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ORIGINAL PAPER

E. Burton · N. Yakandawala · K. LoVetri
M. S. Madhyastha

A microplate spectrofluorometric assay for bacterial biofilms

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Abstract A spectrofluorometric assay was developed for quantification of bacterial biofilms grown on a microtiter plate. The method involved staining biofilms formed by gram-negative and gram-positive bacteria with wheat germ agglutinin-Alexa Fluor 488 conjugate, which selectively binds to *N*-acetylglucosamine residues in biofilms. The fluorescence of stained biofilms was measured with a fluorescent plate reader. This method was compared with a widely used microplate colorimetric assay involving crystal violet staining of biofilms formed by both gram-negative and gram-positive bacteria. A strong linear association existed between the two methods ($r^2 = 0.99/0.94$). Being more sensitive and specific as compared to colorimetric method, the spectrofluorometric assay provides a better alternative for quantification and characterization of bacterial biofilms.

Keywords Biofilm · Spectrofluorometric assay · Colorimetric assay · Crystal violet · WGA-Alexa Fluor 488

Introduction

Biofilms represent the most prevalent type of microbial growth in nature and are crucial to the development of clinical infections [9]. Intense interest in biofilms over the past several years comes largely from the recognition of their roles in protecting microbes from the immune system, antimicrobials, predation, and stresses [1].

The availability of a sensitive, specific and reproducible methodology for quantification of biofilms is essential for the evaluation of biofilm formation. A variety of direct and indirect quantitative methods have been developed for this purpose. Indirect methods with 96-well microtiter plates for quantification of biofilms stained with crystal violet or safranin have been widely used due to their simplicity and sensitivity [2]. In the crystal violet assay, the biofilms are stained with crystal violet, and followed by the removal of unbound stain [4]. These methods present some limits: successive steps may lead to loss of part of the material; solubilization of the exopolymers is dependent on the choice of the extraction fluid; and because the quantity of exopolysaccharides (EPS) present in biofilms is too small, it is often necessary to increase the total area colonized by the cells to quantify biofilms. Furthermore, as the specificity of these cationic dyes to polyanions was empirically established, they are not always reliable detectors of EPS [7].

The binding specificity of lectins toward simple sugars of EPS in biofilms appears to be a specific way to characterize and quantify biofilms. The emergence of fluorochrome-conjugated lectins allowed for the direct visualization of the EPS of biofilms by epifluorescence microscopy [8, 10]. Recently, Thomas and coworkers [11] successfully developed an enzyme-linked lectinsorbent assay (ELLA) to quantify in situ the *N*-acetylglucosamine components of biofilms produced by *Staphylococcus epidermidis*.

In this paper, we describe a spectrofluorometric assay for quantification of biofilms of gram-negative and gram-positive bacteria on a microtiter plate. This method utilizes the specific binding of wheat germ agglutinin-Alexa Fluor 488 conjugate (WGA) to *N*-acetylglucosamine in biofilms. This lectin conjugate also binds to *N*-acetylneuraminic acid in the peptidoglycan layer of gram-positive bacteria. Furthermore, WGA binds specifically to polysaccharide adhesin (poly *N*-acetylglucosamine) involved in biofilm formation by both gram-positive and gram-negative bacteria. We compared colorimetric assay with spectrofluorometric assay for quantification of inhibition of biofilm formation in *Escherichia coli* and *S. epidermidis* by nitrofurazone and dispersin B.

E. Burton · N. Yakandawala · K. LoVetri
M. S. Madhyastha (✉)
Kane Biotech Inc., 5-1250 Waverley Street, Winnipeg, MB, Canada R3T 6C6
E-mail: srim@kanebiotech.com
Tel.: +1-204-4531301
Fax: +1-204-4531314

Materials and methods

Chemicals

Nitrofurazone was purchased from Sigma-Aldrich (St. Louis, MO). Dispersin B was supplied by Dr. Kaplan, Department of Oral Biology, New Jersey Dental School, NJ. Wheat germ agglutinin-Alexa Fluor 488 was purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals used were of analytical grade and were obtained from standard sources.

