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Transcriptional analysis of mutacin I (*mutA*) gene expression in planktonic and biofilm cells of *Streptococcus mutans* using fluorescent protein and glucuronidase reporters

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Streptococcus mutans is implicated as the primary pathogen involved in the development of dental caries. The production of specific bacteriocins (called mutacins) by *S. mutans* is one of the major virulence factors which facilitate the dominance of the bacterium within dental plaque. While much has been revealed about the biochemical structures of mutacins, little is known about the expression and regulation of mutacin genes, largely due to the lack of proper methods to monitor mutacin gene expression, especially under biofilm conditions. In this study, a set of reporter systems with the green fluorescent protein (*gfp*), the monomeric red fluorescent protein (*mrfp*1), and the glucuronidase (*gusA*) are introduced to *S. mutans* to study the transcriptional activities of the mutacin I gene (*mutA*). Although the *mutA*-reporter fusions are in single copy on the chromosome, these reporter systems display strong signals that allow us to effectively monitor *mutA* gene expression in *S. mutans*. Using these reporter systems, we show that *mutA* is expressed in both planktonic and biofilm cells, even though mutacin activities are normally detected only in biofilm cells. Furthermore, we confirm that *mutR*, the gene upstream of the mutacin operon, is required for mutacin I gene expression. The success of this study validates the feasibility of using these reporter systems to study gene expression and regulation in *S. mutans*.

Key words: Gfp; GusA; mRfp; mutacin; reporter genes; *Streptococcus mutans*

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The oral bacterium *Streptococcus mutans* is regarded as the principal etiologic agent in the development of dental caries (14). In the oral cavity, *S. mutans* resides in a multispecies biofilm, the dental plaque, which is composed of over 400 bacterial species (16). *S. mutans* exists as part of the typical oral flora within the biofilm community; however, it becomes predominant in severe caries lesions. One of the major virulence factors contributing to this ecologic switch are bacteriocins (mutacins) produced by *S. mutans* for defensive and/or offensive purposes (17). Despite the extensive studies on the biochemical structures of these mutacin molecules, little is known about the regulatory mechanisms for their production. Our previous studies demonstrated that mutacin I was produced by cells grown in a biofilm or in large aggregates, but not by planktonic cells grown in liquid (22), indicating that mutacin production may be regulated. However, such a hypothesis could not be

effectively tested due to the lack of proper tools to effectively study mutacin production at transcriptional or post-transcriptional levels. This study aims to develop reporter systems to analyze mutacin production at the transcriptional level.

Reporter gene fusions have been routinely used to study gene expression in prokaryotic and eukaryotic organisms. Examples of reporter genes include the *Escherichia coli* β-galactosidase (*lacZ*), which is the most widely used reporter gene in microorganisms (27), the chloramphenicol acetyl transferase (*cat*) (13), the β-glucuronidase (*gusA*) (19), the firefly luciferase (*luc*) (11), and the jelly fish green fluorescent protein (*gfp*) (5), which is commonly used for visualization of gene expression and localization of proteins in live cells (5, 6). Although widely used in other bacteria, some of these reporter systems have not been successfully adapted for utilization in *S. mutans*. For example, *lacZ* has been utilized only to a limited extent for studies in *S. mutans*, and there is currently only one report of using *gfp* delivered via a multiple copy shuttle plasmid (8–10, 25, 29). To develop the capacity to effectively and accurately monitor mutacin gene expression on the chromosome, in this study, we constructed a set of reporter systems with *gfp*, the monomeric red fluorescent protein gene (*mrfp1*) (4), and *gusA* (19) to monitor *mutA* gene expression.

