00-030

5 Gene Expression of Cells Attached to Surfaces

Amanda E. Goodman and Gill G. Geesey

Gene expression in biofilm microbial populations can now be assessed at the single cell level. Studies of genetically engineered bacterial populations attached to surfaces have revealed a variety of genes that are up-expressed when cells exist in biofilms. Real-time studies have shown that gene expression within a cell may be transient during residence on a surface. Within isogenic, surface-associated populations, gene expression can be heterogeneous, possibly reflecting microscale variations in environmental conditions. Since a significant proportion, if not the majority of microbial life forms spend some portion of their existence in association with surfaces, it is likely that many genes and cell functions yet to be discovered will be detected in microbial biofilm populations. The genetic capacity of microbial life cannot be realized and the emerging field of genomics cannot achieve full potential until a better understanding is gained of gene expression in cells on surfaces.

KEY WORDS: biofilm, reporter genes, green fluorescent protein

INTRODUCTION

Microbial biofilms represent a complex assemblage of individual cells that are associated with surfaces. Unlike microbial cells freely dispersed in an aqueous phase, biofilm cells associated with surfaces develop spatial relationships to each other that permit interactions approaching those of multicellular organisms. Because biofilm cells are fixed in space for at least short periods of time, their behavior can be evaluated on an individual cell basis. This provides the opportunity to determine intra-population variations as well as inter-population interactions in mixed species biofilm communities.

Spatial relationships between biofilm cells have been observed microscopically for decades (Mack *et al.*, 1975; Kudo *et al.*, 1987). Post-treatment of fixed populations with specific fluorescent-labeled antibodies has been used to reveal the locations and associations between cells of different microbial species. Fluorescently-labeled-oligonucleotide probes that hybridize *in situ* with specific sequences in the ribosomal RNA molecule in intact cells have been used to identify specific microbial populations and as indicators of overall cell activity in biofilm populations (Poulsen *et al.*, 1993; Møller

et al., 1996). While these microscopic approaches have been used to characterize activity and spatial relationships between biofilm cells at a single point in time, there is growing interest in following the development of spatial relationships and cell-cell interactions in real time (Caldwell *et al.*, 1992).

REPORTER GENES

Recent investigations have shown that communication, *via* chemical signals can occur between bacteria in biofilms. Reporter genes were used in this regard. Here the expression of a gene of interest is coupled to a promoterless gene (reporter gene) whose product is readily detectable by microscopy or other analytical instrumentation. Genes that have been used as reporters include *cat*, *xylE*, and *galK*. The most popular reporter gene in bacteria is *lacZ*, which codes for the enzyme β-galactosidase (β-gal). Chromogenic and fluorogenic substrates (chromophore and fluorophore-galactose conjugates which are colorless or non-fluorescent until cleavage) are used to detect *lacZ* expression and hence expression of the gene of interest. When used in conjunction with a fluorogenic substrate, this reporter gene can be used to detect and localize gene expression at the cellular level by fluorescence microscopy and flow cytometry. Quantification of the amount of enzyme produced can be achieved using fluorimetry or colorimetry.

Use of *lacZ* as a Reporter of Biofilm Activities

There is growing interest in evaluating the activity and, in particular, the expression of specific genes in individual biofilm cells non-destructively in real time. This has been made possible in recent years through the use of a combination of bioreactors, molecular techniques, and microscopic imaging systems (Davies *et al.*, 1993). An indirect approach that has been used for decades is assessment of specific activities in populations of cells by following the expression of genes encoding the enzymes associated with biochemical pathways linked to the activities. A more direct approach is to use reporter genes to assess particular gene activity in individual bacterial cells in biofilms.

Extracellular polymer production

The *lacZ* reporter has been used to follow the expression of *algC*, a house-keeping gene also involved in alginate biosynthesis in *Pseudomonas aeruginosa*. Since members of the genus *Pseudomonas* are naturally *lacZ*$^-$, the level of expression of this reporter gene does not have to be corrected for background expression. In *P. aeruginosa* strain 8830, *lacZ* was put under the control of the *algC* promoter in the *algC*-*lacZ* transcriptional fusion plasmid pNZ63 (Davies *et al.*, 1993). When a comparison was made between a mature biofilm population growing on a Teflon substratum and a suspended cell population, *algC* expression was nearly 20-fold higher and alginate levels were over 2-fold higher in the former compared to the latter population. Cells shed from

the biofilm into the bulk aqueous medium displayed *algC* expression levels in between those of the biofilm and original suspended cell populations.

