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On-line detection of bacterial adhesion in a shear gradient with bioluminescence by a *Pseudomonas fluorescens (lux)* strain

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Summary

On-line detection of bacterial adhesion in a shear flow gradient was measured with an engineered bioluminescent bacterium. Parallel plate flow cells showed colonization by a *Pseudomonas fluorescens* strain containing the *Vibrio fischeri (lux)* bioluminescent operon. When induced by sodium salicylate, bulk phase and sessile cells were assayed quantitatively with an ammeter-photomultiplier-fiber optic system. The detection limit in a shear force gradient was 2×10^5 attached cells·cm⁻² on glass coupons. Light production was found to correlate with biofilm lipid synthesis on a per unit area basis (1-¹⁴C-acetate incorporation). Light correlated directly with sessile bacterial acridine orange direct counts in the range of 10^5 – 10^7 cells·cm⁻², providing on-line detection of both biofilm and bulk phase biomass and specific metabolic activity when induced.

Key words: Bacterial adhesion; Bacterial detection; Biofilm; Bioluminescence; Monitoring

Introduction

Conditions affecting bacterial adhesion with the formation of biofilms are important considerations in many industrial applications. Microbial biofouling leads to increased corrosion rates [1], impedance of heat transfer [2], and detrimental changes in fluid hydraulics [3]. Microbial biofilms are the primary reservoir for planktonic bacteria which contaminate pharmaceutical products [4], medical devices [5], and microelectronics [6].

Recently employed methods for detecting in situ biofilm activity include confocal laser microscopy [7], electrochemical impedance spectroscopy (EIS) [8], and attenu-

ated total reflectance Fourier transform spectroscopy (ATR-FTIR) [9]. These evolving technologies are complex, however, and involve relatively expensive instrumentation. In addition, they fail to distinguish between viable and nonviable biofilm bacteria and thus are limited in their applicability to studies of metabolic activity.

Bacterial bioluminescence as an indicator of cell number and viability has been applied to several test systems [10]. Jassim et al. [11] have described an *in vivo* bioluminescence technique for evaluating biocide effects on planktonic bacterial populations. King et al. [12] utilized a bioluminescent reporter plasmid to evaluate aromatic hydrocarbon utilization in contaminated soils. Vashitz et al. [13] optimized continuous culture conditions in a bioreactor using bioluminescence as a biosensor for activity. A US patent [14] has been issued for the application of bioluminescence to microbial detection and identification.

Our goal in the present investigation was the development of a real-time system for measuring bacterial adhesion under conditions which simulate those of *in situ* environments. A number of cell adhesion experiments were performed which were designed to evaluate the potential utility of bioluminescent bacteria in adhesion studies. The system described herein utilized an engineered light emitting strain to provide not only detection of adhesion, but also defined metabolic activity in the biofilm.

Materials and Methods

Bioluminescent Strain Construction

The details of the bioluminescent construct, *P. fluorescens* 5RL, have been previously described by King et al. [12]. A bioluminescent reporter plasmid for naphthalene catabolism designated PUTK21 was constructed by transposon mutagenesis of a *P. fluorescens* isolate using the *lux* transposon, Tn4431 [15]. Southern hybridization studies demonstrated that the *lux* transposon was inserted adjacent to the *nahH* gene in a 1.6 kb *Pst*I fragment and within the *nahG* (salicylate hydroxylase) gene [12]. Luciferase-mediated light production was induced upon exposure to sodium salicylate. Salicylate was not, however, utilized by this construct as a carbon source.

