

Investigating the effect of antibiotics on quorum sensing with whole-cell biosensing systems

Anjali K. Struss · Patrizia Pasini ·
Deborah Flomenhoft · Harohalli Shashidhar ·
Sylvia Daunert

Received: 1 September 2011 / Revised: 21 December 2011 / Accepted: 4 January 2012 / Published online: 31 January 2012
© Springer-Verlag 2012

Abstract Quorum sensing (QS) allows bacteria to communicate with one another by means of QS signaling molecules and control certain behaviors in a group-based manner, including pathogenicity and biofilm formation. Bacterial gut microflora may play a role in inflammatory bowel disease pathogenesis, and antibiotics are one of the available therapeutic options for Crohn's disease. In the present study, we employed genetically engineered bioluminescent bacterial whole-cell sensing systems as a tool to evaluate the ability of antibiotics commonly employed in the treatment of chronic inflammatory conditions to interfere with QS. We investigated the effect of ciprofloxacin, metronidazole, and tinidazole on quorum sensing. Several concentrations of individual antibiotics were allowed to interact with two different types of bacterial sensing cells, in both the presence and absence of a fixed concentration of *N*-acylhomoserine lactone (AHL) QS molecules. The antibiotic effect was then determined by monitoring the biosensor's bioluminescence response. Ciprofloxacin, metronidazole, and

tinidazole exhibited a dose-dependent augmentation in the response of both bacterial sensing systems, thus showing an AHL-like effect. Additionally, such an augmentation was observed, in both the presence and absence of AHL. The data obtained indicate that ciprofloxacin, metronidazole, and tinidazole may interfere with bacterial communication systems. The results suggest that these antibiotics, at the concentrations tested, may themselves act as bacterial signaling molecules. The beneficial effect of these antibiotics in the treatment of intestinal inflammation may be due, at least in part, to their effect on QS-related bacterial behavior in the gut.

Keywords Bacterial whole-cell biosensing systems · Quorum sensing · Antibiotics · Bioluminescence · Inflammatory bowel disease

Introduction

It is well established that bacteria communicate with one another and control certain behaviors such as pathogenicity and biofilm formation in a population density dependent manner [1]. Microorganisms estimate their population size by producing and responding to certain chemical signaling molecules in a process known as quorum sensing (QS). The concentration of these chemical signaling molecules directly reflects the bacterial population size. The QS signaling molecules are mainly *N*-acylhomoserine lactones (AHLs) in gram-negative bacteria and oligopeptides in gram-positive bacteria. Another group of molecules, collectively named autoinducer-2, is shared by both gram-positive and gram-negative types of bacteria. When the QS signaling molecules reach a threshold concentration, expression of certain genes occurs. Thus, the phenomenon of QS allows

Published in the topical collection *Biomimetic Recognition Elements for Sensing Applications* with guest editor María Cruz Moreno-Bondi

A. K. Struss · P. Pasini · S. Daunert
Department of Chemistry, University of Kentucky,
Lexington, KY 40506, USA

P. Pasini · S. Daunert (✉)
Department of Biochemistry and Molecular Biology,
Miller School of Medicine, University of Miami,
R. Bunn Gautier Bldg., 1011 NW 15th Street,
Miami, FL 33136, USA
e-mail: SDaunert@med.miami.edu

D. Flomenhoft · H. Shashidhar
Division of Gastroenterology and Nutrition,
Department of Pediatrics, University of Kentucky,
Lexington, KY 40506, USA

bacteria to regulate their gene expression and coordinate their behavior in a group-based manner.

The association of bacteria with a wide variety of disorders has been demonstrated. Studies involving both animal models and humans showed that the gut bacterial flora may play an important role in the pathogenesis of inflammatory bowel disease (IBD), a chronic inflammatory condition of the gastrointestinal (GI) tract presenting outbursts of acute inflammation [2–4]. Specifically, animal models raised in germ-free conditions did not develop colitis but did so when colonized with their normal gut flora [3]. Quorum sensing is thought to be important for bacterial colonization in the GI system [5]. The successful colonization of the gut may be governed by several processes, including biofilm formation, production of virulence factors, and microorganism dispersal. The role of bacterial chatter in the formation of biofilms is supported by a plethora of reports [6, 7]. Bacterial communication has also been proven to regulate virulence and dispersal mechanisms, such as motility, in enteric pathogens [8, 9]. Additionally, we have shown the presence of AHL QS signaling molecules in stool samples of infants in the Newborn Intensive Care Unit by using genetically engineered bacterial whole-cell sensing systems [10]. Furthermore, the successful use of antibiotics in patients with IBD, mainly imidazole antibiotics for Crohn's disease (CD), also supports the involvement of bacteria in IBD [3, 11].

Given the association of QS with bacterial pathogenicity, interference with QS may be an alternative powerful strategy to alter bacterial interactions and behavior, and, therefore, combat bacterial infections. To that end, it has been already demonstrated that the macrolide antibiotic azithromycin inhibits QS. Specifically, this antibiotic reduced the production of virulence factors and prevented biofilm formation by inhibiting QS regulation in *Pseudomonas aeruginosa* [12, 13]. Previously, we have designed and developed a method, based on whole-cell sensing systems, and have demonstrated their efficacy as an analytical tool for sensitive, selective, and rapid detection of AHLs in biological matrices such as stool [10] and saliva [10, 14, 15]. Whole-cell biosensing systems are engineered to harbor a plasmid that contains genes which encode for a regulatory protein capable of molecular recognition coupled to those of a reporter protein able to produce a detectable signal. In the presence of an analyte, the regulatory protein recognizes it and activates reporter gene transcription. The signal generated by the reporter protein is produced in a dose-dependent manner and is a measure of the concentration of the analyte. In addition to the detection of AHLs in physiological specimens, this valuable analytical tool can be employed for screening of molecules for their QS interfering activity in *in vitro* and *in vivo* samples. The purpose of this study was to employ whole-cell sensing systems to test certain antibiotics for their ability to affect bacterial QS. Specifically, we investigated

antibiotics that are commonly used in the treatment of patients with IBD, that is, ciprofloxacin, metronidazole, and tinidazole. Azithromycin, which has been previously demonstrated as a QS inhibitor/antagonist, was employed as a reference compound.