The *lacZ* reporter has also been used to follow expression of *algD*, the gene encoding GDP-mannose dehydrogenase, which catalyzes the conversion of GDP-mannose to GDP-mannuronic acid in the alginate biosynthesis pathway in biofilms of *P. aeruginosa* (Hoyle *et al.*, 1993). *P. aeruginosa* 579 was transformed with plasmids pSDF13 and pSDF14 containing *lacZ* under the control of the *algD* promoter and conferring gentamicin resistance. β-Gal activity in extracts of suspended cell populations was significantly less than that of 1- and 4-day biofilm populations containing equivalent numbers of viable cells. Cells of 7-day biofilms displayed reporter activity that was not significantly different from that of suspended cells, suggesting that alginate production drops with biofilm maturation. The presence of NaCl appeared to depress but not completely eliminate *algD* expression. Hoyle *et al.* (1993) suggested that the decrease in *algD* expression in biofilm populations after day 4 was consistent with a decrease in production of mucoexopolysaccharide, based on the establishment of a plateau after day 1 in accumulation of neutral hexose in the suspended population as assayed by the method of Dubois *et al.* (1956). Electron transport activity, based on reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to INT-formazan was found to follow *algD* expression more closely than neutral hexose accumulation. The results were interpreted as strong evidence for enhanced but transient production of mucoexopolysaccharide by *P. aeruginosa* 579 following attachment to a surface.

Expression of the *P. aeruginosa algC* gene was also monitored during the initial phase of biofilm development in individual bacteria which had detached from an upstream biofilm and subsequently reattached to a glass substratum in the presence of the fluorogenic substrate methylumbelliferyl-β-D-galactoside (MUG) in a flow-through, flat plate, channel reactor with a viewing window that permitted fluorescent and phase contrast microscopic observation of the attached cells (Davies and Geesey, 1995). Using this approach it was found that the expression of *algC* was temporally related to surface attachment and colonization of individual bacterial cells. Although *algC* was down-expressed in the majority of the cells (>93%) that had been attached to the substratum for less than 15 min, during the subsequent 165 min period, *algC* expression in the attached cells increased from 26 to 50% of the total attached population, with 89% of the total attached population up-expressed for *algC* at the end of this observation period. Many of the attached cells displayed transient expression of *algC* and became detached from the substratum over the observation period. Over 70% of the cells that detached became down-expressed for *algC* just prior to detachment. These results suggest a relationship between the expression of certain genes and the ability of cells to remain associated with a surface.

The use of the *lacZ* reporter system in this application yielded new insight into biofilm bacterial cell behavior. The results demonstrated 1) variation in gene expression among cells of an isogenic population, 2) up-expression of *algC* in cells shortly after they become associated with a substratum, 3) transient expression of *algC* in cells during attachment to a substratum, and 4) bacteria that were down-expressed for *algC* while associated with the substratum showed a higher propensity to detach from the substratum than did bacteria which were up-expressed for *algC*.

Cell-cell signaling

Fuqua and colleagues used a *lacZ* reporter gene system to show that bacteria in biofilms produce cell-cell signaling molecules. In one study (McLean *et al.*, 1997) a community of freshwater bacteria, including strains of *Pseudomonas putida* and *Pseudomonas fluorescens*, growing as biofilms on rocks in a river, were found to produce acylated homoserine lactone (HSL) type chemicals. Stickler *et al.* (1998) found that pure cultures of *P. aeroginosa* biofilms colonizing model catheters produced acylated homoserine lactones, and showed that these compounds were produced in about 50% of biofilm-colonized catheters recovered from hospital patients. The three dimensional (3D) biofilm structure formed by *P. aeruginosa* cells has been found to rely on chemical communication occurring between cells. Davies *et al.* (1998) compared the 3D biofilm structures formed by a *lasI* mutant strain of *P. aeruginosa* and the wild-type. *LasI* directs synthesis of the cell-signaling molecule *N*-(3-oxododecanoyl)-L-homoserine lactone. Cells unable to produce *lasI* formed flat, non-structured biofilms that were sensitive to the detergent sodium dodecyl sulphate and produced as much extracellular polymer as the wild type. When exogenous HSL was supplied to the *las1* mutant strain, a thick, differentiated biofilm developed, similar to that produced by the wild type, which consisted of mushroom- and pillar-like structures attached to the substratum between liquid-filled spaces (Davies *et al.*, 1998). Another HSL molecule produced by *P. aeruginosa*, *N*-buytryl-L-homoserine lactone, did not appear to have any effect on the structure of the biofilm produced on a glass substratum (Davies *et al.*, 1998).