Material and methods

Bacterial strains and media

E. coli strain Top10 (Invitrogen; Carlsbad, CA) was used for cloning as well as plasmid amplifications. The bacteria were grown on Luria-Bertani broth (LB, Difco, Sparks, MD) or LB agar aerobically at 37°C. Transformants were selected on LB medium with ampicillin (100 μg/ml) or spectinomycin (100 μg/ml). *S. mutans* strain UA140 was routinely cultured in brain heart infusion (BHI, Difco) or BHI agar at 37°C under anaerobic conditions. BHI medium supplemented with spectinomycin (800 μg/ml) was used for the selection of transformants carrying the reporter gene plasmids. For the indication of β-glucuronidase activity in *S. mutans*, BHI plates were supplemented with 0.30 mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-glucoronide (X-glcA) (Sigma; St. Louis, MO).

DNA manipulations

Restriction enzymes were obtained from Promega (Madison, WI), Fisher (Pittsburgh, PA) or NEB (Beverly, MA). Ligations were performed using the Quick Ligation™ Kit from NEB. Polymerase chain reaction (PCR) products were cloned into the TOPO TA CLONING® Kit from Invitrogen. All plasmids were extracted and purified from *E. coli* with the QIAGEN (Valencia, CA) Miniprep Kit. DNA extracted from agarose gels (1%) was purified with the QIAGEN QIAquick® Gel Extraction Kit. DNA transformation into competent *E. coli* was performed following routine methods (24). Transformation of *S. mutans* was done as described previously (26). PCR was performed with a MyCycler™ thermocycler (Bio-Rad; Hercules, CA) using a protocol supplied with the thermocycler. Taq-DNA Polymerase was obtained from Promega and dNTPs from Fisher.

Construction of the reporter gene fusions

The backbone vector for the construction of all reporter gene fusions was pFW5 (20), which contains a spectinomycin resistance marker (gene *aad9*) that works in both gram-negative and gram-positive bacteria (20). To construct the *gfp* gene fusion, the *gfp-mut2* gene with its own ribosome binding site was excised from vector pKEN (7) with *Bam*HI and *Hin*dIII, and inserted into pFW5 at the *Bam*HI and *Hin*dIII sites (Fig. 1) to generate plasmid pFW5::*gfp*. To construct the *gusA* gene reporter, the *gusA* gene was excised from plasmid pLB85 (3) with *Hin*dIII and *Sal*I and cloned into pFW5 at the *Hin*dIII and *Xho*I sites, which was compatible with *Sal*I, to generate plasmid pFW5::*gusA*.

To insert the *mutA* promoter in front of the reporter genes, the *mutA* promoter region was amplified by PCR from chromosomal DNA of strain UA140 (21), using primers mutApF (5′-GAATTAG GTGAACTTTATCGAG-3′) and IAR1 (5′-ggggaTCCTCCTTTTTCATGTGTGT AAC-3′). Primer IAR1 had a *Bam*HI site incorporated at its 5′ end (underlined sequence). The PCR product was cloned into pCR®2.1-Topo® (Invitrogen) to generate pCR2.1::*mutAp*. The plasmid pCR2.1::*mutAp* was digested with *Bam*HI and *Eco*RV, and the DNA fragment containing the *mutA* promoter was purified from an agarose gel. The *mutA* promoter region was then inserted upstream of the reporter genes, *gfp* and *gusA*, at the *Bam*HI and an upstream *Stu*I site to form plasmid pFW5ϕ (*mutAp-gfp*) and pFW5ϕ (*mutAp-gusA*), respectively.