Bacterial strains, inoculum preparation, and biofilm assay

Staphylococcus epidermidis 1457 and *E. coli* TRMG1655 ($CsrA^-$) were obtained from Dr. Romeo, Department of Microbiology and Immunology, Emory University School of Medicine (Atlanta, GA). Inoculum of *S. epidermidis* and *E. coli* was prepared in Luria-Bertani (LB) broth and tryptic soy broth (TSB), respectively, and incubated for 18 h at 37°C on a shaker at 200 rpm. The inoculum was diluted 1:20 times in either colony forming antigen (CFA) medium for *E. coli* or TSB for *S. epidermidis* and 190 µl each was aliquoted per well on a 96-well polystyrene microtiter plate. For biofilm inhibition assay, 10 µl each of appropriately concentrated nitrofurazone (0, 3.12, 6.25, 12.5, 25, and 50 µg/ml) or dispersin B (0, 0.2, 0.4, 0.8, 1.6 and 3.2 µg/ml) was added to each well of 96-well plate. The microtiter plates for *E. coli* and *S. epidermidis* were incubated at 26 and 37°C, respectively for 24 h, and used for the colorimetric and spectrofluorometric assays.

Colorimetric assay

The colorimetric assay of biofilms with crystal violet staining was performed, as described previously [4]. After 24-h incubation, the medium was gently removed and the microtiter plate wells were washed three times with 200 µl of PBS (0.1 M, pH 7.4) buffer using a multichannel pipette, and allowed to dry for 15 min. The microtiter plate wells were stained with 200 µl of 0.4% crystal violet for 15 min at room temperature. The unbound crystal violet stain was removed and the wells were washed gently three times with 200 µl of PBS buffer. The wells were air-dried for 15 min and the crystal violet in each well was solubilized by adding 200 µl of 33% acetic acid. The plate was read at 630 nm using a microtiter plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland).

Spectrofluorometric assay

Escherichia coli and *S. epidermidis* biofilms were grown on the 96-well plate as described previously. The biofilms were washed and stained with 200 µl of 5 µg/ml WGA in PBS for 2 h at 4°C in the dark. The dye was removed, and each well was gently washed three times with PBS buffer. The plates were air-dried for 15 min and the WGA was solubilized with 200 µl of 33% acetic acid per well. The plates were sonicated in a water sonicator (FS20, Fisher Scientific, Pittsburgh, PA) for 30 s, and incubated at 37°C for 1 h. The plates were sonicated again for 30 s and 150 µl from each well was transferred to a microtiter plate for bottom reading spectrofluorometer. The sensitivity of the detector was standardized using 150 µl of fresh 5 µg/ml WGA. The fluorescence of the samples was read from the bottom of the plate at an excitation wavelength of 495 nm and an emission wavelength of 520 nm using a spectrofluorometer (FLUOstar OPTIMA, BMG Labtech Inc., Durham, NC).

The values were expressed as mean of six replicates ± SD. The results of spectrofluorometric assay were plotted against that of colorimetric assay to determine the correlation between the two assays using Microsoft Excel software.

Results and discussion

The colorimetric assay involving crystal violet staining of biofilms and the spectrofluorometric assay involving WGA staining of biofilms were used for quantification of inhibition of biofilm formation in *E. coli* and *S. epidermidis* by nitrofurazone and dispersin B. These two bacterial strains represent both gram-negative and gram-positive species. Nitrofurazone is a broad-spectrum antimicrobial compound, which inhibits biofilm formation in bacteria by inhibiting the growth [5]. Dispersin B is an antibiofilm enzyme, which inhibits biofilm formation without affecting the growth by interfering with the bacterial adherence to surfaces [3, 6]. Adherence of cells

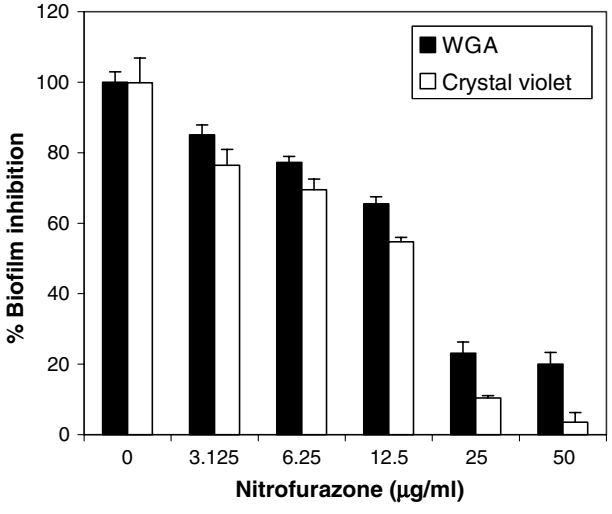

Fig. 1 Comparison of colorimetric (crystal violet) and spectrofluorometric (WGA) assays for quantification of *E. coli* biofilm inhibition by nitrofurazone