It should be noted that other biofilm-forming bacteria develop biofilm architectures completely different from that produced by *P. aeruginosa*. The structures of bacterial biofilms often depend on the nature of the substratum (reviewed in Dalton *et al.*, 1994; 1996; Lawrence *et al.*, 1995; Stretton *et al.*, 1998) as well as the nutrient concentration and composition of the aqueous phase (Lawrence *et al.*, 1995).

Use of the lacZ reporter gene to evaluate conditions of biofilm environment

Reporter genes have been used to probe the condition of the environment at the microscale. A *lacZ* reporter gene was used to show that individual *E. coli* cells (previously inactivated for their natural β-gal production) incorporated into a drinking water biofilm, expressed the anaerobically-induced *nirB* promoter in microcolonies when examined after 13 d of biofilm growth (Robinson *et al.*, 1995). This demonstrated that cells within the microcolonies were experiencing anaerobic conditions, whereas planktonic cells, which did not express the *nirB* promoter, were not.

Limitations of lacZ as a reporter of bacterial activity

The success of the *lacZ* gene in reporting gene expression in individual cells of a biofilm population depends on accessibility of the fluorogenic substrate to the cells, the uptake of the substrate by the cells, and the retention of sufficient quantities of the fluorescent product by the cells to elicit a detectable fluorescent signal. Many types of bacteria do not take up the substrate or retain the product in sufficient amount to produce a fluorescent cell, thereby precluding use of the *lacZ* reporter gene in studies

of gene expression in individual cells. Furthermore, these substrates are costly to use in flow-through systems such as described above. In such situations, other reporter genes offer advantages over *lacZ*.

The use of *lacZ* to report expression of other genes in a bacterial cell is usually restricted to those strains that normally lack a functional copy of this gene or have had the normal gene deleted. Otherwise, the β-gal assay will report the combined activity of the reporter gene as well as that of the normal gene that is present.

Use of *lux* Genes as a Reporter of Biofilm Activities

Biofilm activities have also been evaluated using a *lux* gene cassette composed of 5 genes, *luxCDABE*. The *lux*AB genes encode a heterodimeric luciferase enzyme. The enzyme requires no fluorophore/enzyme substrate, and oxidizes a tetradecanol to a tetradecanoic acid using oxygen and reduced flavin mononucleotide ($FMNH_2$), yielding light as a byproduct (Meighen, 1991). The light is detected by an extremely sensitive photon-counting camera, producing spatially resolved quantitative images of photon flux at the level of resolution necessary to assay *lux* expression within single bacterial cells (Palmer *et al.*, 1996). Since most bacteria cannot make sufficient tetradecanol for prolonged light production, it must either be added as a supplement to the medium or the genes necessary for *in vivo* production (*luxCDE*), included in the cassette.

The *lux* reporter cassette has been inserted by transposon mutagenesis into the plasmid PUTK21 carrying genes for naphthalene catabolism to report *nahG* (salicylate hydrolase) gene expression in cells of *P. fluorescens* strain 5RL growing as a biofilm in a cell adhesion measurement module (CAMM) (Mittelman *et al.*, 1992). Luciferase-mediated light production was induced upon exposure to sodium salicylate, collected with a flexible liquid light cable and collimated beam probe, and detected as a photoelectric-induced current using a photomultiplier-digital readout system. Upon addition of sodium salicylate, induction of *nah*G based on light production from the *lux* reporter was similar in cells attached to glass and stainless steel. Light production was positively correlated with total attached cell densities. Such a light-based approach was used to relate substratum colonization rate to surface shear force.

