the fluorescence of the SNAP-tag can be readily turned on with the addition of a variety of fluorescent probes directly to the culture media. A labeled SNAP-tag is very stable and retains signal intensity, in contrast to some GFP spectral variants.

SNAP-tag fusion proteins were expressed previously in E. coli, yeast, and mammalian cells (Keppler et al., 2003; Kindermann et al., 2003). However, this enzyme has never been used alone in labeling bacteria and visualizing in biofilm. This study is the first to use the SNAP-tag alone without a tagged host protein. Unlike oxygen-dependent autofluorescent proteins, such as GFPs, this innovative technology is perfectly adapted to anaerobic periodontopathogens such as P. gingivalis. The SNAP-tag displays robust properties for monitoring flow-cell anaerobic biofilm growth and the precise localization of bacteria in biofilms. This study is also the first in which SNAP-tag has been cloned, expressed, and successfully labeled with several specific fluorophores in P. gingivalis. This new tag will have a significant impact on the specific and selective labeling of anaerobic bacteria in biofilm research. The SNAP-tag could be a valuable and highly efficient tool to study in situ bacterial interactions and the regulation of virulence factor expression inside the biofilm.

Finally, this tool could also be used in other applications to characterize host–bacteria interactions, specifically bacterial localization within the different cellular compartments during phagocytosis or the mechanisms of bacterial migration and tissue invasion.

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Fig. 3. CLSM z-projections of the bispecies biofilm formed by Streptococcus gordonii DL1 (GFPmut3*) (green) (a and d) with Porphyromonas gingivalis ATCC 33277 (SNAP26) stained with TMR-Star (red) (b and e) after 4 h (a–c) and 24 h (d–f). (c and f) The merged images of the two species for the S. gordonii-GFPmut3*/P. gingivalis-SNAP26 biofilm. Scale bars = 10 μm.
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O.N. and A.R. contributed equally to this work.
Authors'contribution
O.N. and A.R. contributed equally to this work.

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**New tool for labeling live Porphyromonas gingivalis**

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