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Reporter genes and fluorescent probes for studying the colonisation of biofilms in a drinking water supply line by enteric bacteria

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Abstract

Biofilms containing diverse microflora were developed on bitumen-painted steel and glass tiles suspended in a chemostat model of a water distribution system. *Escherichia coli*, taken from a naturally occurring biofilm, was transformed with a plasmid containing the anaerobically induced *nirB* promoter fused to the *lacZ* reporter gene. The resulting transformant, PRB1, was introduced into the chemostat. After 7 and 13 days, an *E. coli* strain with an anaerobically induced Lac^+ phenotype was present in the biofilm. Development of an episcopic differential interference contrast technique combined with UV fluorescence microscopy enabled the simultaneous visualization of *E. coli* in the biofilm using a fluorescent probe to detect expression of the *gusA* reporter gene and a *lacZ* fluorescent probe to monitor anaerobic expression of β-galactosidase from *pnirB*.

Keywords: *Escherichia coli*; Biofilm; Differential interference contrast microscopy; Fluorescence microscopy; *Escherichia coli nirB* promoter; *lacZ* reporter gene; *gusA* reporter gene; Anaerobic regulation

1. Introduction

Water treated and supplied for drinking and domestic purposes is not sterile because biofilms are found on pipework used in its distribution [1]. Biofilms occur as a result of the attachment of microorganisms to surfaces followed by growth, formation of products such as extracellular polysaccharide and eventually sloughing of bacteria into the system. Extracellular polysaccharides, formed at the surface of the biofilm, are involved in the entrapment of nutrients creating a favourable environment in a situation otherwise deficient in nutrients [2]. These extracellular polysaccharides not only protect the biofilm itself from residual chemical disinfectants but also allow the attachment of other bacteria to the developing biofilm. Contamination of drinking water is a public health concern because it is assumed that the presence of microorganisms such as *Escherichia coli* is the result of faecal contamination of human or animal origin. The presence of *E. coli* in biofilms on water distribution pipes has attracted particular inter-

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est because of the risk they pose to the quality of drinking water.

Although the availability of fluorescent substrates to detect gene expression has allowed non-invasive methods to be developed to study the presence of particular strains or species of bacteria within a biofilm [3], no-one has reported the use of reporter genes such as *lacZ* and *luxAB* fused to nutrient-sensitive promoters as environmental sensors in biofilm studies. In this work a recombinant plasmid has been exploited which carries the *lacZ* gene fused downstream of the anaerobically induced *nirB* promoter. Transcription from the *nirB* promoter is repressed during aerobic growth, induced by anaerobiosis and further induced by the presence of nitrite or nitrate in the growth environment [4,5]. This induction is dependent on the regulatory protein, FNR, a factor essential for the transcription of a number of genes expressed during anaerobic growth [4,6,7]. Expression from *pnirB* is also activated by NarL in response to nitrate and nitrite and activated by NarP in response to nitrate but not nitrite [8].

The aims of this work were to introduce the anaerobically induced *nirB* promoter, fused to a *lacZ* reporter gene, into an *E. coli* strain which had been isolated from a natural biofilm, to demonstrate the re-attachment of this *E. coli* to the biofilm and to investigate whether this O_2-sensitive, plasmid-encoded gene was expressed within the biofilm.

2. Materials and methods

2.1. Strains and plasmids used and their source

Strains and plasmids used in this work are listed in Table 1 together with details of their source or method of construction. The plasmid, pAA182, is a pBR322-based promoter probe vector [5].

2.2. Screening and maintenance of strains

Bacteria purified from biofilms were screened for Amp^R or Tet^R by plating onto minimal medium or nutrient agar supplemented with either 15 mg/ml tetracycline or 80 mg/ml ampicillin.

Stock cultures were maintained at −70° C in Lennox Broth supplemented with 15% (v/v) glycerol. Working cultures were maintained for up to 8 weeks on nutrient agar at 4° C.