For the construction of the monomeric red fluorescent protein reporter pFW5ϕ (*mutApo-mrfp1*), a three-piece ligation strategy was used because of naturally occurring restriction sites for *Hin*dIII in the *mutA* promoter region and *Bam*HI in the *mrfp1* gene. The *mrfp1* gene was amplified by PCR from vector pRSET$_B$-*mrfp1* (kindly provided by R.Y. Tsien) (4) using primers pRSET-F-*Bgl*II (CAACGGTTT agaTCTAGAAATAATTTTG) and pRSET-R (AGCCAACTCAGCTTCCTTTC). Primer pRSET-F-*Bgl*II contained an artificial *Bgl*II site at its 5′ end (underlined sequence). The PCR product was cleaved with *Bgl*II and *Hin*dIII, which was present in the vector downstream of the *mrfp1* coding region. The *Bgl*II and *Hin*dIII-digested DNA fragment containing the *mrfp1* gene was then ligated with *Eco*RV and *Bam*HI-digested DNA containing the *mutA* promoter region and the pFW5 vector restricted with *Stu*I and *Hin*dIII. The ligation mixture was transformed into *E. coli* and the plasmid containing the three pieces in the right order was confirmed and named pFW5ϕ (*mutAp-mrfp*1).

To construct strains expressing the reporter gene fusions, strain UA140 was transformed with pFW5ϕ (*mutAp-gfp*), pFW5ϕ (*mutAp-gusA*), and pFW5ϕ (*mutAp-mrfp*1). Transformants were selected on BHI plates supplemented with

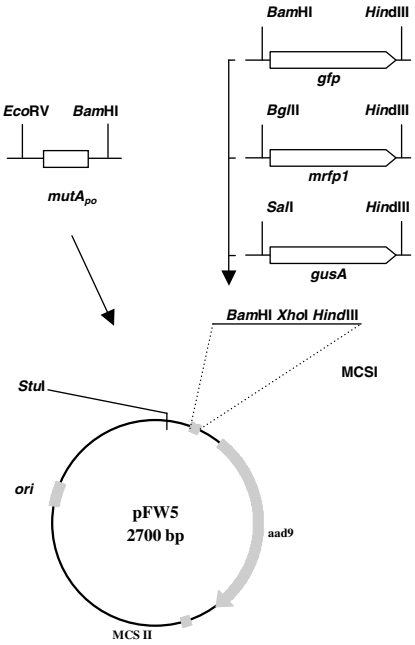

Fig. 1. Construction of reporter gene plasmids pFW5ϕ (*mutAp-gfp*), pFW5ϕ (*mutAp-mrfp1*) and pFW5::ϕ (*mutAp-gusA*). The cloning sites for *gfp* are *Bam*HI and *Hin*dIII, for *gusA* are *Xho*I and *Hin*dIII, and for *mrfp1* are *Bgl*II and *Hin*dIII. The cloning sites for the *mutA* promoter region are *Eco*RV and *Bam*HI. MCS, multiple cloning site; *ori*, origin of replication; *aad9*, spectinomycin resistance cassette.

800 μg/ml spectinomycin. Confirmed reporter strains had both reporter gene activity as well as wild-type levels of mutacin production.

Biofilm growth and fluorescence microscopy

S. mutans biofilms were grown in BHI medium with 0.05% sucrose for 2 days on a microscope cover slide inside a Petri dish as a static culture. Fresh medium was added after 24 h of incubation. Mature biofilms were exposed to air for 5–10 min at room temperature to promote folding of the fluorophore of *gfp* and *mrfp1* and then washed with PBS buffer. The biofilms were visualized using fluorescent microscopy (Nikon ECLIPSE E400 microscope) with appropriate filters for Gfp and mRfp fluorescence. Confocal laser scanning microscopy (CLSM) was performed using LSM 5 PASCAL with LSM 5 PASCAL software (Carl Zeiss). The microscope was equipped with detectors and filter sets for monitoring of Gfp and mRfp fluorescence. Images were obtained with a 63 × 1.4 Plan-Neofluar oil objective.

β-glucuronidase activity assay

Cells in late exponential growth phase were collected and resuspended in test buffer (50 mM $NaPO_4$, pH 7). The cells were adjusted to an $Abs._{600}$ of 0.5, and 0.5 ml of the cell suspension was mixed with 10 μl of toluene for 60 s by vortexing. The reaction tube was allowed to stand for 5 min at 37°C, and the reaction was initiated with the addition of 50 μl 4-nitrophenyl-β-1, 4-glucuronide (β-NPG) to a final concentration of 2 mM. The reaction was terminated with the addition of 0.7 ml sodium carbonate (400 mM) after a clear, yellow color appeared (150 min to 180 min). The reaction product (4-nitrophenol) was detected by its absorption at 405 nm (molar extinction $\epsilon = 18{,}500$ per M per cm). The enzyme activity was calculated as nmol per minute per $Abs._{600}$ according to the equation adapted from (12).

