

# Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration

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**Use of green fluorescent protein (GFP) as a molecular reporter is restricted by several environmental factors, such as its requirement for oxygen in the development of the fluorophore, and its poor fluorescence at low pH. There are conflicting data on these limitations, however, and systematic studies to assess the importance of these factors for growing bacterial cultures are lacking. In the present study, homogeneous expression of the *gfpmut3\** gene directed by a synthetic constitutive lactococcal promoter was demonstrated in batch cultures and in biofilms of *Streptococcus gordonii* DL1. A lower limit of oxygen concentration for maturation of the GFP fluorophore was determined: fluorescence was emitted at 0.1 p.p.m. dissolved oxygen (in conventionally prepared anaerobic media lacking reducing agents), whereas no fluorescence was detected in the presence of 0.025 p.p.m. dissolved oxygen (obtained by addition of L-cysteine as reducing agent). When an anaerobically grown (non-fluorescent) >50 µm thick biofilm was shifted to aerobic conditions, fluorescence could be detected within 4 min, reaching a maximum over the next 16 min. It was not possible to detect any fluorescence gradients (lateral or vertical) within the >50 µm thick biofilm, and fluorescence development after the shift to aerobic conditions occurred throughout the biofilm (even at the substratum). This suggests that oxygen gradients, which might result in reduced GFP fluorescence, did not exist in the >50 µm thick biofilm of this organism. Production of lactic acid and the subsequent acidification in batch cultures of *S. gordonii* DL1 led to a decrease in fluorescence intensity. However, severe pH reduction was prevented when the bacterium was grown as a biofilm in a flowcell, and a homogeneous distribution of a strong fluorescence signal was observed. These findings show that GFP can be applied to studies of oxygen-tolerant anaerobic bacteria, that densely packed, flowcell-grown biofilms of *S. gordonii* do not develop oxygen gradients inhibitory to GFP fluorescence development, and that the often transient nature of GFP fluorescence in acid-producing bacteria can be overcome in flowcells, probably by the elimination of metabolic by-product accumulation.**

Keywords: Gram-positive bacteria, biofilms, green fluorescent protein tagging, anaerobic growth

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Abbreviation: GFP, green fluorescent protein.

## INTRODUCTION

Green fluorescent protein, GFP, has been widely used for clonal tagging or as a reporter for specific gene expression in both bacteria and higher organisms. Autocatalytic formation of the fluorophore in the polypeptide chain, along with the advantages of fluorescence in advanced microscopy and image analysis, have driven the rapid development of *in situ* methods for investigations of cellular activity in complex scenarios such as mixed-species biofilm communities. In microbiology, by far the greatest impact of GFP has been in studies of Gram-negative bacteria, partly due to the availability of a broad range of expression systems derived from these bacteria, and partly because the majority of *in situ* studies so far have been concerned with Proteobacteria. Fewer reports have described the applications of GFP to analyses of Gram-positive bacterial (e.g. Bubert *et al.*, 1999; Fernández de Palencia *et al.*, 2000; Freitag & Jacobs, 1999; Geoffroy *et al.*, 2000; Lewis & Errington, 1996; Lewis & Marston, 1999; Scott *et al.*, 1998).

The GFP fluorophore has been characterized in detail. It consists of a *p*-hydroxybenzylideneimidazolinone formed autocatalytically by cyclization and oxidation of the Ser-Tyr-Gly sequence at positions 65–67 with a time constant of approximately 2–4 h at 22 °C and atmospheric pO<sub>2</sub> (Heim *et al.*, 1994, 1995). The final step in protein maturation is dehydrogenation by molecular oxygen of residue 66 (Cubitt *et al.*, 1995; Heim *et al.*, 1994; Reid & Flynn, 1997). In order to enhance the fluorescence of GFP, to broaden the useful pH and temperature ranges of the protein, to increase the rate of fluorophore formation, and to shift wavelengths of excitation and emission, several mutations of the protein have been introduced (Cormack *et al.*, 1996; Cramer *et al.*, 1996; Delagrave *et al.*, 1995; Ehrig *et al.*, 1995; Elsliger *et al.*, 1999; Heim *et al.*, 1994; Heim & Tsien, 1996; Kimata *et al.*, 1997; Patterson *et al.*, 1997; Siemering *et al.*, 1996; Ward, 1998). One variant (Gfpmut3b) with two mutations in the chromophore region (S65G, S72A) fluoresces 21 times more intensely than wtGfp, and was observed to mature with a time constant of approximately 45 min (Cormack *et al.*, 1996). Throughout the present study, a variant of this protein (Gfpmut3\*) was used, which has an additional mutation at position 2 (S2R) that permits introduction of a *SphI* site in the gene (Andersen *et al.*, 1998).