Materials and methods

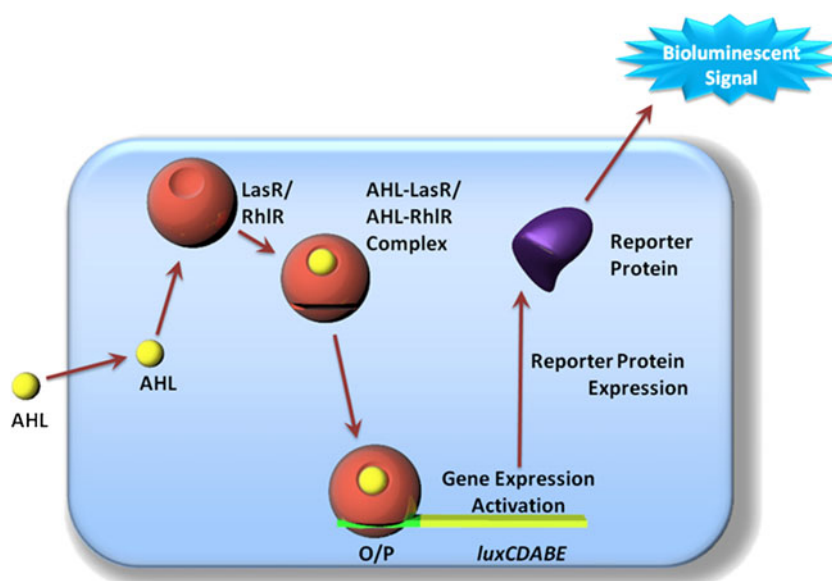
Previously, we designed and developed genetically engineered bioluminescent whole-cell-based sensing systems for the quantitative detection of QS signal molecules, AHLs [10]. Briefly, we employed two *Escherichia coli* sensing systems harboring either plasmid pSB406 or pSB1075 that contain the QS regulatory systems of *P. aeruginosa* as the recognition elements and the *luxCDABE* gene cassette as the reporter [16]. The recognition elements consist of regulatory genes *rhlR* and *lasR* in the plasmids pSB406 and pSB1075, respectively. The expression of the luciferase reporter protein is under transcriptional control of the promoters, *P_{rhl}* and *P_{las}* in the plasmids pSB406 and pSB1075, respectively (Fig. 1). Thus, the regulatory proteins RhlR and LasR form a regulatory protein-AHL complex, when AHLs are present in the environment of the whole sensing cells. The resulting complex then binds to its corresponding promoter and activates transcription of the *luxCDABE* reporter gene cassette. As a result, the reporter protein with an associated bioluminescent signal that is directly proportional to the amount of the AHL molecule present in the environment of the sensing cells is produced. In the present study, we employed these two whole-cell sensing systems to investigate the ability of antibiotics to interfere with QS. The chemical structures of the AHLs and antibiotics tested are shown in Table 1.

Materials Luria Bertani (LB) agar and broth were purchased from Difco (Sparks, MD). Ampicillin, AHL standard compounds, and test antibiotics were obtained from Sigma (St. Louis, MO). The AHLs used were *N*-hexanoyl-DL-homoserine lactone (C6-HSL) and *N*-dodecanoyl-DL-homoserine lactone (C12-HSL). The antibiotics tested were azithromycin, ciprofloxacin, metronidazole, and tinidazole. The Live/Dead BacLight Bacterial Viability Kit was from Invitrogen (Carlsbad, CA).

Bioluminescent measurements were performed using the FLUOstar OPTIMA microplate reader from BMG Labtech (Durham, NC). Fluorescent measurements were carried out using the CYTOFLUOR multi-well plate reader series 4000 from Applied Biosystems (Foster City, CA).

Plasmids, bacterial strains, and culture conditions The plasmids pSB406 and pSB1075 were provided by Prof. Paul Williams (University of Nottingham, Nottingham, UK).

Fig. 1 Schematic of genetically engineered bioluminescent whole-cell-based sensing systems for the detection of AHLs. At a certain critical threshold concentration of AHLs, these molecules bind to the regulatory proteins RhlR/LasR. The resulting AHL-RhlR and AHL-LasR complexes then bind to their respective promoters, P_{lasI} and P_{rhlI} , resulting in the expression of the reporter protein, which then produces a corresponding bioluminescent signal response



Each plasmid was transformed into competent *E. coli* JM109 cells (Stratagene, Cedar Creek, TX) using the standard protocol provided by the manufacturer. The transformed cells were grown at 37 °C overnight on LB agar plates containing 100 µg/mL ampicillin. Cells from a single colony were grown, overnight in an orbital shaker at 37 °C and 250 rpm, in LB broth containing the same amount (*w/v*) of ampicillin. Finally, glycerol stocks were prepared from these cell cultures and stored at 80 °C. Fresh cell cultures were obtained from the glycerol stocks and grown in an orbital shaker at 37 °C and 250 rpm until an optical density at 600 nm (OD_{600nm}) of 0.45–0.50 was reached.

Effect of antibiotics on the bacterial sensing systems in the presence of quorum sensing molecules The bacterial *E. coli* JM109 cells harboring either plasmid pSB1075 or pSB406 were employed as whole-cell sensing systems in the present study. Commercially available C12-HSL and C6-HSL were used as standards in the development of dose-response curves employing the sensing cells containing the plasmid pSB1075 and pSB406, respectively. Several concentrations of individual antibiotics were allowed to interact with both types of bacterial sensing cells in the presence of a fixed concentration of the respective AHL for a period of time, and then the bioluminescence signal was measured.