Results

Construction of *mutAp-gfp* and *mutAp-mrfp1* gene fusions

For promoter activity and gene regulation studies, it is preferable to use reporter genes fused to the promoter as a single copy on the chromosome, because gene dosage effects or plasmid supercoiling could complicate the result. To create single-copy *mutAp-gfp* and *mutAp-mrfp1* reporter gene fusions, we used the suicide vector pFW5 as a backbone vector. Vector pFW5 has a ColE1 *ori* for replication in *E. coli* but lacks an *ori* for replication in streptococci. In addition, it contains a constitutively expressed spectinomycin-resistance gene (*aad9*), which made it suitable for selection in both *E. coli* and *S. mutans* (20). The structural gene *gfp* with its ribosome binding site was cloned into pFW5, resulting in pFW5::*gfp* (see Material and methods). The *mutA* promoter region was then inserted in a multiple cloning site of the vector. The resulting plasmid was transformed into *S. mutans* and integrated into the chromosome via single crossover recombination conferred by the homologous promoter region (Fig. 1). The *mrfp* reporter plasmid was constructed via fusing the *mrfp1* gene with the *mutA* promoter (see Material and methods). The resulting plasmid was transformed into *S. mutans* to create the *mutAp-mrfp1* reporter strain. Both reporter strains were confirmed with Southern blotting or PCR (data not shown).

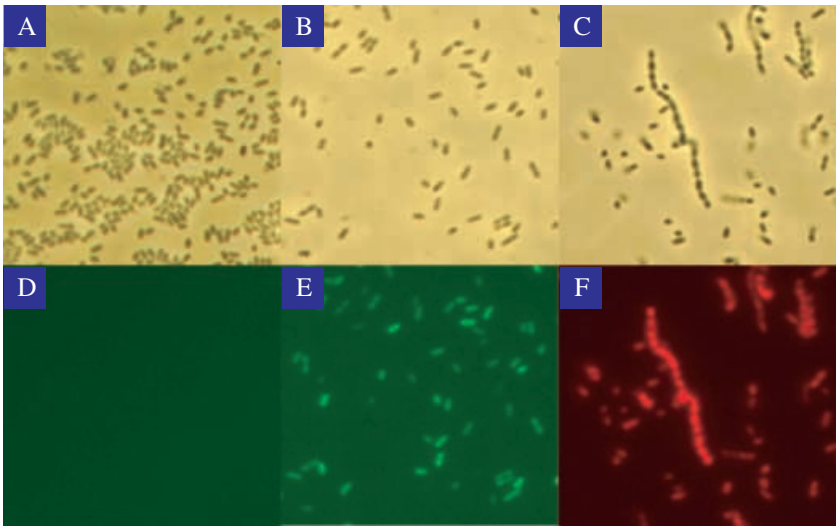

Fig. 2. Expression of the *mutAp-gfp* and *mutAp-mrfp1* fusions in single cells. A–C, phase-contrast photographs of UA140, UA140::ϕ (*mutAp-gfp*), and UA140::ϕ (*mutAp-mrfp1*) single cells. D–F, Fluorescent photographs of UA140, UA140::ϕ (*mutAp-gfp*), and UA140::ϕ (*mutAp-mrfp1*) single cells. Pictures were taken with a total magnification of 1000×.