Many Gram-positive bacteria, which are interesting in relation to industrial applications or as human and animal pathogens, are fermentative and microaerophilic. It has generally been assumed that GFP was less useful as a reporter in these organisms because of the requirement for oxygen in fluorophore formation (Cody *et al.*, 1993; Cubitt *et al.*, 1995; Heim *et al.*, 1994; Prasher *et al.*, 1992; Reid & Flynn, 1997). In addition, there are fewer handy genetic tools available for Gram-positive bacteria compared to those designed for enterics and other Proteobacteria. It is therefore understandable that

the current literature concerning GFP as a reporter in, for example, streptococci and related bacteria is quite limited. Besides the oxygen requirements, the actual rate of oxidation, and the stability of GFP under different conditions, are uncertain (Cormack *et al.*, 1996; Heim *et al.*, 1994; Reid & Flynn, 1997). The design of standardized GFP test plasmids (a broad-host-range shuttle vector carrying the *gfp* gene under control of a strong constitutive promoter) and clarification of the limitations of the specific growth conditions of these organisms in relation to obtainable fluorescence signals are therefore warranted.

The Gram-positive *Streptococcus gordonii* is one of many bacterial species involved in the formation of dental plaque. In the oral environment, *S. gordonii* is an initial colonizer, which grows attached to the tooth surface (Kolenbrander & London, 1993). It is important to characterize the general performance of this bacterium when growing in biofilms in addition to what has been investigated for cells in the planktonic state, since sessile populations represent *in vivo* conditions more accurately than planktonic bacteria. Cultivation of bacteria in flow-chambers makes it possible to study biofilm communities, and this model system approach has proven to be much more representative of conditions in the oral cavity than are static systems (Palmer & Caldwell, 1995).

The aims of this study were (i) to design and characterize genetic tools targeting Gram-positive bacteria in general and microaerophilic species in particular; (ii) to test the limits of the use of GFP as a marker under suboptimal maturation conditions (low pH, low O<sub>2</sub>), and thereby to address these limitations in a practical application of GFP – the use of the *gfpmut3\** gene (Andersen *et al.*, 1998) as a molecular marker in the Gram-positive bacterium *S. gordonii* DL1 grown in batch cultures and in biofilms under aerobic and anaerobic conditions; and (iii) to determine which growth conditions allow sufficient GFP reporter signals to be useful in connection with *in vitro* as well as *in situ* studies.

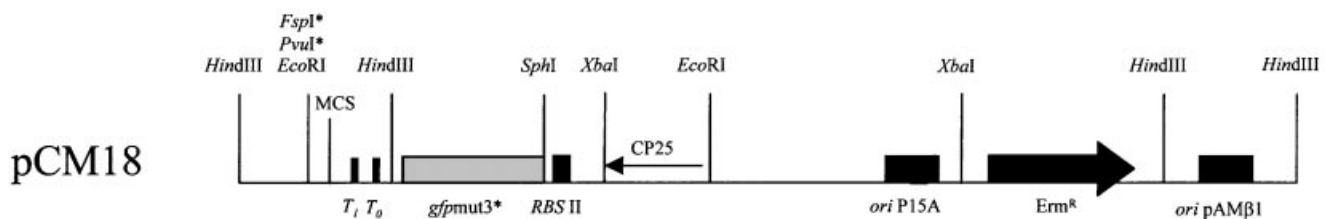
## METHODS

**Bacterial strains and growth conditions.** Bacterial strains and plasmids are listed in Table 1. *S. gordonii* DL1 (kindly donated by P. Kolenbrander, NIDCR/NIH) was grown at 34 °C in CAMG broth (per litre: 5 g tryptone, 5 g yeast extract, 5 g dibasic potassium phosphate) supplemented with either 2 g or 5 g glucose, as batch cultures in anaerobic culture tubes (Bellco Labs). The medium was prepared using strict anaerobic methods (boiling under sparging with 95:5 N<sub>2</sub>/CO<sub>2</sub> for 1 h prior to tubing, head-gas replacement and autoclaving). Resazurin (0.1%, w/v, stock solution) was added to a final concentration of 0.0001% as an E<sub>h</sub> (redox potential) indicator and visual check of reduced conditions. Growth medium for the biofilms was either a 1/10 diluted version of anaerobic CAMG broth (per litre: 0.5 g tryptone, 0.5 g yeast extract, 5 g dibasic potassium phosphate, 0.2 g glucose), or 1/10 diluted