The AHLs were dissolved in acetonitrile to obtain 1×10^{-2} M stock solutions, which were then serially diluted with water to achieve 1×10^{-5} M AHL standard solutions. Upon addition to the cell suspensions, the AHL final concentration was 8.3×10^{-7} M. The preparation of the antibiotic solutions and the concentration range analyzed in the present study are described below.

Azithromycin (1.92 mg) was dissolved in 1 mL of acetonitrile and serially diluted with water so that, upon

addition of the obtained solutions (3–24 µg/mL) to the cell suspensions, the final azithromycin concentrations tested were 0.25, 0.5, 1.0, and 2.0 µg/mL. Ciprofloxacin (24 mg) was dissolved in 1 mL of 0.1 N HCl in water and serially diluted with water so that, upon addition of the obtained solutions (0.094–7.5 µg/mL) to the cell suspensions, the final ciprofloxacin concentrations tested were 0.008, 0.016, 0.156, 0.313, and 0.625 µg/mL. Metronidazole (30 mg) was dissolved in 1 mL of 50% (*v/v*) acetonitrile in water and serially diluted with water so that, upon addition of the obtained solutions (0.15–1.8 mg/mL) to the cell suspensions, the final metronidazole concentrations tested were 12.5, 25, 50, 100, and 150 µg/mL. Tinidazole (30 mg) was dissolved in 1 mL of acetonitrile and serially diluted with water so that, upon addition of the obtained solutions (0.15–1.8 mg/mL) to the cell suspensions, the final tinidazole concentrations tested were 12.5, 25, 50, 100, and 150 µg/mL.

One hundred microliters of each of the antibiotic solutions at various concentrations, in triplicate, 100 µL of 10^{-5} M C12-HSL and 1 mL of *E. coli* JM109 cells harboring plasmid pSB1075 ($OD_{600nm}=0.45-0.50$) were incubated in culture tubes in an orbital shaker at 37 °C and 250 rpm for three hours. Similarly, 100 µL of each of the antibiotic solutions at various concentrations, in triplicate, 100 µL of 10^{-5} M C6-HSL and 1 mL of *E. coli* JM109 cells harboring the plasmid pSB406 ($OD_{600nm}=0.45-0.50$) were incubated in culture tubes at 37 °C at 250 rpm for three hours. For both sensing systems, 100 µL of each antibiotic solvent, appropriately diluted, was employed as a blank. Specifically, the blanks were 1% acetonitrile in water for azithromycin, metronidazole, and tinidazole and 0.01 N HCl for ciprofloxacin. The induced bioluminescence

Table 1 Chemical structures of the *N*-acylhomoserine lactones (AHLs) and antibiotics tested

	Structure
<i>N</i> -hexanoyl-homoserine lactone (C6-HSL)	
<i>N</i> -dodecanoyl-homoserine lactone (C12-HSL)	
Azithromycin	
Ciprofloxacin	
Metronidazole	
Tinidazole	

was then measured using the microplate reader after transferring 200- μ L aliquots in triplicate from each culture tube into a 96-well microtiter plate. The total measuring time per well was 0.20 s. The light intensity was expressed in relative light units (RLU).

Effect of antibiotics on the bacterial sensing systems in the absence of quorum sensing molecules Individual antibiotic solutions at various concentrations prepared as described above were also allowed to interact with both types of bacterial sensing cells in the absence of AHLs for a pre-determined period of time prior to measurement of the bioluminescence signal generated.

One hundred μ L of each of the antibiotic solutions at various concentrations, in triplicate, 100 μ L of water and 1 mL of *E. coli* JM109 cells harboring plasmid pSB1075 or pSB406 ($OD_{600nm}=0.45-0.50$) were

incubated in culture tubes in an orbital shaker at 37 °C and 250 rpm for three hours. The bioluminescence measurements were performed as described in the above section.

Cell viability assay The direct toxicity of the examined concentrations of antibiotics on the bacterial sensing cells was evaluated. For that, we employed the LIVE/DEAD[®]BacLight[™] Bacterial Viability Kit from Molecular Probes, Inc. (Eugene, OR), using the protocol provided by the manufacturer. Upon incubation of the sensing cells with individual antibiotics at various concentrations, fluorescence was measured at the dyes' respective emission maxima using the CYTOFLUOR microplate fluorometer. Ratios of the measured green/red fluorescence intensities were calculated. Percentages of live bacteria were determined by interpolating the

Table 2 Effect of azithromycin on the whole-cell sensing system response in the presence of AHL

Azithromycin ($\mu\text{g/mL}$)	Signal change (%)	% RSD
pSB406		
0.00	100	4.55
0.25	110	3.64
0.50	12	3.68
1.00	-17	1.63
2.00	-20	1.60
pSB1075		
0.00	100	7.35
0.25	119	1.03
0.50	49	3.12
1.00	43	4.65
2.00	31	2.54

Light signals are reported as percent changes with respect to the signals measured in the absence of azithromycin. The signals have been corrected with respect to the blank and normalized according to the cell viability data. Data shown are the average \pm one standard deviation ($n=3$)

% RSD, percent relative standard deviation

calculated ratios in a calibration plot obtained with known ratios of live/dead bacteria. The calibration plot was defined by a least-square fit equation.