Table 1
E. coli strains and plasmids used and their source

Strain or plasmid	Description	Reference or source
Strain		
M182	*lac*.fnr$^+$	[15]
PRB1	Amp^S Lac^-	Thames Water Utilities plc
Plasmid		
pR150	pRB322-based promoter probe vector with *pnirB*	[18]

2.3. Media used and growth conditions for culturing bacteria in the biofilm

Heterotrophic biofilm bacteria were counted on the low-nutrient R2A minimal medium [9] which minimizes substrate shock for nutrient stressed organisms. *E. coli* PRB1 was reisolated from the biofilm by selecting blue Lac^+ Amp^R colonies on minimal medium supplemented with 80 μg/ml ampicillin and 1% 5-bromo-4-chloro-3-indolyl galactopyranoside (X-Gal, Novobiochem; A11311). Cultures were grown at 30° C for 7 days.

2.4. Continuous-culture model for studying biofilm formation

A chemostat was inoculated with a natural microbial flora obtained from a biofilm formed from water supplied by Thames Water Utilities plc. The microbial flora, partially identified by the API 20NE and API 20E system (API Biomeriux, Basingstoke, UK) according to the manufacturer's instructions, included *Pseudomonas* sp., *Aeromonas* sp., *Serratia* and *Methylobacteria* sp. The *E. coli* was an environmental isolate obtained from Thames Water Utilities plc and was inoculated as 10 ml suspensions of 10^6 cfu/ml of culture into the model when it was at steady state. The culture medium was Birmingham tap water which was filter-sterilized through a nylon membrane. Previous studies had shown that this simple membrane filtration technique provided water

that was both sterile and chemically unaltered [10]. The biofilm model originally consisted of two continuous-culture glass vessels in series, each with a working volume of 500 ml [11]. The first vessel contained the Thames Water inoculum and was supplied with filter-sterilized Birmingham tap water. The effluent from this fermenter was used to supply the second vessel. After creating biofilms with a sufficiently diverse microflora in the second vessel, the effluent line from the first vessel feeding the second was replaced by a line supplying filter-sterilized Birmingham tap water at a dilution rate of 0.02/h, and both chemostats were run in tandem. The top plate and sampling ports were made of titanium or stainless steel and all connectors and probes were made of glass so that there was no leaching of metal ions into the culture. The cultures were maintained aerobically at ambient temperature, between 20° C and 25° C. The pH was monitored but not controlled.

2.5. Biofilm formation

Biofilms were formed on 1 cm^2 bitumen-painted mild steel, as used in line distribution mains, or on 1 cm^2 glass tiles [11]. Tiles were suspended in vessels by titanium wire and sampled after immersion for various times in the culture. The tiles were removed aseptically, washed in sterile water and the biofilm was transferred with a dental probe into 4 ml of distilled water. The biofilm sample was vigorously vortexed to break up aggregates of cells; aliquots of serial dilutions were then plated onto selective or non-selective agar. A sample of the planktonic (liquid) phase was also taken at the same time in order to determine viable counts of bacteria.

2.6. Fluorescent probes for β-galactosidase and β-glucuronidase

The fluorescent probes, obtained from Molecular Probes Inc., Eugene, Oregon, USA, were Imagene Green C_8 FDG *lacZ*(I-2896) and Imagene Red C_{12}RGlcU GUS (I-2910) diluted to 33 and 50 μM, respectively, with sterile tap water and prewarmed to 37° C. Tiles were secured firmly onto microscope slides and 100 μl of each probe was added to the upper side of the tile. Tiles were viewed after 30 min at 37° C.

2.7. Differential interference contrast (DIC) microscopy

A Nikon Labophot-2 microscope equipped with both episcopic differential interference contrast (DIC) and epifluorescence was used to view biofilms on tiles [10,12]. An immunogold staining block (IGS) was used for DIC microscopy and rhodamine and fluorescein filter blocks were used for epifluorescence studies to detect the hydrolysis products of the GUS and β-galactosidase substrates, respectively. The objective lens was a non-contact M Plan Apo 40× and the light source was a 100 W halogen lamp. Neutral density filters were used where required to suppress high background fluorescence. Images were captured using a black and white charged-couple device camera and further enhanced and magnified using the Digithurst Microeye TC image analysis transputer card and software running on PC-compatible computers.

2.8. Assays for β-galactosidase

Bacteria for β-galactosidase determinations were grown aerobically or anaerobically in Lennox Broth supplemented with 0.4% (w/v) glucose, 80 mg/ml ampicillin and 20 mM nitrate or 2.5 mM nitrite. The β-galactosidase activities were assayed as described by Jayaraman et al. [5]. Activities are expressed as nmol *o*-nitrophenyl β-D-galactosidase hydrolysed min^{-1} (mg dry cell mass)$^{-1}$ and are the means of at least two independent, duplicated determinations that differed by no more than 5%.