**Table 1.** Strains and plasmids

Strain or plasmid	Description†	Reference or source
<b>Strains</b>		
<i>E. coli</i> JM105	<i>thi rpsL</i> (Str <sup>r</sup> ) <i>endA sbcB15 sbcC hsdR4</i> ( $r_{\text{K}}$ $m_{\text{K}}$ ) $\Delta(lac-proAB)$ [F' <i>traD36 lacI<sup>a</sup></i> $\Delta(lacZ)M15 proA^+B^+$ ]	Yanisch-Perron <i>et al.</i> (1985)
<i>S. gordonii</i> DL1		P. Kolenbrander (NIDCR/NIH)
<b>Plasmids</b>		
pJBA25	Ap <sup>R</sup> ; pUC18Not-RBSII- <i>gfpmut3*</i> -T <sub>0</sub> -T <sub>1</sub>	Andersen <i>et al.</i> (1998)
pCP25	Em <sup>R</sup> ; pAK80-P <sub>CP25</sub>	Jensen & Hammer (1998)
pTRKL2	Em <sup>R</sup> ; <i>lacZ oriP15A oripAMβ1</i>	O'Sullivan & Klaenhammer (1993)
pCM18	Em <sup>R</sup> ; pTRKL2-P <sub>CP25</sub> -RBSII- <i>gfpmut3*</i> -T <sub>0</sub> -T <sub>1</sub>	This study

† Ap<sup>R</sup>, ampicillin resistance; Em<sup>R</sup>, erythromycin resistance; RBSII, synthetic ribosome-binding site; T<sub>0</sub>-T<sub>1</sub>, transcriptional terminators from phage lambda and from the *rrnB* operon of *E. coli*.



**Fig. 1.** Genetic and physical map of the pCM18 plasmid vector (not to scale). Only relevant restriction sites are indicated (sites marked \* are unique). Abbreviations: MCS, multiple cloning site (*EcoRI*, *SmaI*\*, *Sall*\*, *HindIII*, *NruI*\*, *XbaI*); T<sub>1</sub>, transcriptional terminator from the *rrnB* operon of *E. coli*; T<sub>0</sub>, transcriptional terminator from phage lambda; *gfpmut3\**, gene encoding Gfpmut3\* (S2R, S65G, S72A); RBSII, synthetic ribosome-binding site; CP25, synthetic constitutive lactococcal promoter; *ori*, origin of replication; Erm<sup>R</sup>, erythromycin resistance gene.

anaerobic CAMG broth supplemented with 0.5 g L-cysteine l<sup>-1</sup> as a reducing agent. Erythromycin (5 mg ml<sup>-1</sup>) was added to a final concentration of 5 µg ml<sup>-1</sup> in the medium, in the experiments where a selection pressure for the maintenance of plasmid pCM18 was applied. Growth medium for the transformation of *S. gordonii* DL1 was Todd-Hewitt broth (Difco) supplemented with 5% (v/v) heat-inactivated horse serum. *Escherichia coli* JM105(pCM18) was maintained at 37 °C in LB broth containing 4 g NaCl l<sup>-1</sup> and supplemented with erythromycin to a final concentration of 150 µg ml<sup>-1</sup>.

**Plasmid construction and transformation of *S. gordonii*.** The synthetic constitutive lactococcal promoter, CP25 (Jensen & Hammer, 1998), was excised from plasmid pCP25 with *XbaI* and ligated into *XbaI*-digested pJBA25 (Andersen *et al.*, 1998) upstream of the promoter-less *gfpmut3\**. The fragment from this new plasmid containing CP25, the synthetic ribosome-binding site (RBSII), the *gfpmut3\** gene and two terminators (the transcriptional terminator T<sub>0</sub> from phage lambda and the transcriptional terminator T<sub>1</sub> from the *rrnB* operon of *E. coli*) was excised with *XbaI* and *NotI*, made blunt ended, and ligated into the unique *EcoRV* site of the vector pTRKL2 (O'Sullivan & Klaenhammer, 1993). The construct was verified by agarose gel electrophoresis of analytical digestions. A linearized form of the plasmid pCM18 is shown in Fig. 1.