Results

The lowest minimum inhibitory concentration (MIC) reported in the literature for azithromycin against *E. coli* is 2 $\mu\text{g/mL}$ [17]. When various sub-inhibitory concentrations of this antibiotic were incubated with the whole-cell sensing systems containing either plasmid pSB1075 or pSB406 in the presence of 10^{-5} M C12-HSL or C6-HSL, respectively, we observed a dose-dependent decrease in the light production, as shown in Table 2. The cell-viability studies showed a slight degree of toxicity of azithromycin on the bacterial sensing cells in the concentration range tested, manifested by an increase in cell death, as shown in Table 3. Therefore, the signal intensities obtained at various azithromycin concentrations were normalized taking into account the respective live cell percentages. The observed decrease in signal implies that the macrolide antibiotic azithromycin does interfere with the response to AHLs of both the *las* and *rhl* QS systems of *P. aeruginosa* carried by our biosensing systems.

One of the lowest MICs of ciprofloxacin reported in the literature for *E. coli* is 0.016 $\mu\text{g/mL}$ [18]. When sub-inhibitory concentrations of ciprofloxacin were incubated with the two whole-cell sensing systems, harboring either the plasmid pSB1075 or pSB406, in the presence of 10^{-5} M C12-HSL or C6-HSL respectively, no change in light signal

Table 3 Percentage of live bacteria for the chemical compounds analyzed

Antibiotic	Live bacteria (percentage)
Azithromycin ($\mu\text{g/mL}$)	
Blank	100
0.0	100
0.25	93
0.50	97
1	85
2	78
4	67
8	78
16	72
Ciprofloxacin ($\mu\text{g/mL}$)	
Blank	100
0.0	100
0.0078	100
0.0156	100
0.1560	66
0.3125	57
0.6250	51
1.25	46
2.50	32
Metronidazole ($\mu\text{g/mL}$)	
Blank	100
0.0	100
50	100
100	94
150	90
200	89
250	87
Tinidazole ($\mu\text{g/mL}$)	
Blank	100
0.0	100
50	100
100	93
150	91
200	93
250	95

Data shown are the average \pm one standard deviation ($n=3$). A calibration plot of percent live bacteria was also obtained for each antibiotic using the protocol provided by the manufacturer. The coefficients of determination (r^2) of the linear regression obtained for these calibration plots were all $r^2=0.99$

was observed (data not shown). When concentrations of ciprofloxacin \geq MIC were allowed to interact with both whole-cell sensing systems in the presence of 10^{-5} M of the appropriate AHL, a dose-dependent increase in light production was observed, as shown in Table 4. When ciprofloxacin at the same concentrations (0.008–0.625 $\mu\text{g/mL}$) was allowed to interact with both whole-cell sensing systems, in the absence of the respective AHLs, a dose-dependent increase in light production was also observed (Fig. 2). All the reported signal intensity values were normalized according to the cell viability data obtained, as

Table 4 Effect of individual antibiotics on the whole-cell sensing system response in the presence of AHL: ciprofloxacin

Ciprofloxacin ($\mu\text{g/mL}$)	Signal change (%)	% RSD
pSB406		
0.000	100	9.38
0.008	118	6.18
0.016	167	1.11
0.156	371	4.88
0.313	505	1.23
0.625	562	1.13
pSB1075		
0.000	100	5.55
0.008	99	5.80
0.016	105	3.97
0.156	233	2.26
0.313	334	1.19
0.625	287	1.44

Light signals are reported as percent changes with respect to the signals measured in the absence of antibiotics. The signals have been corrected with respect to the blank and normalized according to the cell viability data. Data shown are the average \pm one standard deviation ($n=3$)

% RSD, percent relative standard deviation

shown in Table 3. Overall, these results show that ciprofloxacin demonstrates an augmentation in the response of both biosensing systems in the presence of AHLs when compared with their response to AHLs alone. Interestingly, ciprofloxacin alone is also able to activate both the QS based whole-cell sensing systems in a dose-dependent manner. Hence, ciprofloxacin itself demonstrates an AHL-like effect.

The MIC of metronidazole reported in the literature for *E. coli* is $\sim 500 \mu\text{g/mL}$ [19, 20]. When sub-inhibitory concentrations of metronidazole were allowed to interact with the whole-cell sensing system, harboring the plasmid pSB1075,

Table 5 Effect of individual antibiotics on the whole-cell sensing system response in the presence of AHL: metronidazole

Metronidazole ($\mu\text{g/mL}$)	Signal change (%)	% RSD
pSB406		
0.00	100	3.27
6.25	99	5.20
12.50	110	4.61
25	135	3.36
50	148	2.88
100	152	5.19
150	161	2.79
pSB1075		
0.00	100	1.57
6.25	90	2.43
12.50	121	3.32
25	127	3.27
50	147	1.29
100	195	4.28
150	218	4.11

Light signals are reported as percent changes with respect to the signals measured in the absence of antibiotics. The signals have been corrected with respect to the blank and normalized according to the cell viability data. Data shown are the average \pm one standard deviation ($n=3$)

% RSD, percent relative standard deviation

in the presence of 10^{-5} M C12-HSL, a dose-dependent increase in light production was observed, as shown in Table 5. When the same metronidazole concentrations were allowed to interact with the whole-cell sensing system, harboring the plasmid pSB406, in the presence of 10^{-5} M C6-HSL, a similar dose-dependent change in light production was detected, although to a lesser extent when compared with that for the pSB1075 system, as shown in Table 5. Interaction of metronidazole (25–150 $\mu\text{g/mL}$) with

Fig. 2 Dose-dependent response of the biosensing systems employing plasmid pSB406 (gray) and pSB1075 (white). Bacterial sensing cells were incubated with various concentrations of the antibiotic ciprofloxacin for three hours at 37°C with continuous shaking, and then bioluminescence was measured. Data shown are the average \pm one standard deviation ($n=3$)