Light emission from cells of the marine bacterium *Vibrio harveyi,* which carries *lux* genes naturally on the chromosome, was used to evaluate the efficacy of marine antifouling coatings on bacterial surface colonization (Mittelman *et al.*, 1993). Since light flux correlated positively with the surface densities of both viable and total direct counts, *lux* gene expression offered a simple, non-destructive, real time measure of the extent of bacterial surface colonization. Two copper-based coatings, Navy F-121 and International Paints BRA-640, were colonized less readily than a 15% dinitrophenol coating. The expression of *lux* genes in *V. harveyi* was found to be a useful indicator of antifouling efficacy under dynamic-flow conditions.

While the lux reporter system avoids the need for a fluorogenic enzyme substrate, its dependence on oxygen and sensitivity to oxygen concentration limits it use to environments of high, constant oxygen concentrations. This feature of the *lux* reporter has been exploited to measure oxygen mass transfer between the bulk liquid and bacterial cells growing on the surface of a hollow fiber reactor (Sheintuch *et al.*, 1992).

Since the respiratory activities of bacteria in biofilms generate strong oxygen gradients within the biofilm (Abrahamson *et al.*, 1996) gene expression is difficult to interpret from photon flux using the *lux* reporter system. The luciferase enzyme is also sensitive to other factors such as ATP and metal concentrations, as well as the ability of a cell to produce or regenerate FMNH. Any variation in the availability of these factors can complicate enzyme activity interpretation (Jacobs *et al.*, 1991).

Use of the *gfp* Gene to Report Biofilm Activities

Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* fluoresces upon transfer of energy from the Ca^{2+}-activated photoprotein, aequorin. The energy transfer is thought to proceed *via* direct interaction between these two proteins. Aequorin is a "precharged" quasi-stable enzyme peroxide intermediate formed by reaction of the coelenterate luciferase and luciferin with oxygen (Hastings, 1996). Apo-aequorin is, thus, coelenterate luciferase, which binds the substrate coelenterazine (luciferin) and reacts with oxygen to form aequorin, which is then stored until its further reaction is triggered by calcium. GFP emits green light (l_{max} = 510 nm) when excited with ultraviolet or blue light (l_{max} = 395 nm with a minor peak at 470 nm). GFP fluorescence can be monitored non-invasively by fluorescence microscopy and flow cytometry.

While full-length GFP is required for fluorescence, the minimal chromophore needed for light absorption is located within a hexapeptide at amino acid position 64 through 69. This region of the protein contains a ser^{65}-dehydrotyr^{66}-gly^{67} trimer which cyclizes to yield the chromophore. Mutant GFP proteins have been reported in which the excitation maximum is shifted from 395 nm to around 490 nm, and this causes increased intensity of protein fluorescence by changing the ser^{65} to thr (Heim *et al.*, 1995), or by various changes to amino acids at positions 64, 65, 68 or 69 (Delagrave *et al.*, 1995). Mutations both within the chromophore and at distal positions in the protein yield functional GFP mutants with altered fluorescence spectra. Red- and blue-shifted GFP mutants are available as reporters as are the filters needed to separate their fluorescence (Delagrave *et al.*, 1995).

The wild type *gfp* gene did not appear useful in prokaryotes, as the intensity of GFP fluorescence was so weak that cell populations of about 10^5–10^6 ml^{-1} were necessary for detection. Falkow and colleagues mutagenised the cloned *gfp* gene in *E. coli* and selected mutant proteins, GFPmut1-3, that produced high levels of fluorescence such that single bacterial cells were easily visualised (Cormack *et al.*, 1996). These mutant GFP proteins remained soluble in the bacterial cell, had their excitation maxima shifted to 481–501 nm (with negligible emission occurring when excited at 395 nm), and yielded about 100 times greater fluorescence compared to the wild type GFP. Cormack *et al.* (1996) suggested that the maximal fluorescence produced by these mutant GFPs results from simultaneous double mutations at amino acid positions 65 and 72. The expression of *gfp* does not adversely affect bacterial survival (Valdivia *et al.*, 1996) and requires no cofactors or addition of exogenous substrates or other factors. It is ideally suited, therefore, for use as a reporter gene, and constructs for use in bacteria have been developed (for example Matthysse *et al.*, 1996; Stretton *et al.*, 1998).