Media composition and adhesive strain selection

Preliminary studies utilized collar formation in shake flasks incubated 48 h at 25 °C as a criterion for adhesive selection. A yeast extract-polypeptone-glucose medium (YEPG), containing ($\text{g}\cdot\text{l}^{-1}$) yeast extract, 0.20; polypeptone, 2.0; NH_4NO_3 , 0.20 at pH 7.1 produced a significant collar from which an adherent strain was selected. Tetracycline HCl was added at $14\text{ mg}\cdot\text{l}^{-1}$ to maintain selective pressure on the organisms. The light induction medium consisted of $0.2\text{ g}\cdot\text{l}^{-1}$ sodium succinate in a minimal medium composed of ($\text{g}\cdot\text{l}^{-1}$) sodium succinate, 0.10; D-glucose, 0.10; yeast extract (Difco, Detroit, Michigan), 0.10; NH_4NO_3 , 0.023; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.10; KH_2PO_4 , 0.68; K_2HPO_4 , 1.73; Wolfe's complex vitamins-minerals, 0.1 ml. The complete medium had a pH of 7.1–7.2. Viable plate counts were performed on YEPG agar with $14\text{ mg}\cdot\text{l}^{-1}$ tetracycline. All reagents employed in this study were obtained from Sigma Chemical (St Louis, Missouri) or Difco Laboratories (Detroit, Michigan) and were of analytical reagent grade, unless otherwise noted.

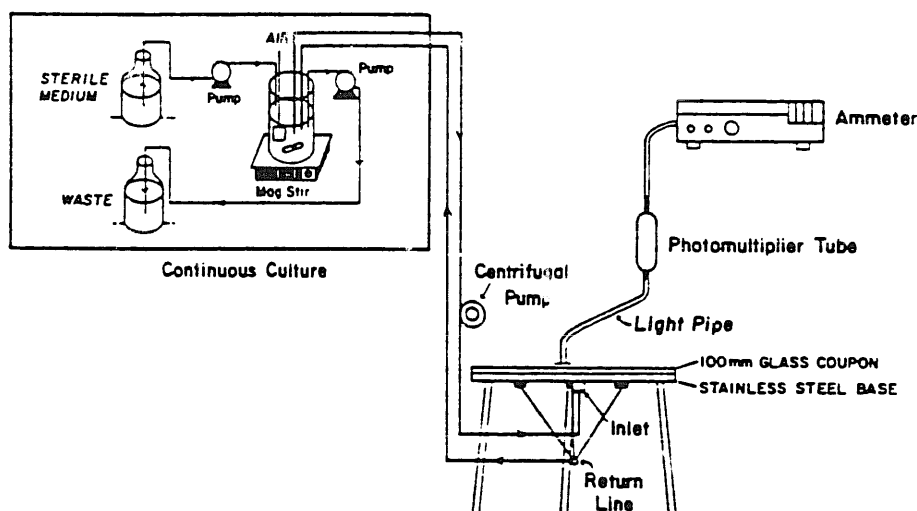


Fig. 1. Schematic of in situ CAMM biofilm monitoring system in which the shear force is directly proportional to the flow rate and inversely proportional to the radius of the interaction. The bioluminescence is measured through a glass plate on the CAMM upper surface.

Continuous culture-adhesion cell system

A series of 2-l reaction vessels containing 1-l working volume were connected in parallel to cell adhesion measurement modules (CAMM) fitted with 100 mm diameter (5.7 mm thick) borosilicate glass coupons. The CAMM system provided a means by which fluid shear across the coupons could be continuously varied in direct proportion to flow rate. The system as described by Mittelman et al. [16] was modified by inclusion of a glass coupon to permit in situ light detection (Fig. 1). Glass coupons were washed in a detergent solution (Alconox, New York) followed by a chloroform-acetone-methanol-deionized water solvent series prior to use. With the exception of heat-labile pump heads and flow meters which were ethylene oxide-sterilized, all system components were steam sterilized prior to media introduction and inoculation.