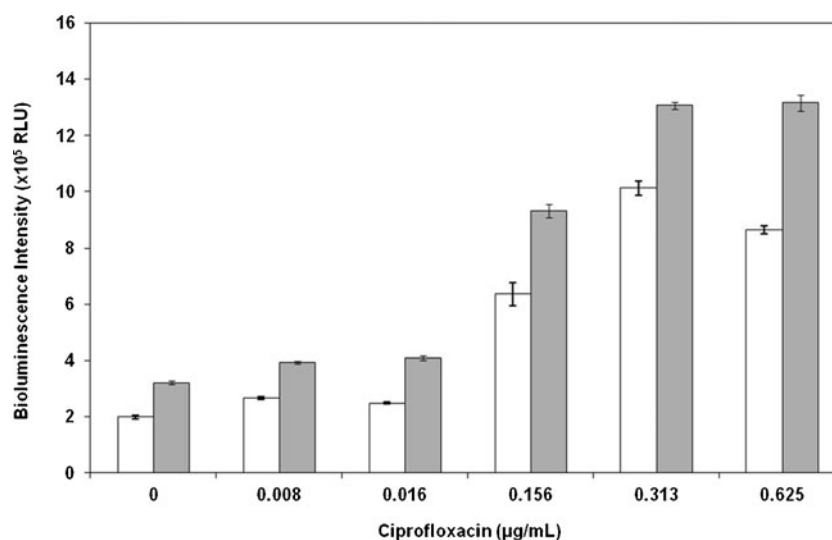
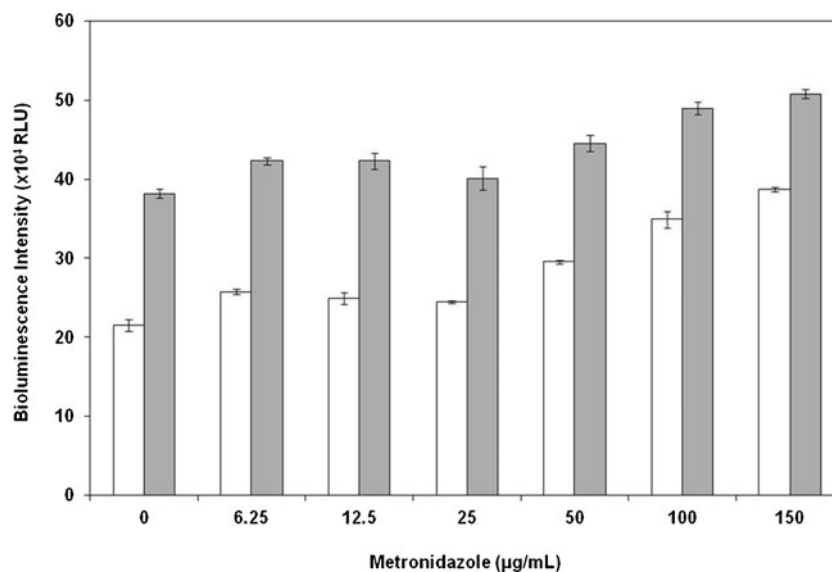


Fig. 3 Dose-response of the biosensing systems employing plasmid pSB406 (*gray*) and pSB1075 (*white*). Bacterial sensing cells were incubated with various concentrations of the antibiotic metronidazole for three hours at 37 °C with continuous shaking, and then bioluminescence was measured. Data shown are the average \pm one standard deviation ($n=3$)



both whole-cell sensing systems, in the absence of the corresponding AHLs, also caused a dose-dependent increase in bioluminescence intensity (Fig. 3). Although metronidazole toxicity is relatively low at the concentrations tested, all the reported signal intensity values were normalized according to the cell viability data, as shown in Table 3. Thus, metronidazole was able to augment the response of both biosensing systems in the presence of AHLs when compared with their response to AHLs alone. Metronidazole alone was also able to activate both the QS based whole-cell sensing systems, which was observed by a dose-dependent increase in light production. Thus, metronidazole itself demonstrates an AHL-like effect.

Tinidazole is similar to metronidazole in structure and activity. Thus, the same concentration range as metronidazole was analyzed for tinidazole. When these concentrations of the antibiotic were incubated with both whole-cell sensing systems, in the presence of 10^{-5} M of the appropriate AHLs, we observed a dose-dependent increase in light production, as shown in Table 6. It should be noted that the dose-dependent change in light production was to a lesser extent in the pSB406 system. Incubation of tinidazole with both whole-cell sensing systems, in the absence of AHLs, also produced a dose-dependent increase in bioluminescence intensity (Fig. 4). Similarly to metronidazole, tinidazole toxicity is relatively low at the concentrations tested. However, all the reported signal intensity values were normalized according to the cell viability data, as shown in Table 3. As with metronidazole, tinidazole increased the response of both biosensing systems in the presence of AHLs compared with their response when the sensing systems were allowed to interact with AHLs alone. As for metronidazole and ciprofloxacin, tinidazole alone was also able to activate both the QS based whole-cell sensing

systems, which was directly observed by dose-dependent increase in light production. Hence, tinidazole itself also demonstrates an AHL-like effect. The responses of our biosensing systems to metronidazole and tinidazole were indeed very similar.