The transformation procedure was essentially as described by Perry *et al.* (1983). The plasmid pCM18 was purified from 2 ml of an overnight culture of *E. coli* JM105(pCM18) using the QIAprep Spin Miniprep Kit (QIAGEN) and eluted in 200 µl H<sub>2</sub>O. An overnight culture of *S. gordonii* DL1 grown in Todd-Hewitt broth supplemented with 5% (v/v) heat-inactivated horse serum was diluted 100-fold and incubated for 3.5 h at 34 °C. Sixty-four microlitres of the culture was mixed with 32 µl of the plasmid preparation and incubated for 1.5 h. The cells were spread on Todd-Hewitt agar plates containing 5 µg erythromycin ml<sup>-1</sup>, and transformants were scored after 2 d incubation at 34 °C.

**Flowcell system.** *S. gordonii* DL1 was grown as biofilms in glass flowcells (channel dimensions 3 mm wide and 1 mm high) for microscopy (Palmer & Caldwell, 1995). Medium for the flowcells was supplied from a reservoir through silicone tubing. A peristaltic pump (Watson Marlow set at rate 12 ml h<sup>-1</sup>) pulled medium through the system and led the effluent to a waste container.

**Glove bag.** In order to obtain anaerobic conditions for the flowcells, an Inflatable Glove Bag (model X-17-17, Instruments for Research and Industry, Cheltenham, PA, USA) was used to

contain the experimental set-up (Hansen *et al.*, 2000). The gas environment in the glove bag was 95:5 N<sub>2</sub>/CO<sub>2</sub>.

**Measurement of optical density.** A Shimadzu spectrophotometer model UV-1201 (SpectraChrom, Brøndby, Denmark), was used for measurements of OD<sub>600</sub>.

**Measurement of pH.** A Sentron pH meter model 1001 was used for measurements of pH. Calibration was performed using pH 4.0 and pH 7.0 buffers.

**Measurement of dissolved oxygen.** The concentration of dissolved oxygen in the anaerobic medium in anaerobic culture tubes (Bellco) and in the reduced flowcell medium was determined colorimetrically using CHEMets Dissolved Oxygen Ampoules (CHEMetrics, Calverton, VA, USA). All measurements were done in the glove bag. Tubed anaerobic medium without L-cysteine contained 0.1 p.p.m. dissolved oxygen. Medium with L-cysteine for the flowcells contained  $\leq 0.025$  p.p.m. dissolved oxygen (limit of detection).

**Scanning confocal laser microscopy.** Flowcells were removed from the glove bag prior to microscopic examination. In the shift experiment from anaerobic to aerobic medium, the medium supply was changed as soon as the flowcell was removed from the glove bag, and the biofilms were immediately observed using a Leica TCS-NT confocal laser microscope. The microscope settings were as follows: excitation at 488 nm, emission at 530/30 BP into channel 1 to record GFP fluorescence, 100 × 1.4 NA oil-immersion lens at an Airy disc setting of 0.9, 1 µm steps collected with four frames averaged at each step. When an increase in fluorescence intensity was expected, the PMT gain was deliberately reduced in order to be able to keep this setting throughout the entire experiment and still avoid saturation in the brightest images. Biofilms were also subjected to microscopic examination after 18 h and after 2 d of growth.

**Epifluorescence microscopy.** Fluorescence in suspended cells from liquid cultures was observed by applying 4 µl of culture on a microscope slide followed by examination using either the confocal laser microscope as described or a Carl Zeiss Axioplan epifluorescence microscope. For the latter, the excitation source was a 100 W HBO bulb, and digital images were captured with a 12-bit cooled slow-scan charge-coupled-device camera (KAF 1400 chip; Photometrics). The charge-coupled-device camera was controlled by the PMIS software (Photometrics), and an FITC filter set was used for the excitation and detection of GFP.

## RESULTS AND DISCUSSION

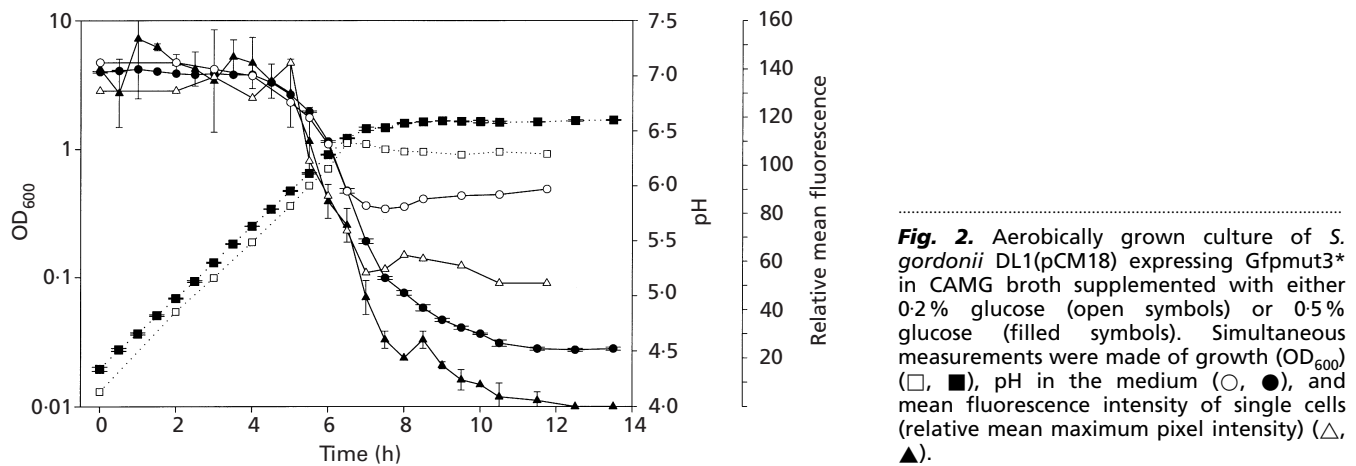
### Construction and characterization of pCM18 – a broad-host-range GFP test plasmid