Following inoculation of the reaction vessels with 1 ml of an 18-h broth culture, the suspension was allowed to recirculate through the CAMM without dilution for 4 h at volumetric flow rates and fluid shear stresses ranging from 0.25 to 1.3 $\text{l}\cdot\text{min}^{-1}$ and 1.0 to 40 $\text{dyn}\cdot\text{cm}^{-2}$, respectively. At the end of the 4-h period, the YEPG dilution rate was maintained at 0.2 h^{-1} . Aeration of the vessels was maintained at $\approx 0.25 \text{ l}\cdot\text{min}^{-1}$. Following recirculation periods ranging from 18 to 87 h, the CAMM were flushed at the in situ flow rates with 1.5 l of the salicylate induction medium. Cell-free salicylate medium was then recirculated through the CAMM for 3 h, with replacement of the medium at 1-h intervals. All experiments were conducted at 25 °C in a laminar-flow hood.

Light measurement

Bioluminescence was measured in situ through the glass coupons as well as via grab samples of the bulk phase. A flexible liquid light cable and collimating beam probe (0.25- cm^2 collection area) were utilized for light acquisition. Detection of photoelec-

tric-induced current was made with an Oriel (Stratford, Connecticut) photomultiplier-digital readout system (Model 77340;7070). The light detection system was calibrated electronically in a light-proof container prior to and following each sampling. In situ coupon measurements were obtained by inserting the beam probe through a light-proof fabric placed at the top of each CAMM (Fig. 1). Light measurements were taken in triplicate at several radii on each coupon by manually directing the probe across the coupon surfaces.

Immediately following the coupon measurements, 2-ml samples were taken from the bulk liquid phase and placed in 1-cm pathlength polystyrene cuvettes; light measurements were made within 30 s of sampling. The probe was placed directly in contact with the cuvettes which were contained within light-proof cuvette holder assemblies.

Enumeration of bacteria

Viable plate counts of bulk phase organisms were performed on YEPG agar. Plates were incubated at 25 °C for 72 h prior to counting.

Extracts from glass coupons were obtained via sonication of defined areas in a 10 mM, pH 7.2, disodium pyrophosphate buffer solution. Glass O-ring extractors (Kontes Glass, Vineland, New Jersey) facilitated removal of cells and biomass from the glass surfaces. The CAMM stainless steel surfaces underlying the glass coupons were quantitatively scraped with cell-free Teflon scrapers and sterile cotton-tipped swabs saturated with 10 mM, pH 7.2, disodium pyrophosphate buffer solution. The swabs were then placed in small volumes of pyrophosphate buffer containing 2% (v/v) glutaraldehyde as a preservative, and sonicated for several seconds to disaggregate cells from the swab surfaces.

The coupon and CAMM extracts were processed for AODC by filtration through 0.2- μm polycarbonate filters (Nuclepore, Pleasanton, California), staining for 5 min in 0.1 mg·ml⁻¹ acridine orange (AO) in 100 mM phosphate buffer, pH 7.2, then examination under epifluorescent illumination. Direct epifluorescent microscopic examination of AO stained coupons following extraction revealed that >95% of the adherent cells were removed during the sonication procedure.

Metabolic activity

A 200 ml volume of the cell-free succinate medium containing 0.25 $\mu\text{Ci}\cdot\text{ml}^{-1}$ of D-[¹⁴C(U)]glucose (New England Nuclear, Wilmington, Delaware) with a specific activity of 340 mCi·mmol⁻¹ was recirculated through the CAMM at the in situ flow rates. The cells in the CAMM were induced prior to labeling as described above. Following a 60-min labeling period, the coupons were immediately removed from the CAMM, drained of residual bulk phase liquid, placed in a sealed 150 mm Petri dish containing 1 ml 25% (v/v) glutaraldehyde, then refrigerated overnight.

The pulse-labeled coupons and CAMM underlying stainless steel surfaces were processed as described above for cell counts. A Bligh-Dyer lipid extraction protocol, which employed chloroform-methanol-water extraction reagents [17], was utilized for radiolabeled lipid extraction. Lipid extracts were counted in 4 ml Ecolume (ICN Biomedicals, Irvine, California) on a LKB model 1212 (Gaithersburg, Maryland) liquid scintillation counter. The counts were quench corrected using a series of external standards (Packard, Downers Grove, Illinois). Further details of this procedure may be found in Mittelman et al. [16].