2.9. Recombinant DNA techniques

Small-scale DNA preparations, restriction digests, agarose gel electrophoresis, the isolation of DNA fragments, the preparation and transformation of competent M182 and other routine DNA manipulations were as described by Maniatis et al. [13]. Competent cells of the biofilm isolate of *E. coli* were prepared and transformed as described by Hanahan [14].

3. Results and discussion

3.1. Introduction of Escherichia coli into a developing biofilm

Biofilms were established on tiles suspended in a 1 l fermenter fed for 14 days with the planktonic phase of a second fermenter containing biofilms of bacteria isolated from a Thames Water Utilities plc supply pipe. Although both Amp^R Lac^- and Amp^S Lac^+ bacteria were present in both the biofilms and the planktonic phase at this stage, no Amp^R Lac^+ strains could be detected. *E. coli* strain PRB1 was then introduced to a final density of 2×10^4 ml^{-1}. After a further 7 days, both the planktonic phase and the biofilms were screened for the presence of Amp^R Lac^+ bacteria. The density of such bacteria in the planktonic phase, identified as *E. coli* by the API 20E identification kit, had increased to 5×10^7 ml^{-1}. Typical counts of *E. coli* reisolated from 1 cm^2 tiles were 1.6×10^4 after 7 days and 1×10^3 after 13 days.

The presence of the NirB′LacZ fusion plasmid in strain PRB1 was exploited to confirm that the *E. coli* isolated from the biofilms were indistinguishable from those originally introduced into the fermenter. Plasmid DNA was purified from the colonies isolated from the biofilm and transformed into *E. coli* strain M182. The original *E. coli* PRB1, the strain isolated from the biofilm and M182 transformants were grown aerobically, or anaerobically in the presence and absence of nitrate or nitrite, and β-galactosidase activities were determined to estimate rates of transcription from the *nir* promoter. Transcription from the *nir* promoter in all three strains was repressed during aerobic growth, partially induced during anaerobic growth in the absence of an added electron acceptor and fully induced during anaerobic growth in the presence of nitrate or nitrite (Fig. 1), as expected [5]. These data confirmed that the *E. coli* strain recovered from the biofilm were identical to those introduced into the planktonic phase 7 days earlier. We conclude that *E. coli* can become established in biofilms on tiles supplied with domestic tap water and survive in the biofilm, at least for the duration of these experiments.

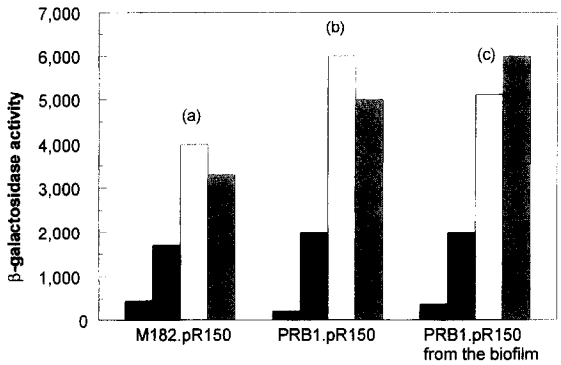

Fig. 1. Activity of the *nir* promoter during growth in different media. Bacteria were grown either aerobically in Lennox broth (solid bars), or anaerobically in Lennox broth supplemented with 0.4% (w/v) glucose as the carbon and energy source. Anaerobic cultures were grown with glucose alone (shaded bars), glucose plus 2.5 mM nitrite (open bars) or glucose plus 20 mM nitrate (hatched bars). The heights of the histogram indicate β-galactosidase expression from the *nir* promoter in *E. coli* strain M182.pR150 (a), PRB1.pR150 (b) and *E. coli* re-isolated from the biofilm (c). Units are nmol of ONPG hydrolysed min^{-1} (mg dry bacteria)$^{-1}$.