Plasmid pCM18 carries a fusion between the synthetic strong lactococcal constitutive promoter CP25 (Jensen & Hammer, 1998) and the *gfpmut3\** gene (see Fig. 1). It is a low-copy-number (6–9 per chromosome equivalent) broad-host-range plasmid, which is easily introduced and maintained in a variety of bacteria due to its relatively small size (8.2 kb including the 1.8 kb *gfpmut3\** gene insert) and the presence of pAMβ1

(Gram-positive) and P15A (Gram-negative enterics) replicons. Expression of the *gfpmut3\** gene from pCM18 yielded green fluorescence in both *S. gordonii* DL1 and *E. coli* JM105. The CP25–*gfpmut3\** fusion was also introduced into *Bacillus subtilis* and *Lactococcus lactis*, yielding green fluorescence in both organisms (unpublished results). The gene dosage carried by the low-copy-number plasmid pCM18 was expected to be relatively constant, in contrast to many high-copy-number plasmids, due to a reduced probability of adverse effects on cellular growth caused by excess heterologous gene expression. Furthermore, pCM18 was predicted to be segregationally stable due to the replication mechanism of the pAMβ1 replicon, which proceeds by the theta (θ) mechanism in Gram-positive bacteria, and therefore does not lead to accumulation of single-stranded intermediates as in replication by the rolling-circle replication mechanism (Ehrlich *et al.*, 1991). This was confirmed in growth experiments in batch cultures, in which no decrease in fluorescence was observed in a culture growing for ten generations in the absence of selection pressure. Parallel growth experiments were performed, in which biofilms were grown without selection pressure to monitor loss of the plasmid. Biofilms were grown in the absence or presence of 5 µg erythromycin ml<sup>-1</sup>. It was found that fluorescence was homogeneously distributed in both types of biofilms, and that the pattern of biofilm development was the same (data not shown). This implies that pCM18 can be used as a reliable vehicle for single-cell reporters under a variety of growth conditions, and furthermore that the construct provides an easy way of introducing the CP25–*gfpmut3\** fusion into a variety of Gram-negative and Gram-positive bacteria.

### Expression of GFP in batch cultures of *S. gordonii* DL1(pCM18)

The CAMG broth medium used for growth of *S. gordonii* DL1 was prepared either anaerobically or aerobically. The conventionally prepared anaerobic medium contained 0.1 p.p.m. (0.1 mg l<sup>-1</sup>, approximately 3 µM) dissolved oxygen. There have been several reports on the oxygen requirements of GFP for the final oxidation of the mature, cyclized chromophore (Cody *et al.*, 1993; Heim *et al.*, 1994, 1995; Inouye & Tsuji, 1994; Prasher *et al.*, 1992), and describing the kinetics of the steps involved in chromophore formation including the final oxidation of the tripeptide (Reid & Flynn, 1997). We found that exponentially growing cells were fluorescent in anaerobic medium, i.e. in the presence of 0.1 p.p.m. dissolved oxygen, even after several transfers to secure sufficient dilution of the initial inoculum (data not shown). This implies that a significant proportion of GFP was oxidized by the trace amounts of oxygen found in our anaerobic pressure tubes. Several applications of GFP have faced troublesome interpretations of the fluorescence due to potentially limiting concentrations of oxygen, e.g. cells in the centre of microcolonies in otherwise aerobic environments. The present demon-



stration that very low amounts of oxygen are needed for maturation of GFP opens the possibility of using this reporter under conditions of near-anaerobiosis.