In summary, when various concentrations of azithromycin were analyzed with two different whole-cell-based QS

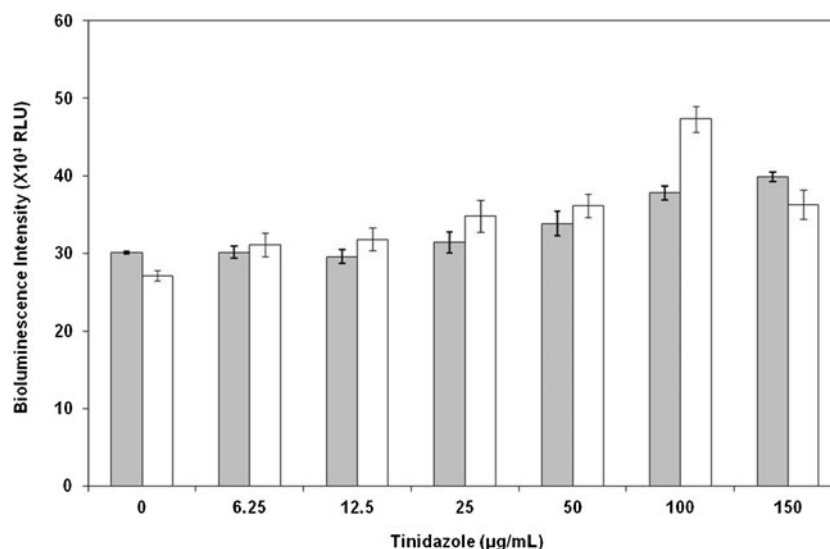
Table 6 Effect of individual antibiotics on the whole-cell sensing system response in the presence of AHL: tinidazole

Tinidazole (µg/mL)	Signal change (%)	% RSD
pSB406		
0.00	100	6.95
6.25	98	0.24
12.50	102	6.69
25	110	2.03
50	126	5.07
100	151	6.31
150	128	4.80
pSB1075		
0.00	100	3.96
6.25	106	3.11
12.50	164	3.30
25	196	2.71
50	230	1.32
100	265	1.74
150	250	4.52

Light signals are reported as percent changes with respect to the signals measured in the absence of antibiotics. The signals have been corrected with respect to the blank and normalized according to the cell viability data. Data shown are the average \pm one standard deviation ($n=3$)

% RSD, percent relative standard deviation

Fig. 4 Dose-response of the biosensing systems employing plasmid pSB406 (gray) and pSB1075 (white). Bacterial sensing cells were incubated with various concentrations of the antibiotic tinidazole for three hours at 37 °C with continuous shaking, and then bioluminescence was measured. Data shown are the average \pm one standard deviation ($n=3$)



sensing systems, in the presence of AHLs, a dose-dependent decrease in bioluminescence signal intensity was observed, which implies an anti-AHL-like effect. When ciprofloxacin, metronidazole, and tinidazole at various concentrations were analyzed with the two sensing systems, in the presence and absence of AHLs, a dose-dependent increase in bioluminescence signal intensity was seen, which suggests an AHL-like effect.

Discussion

The effectiveness of antibiotics in the treatment of IBD appears to be not solely through their bacteriostatic/bactericidal mechanisms of action. Previously, it has been demonstrated that the macrolide antibiotic azithromycin, at sub-inhibitory concentrations, inhibits QS in *P. aeruginosa* [12, 13]. The antibiotic was shown to reduce the production of virulence factors and biofilm formation, which are both regulated by QS. Furthermore, azithromycin proved to be effective against *P. aeruginosa* respiratory infections associated with cystic fibrosis, despite its poor anti-pseudomonal activity. The mechanism of action is not fully understood. However, anti-inflammatory effects or interference with QS are thought to be involved [12, 21, 22]. Also, there are several reports in the literature that cite the association of bacterial pathogenicity with QS [7–9, 23]. Given the ever increasing number of microbes resistant to antibiotics, there is a need for finding new drugs that target the QS mechanism as an alternative treatment for microbial diseases. Thus, it may prove significant to design and screen for chemicals that may interfere with QS. Moreover, compounds known for interfering with QS regulation may have the effect of either an agonist/stimulator or antagonist/inhibitor [24, 25].

Towards that end, in the present study we employed genetically engineered bioluminescent bacterial whole-cell sensing systems to evaluate the ability of the selected antibiotics to interfere with QS. The main advantage of using whole-cell biosensing systems engineered with elements of certain regulatory/signaling pathways is that they afford important information on effects of the chemicals tested other than those related to bactericidal/bacteriostatic activities. They may also provide insight into the interaction at the molecular level of the target compounds with the QS regulatory system present in the genetically engineered sensing cells. Further, whole-cell biosensing systems are easy to use, cost-effective, and amenable to high-throughput screening.

Two different whole-cell sensing systems able to respond to long (plasmid pSB1075) or short (plasmid pSB406) chain AHLs were used. These were previously characterized and shown to be quantitative, sensitive, and reproducible [10]. C6-HSL and C12-HSL induced the maximum bioluminescence responses in the bacterial whole-cell sensing systems harboring plasmid pSB406 and pSB1075, respectively. Thus, in this work, we used these two compounds for all the interaction/interference studies of antibiotics with the whole-cell sensing systems' response. Specifically, various concentrations of individual antibiotics were allowed to interact with the bacterial sensing cells in the presence of a fixed concentration of the appropriate AHL. Such fixed AHL concentration of 1×10^{-5} M was selected based on inducing a high bioluminescence response and falling within the linear region of the sensing system's dose-response curves.

The results obtained in the present study with azithromycin confirm the QS inhibitory effect of this antibiotic, as reported in the literature, thus supporting the suitability of our whole-cell sensing systems for evaluating QS interference. In order to explain the biosensing systems' responses to ciprofloxacin, metronidazole, and tinidazole, in the

absence of AHLs, as well as the increase in signals observed when the antibiotics were added to AHLs, we hypothesize that these antibiotics themselves may act as bacterial signaling molecules. It has previously been reported that certain antibiotics, at concentrations below their MIC, may act as cell–cell signaling molecules. For instance, Goh et al. demonstrated that erythromycin and rifampicin activated gene transcription at sub-MIC concentrations [26]. Other studies discussed the contrasting effects of antibiotics, exhibiting stimulatory and inhibitory effects on global transcription, at low and high doses, respectively [27, 28]. The mechanisms of action underlying these effects have not been elucidated. One could speculate that antibiotics at low concentrations bind to certain molecular targets and activate transcription of certain genes.