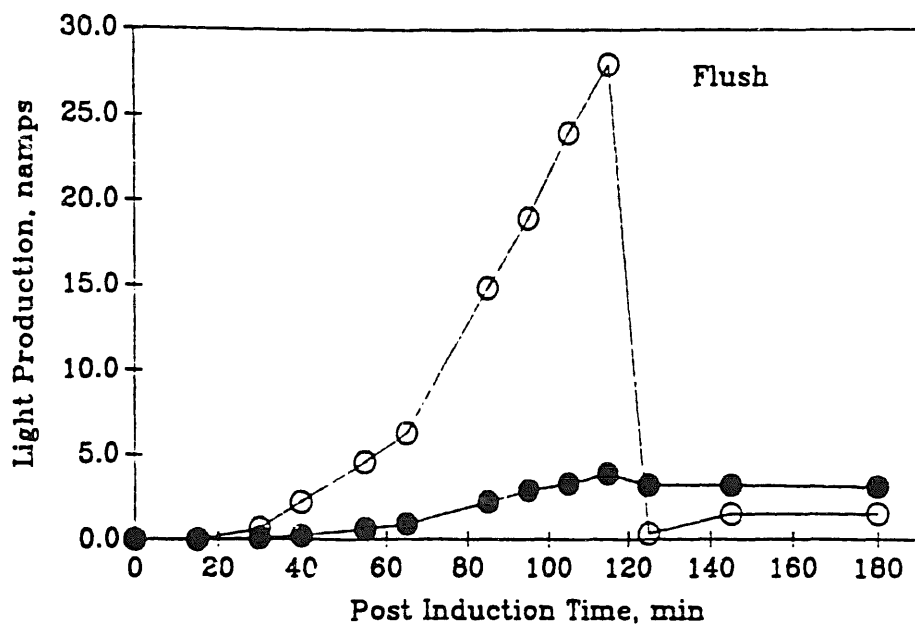


Fig. 2. Effect of flushing procedure on bulk phase (\circ) and biofilm (\bullet) bioluminescence. Coupons were flushed with cell-free medium containing sodium salicylate 2-h following induction.

Results and Discussion

Initial light production in the bulk phase was evident within ≈ 25 min after salicylate induction of an 18-h continuous culture (Fig. 2). After ≈ 2 h following onset of induction, bulk phase light production increased to ≈ 28 nA. Replacement of the

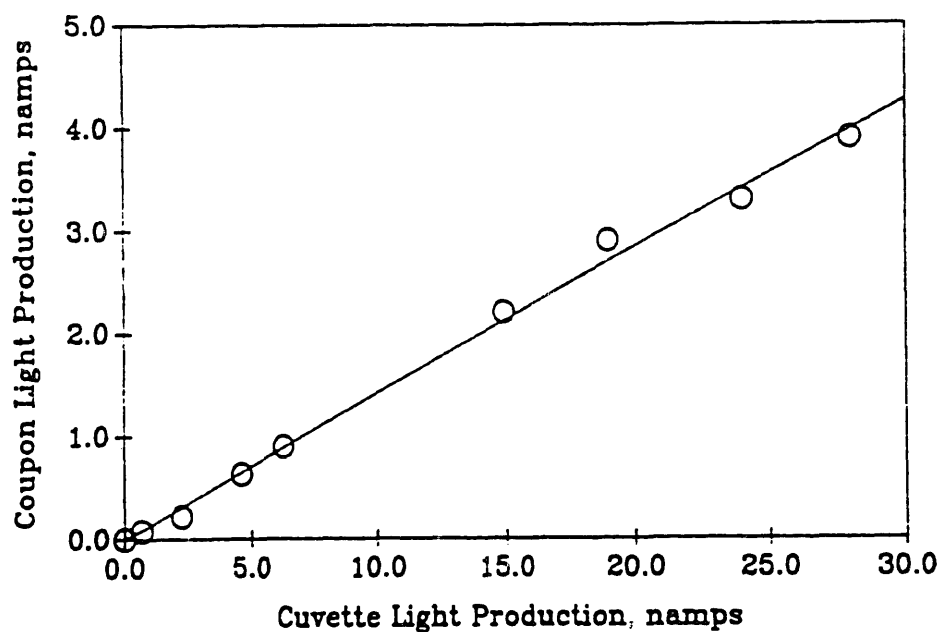


Fig. 3. Relationship between bulk phase and biofilm bioluminescence under flowing conditions.