*S. gordonii* is a homofermentative bacterium: glucose is fermented to twice the equimolar amount of lactate. The production of acid reduces pH in the medium as lactate accumulates. As shown in the initial characterization of GFP (Prasher *et al.*, 1992), the fluorescence of the protein is significantly influenced by changes in pH. Elsliger *et al.* (1999) argued that the quenching of fluorescence at low pH is due to the formation of a protonated form of the chromophore phenolate in the GFP S65T variant. This pH sensitivity of GFP was exploited by Robey *et al.* (1998) and Miesenböck *et al.* (1998), using mutants of GFP as *in vivo* reporters of cytoplasmic pH. The pH sensitivity becomes problematic when GFP is used in lactic acid bacteria in batch cultures. For several lactic acid bacteria, it has been found that the intracellular pH decreased with the extracellular pH, maintaining a constant  $\Delta$ pH of 0.5–0.8 units (Cook & Russell, 1994; Siegmund *et al.*, 2000). Similarly, epifluorescence microscopy of single cells from an aerobic exponentially growing culture of *S. gordonii* DL1 showed that fluorescence was equally distributed among the cells in the population. As pH decreased in the medium, fluorescence intensity decreased. If the growth medium is supplemented with different amounts of glucose (0.2 and 0.5%), the cultures reach stationary phase at different pH values. This makes it possible to compare the pH effect unbiased by the change in growth phase. In medium with 0.2% glucose, the pH decreased from 7 to 6, resulting in a corresponding drop in fluorescence intensity. However, when the medium was supplemented with 0.5% glucose, the pH decreased to 4.5, with a further decrease in fluorescence. Fluorescence from cells at or below about pH 5.5 was only detectable through a strong amplification of the signal, making the fluorescence practically useless, as the cells lost edge definition (they were difficult to spot). These data are summarized in Fig. 2. For each sample point, a set of epifluorescence micrographs was obtained, and the fluorescence intensity was estimated using the maximum pixel intensity option

from the PMIS (Photometrics) software. Although streptococci to some extent may control their internal pH (Cook & Russell, 1994), Fig. 2 clearly shows the effect of external pH on GFP fluorescence *in vivo*. This phenotype was independent of oxygen supplied by vigorous shaking of the culture.

It was attempted to reverse the acid-induced reduction in fluorescence by shifting stationary-phase GFP-tagged cells to fresh medium or to a buffer at neutral pH. As summarized in Table 2, it was possible to regain fluorescence from cells in stationary phase (at pH below 5) for up to 13 h. However, it was not possible to regain fluorescence from cells after 23 h in stationary phase at low pH. These results were independent of resuspension medium and incubation time, as resuspension of cells was performed in either fresh medium or a buffer at neutral pH for either 5 min or 60 min. These findings add to a recent observation in GFP-tagged lactococci (Fernández de Palencia *et al.*, 2000), which indicated that the acid-induced reduction of fluorescence was reversible upon resuspension in PBS buffer. Thus, GFP does not seem to be degraded upon low pH and starvation conditions for up to 13 h by induced proteases, as the fluorescence can be regained by a shift to neutral pH. However, the prolonged stationary phase (23 h) of *S. gordonii* is critical, as the cells lose fluorescence permanently.

In a shift experiment from anaerobic to aerobic conditions, Scott *et al.* (1998) observed heterogeneous expression of GFP in colonies of *L. lactis* IL1403 on GM17 agar plates after 22 h exposure to air. Since GFP fluorescence was restricted to the outer zone of the colonies, it was argued that oxygen diffusion through the colonies was restricted. Parallel experiments with *E. coli* V850 on LB plates showed homogeneous GFP expression in the colonies, indicating that for this organism there was no diffusion barrier. However, the external pH on colonies of lactic acid bacteria (e.g. *L. lactis* IL1403) on a GM17 (0.5% glucose) agar plate is approximately 5 (depending on the distribution and size of colonies) (unpublished data). This fact, together with the findings presented above, suggests that an acidifi-

**Table 2.** Fluorescence in batch cultures of *S. gordonii* DL1(pCM18)

Time (h)*	Condition	pH	Fluorescence†	Fluorescence† after resuspension‡	
				5 min	60 min
-1	no cysteine, 0.1 p.p.m. O <sub>2</sub>	7	+		
-1	with cysteine, ≤ 0.025 p.p.m. O <sub>2</sub>	7	-		
-1	aerobic	7	+		
0	aerobic	6	+		
5	aerobic	4.5	-	+	+
13	aerobic	4.5	-	+	+
23	aerobic	4.5	-	-	-

\* Time after entry into stationary phase.