gfp as a reporter of chitinase gene activity

Using a vector construct designed for use with marine bacteria, Stretton *et al.* (1998) placed a *gfp* reporter gene under the control of a chitinase encoding gene, *chiA*, in the marine bacterium *Pseudoalteromonas* sp. S9. S9 *chiA-gfp* cells were grown on squid pen (a natural marine biodegradable polymer consisting of about 60% protein and 40% chitin by weight, Gooday, 1990) and found to colonize patches of the surface in small microcolonies. After 7 d, surface colonization still appeared to be patchy although microcolony volume had increased substantially. Visualization by laser scanning confocal microscopy showed that the *chiA* gene was strongly expressed in individual bacterial cells within microcolonies (Stretton *et al.*, 1998).

More recently, Baty and Geesey (unpublished results) have followed colonization of starved cells of *Pseudoalteromonas* sp. S9 on a thin film of pure chitin cast on an optically smooth silicon substrate. In the absence of other carbon, nitrogen and energy sources, cells colonized the substratum in a random manner. Following initial colonization, it was determined that a portion of the attached population synthesized chitinase enzyme(s), which permitted the utilization of the solid chitin film for attached cell growth and replication. Under these conditions, a biofilm formed over a 200-h period that consisted predominantly of a monolayer of evenly distributed cells across the surface. That not all cells of the isogenic population attached to the chitin surface produced the chitinase enzyme was demonstrated by incorporation of a *gfp* reporter gene under the control of a promoter for the chitinase-encoding gene, *chiA*. Through a combination of reflected differential interference contrast and epifluorescence microscopies, total attached cells and chitinase-producing cells could be located in the same field of view of the chitin surface. Although total cells were randomly distributed across the surface, chitinase-producing cells were clearly aggregated. Thus, reporter genes are useful in evaluating metabolic heterogeneity among cells within isogenic populations.

gfp as a reporter of contaminant biodegradation

Møller *et al.* (1998) developed a system to simulate the biodegradation of toluene and other related aromatic compounds by microbial biofilms. *P. putida* strains were constructed in which each of the promoters of the two operons, as well as appropriate activator genes, involved in the toluene degradation pathway were fused independently to a *gfp*-reporter and inserted into the chromosome. The *Pu* and *Pm* promoters drive the operons encoding genes for the oxidation of toluene to catechol and the subsequent transformation of catechol to Krebs cycle intermediates, respectively. In pure culture biofilms, growing in once through flow chambers supplied with benzyl alcohol as the carbon source, it was found that the *Pu* promoter was homogeneously expressed in all cells, and that the *Pm* promoter was strongly expressed in only a sub-population (<0.01%) of cells. Twenty four h after addition of benzoate (a known inducer of the *Pm* promoter) strong expression of the *Pm* promoter in all cells was observed. This showed that biofilm cells degrading benzyl alcohol (or toluene) did not accumulate benzoate.

In mixed culture biofilms, similar results were found for the *Pu* promoter, although it showed heterogeneous expression after 24 h, with *P. putida* cells immediately surrounding an *Acinetobacter* microcolony displaying the highest *Pu* activity. It was found that the *Acinetobacter* sp. produced a diffusible "inducing agent" which spread through the biofilm with time so that in a 3-day old biofilm expression of *Pm* in the *P. putida* cells was relativley strong and homogenous. It was suggested that either the "inducing agent" leaching from the *Acinetobacter* cells (which also contain genes for degradation of benzyl alcohol) was benzoate or that the *Acinetobacter* cells caused a change in the way the *P. putida* cells were degrading benzyl alcohol such that the benzoate accumulated in these cells (Møller *et al.*, 1998).

gfp as a reporter of the dynamics of gene expression

Further variants of GFP have now been constructed by the addition of a peptide sequence to the C-terminal end of the molecule that renders it susceptible to protease degradation (Andersen *et al.*, 1998). These protein variants have a half-life of from 40 min to a few hours when synthesized in *E. coli* and *P. putida*, making them useful reporters of temporal gene expression.