TABLE 1

EFFECT OF BIOFILM METABOLIC ACTIVITY ON LIGHT PRODUCTION

Substratum	uptake $\cdot \text{cm}^{-2\text{a}}$	light production (μA)
Glass	3.0×10^{-8} (7.1×10^{-9}) ^b	5.2×10^{-4} (1.0×10^{-4})
Stainless	4.0×10^{-7} (2.0×10^{-7})	7.6×10^{-4} (1.2×10^{-4})
	cells $\cdot \text{cm}^{-2}$	uptake ($\mu\text{mol} \cdot \text{cell}^{-1}$)
Glass	2.6×10^5 (2.5×10^5)	1.2×10^{-13} (1.2×10^{-13})
Stainless	3.3×10^6 (1.3×10^6)	1.2×10^{-13} (3.1×10^{-13})

^a $\mu\text{mol glucose} \cdot 60 \text{ min}^{-1}$ pulse-labeling. ^b () = SD.

bulk phase with cell-free media resulted in an immediate drop in light production. Light production in the biofilm, however, was not significantly influenced by the flushing procedure (Fig. 2). Biofilm light production stabilized within 2 h following sodium salicylate induction.

The contribution of bioluminescent bulk phase bacteria to the measured light was eliminated by the flushing process. Fig. 3 illustrates the relationship between light measured in the 1-cm lightpath cuvette and that measured in situ through the coupon during a 2-h recirculation at $1 \text{ l} \cdot \text{min}^{-1}$. A linear equation describing the influence of bulk phase light production on the in situ measurements was obtained from a least squares linear regression of the data ($r^2=0.998$). The equation, $y=0.142x - 5.44 \times 10^{-12}$, was employed as a correction factor to compensate for the bulk phase

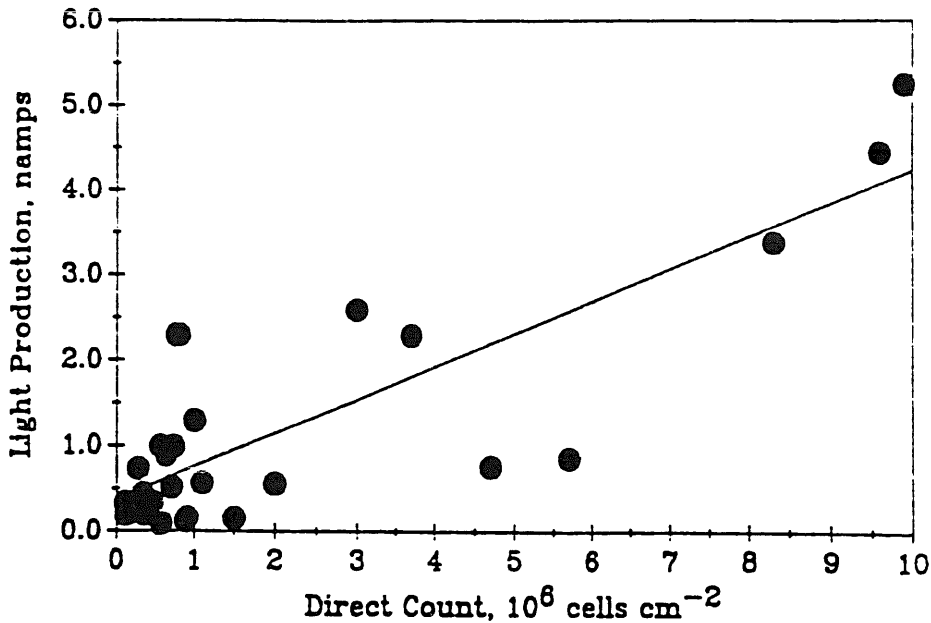


Fig. 4. Relationship between bioluminescence and AODC of induced *P. fluorescens (lux)* attached to glass under flowing conditions. Average shear stress was calculated to be $\approx 20 \text{ dyn} \cdot \text{cm}^{-2}$.

effect on biofilm light production values (x).