† +, Fluorescence; -, absence of fluorescence.

‡ Cells were resuspended in either fresh growth medium or phosphate buffer pH 7.0; fluorescence was determined 5 or 60 min after resuspension.

cation of the medium rather than oxygen limitation in the *L. lactis* IL1403 colonies is the primary cause of fluorescence reduction. This explanation is in agreement with our demonstration that maturation of GFP still occurs at very low levels of oxygen, and with the fact that no oxygen consumption occurs in colonies of *L. lactis*.

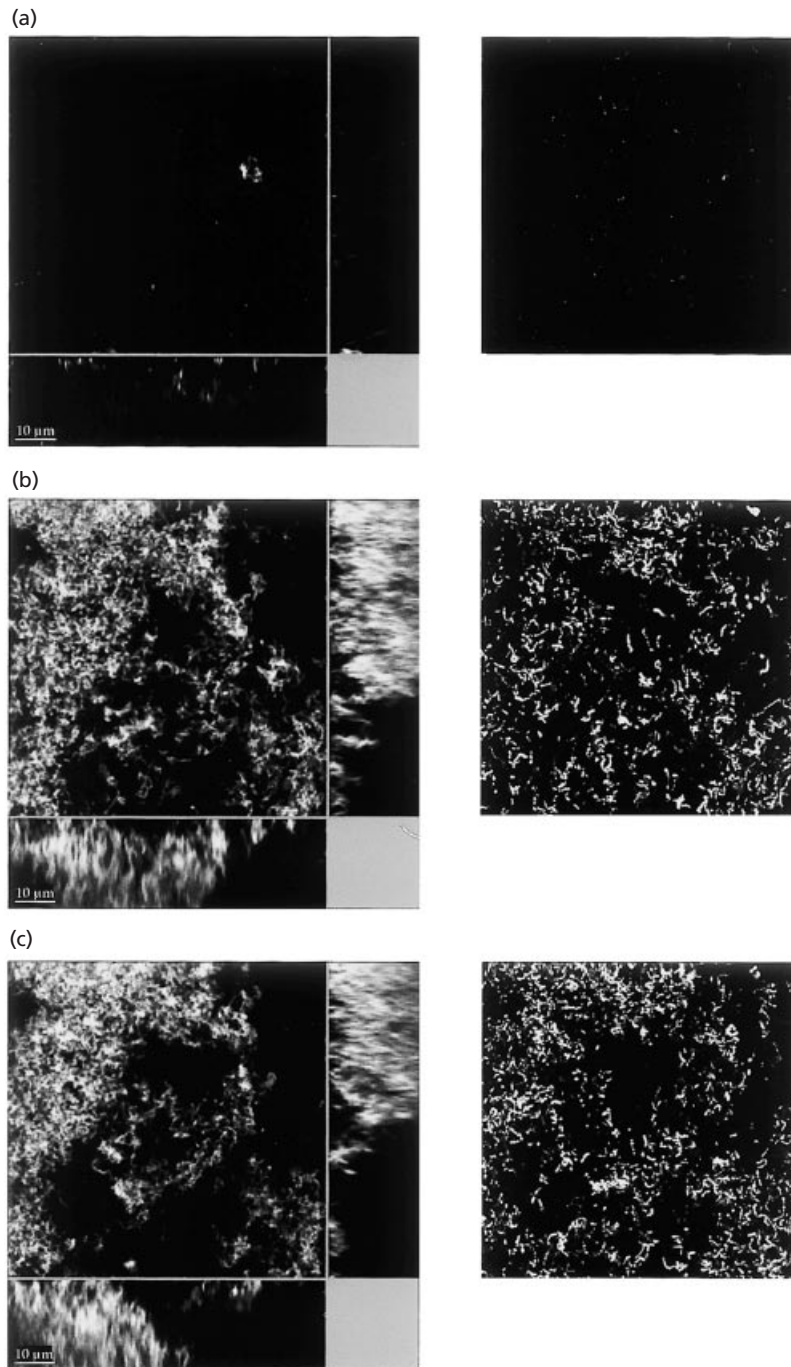
### Expression of GFP in biofilms

As in batch cultures, green fluorescence in biofilms was observed in medium containing approximately 0.1 p.p.m. oxygen. The pH in the effluent from a biofilm grown for 2 d was approximately 7 (data not shown). GFP fluorescence in aerobic biofilms of *S. gordonii* DL1(pCM18) was homogeneously distributed throughout the biofilm until a very thick cell-layer (> 50 µm) quenched the fluorescence signal. The continuous flow of medium through the flowcell system prevents accumulation of lactic acid and a resultant drop in pH, and consequently the GFP fluorescence signal intensity allowed excellent recordings of the cells by scanning confocal laser microscopy throughout the course of the biofilm development (up to 4 d).