In the specific case of the QS systems employed in the present study, possible targets for the tested antibiotics could be the regulatory proteins RhlR and LasR. Although the complete X-ray crystallographic structures of these proteins are unknown, the crystal structure of the ligand binding domain (LBD) of the LasR protein was recently solved [29]. In a study aimed at screening antibiotics, including azithromycin and ciprofloxacin, for their QS inhibitory activity, an *in silico* docking of these antibiotics against the LBD of the LasR protein was performed [30]. It was found that neither azithromycin nor ciprofloxacin demonstrated a high score in the docking. Therefore, it appears that these antibiotics do not bind to the AHL binding site of the LasR regulatory protein. In another study, it was suggested that halogenated furanones, which are known to inhibit AHL-mediated QS, do not compete with *N*-3-oxohexanoyl-L-homoserine lactone for the binding site of the LuxR regulatory protein in a traditional way, but they may rather bind in a non-agonist manner [31]. In the cited study, several LuxR mutant proteins were designed using site-directed mutagenesis of the amino acid residues comprising the AHL receptor site of the LuxR regulatory protein. It was observed that certain mutations caused a minor effect on the sensitivity of the LuxR mutant proteins to halogenated furanones, thus suggesting that such sensitivity is not dependent on the furanone binding to the AHL receptor site.

It should be pointed out that our results on ciprofloxacin are in contrast with those reported by Skindersoe et al. in the above antibiotic screening study [30]. Specifically, we observed a dose-dependent QS stimulatory effect for ciprofloxacin in both the presence and absence of AHLs, in the concentration range 0.016–0.625 $\mu\text{g}/\text{mL}$ while in the cited paper a QS inhibitory effect was observed at the ciprofloxacin tested concentration of 0.04 $\mu\text{g}/\text{mL}$. The main reason for this discrepancy possibly lies in the different types of cells used. While we used *E. coli* expressing *P. aeruginosa* QS systems, Skindersoe et al. employed *P. aeruginosa* PAO1. This difference is significant because our sensors

allowed evaluating effects of the antibiotic specifically on the QS regulatory protein/promoter systems, while the antibiotic effects observed in *P. aeruginosa* may be mediated by other cell components. To support this hypothesis, it has recently been demonstrated that sub-inhibitory concentrations of azithromycin did not directly affect expression of QS-related genes, such as those coding for AHL synthases in *P. aeruginosa*, but lowered expression of genes upstream of the synthase ones, which may eventually lead to a decrease in AHL production [32].

Finally, it is noteworthy to report that the antibiotic concentrations tested in our study are clinically relevant. In fact, the peak plasma concentrations of azithromycin (250–500 mg dose/day), ciprofloxacin (250–750 mg dose/day), metronidazole (500 mg dose/day), and tinidazole (2 g for 1/2 days) are 0.24–0.4, 1.1–6.4, 8–13, and 40–54 $\mu\text{g}/\text{mL}$, respectively [33]. For azithromycin, metronidazole, and tinidazole, the QS interfering effects demonstrated in our study were observed in concentration ranges that included their peak plasma concentrations. For ciprofloxacin, data are presented in a concentration range immediately below the peak plasma levels because this compound at a concentration around 1.1 $\mu\text{g}/\text{mL}$ was significantly toxic to our cell sensing systems (46% cell viability).

In summary, we employed genetically engineered bacterial whole-cell sensing systems that are capable of rapid, sensitive and quantitative detection of different types of bacterial signaling molecules, specifically, long and short chain AHLs. Previously, we have demonstrated the efficacy of the sensing systems as a tool for the detection of these long and short chain AHLs in biological matrixes such as saliva and stool. In the present work, we have shown that these cell-based sensing systems are a valuable analytical tool for the screening of compounds potentially able to interfere with QS. Specifically, we tested a few selected antibiotics, which are often used for the treatment of patients with IBD, for their capabilities as QS modulators. The AHL-like effect of ciprofloxacin, metronidazole, and tinidazole on our biosensing systems' response indicates that the tested antibiotics may interfere with these bacterial QS systems as QS analogues. Such an observation led us to postulate that the beneficial effect of these antibiotics in the treatment of intestinal inflammatory conditions may, at least in part, be due to their ability to alter QS-related gut bacterial behavior. However, further tests are needed to verify if this increased response caused by antibiotics may underlie their efficacy in certain clinical settings.

Acknowledgments This work was partly supported by the National Science Foundation (Grant CHE-0416553), the Children's Miracle Network, and the Broad Foundation, Broad Medical Research Program (BMRP), Grant IBD-0198R. S.D. is grateful for support from the Lucille P. Markey Chair in Biochemistry and Molecular Biology of the Miller School of Medicine of the University of Miami, as well as

from a Gill Eminent Professorship from the University of Kentucky. A. K.S. acknowledges support from a Gill Fellowship and a Research Challenge Trust Fund Fellowship from the University of Kentucky. We also thank Prof. Paul Williams (University of Nottingham, Nottingham, UK) for providing plasmids pSB406 and pSB1075.