Bioluminescence detected through the glass coupon was a function of attachment to both the CAMM glass and stainless steel substrata. Light output, cell counts, and per cell metabolic activity were shown in Table 1 for 72 h biofilms. On a per cell basis, the activities of the colonized cells were equivalent on the two surfaces. Bioluminescence originating in the stainless steel biofilms was distinguished from that produced on glass coupons by replacing the colonized glass with a clean coupon prior to light measurement.

A comparison of light production between bulk phase and glass biofilm organisms revealed that per cell, light production was approximately the same, from $1.1 - 2.4 \times 10^{-7}$ and $2.1 - 4.0 \times 10^{-7}$ nA \cdot cell $^{-1}$, respectively.

Both the glass and stainless steel substrata were colonized to approximately the same extent. Colonization decreased with exposure time in a shear force gradient, ranging from 7.7×10^6 to 3.9×10^5 cells \cdot cm $^{-2}$ at 18 and 87 h, respectively. A total of 36 light and AODC measurements were taken from 18 - 87 h induced biofilms exposed to 1 - 40 dyn \cdot cm $^{-2}$ fluid shear. Biofilm light production was positively correlated ($r^2 = 0.826$; significant at $P < 0.01$) with biofilm cell number on glass substrata in the range of 1×10^5 to 1×10^7 cells \cdot cm $^{-2}$ (Fig. 4). The cell counts obtained represent extracts from glass coupons exposed to different shear forces and exposure times. The threshold light detection value in this test system was 0.05 nA, which corresponded to 10^5 cells \cdot cm $^{-2}$ on the glass coupon with an equivalent contribution from the stainless steel plate. A similar threshold value, 0.05 nA, was obtained for 10^5 cells \cdot ml $^{-1}$ bulk phase organisms detected in a 1-cm pathlength cuvette.

This threshold value is based upon total cell counts rather than viable counts. Recovery of viable cells from intact biofilms is inefficient and, for fastidious and stressed environmental isolates, may be negatively biased [18]. The use of direct counts in establishing calibration curves provides a conservative estimate of biofilm bacterial numbers.

The results reported herein compare favorably with those obtained with other, on-line biofilm detection methods. Nivens et al. [19] showed that 10^4 and 5×10^5 cells \cdot cm $^{-2}$ could be detected using ATR-FTIR and a piezoelectric quartz crystal microgravimetric technique, respectively. Detection of bioluminescent reporter strains should improve with the application of more sophisticated light measuring systems such as the CCD imaging device described by Hooper et al. [20].

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References

- 1 Costerton, J. W. and Boivin, J. (1988) Microbially influenced corrosion. In: *Biological Fouling of Industrial Water Systems: A problem Solving Approach* (Mittelman, M. W. and Geesey, G. G., eds.), pp. 56 - 76, Water Micro Association, San Diego.
- 2 McCoy, W. F. and Costerton, J. W. (1982) Fouling biofilm development in tubular flow systems. *Dev. Ind. Microbiol.* 23, 551 - 558.