When the entire flowcell set-up was placed in a glovebag as described by Hansen *et al.* (2000), and L-cysteine was added to the medium, the biofilms could be maintained under strictly anaerobic conditions (≤ 0.025 p.p.m. oxygen, equivalent to ≤ 0.8 µM). Under these conditions the biofilms increased in biomass faster than parallel aerobic biofilms, but similar biofilm characteristics (distribution of biomass and cellular morphologies) were developed under the two sets of conditions. However, green fluorescence was not observed in these biofilms until after a shift to aerobic medium. Inspection of the fluorescence development in a shift from anaerobiosis to aerobiosis using the scanning confocal microscope showed that fluorescence was detectable 4 min

after oxygen was supplied (Fig. 3). During the next 16 min, a further increase in fluorescence intensity occurred, but after an additional 20, 40, 60 and 100 min, little or no increase in fluorescence could be observed. In Fig. 3, single optical sections directly at the substratum show the fluorescence increase at the deepest regions of the biofilm (in the middle of microcolonies) as well as in cells exposed to pore-space fluid (cells at the edge of those microcolonies), and are displayed in conjunction with images of the entire biofilm biomass (maximum projections). These micrographs of single sections indicate the uniform increase of fluorescence in this biofilm as do the side panels (*zx* and *zy* scans) on the entire stack of images. Neither vertical nor horizontal gradients of fluorescence through the biofilm were observed – fluorescence increased the same way in cells from the middle of a micro-colony as in those on the surface, indicating a rapid distribution of oxygen through the flowcell and through the *S. gordonii* biomass.

Previous determinations of the ordered reaction kinetics of the three distinct steps in the chromophore formation of purified GFP (S65T) showed that oxidation of the mature and cyclized protein had a time constant  $k = 1.51 \times 10^{-4} \text{ s}^{-1}$ , corresponding to  $T_{1/2} = 76 \text{ min}$  (Reid & Flynn, 1997). In an experiment where anaerobically grown *E. coli* cells were shifted to aerobic conditions, Heim *et al.* (1995) found that the S65T mutant GFP formed four times faster ( $T_{1/2} = 27 \text{ min}$ ) than wild-type GFP. Unfortunately, measurements of the kinetics based on the S65T GFP makes comparisons with Gfpmut3\* (S2R, S65G, S72A) difficult, as certain mutations have been observed to affect the rate of chromophore formation (Cormack *et al.*, 1996; Heim *et al.*, 1995). The time constant measured *in vivo* by Heim *et al.* (1995), however, shows a much faster oxidation step than wild-type GFP, which corresponds to results obtained for several enhanced mutants (Cubitt *et al.*, 1995; Reid & Flynn, 1997; Scott *et al.*, 1998). The *in vivo* examinations



**Fig. 3.** Confocal micrographs of *S. gordonii* DL1(pCM18) in a biofilm shifted from anaerobic ( $\leq 0.025$  p.p.m. oxygen) to aerobic conditions. Green fluorescence was monitored 4 min (a), 20 min (b), and 40 min (c) after a shift to aerobic medium. All scanning variables (e.g. PMT settings) were held constant over the entire observation period. The panels below and on the right side of the main frame illustrate zx and zy scans, respectively. Next to each confocal micrograph is shown one slice (approx.  $0.3 \mu\text{m}$  thick) closest to the substratum. No fluorescence was detectable at time zero.

in this study demonstrate an even faster maturation of the Gfpmut3\* fluorophore, which implies that this reporter protein can indeed be used effectively for assessments of up-regulated promoter activity, and that even brief exposures to oxygen should suffice for maturation of GFP synthesized under anaerobic conditions.

The actual *in vivo* requirements of oxygen for GFP fluorescence under limiting oxygen tensions have been determined. Maturation of the GFP fluorophore occurs

at levels of oxygen as low as  $0.1$  p.p.m., whereas no fluorescence was detectable at  $0.025$  p.p.m. oxygen. This implies that the use of GFP is not restricted to aerobic bacteria and well-aerated systems – microaerophilic organisms may also be characterized using GFP. Oxygen-dependent maturation is very fast, since a fully fluorescent biofilm was obtained in less than 20 min. A low pH in the environment results in low fluorescence yield, and for organisms which most often grow fermentatively it is highly important to control external pH.

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