3.2. Visualization of E. coli in the biofilm using fluorescence microscopy

Thirteen days after *E. coli* PRB1 had been introduced into the chemostat, tiles were removed for microscopic examination after staining them with the β-galactosidase and GUS fluorescence probes. Tiles from the original feed culture to which no *E. coli* had been added served as controls. The microscope was initially used in DIC mode to observe the total biofilm flora [16]. It was then switched to epifluorescence mode to detect fluorescence from the hydrolysis products of the GUS and β-galactosidase substrates [16]. Switching between the rhodamine and fluorescein filters revealed identical areas of fluorescence due to the presence of the Lac^+ GUS^+ strain, *E. coli* PRB1 (Fig. 2a,b). Similar experiments with tiles from the control culture revealed areas of biofilm containing Lac^+ GUS^- bacteria, but, as expected, no areas of the biofilm were simultaneously stained with both substrates (Fig. 2c,d). These data confirmed that *E. coli* PRB1 was present in the biofilm and that at least some of them were in an environment that was sufficiently anaerobic for the *nirB* promoter to direct the synthesis of high levels of β-galactosidase.

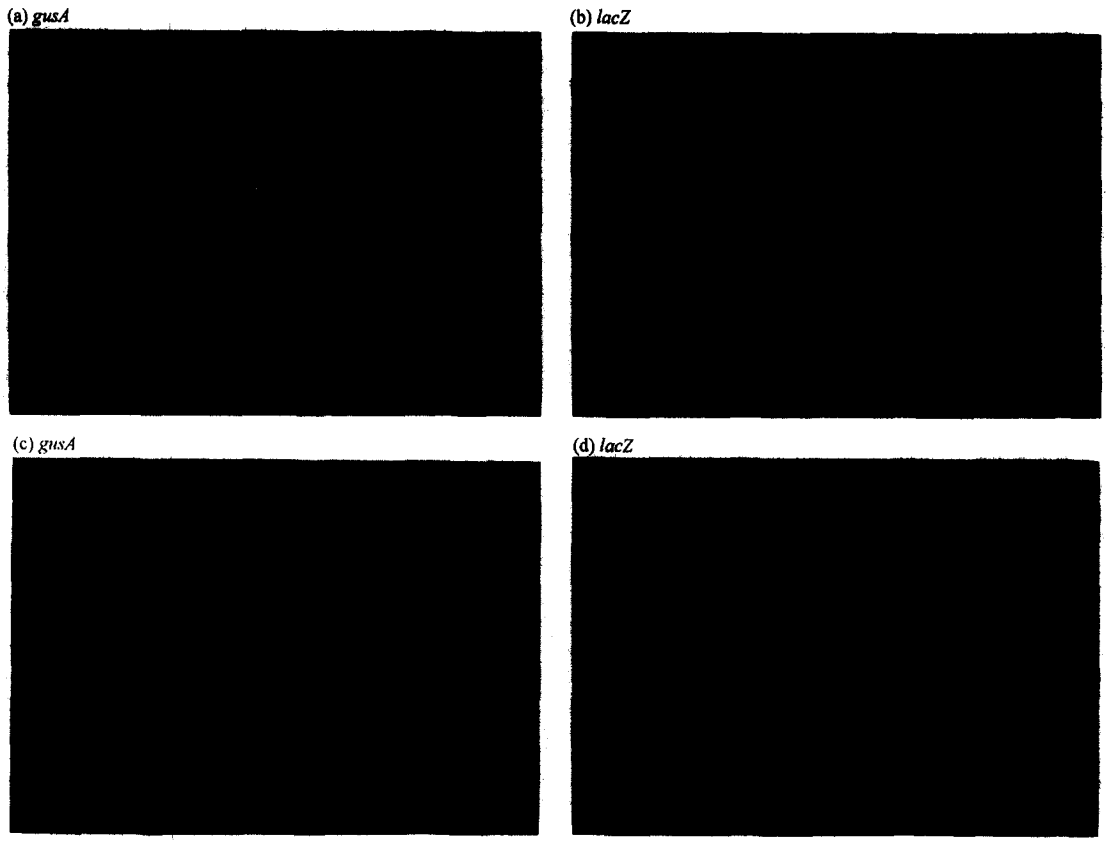

Fig. 2. Simultaneous visualization of fluorescence due to β-glucuronidase (**a**) and (**c**) and β-galactosidase (**b**) and (**d**) activity from bacteria in a 13-day-old biofilm using episcopic DIC microscopy in UV flourescence mode. The biofilm shown in (**a**) and (**b**) was removed from the chemostat that had been seeded with *E. coli* PRB1.pR150; the tile in (**c**) and (**d**) was taken from the initial chemostat used as a source of diverse microbial flora. Note the strong fluorescence with both probes only from areas of the biofilm in (**a**) and (**b**), indicative of the presence of Lac^+ GUS^+ *E. coli*.