- 3 Characklis, W.G. (1973) Attached microbial growths i. Attachment and growth. *Water Res.* 7, 1113 – 1127.
- 4 Mittelman, M.W., Islander, R. and Platt, R.M. (1987) Biofilm formation in a closed-loop purified water system. *Med. Device Diagn. Ind.* 9, 50 – 55.
- 5 Passerini, L., Phang, P.T., Jackson, F.L., Lam, K., Costerton, J.W. and King, E.G. (1990) Biofilms on right heart flow-directed catheters. *Chest* 92, 440 – 446.
- 6 Craven, R.A., Ackerman, A.J. and Tremont, P.L. (1986) High purity water technology for silicon-wafer cleaning technology. *Microcontam.* 4, 14 – 21.
- 7 Lawrence, J.R., Korber, D.R., Hoyle, B., Costerton, J.W. and Caldwell, D.E. (1990) Visualization of microbial biofilm architecture using confocal scanning laser microscopy. *Abstr. Q-28*, p. 293. *Abstr. 90th Annu. Meet. Am. Soc. Microbiol.* 1990.
- 8 Dowling, N.J.E., Guezennec, J., Lemoine, M.L., Tunlid, A. and White, D.C. (1988) Corrosion analysis of carbon steel affected by aerobic and anaerobic bacteria in mono and co-culture using AC impedance and D.C. techniques. *Corrosion* 44, 869 – 874.
- 9 Jolley, J.G., Geesey, G.G., Hankins, M.R., Wright, R.B. and Wichlacz, P.L. (1989) In situ real-time FT-IR-CIR-ATR study of the biocorrosion of copper by gum arabic acid bacterial culture supernatant and *Pseudomonas atlantica* exopolymer. *Appl. Spectrosc.* 43, 1062 – 1067.
- 10 Stewart, G.S.A.B. (1990) In vivo bioluminescence: New potentials for microbiology. *Lett. Appl. Microbiol.* 10, 1 – 8.
- 11 Jassim, S.A.A., Ellison, A., Denyer, S.P. and Stewart, G.S.A.B. (1990) In vivo bioluminescence: a cellular reporter for research and industry. *J. Bioluminesc. Chemiluminesc.* 5, 115 – 122.
- 12 King, J.M.H., Digrazia, P.M., Applegate, B., Burlage, R., Sanseverino, J., Dunbar, P., Larimer, F. and Saylor, G.S. (1990) Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. *Science* 249, 778 – 781.
- 13 Vashitz, O., Ulitzur, S. and Sheintuch, M. (1990) Forced bioluminescence fluctuations – a tool for feed optimization. *Appl. Microbiol. Biotechnol.* 33, 154 – 160.
- 14 Ulitzer, S.Y. and Kuhn, J.C. (29 August 1989) US Patent 4861709.
- 15 Shaw, J.J. and Kado, C.I. (1987) Direct analysis of the invasiveness of *Xanthomonas campestris* mutants generated by Tn4431, a transposon containing a promoterless luciferase cassette for monitoring gene expression. *Proc. 3rd Int. Symp. Mol. Genet. Plant – Microe Assoc.*, pp. 57 – 60, Montreal.
- 16 Mittelman, M.W., Nivens, D.E., Low, C. and White, D.C. (1990) Differential adhesion, activity, and carbohydrate:protein ratios of *Pseudomonas atlantica* monocultures attaching to stainless steel in a linear shear gradient. *Microb. Ecol.* 19, 269 – 278.
- 17 Bligh, E.G. and Dyer, E.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911 – 917.
- 18 Colwell, R.R., Brayton, P.R., Grimes, D.J., Roszak, D.B., Huq, S.A. and Palmer, L.M. (1985) Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for release of genetically engineered organisms. *Biotechnol.* 3, 817 – 820.
- 19 Nivens, D.E., Chambers, J.Q. and White, D.C. (1990) Monitoring microorganisms at solid surfaces using on-line devices. In: *Microbially Influenced Corrosion and Biodeterioration* (Dowling, N.J.E., Mittelman, M.W. and Danko, J.C., eds.), pp. 547 – 556, University of Tennessee, Knoxville.
- 20 Hooper, C.E., Ansoerge, R.E., Browne, H.M. and Tomkins, P. (1990) CCD imaging of luciferase gene expression in single mammalian cells. *J. Bioluminesc. Chemiluminesc.* 5, 123 – 130.