Limitations of gfp as a reporter under low oxygen conditions

Because GFP requires molecular oxygen for fluorescence, it has not been used in anaerobic bacteria. Recent studies suggest, however, that only small amounts of oxygen are required for the fluorescence reaction in the cell (Gorby, Weaver, Brown, Romine, Neal, unpublished results). GFP fluorescence has been detected in individual cells of the facultatively anaerobic, dissimilatory iron reducing bacterium *Shewanella putrefaciens* MR-1, carrying a plasmid with a constitutively-expressed *gfp* gene in the presence of less than 0.7 mg l^{-1} dissolved oxygen in the bulk aqueous medium. GFP fluorescence has also been detected in actively-growing, individual cells of the dissimilatory sulfate-reducing bacterium *Desulfovibrio desulfuricans*, carrying a plasmid with a constitutively-expressed *gfp* gene (Neal, Techkarnjanaruk, Mead, unpublished results). Thus, it appears that the *gfp* gene can be transcribed and the GFP product induced to fluoresce in bacteria cultured under conditions of low oxygen concentration.

SUMMARY

Gene expression of cells attached to surfaces has received increased attention in the past few years. Gene fusions involving *lux*, *lacZ* and *gfp* have facilitated the detection of cells and changes in cell metabolism following attachment to a variety of surfaces. There is growing evidence that cells on surfaces, and in particular, those within biofilms, display unique physiologies different from those displayed by cells in suspension. There appears to be considerable heterogeneity in gene expression among isogenic population of cells on surfaces. The significance of this phenomenon may relate to survival and fitness of the population. What remains to be determined is

how gene expression in one population affects gene expression and cell fitness in surrounding microbial populations within biofilms.

ACKNOWLEDGMENTS

The authors wish to acknowledge NSF grants No. OCE-9720151 and EEC-8907039, and U.S. Department of Energy grant No. ER-62719 and subcontract GOOO668 from Washington State University. Part of this work was supported by the Australian Research Council and The Flinders University of South Australia.

REFERENCES

Abrahamson M., Lewandowski Z., Geesey G., Skjak-Braek G., Strand W., Christensen B.E. (1996). Development of an artificial biofilm to study the effects of a single microcolony on mass transport. *J Microbiol Methods*, 26, 161–169.

Andersen J.B., Sternberg C., Poulsen L.K., Bjørn S.P., Givskov M., Molin S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol*, 64, 2240–2246.

Caldwell D.E., Korber D.R., Lawrence J.R. (1992). Confocal laser microscopy and digital image analysis in microbial ecology. *Adv Microb Ecol*, 12, 1–67.

Cormack B.P., Valdivia R.H., Falkow S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene*, 173, 33–38.

Dalton H.M., Goodman A.E., Marshall K.C. (1996). Diversity in surface colonization behavior in marine bacteria. *J Ind Microbiol*, 17, 228–234.

Dalton H.M., Poulsen L.K., Halasz P., Angles M.L., Goodman A.E., Marshall K.C. (1994). Substratum-induced morphological changes in a marine bacterium and their relevance to biofilm structure. *J Bacteriol*, 176, 6900–6906.

Davies D.G., Geesey G.G. (1995). Regulation of the alginate biosynthetic gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Appl Environ Microbiol*, 61, 860–867.

Davies D.G., Chakrabarty A.M., Geesey G.G. (1993). Exopolysaccharide production in biofilms, substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl Environ Microbiol*, 59, 1181–1186.

Davies D.G., Parsek M.R., Pearson J.P., Iglewski B.H., Costerton J.W., Greenberg E.P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*, 280, 295–298.

Delagrave S., Hawtin R.E., Silva C.M., Yang M.M., Youvan D.C. (1995). Red-shifted excitation mutants of the green fluorescent protein. *Bio/Technology*, 13, 151–154.

Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A., Smith F. (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem*, 28, 350–356.

Gooday G.W. (1990). The ecology of chitin degradation. *Adv Microb Ecol*, 11, 387–430.

Hastings W. (1996). Chemistries and colors of bioluminescent reactions, a review. *Gene*, 173, 5–11.

Heim R., Cubitt A.B., Tsien R.Y. (1995). Improved green fluorescence. *Nature (Lond)*, 373, 663–664.