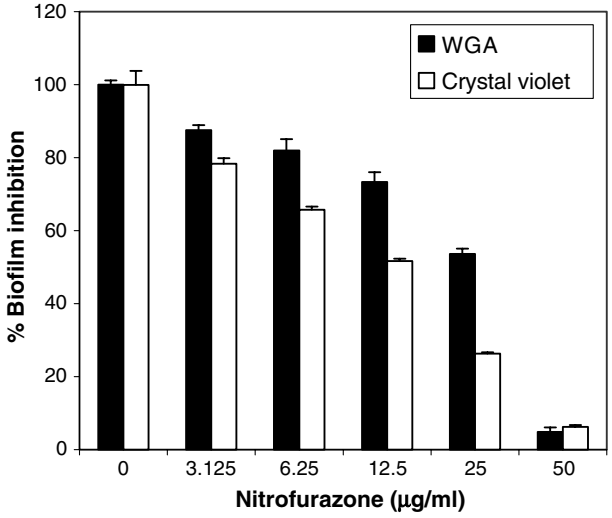

Fig. 2 Comparison of colorimetric (crystal violet) and spectrofluorometric (WGA) assays for quantification of *Staphylococcus epidermidis* biofilm inhibition by nitrofurazone

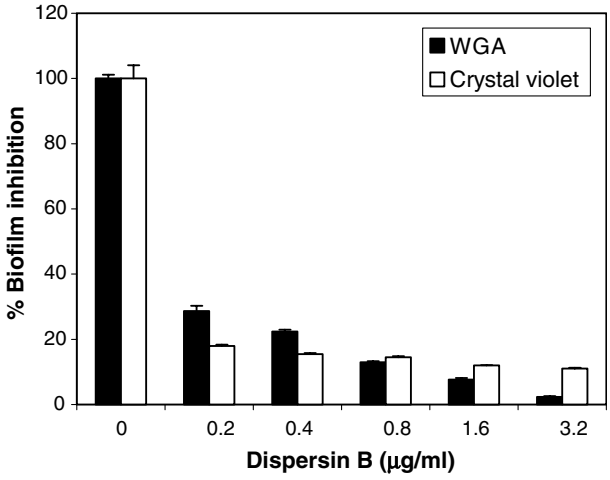

Fig. 4 Comparison of colorimetric (crystal violet) and spectrofluorometric (WGA) assays for quantification of *Staphylococcus epidermidis* biofilm inhibition by dispersin B

to surfaces and to each other involving polysaccharide adhesin is critical for biofilm formation [12]. The results of studies on the effects of nitrofurazone (Figs. 1, 2) and dispersin B (Figs. 3, 4) showed a dose-dependent anti-biofilm activity of both the compounds against the test organisms. The spectrofluorometric assay of biofilms in *E. coli* and *S. epidermidis* treated with dispersin B showed slightly more biofilm at lower concentrations and less biofilm at higher concentrations as compared to the colorimetric assay. The results from both the colorimetric and spectrofluorometric assays illustrate that WGA staining can be a more specific means of biofilm detection and quantification. Furthermore, this assay is highly specific for gram- negative bacterial biofilms because of the inability of WGA molecule to penetrate the outer membrane in order to bind to peptidoglycan. While WGA staining of biofilms treated with the higher concentrations of dispersin B had near zero readings for both *E. coli* and *S. epidermidis*, the colorimetric assay still showed the presence of biofilms. An effort was also made to correlate the results of the spectrofluorometric and colorimetric assays (Figs. 5, 6). The correlation analysis showed a significant positive relationship between the two methods ($r^2 = 0.99/0.94$), despite the one method being more specific to biofilms than the other.

In conclusion, although there was a strong correlation between the two assay methods, we found the spectrofluorometric biofilm assay more sensitive and specific than the colorimetric method. As this method takes advantage of the selective binding of WGA to *N*-acetylglucosamine, it is more specific to gram-negative bacterial biofilms. Further, the method allows the direct quantification of specific components of biofilms such as *N*-acetylglucosamine, instead of nonspecific staining of

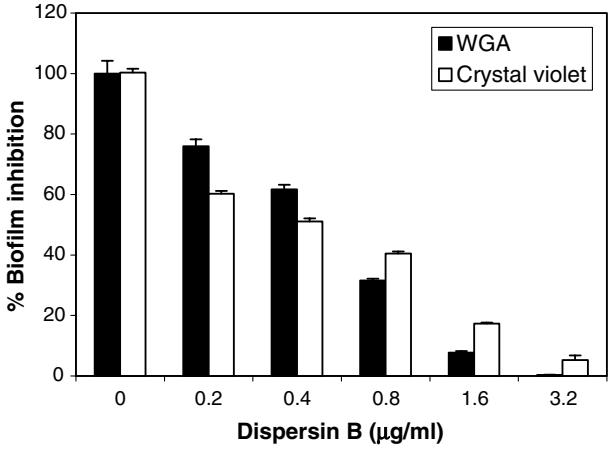

Fig. 3 Comparison of colorimetric (crystal violet) and spectrofluorometric (WGA) assays for quantification of *E. coli* biofilm inhibition by dispersin B