Expression of *mutAp-gfp* and *mutAp-mrfp1* gene fusions in planktonic cells

Since mutacin I could not be produced in planktonic cells, we wanted to examine whether the mutacin promoter could be expressed under the same conditions. We tested both *mutAp-gfp* and *mutAp-mrfp1* reporter strains for *mutA* gene expression using fluorescent microscopy to see if single cells were fluorescent relative to their parental strain UA140. As shown in Fig. 2, after 24 h growth, single cells became fluorescent only in strains UA140::ϕ (*mutAp-gfp*) and UA140::ϕ (*mutAp-mrfp1*), but not in UA140. These results indicate that although the mutacin I activity is not detected in planktonic cells, the mutacin promoter is expressed under the same conditions. This result also demonstrates the utility of the *gfp* and *mrfp1* as a single copy reporter on the chromosome for monitoring gene expression in general.

Expression of *mutAp-gfp* and *mutAp-mrfp1* gene fusions in biofilm

To test if the mutacin promoter can be expressed in biofilms, strains of UA140::ϕ (*mutAp-gfp*) and UA140::ϕ (*mutAp-mrfp1*) were grown in BHI plus 0.05% sucrose as

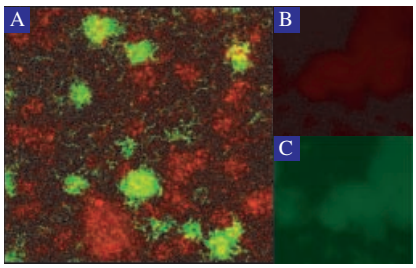

Fig. 3. Mixed culture biofilm with strains UA140::ϕ (*mutAp-gfp*) and UA140::ϕ (*mutAp-mrfp1*). A, CLSM image of biofilm development (see Material and methods section). Picture was taken with a total magnification of 630×. B and C, fluorescence microscopy pictures showing autofluorescence of a UA140 biofilm taken with filters for visualizing green and red fluorescence, respectively. Pictures were taken with a total magnification of 400×.

mixed cultures. The cultures were incubated aerobically as static cultures at 37°C for 2 days, and the formed biofilms were analyzed. As shown in Fig. 3, UA140::ϕ (*mutAp-gfp*) and UA140::ϕ (*mutAp-mrfp1*) displayed green and red fluorescent biofilms, respectively, where they grew as separate microcolonies, and orange fluorescent biofilms where the two strains were intermingled (Fig. 3, A). As a control, UA140 cells only showed weak autofluorescence (Fig. 3B, C). These results indicate that the mutacin promoter is effectively expressed in biofilm cells.

Quantification of the mutacin promoter activity by using the β-glucuronidase reporter

While the *gfp* and *mrfp* reporters are ideal for imaging mutacin gene expression in planktonic and biofilm cells, quantification of promoter activity using these reporters would be less accurate. For quantification of mutacin gene expression, we fused the mutacin promoter with the *gus*A reporter. After integrating into the chromosome of UA140, the fusion strain, UA140::ϕ (*mutAp-gusA*) was tested for β-glucuronidase activity by colorimetric assays with the substrate β-NPG. Both UA140 and UA140::ϕ (*mutAp-gusA*) were grown until late exponential phase, when the expression of the *mutA* gene reaches its maximum level (unpublished results), and GusA activities were measured. As shown in Table 1, UA140 exhibited only background activity (below 0.02 nmol/min per $Abs._{600}$), whereas UA140::ϕ (*mutAp-gusA*) displayed an activity of 0.86 nmol/min per $Abs._{600}$, a > 40-fold increase over the control strain. The standard deviation of two independent assays performed in triplicates did not exceed 10%, which made the assay suitable for quantification of promoter expression.