Hoyle B.D., Williams L.J., Costerton J.W. (1993). Production of mucoid exopolysaccharide during development of *Pseudomonas aeruginosa* biofilms. *Infect Immun*, 61, 777–780.

Jacobs M., Hill P.J., Stewart G.S.A.B. (1991). Highly bioluminescent *Bacillus subtilis* obtained through high-level expression of a *lux*AB fusion gene. *Mol Gen Genet*, 230, 251–256.

Kudo H., Cheng K.-J., Costerton J.W. (1987). Interactions between *Treponema bryantii* and cellulolytic bacteria in the *in vivo* degradation of straw cellulose. *Can J Microbiol*, 33, 244–248.

Lawrence J.R., Korber D.R., Wolfaardt G.M., Caldwell D.E. (1995). Behavioral strategies of surface-colonizing bacteria. *Adv Microb Ecol*, 15, 1–75.

Mack W.N., Mack J.P., Ackerman A.O. (1975). Microbial film development in a trickling filter. *Microb Ecol*, 2, 215–226.

Matthysse A.G., Stretton S., Dandie C., McClure N.C., Goodman A.E. (1996). Construction of GFP vectors for use in Gram-negative bacteria other than *Escherichia coli*. *FEMS Microbiol Lett*, 145, 87–94.

McLean R.J.C., Whitely M., Stickler D.J., Fuqua W.C. (1997). Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiol Lett*, 154, 259–263.

Meighen E.A. (1991). Bacterial bioluminescence, organization, regulation and application of the *lux* genes. *FASEB J*, 7, 1016–1022.

Mittelman M.W., King J.M.H, Sayler G.S., White D.C. (1992). On-line detection of bacterial adhesion in a shear gradient with bioluminescence by *Pseudomonas fluorescens* (*lux*) strain. *J Microbiol Methods*, 15, 53–60.

Mittelman M.W., Packard J., Arrage A.A., Bean S.L., Angell P., White D.C. (1993). Test system for determining antifouling coating efficacy using on-line detection of bioluminescence and fluorescence in a laminar-flow environment. *J Microbiol Methods*, 18, 51–60.

Møller S., Pedersen A.R., Poulsen L.K., Arvin E., Molin S. (1996). Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assess by quantitative *in situ* hybridization and scanning confocal laser microscopy. *Appl Environ Microbiol*, 62, 4632–4640.

Møller S., Sternberg C., Anderson J.B., Christensen B.B., Ramos J.L., Givskov M., Molin S. (1998). *In situ* gene expression in mixed-culture biofilms, evidence of metabolic interactions between community members. *Appl Environ Microbiol*, 64, 721–732.

Palmer R. Jr., Phiefer C., Burlage R., Sayler G., White D. (1996) Single-cell bioluminescence and GFP in biofilm research. In: Hastings J.W., Kricka L.J., Stanley P.E. (eds) *Bioluminescence and Chemiluminescence, Molecular Reporting with Photons*. John Wiley and Sons, Chichester, UK, pp. 445–450.

Poulsen L.K., Ballard G., Stahl D.A. (1993). Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. *Appl Environ Microbiol*, 59, 1354–1360.

Robinson P.J., Walker J.T., Keevil C.W., Cole J. (1995). Reporter genes and fluorescent probes for studying the colonisation of biofilms in drinking water supply line by enteric bacteria. *FEMS Microbiol Lett*, 129, 183–188.

Sheintuch M., Vashitz O., Wolffberg A. (1992). Engineering applications of bioluminescence: modeling of mass transport in a hollow fiber and in a chemostat. *Chem Eng Sci*, 47, 2615–2620.

Stickler D.J., Morris N.S., McLean R.J.C., Fuqua C. (1998). Biofilms on indwelling urethral catheters produce quorum-sensing signal molecules *in situ* and *in vitro*. *Appl Environ Microbiol*, 64, 3486–3490.

Stretton S., Techkarnjanaruk S., McLennan A.M., Goodman A.E. (1998). Use of green fluorescent protein to tag and investigate gene expression in marine bacteria. *Appl Environ Microbiol*, 64, 2554–2559.

Valdivia R.H., Hromocky A.E., Monack D., Ramakrishnan L., Falkow S. (1996). Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene*, 173, 47–52.