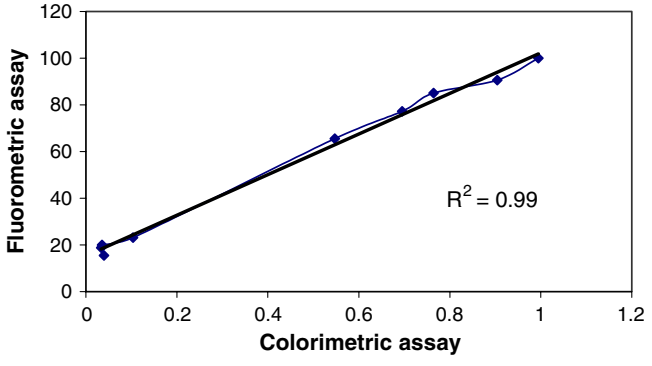

Fig. 5 Correlation between the results of colorimetric and spectrofluorometric assays used for quantification of *E. coli* biofilm inhibition by nitrofurazone

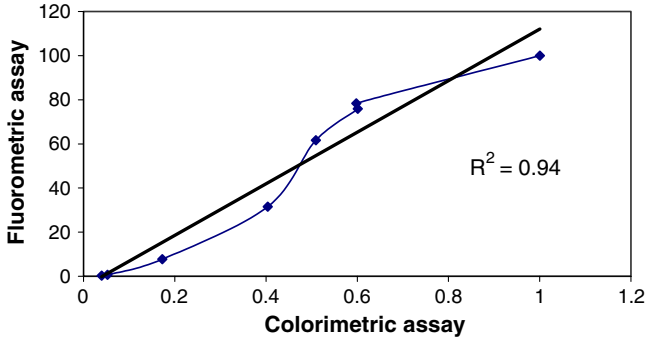

Fig. 6 Correlation between the results of colorimetric and spectrofluorometric assays used for quantification of *E. coli* biofilm inhibition by dispersin B

other extracellular polymeric substances. It is useful in detecting and quantifying biofilm in its early stage of formation as the WGA binds selectively to *N*-acetyl-glucosamine in polysaccharide adhesin involved in the adherence of bacteria to surfaces. In addition, this is the first microplate format spectrofluorometric assay developed to provide a better alternative for quantification and characterization of bacterial biofilms.

References

1. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Ann Rev Microbiol 49:711–745
2. Djordjevic D, Wiedmann M, McLandsborough LA (2002) Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol 68:2950–2958
3. Itoh Y, Wang X, Hinnebusch BJ, Preston JF 3rd, Romeo T (2005) Depolymerization of beta-1,6-*N*-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. J Bacteriol 187:382–7
4. Jackson DW, Suzuki K, Oakford L, Simecka JW, Hart ME, Romeo T (2002) Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. J Bacteriol 184:290–301
5. Johnson JR, Berggren T, Conway AJ (1993) Activity of a nitrofurazone matrix urinary catheter against catheter-associated uropathogens. Antimicrob Agents Chemother 37:2033–2036
6. Kaplan JB, Ragunath C, Velliyagounder K, Fine DH, Ramasubbu N (2004) Enzymatic detachment of *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemother 48:2633–2636
7. Leriche V, Sibille P, Carpenter B (2000) Use of an enzyme-linked lectinsorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms. Appl Environ Microbiol 66:1851–1856
8. Neu TR, Marshall KC (1991) Microbial ''footprints''-a new approach to adhesive polymers. Biofouling 3:101–102
9. O'Toole G, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. Annu Rev Microbiol 54:49–79
10. Sanford BA, De Feijter AW, Wade MH, Thomas VL (1996) A dual fluorescence technique for visualization of *Staphlococcus epidermidis* biofilm using scanning confocal laser microscopy. J Ind Microbiol 16:48–56
11. Thomas VL, Sanford BA, Moreno R, Ramsay MA (1997) Enzyme-linked lectinsorbent assay measures *N*-acetyl-D-glucosamine in matrix of biofilm produced by *Staphylococcus epidermidis*. Curr Microbiol 35:249–254
12. Wang X, Preston JF 3rd, Romeo T (2004) The pgaABCD locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. J Bacteriol 186:2724–34