Using the *mutA-gusA* fusion for gene regulation studies

Glucuronidase (*gusA*) activity can not only be quantified by colorimetric assays, but also be analyzed for blue color colonies on a plate containing X-glcA, similar to experiments with *lacZ* fusions. This property makes *gusA* a very useful reporter for mutagenesis and gene regulation studies. To test the feasibility of this idea, we inactivated the *mutR* gene by insertional inactivation in strain UA140::ϕ (*mutAp-gusA*). The gene *mutR* is located upstream of the mutacin I operon, and is suggested to be involved in the regulation of mutacin operon expression (22). Both UA140::ϕ (*mutAp-gusA*) and UA140::ϕ (*mutAp-gusA*)*mutR* were grown as individual cultures on BHI plates supplemented with 0.30 mg/ml X-glcA, a blue chromogenic substrate for GusA. As shown in Fig. 4, UA140::ϕ (*mutAp-gusA*) produced blue colonies as expected, while UA140::ϕ (*mutAp-gusA*)*mutR* produced white colonies. This result confirmed that the *mutR* gene is indeed required for mutacin promoter activity. As expected, when UA140::ϕ (*mutAp-gusA*) and UA140::ϕ (*mutAp-gusA*)*mutR* were grown as mixed cultures, both blue and white colonies were easily distinguishable. This result indicates that it might be possible after mutagenesis to select for changes in promoter activity based on colony color changes when X-glcA is present in the medium.

Table 1. Expression of *mutA-gusA* gene fusion*

Strain	GusA-activity [a]
UA140	0.02 ± 0.01
UA140::ϕ (*mutA$_{po}$-gusA*)	0.86 ± 0.05

*The activities are given in nmol/min per $Abs._{600}$. [a]Values are the average of two independent experiments performed in triplicate.

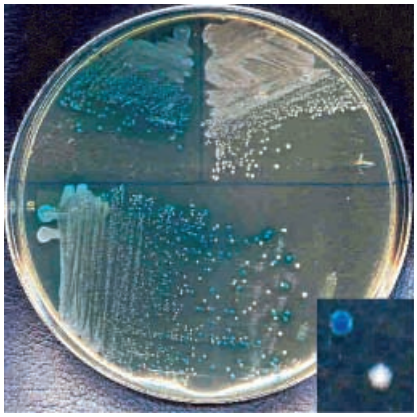

Fig. 4. β-glucuronidase activity monitored with X-glcA as a substrate. Single and mixed cultures of strain UA140::ϕ (*mutAp-gusA*) and UA140::ϕ (*mutAp-gusA*)*mutR* grown on a BHI plate supplemented with 0.30 mg/ml X-glcA. The insert shows two single colonies of UA140::ϕ (*mutAp-gusA*) (blue) and UA140::ϕ (*mutAp-gusA*)*mutR* (white).

Discussion

S. mutans is perhaps the most prevalent oral pathogen in humans, and mutacin production could play an important role in protecting *S. mutans* from competition from other oral streptococci. Despite its importance in the ecology of *S. mutans*, studies on detailed molecular mechanisms of mutacin gene regulation have been difficult due to the lack of proper gene reporter systems. Of particular difficulty is monitoring gene expression in biofilms in real time. Because biofilm formation plays such a vital role in the persistence and pathogenesis of *S. mutans*, understanding the spatial and temporal regulation of mutacin gene expression during biofilm formation will provide crucial insights into the pathogenesis mechanism of *S. mutans*. The reporter systems constructed during the present study made it possible, for the first time, to image mutacin gene expression on the chromosome as a single copy during *S. mutans* biofilm formation in real time.

The advantage of the reporter genes *gfp* and *mrfp1* is that their gene products are fluorescent without the need for substrates. Therefore, these fluorescent protein reporters are ideal for monitoring the temporal and spatial patterns of gene expression during biofilm formation of *S. mutans* in mono species or mixed species biofilms such as the dental plaque. A drawback with the fluorescent proteins, however, is their requirement for oxygen during the development of the fluorophore and its reduced fluorescence at low pH (18, 23, 28). For *Streptococcus gordonii* it was shown that the use of a flow chamber can resolve this problem, because such a system prevents the accumulation of lactic acid produced by *S. gordonii* (15). During our experiments, we found it sufficient to oxygenate the biofilm by exposing it to air at room temperature for several minutes, and to neutralize the acidic pH by replacing the medium with PBS while being exposed to air. Similar findings were also reported for *S. gordonii* (15).