In conclusion, three lines of evidence have been used to demonstrate that *E. coli* can become established in a biofilm. Prior to inoculation of PRB1, no other Lac^+Amp^R *E. coli* had been identified in the biofilm: *E. coli* recovered from the biofilm were indistinguishable from those introduced but were different from pre-existing flora. The presence of *E. coli* in the biofilm has been visualized using the β-glucuronidase marker enzyme which is synthesized by more than 90% of all environmental isolates of *E. coli* [17]. The chromophore, 5-bromo-4-chloro-3-indolyl galactopyranoside and a β-galactosidase fluorescent probe have also been used to detect β-galactosidase activity in these *E. coli*. The presence of high levels of β-galactosidase in microcolonies of *E. coli* within the biofilm suggests that the *nirB* promoter had been activated, so these microcolonies of *E. coli* must have been anaerobic in the biofilm.These studies emphasize the need, when assessing the safety of water for human consumption, to monitor the microbial population of biofilms which accumulate in the pipework of the water distribution system.

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References

[1] Block, J.C., Haudidier, K., Paquin, J.L., Miazga, J. and Levi, Y. (1993) Biofilm accumulation in drinking water distribution systems. Biofouling 6, 333–343.

[2] Ellwood, D.C., Keevil, C.W., Marsh, P.D. and Wardell, J.N. (1982) Surface-associated growth. Phil. Trans. Royal Soc. London B, 297, 513–532.

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

[4] Griffiths, L. and Cole, J. (1987) Lack of redox control of the anaerobically-induced *nirB* gene of *E. coli* K-12. Arch. Microbiol. 147, 364–369

[5] Jayaraman, P.S., Peakman, T.C., Busby, S.J., Quincey, R.V. and Cole, J.A. (1987) Location and sequence of the promoter of the gene for the NADH-dependent nitrite reductase of *Escherichia coli* and its regulation by oxygen, the FNR protein and nitrite. J. Mol. Biol. 76, 781–788.

[6] Cole, J.A. and Ward, F.B. (1973) Nitrite reductase-deficient mutants of *E. coli* K-12. J. Gen. Microbiol. 76, 21–29.

[7] Lambden, P. and Guest, J. (1976) Mutants of *E. coli* K-12 unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. 97, 145–160.

[8] Tyson, K.L., Bell, A.I., Cole, J.A. and Busby, S.J.W. (1993) Definition of nitrite and nitrate response elements at the anaerobically inducible *Escherichia coli nirB* promoter: interactions between FNR and NarL. Mol. Microbiol. 7, 151–157.

[9] Reasoner, D.J. and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1–7.

[10] Keevil, C.W. and Walker, J.T. (1992) Normarski DIC microscopy and image analysis of biofilms. Binary 4, 92–95.

[11] Mackerness, C.W., Colbourne, J.S., Dennis, P.L.J., Rachwal, T. and Keevil, C.W. (1993) Formation and control of coliform biofilms in drinking water distribution systems. In: Microbial Biofilms: Formation and Control. (Denyer, S.P. et al., Eds.), pp. 217–227, Blackwell Scientific Publications, London.

[12] Rogers, J. and Keevil, C.W. (1992) Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic interference contrast microscopy. Appl. Environ. Microbiol. 58, 2326–2330.

[13] Maniatis, T., Fritsch, E., and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

[14] Hanahan, D. (1985) Techniques for transformation of *E. coli*. In: DNA Cloning Volume 1 (Glover, D.M., Ed.), pp. 109–136, IRL Press, Oxford.

[15] Casadaban, M. and Cohen, S. (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. 138, 179-207.

[16] Rogers, J. and Keevil, C.W. (1992) Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. Appl. Environ. Microbiol. 58, 2326–2330.

[17] Shadix, L.C. and Rice, E.W. (1991) Evaluation of β-glucuronidase assay for the detection of *E. coli* from environmental waters. Can. J. Microbiol. 37, 908–911.

[18] Bell, A.I., Cole, J.A. and Busby, S.J.W. (1990) Molecular genetic analysis of an FNR-dependent anaerobically inducible *Escherichia coli* promoter. Mol. Microbiol. 4, 1753-1763