During our biofilm analysis using the *gfp* and *mrfp1* reporters, we noticed uneven fluorescence in the biofilm; some areas were brighter than others (Fig. 3). This unequal distribution may be caused by several factors:

- unequal exposure to oxygen, which was required for chromogenesis of the Gfp and mRfp proteins (15);
- unequal pH distribution;
- different protein turnover rate in different staged cells;
- differential expression of the *mutA* promoter in different cells in the biofilm.

Further investigation with a flow cell chamber will help address the question concerning insufficient oxygen supply and low pH, and a fusion to a housekeeping gene promoter would be a good control to address the issue of regulation of the *mutA* promoter expression in the biofilm. The turnover rate for the fluorescent protein does not seem to be a problem, because previous studies by our group and other have shown

that the protein could remain fluorescent for 2 days after the cell growth is terminated regardless whether the cells are grown in planktonic culture or in biofilms (1).

It is also necessary to point out that *S. mutans* biofilms exhibit autofluorescence (Fig. 3B,C). This may be caused by the formation of exopolysaccharide (glucan), which is required for biofilm formation. While this autofluorescence did not obscure our result with the *mutA-gfp* and *mutA-mrfp* gene fusions, it may become problematic when the expression of the promoter is weak.

In addition to being used for image analysis during biofilm formation, the *gfp* and *mrfp1* reporters could also be used in gene regulation studies. For example, when used with a flow cytometer, these fluorescent reporters could be useful for selecting for mutations that diminish the promoter-reporter gene expression. There is one report describing using flow cytometry to analyze plasmid-borne *gtfB-gfp* reporter gene expression in *S. mutans* (29).

The *gusA* reporter would be ideal for selecting mutants based on blue–white color screening after random mutagenesis. This was nicely demonstrated by expressing the *mutAp-gusA* fusion in the *mutR* background, which formed white colonies due to the inactivation of transcription from the *mutA* promoter (Fig. 4). The enzymatic activity of the *β*-glucuronidase is not oxygen dependent, but oxygen is needed for chromogenesis with X-glcA as substrate (2). This problem could be solved by exposing the plates to air for a couple of hours. In addition to being used for mutant selection, the *gusA* reporter is also ideal for quantification studies, because the enzymatic assay is as easy and as accurate as the *β*-galactosidase assay (Table 1).

In summary, we have constructed a set of reporter systems in *S. mutans* for mutacin gene expression and regulation studies in planktonic as well as in biofilm cells. We have shown that these reporter systems, with the *mutA* promoter fusion, can be expressed effectively as a single copy on the chromosome. Results from this study not only have laid a solid foundation for further detailed molecular analysis of mutacin gene expression and regulation under different conditions, but also provide the tools for gene expression and imaging analysis of other genes in *S. mutans*.

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References

1. Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M, Molin S. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl Environ Microbiol 1998: **64**: 2240–2246.
2. Beneke S, Bestgen H, Klein A. Use of the *Escherichia coli uidA* gene as a reporter in *Methanococcus voltae* for the analysis of the regulatory function of the intergenic region between the operons encoding selenium-free hydrogenases. Mol Gen Genet 1995: **248**: 225–228.
3. Brondsted L, Hammer K. Use of the integration elements encoded by the temperate lactococcal bacteriophage TP901-1 to obtain chromosomal single-copy transcriptional fusions in *Lactococcus lactis*. Appl Environ Microbiol 1999: **65**: 752–758.
4. Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, Tsien RY. A monomeric red fluorescent protein. Proc Natl Acad Sci U S A 2002: **99**: 7877–7882.
5. Chalfie M. Green fluorescent protein. Photochem Photobiol 1995: **62**: 651–656.
6. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994: **263**: 802–805.
7. Cormack BP, Valdivia RH, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 1996: **173**: 33–38.
8. Cote CK, Honeyman AL. The LicT protein acts as both a positive and a negative regulator of loci within the *bgl* regulon of *Streptococcus mutans*. Microbiology 2003: **149**: 1333–1340.
9. Cote CK, Honeyman AL. The transcriptional regulation of the *Streptococcus mutans bgl* regulon. Oral Microbiol Immunol 2002: **17**: 1–8.
10. Cvitkovitch DG, Gutierrez JA, Behari J, et al. Tn*917*-lac mutagenesis of *Streptococcus mutans* to identify environmentally regulated genes. FEMS Microbiol Lett 2000: **182**: 149–154.
11. de Wet JR, Wood KV, Helinski DR, DeLuca M. Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. Proc Natl Acad Sci USA 1985: **82**: 7870–7873.
12. Gallagher R. GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression. New York: Academic Press, 1992.
13. Gorman CM, Moffat LF, Howard BH. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 1982: **2**: 1044–1051.
14. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980: **44**: 331–384.
15. Hansen MC, Palmer RJ, Udsen C, White DC, Molin S. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. Microbiology 2001: **147**: 1383–1391.
16. Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol 2000: **54**: 413–437.
17. Kuramitsu HK. Virulence properties of oral bacteria: impact of molecular biology. Curr Issues Mol Biol 2001: **3**: 35–36.
18. Miesenbock G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 1998: **394**: 192–195.
19. Novel M, Novel G. Regulation of beta-glucuronidase synthesis in *Escherichia coli* K-12: constitutive mutants specifically derepressed for *uidA* expression. J Bacteriol 1976: **127**: 406–417.
20. Podbielski A, Spellerberg B, Woischnik M, Pohl B, Lutticken R. Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). Gene 1996: **177**: 137–147.
21. Qi F, Chen P, Caufield PW. The group I strain of *Streptococcus mutans*, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. Appl Environ Microbiol 2001: **67**: 15–21.
22. Qi F, Chen P, Caufield PW. Purification and biochemical characterization of mutacin I from the group I strain of *Streptococcus mutans*, CH43, and genetic analysis of mutacin I biosynthesis genes. Appl Environ Microbiol 2000: **66**: 3221–3229.
23. Robey RB, Ruiz O, Santos AV, Ma J, Kear F, Wang LJ, Li CJ, Bernardo AA, Arruda JA. pH-dependent fluorescence of a heterologously expressed Aequorea green fluorescent protein mutant: in situ spectral characteristics and applicability to intracellular pH estimation. Biochemistry 1998: **37**: 9894–9901.
24. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratories, 1989.
25. Sato Y, Yamamoto Y, Suzuki R, Kizaki H, Kuramitsu HK. Construction of *scrA*: *lacZ* gene fusions to investigate regulation of the sucrose PTS of *Streptococcus mutans*. FEMS Microbiol Lett 1991: **63**: 339–345.
26. Shah GR, Caufield PW. Enhanced transformation of *Streptococcus mutans* by modifications in culture conditions. Anal Biochem 1993: **214**: 343–346.
27. Shuman HA, Silhavy TJ. Microbial genetics: The art and design of genetic screens: *Escherichia coli*. Nat Rev Genet 2003: **4**: 419–431.
28. Verkhusha VV, Akovbian NA, Efremenko EN, Varfolomeyev SD, Vrzheshch PV. Kinetic analysis of maturation and denaturation of DsRed, a coral-derived red fluorescent protein. Biochemistry (Mosc) 2001: **66**: 1342–1351.
29. Yoshida A, Kuramitsu HK. *Streptococcus mutans* biofilm formation: utilization of a *gtfB* promoter-green fluorescent protein (*PgtfB*: *gfp*) construct to monitor development. Microbiology 2002: **148**: 3385–3394.