References

- Miller MB, Bassler BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55(1):165–199
- Sartor RB (2004) Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 126(6):1620–1633
- Seksik P, Sokol H, Lepage P, Vasquez N, Manichanh C, Mangin I, Pochart P, Dore J, Marteau P (2006) Review article: the role of bacteria in onset and perpetuation of inflammatory bowel disease. *Aliment Pharmacol Ther* 24(s3):11–18
- Frank DN, St. Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci* 104(34):13780–13785. doi:10.1073/pnas.0706625104
- Swift S, Vaughan EE, de Vos WM (2000) Quorum sensing within the gut ecosystem. *Microb Ecol Heal Dis* 12(1 suppl 2):81–92
- Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280(5361):295–298. doi:10.1126/science.280.5361.295
- Kaper JB, Sperandio V (2005) Bacterial cell-to-cell signaling in the gastrointestinal tract. *Infect Immun* 73(6):3197–3209
- Falcao JP, Sharp F, Sperandio V (2004) Cell-to-cell signaling in intestinal pathogens. *Curr Issues Intest Microbiol* 5(1):9–18
- Kendall MM, Sperandio V (2007) Quorum sensing by enteric pathogens. *Curr Opin Gastroenterol* 23(1):10–15
- Kumari A, Pasini P, Deo SK, Flomenhoft D, Shashidhar H, Daunert S (2006) Biosensing systems for the detection of bacterial quorum signaling molecules. *Anal Chem* 78(22):7603–7609
- Khan KJ, Ullman TA, Ford AC, Abreu MT, Abadir A, Marshall JK, Talley NJ, Moayyedi P (2011) Antibiotic therapy in inflammatory bowel disease: a systematic review and meta-analysis. *Am J Gastroenterol* 106(4):661–673
- Nalca Y, Jansch L, Bredenbruch F, Geffers R, Buer J, Haussler S (2006) Quorum-Sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach. *Antimicrob Agents Chemother* 50(5):1680–1688. doi:10.1128/aac.50.5.1680-1688.2006
- Gillis RJ, Iglewski BH (2004) Azithromycin retards *Pseudomonas aeruginosa* biofilm formation. *J Clin Microbiol* 42(12):5842–5845. doi:10.1128/jcm.42.12.5842-5845.2004
- Kumari A, Pasini P, Daunert S (2008) Detection of bacterial quorum sensing *N*-acyl homoserine lactones in clinical samples. *Anal Bioanal Chem* 391(5):1619–1627. doi:10.1007/s00216-008-2002-3
- Struss A, Pasini P, Ensor CM, Raut N, Daunert S (2010) Paper strip whole cell biosensors: a portable test for the semiquantitative detection of bacterial quorum signaling molecules. *Anal Chem* 82(11):4457–4463. doi:10.1021/ac100231a
- Winson MK, Swift S, Fish L, Throup JP, Jorgensen F, Chhabra SR, Bycroft BW, Williams P, Stewart GSAB (1998) Construction and analysis of luxCDABE-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett* 163(2):185–192
- Dinos GP, Michelinaki M, Kalpaxis DL (2001) Insights into the mechanism of azithromycin interaction with an *Escherichia coli* functional ribosomal complex. *Mol Pharmacol* 59(6):1441–1445
- Chalkley LJ, Koornhof HJ (1985) Antimicrobial activity of ciprofloxacin against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* determined by the killing curve method: antibiotic comparisons and synergistic interactions. *Antimicrob Agents Chemother* 28(2):331–342. doi:10.1128/aac
- Tally FP, Goldin BR, Sullivan N, Johnston J, Gorbach SL (1978) Antimicrobial activity of metronidazole in anaerobic bacteria. *Antimicrob Agents Chemother* 13(3):460–465. doi:10.1128/aac
- Ralph ED, Clarke DA (1978) Inactivation of metronidazole by anaerobic and aerobic bacteria. *Antimicrob Agents Chemother* 14(3):377–383. doi:10.1128/aac
- Swords WE, Rubin BK (2003) Macrolide antibiotics, bacterial populations and inflammatory airway disease. *Neth J Med* 61(7)
- Shinkai M, Henke MO, Rubin BK (2008) Macrolide antibiotics as immunomodulatory medications: proposed mechanisms of action. *Pharmacol Ther* 117(3):393–405
- Sircili MP, Walters M, Trabulsi LR, Sperandio V (2004) Modulation of enteropathogenic *Escherichia coli* virulence by quorum sensing. *Infect Immun* 72(4):2329–2337
- Manefield M, Kjelleberg S, Givskov M (2003) Controlling bacterial infection by inhibiting intercellular signalling. *Curr Med Chem-Anti-Infect Agents* 2(3):213
- Geske GD, O'Neill JC, Blackwell HE (2008) Expanding dialogues: from natural autoinducers to non-natural analogues that modulate quorum sensing in Gram-negative bacteria. *Chem Soc Rev* 37(7):1432–1447
- Goh E-B, Yim G, Tsui W, McClure J, Surette MG, Davies J (2002) Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc Natl Acad Sci USA* 99(26):17025–17030. doi:10.1073/pnas.252607699
- Davies J, Spiegelman GB, Yim G (2006) The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 9(5):445–453
- Yim G, Huimi Wang H, Davies Frs J (2007) Antibiotics as signaling molecules. *Philos Trans R Soc B: Biol Sci* 362(1483):1195–1200
- Bottomley MJ, Muraglia E, Bazzo R, Carfi A (2007) Molecular insights into quorum sensing in the human pathogen *Pseudomonas aeruginosa* from the structure of the virulence regulator LasR bound to its autoinducer. *J Biol Chem* 282(18):13592–13600. doi:10.1074/jbc.M700556200
- Skindersoe ME, Alhede M, Phipps R, Yang L, Jensen PO, Rasmussen TB, Bjarnsholt T, Tolker-Nielsen T, Hoiby N, Givskov M (2008) Effects of antibiotics on quorum sensing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 52(10):3648–3663. doi:10.1128/aac.01230-07
- Koch B, Liljefors T, Persson T, Nielsen J, Kjelleberg S, Givskov M (2005) The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. *Microbiology* 151(11):3589–3602. doi:10.1099/mic.0.27954-0
- Kai T, Tateda K, Kimura S, Ishii Y, Ito H, Yoshida H, Kimura T, Yamaguchi K (2009) A low concentration of azithromycin inhibits the mRNA expression of *N*-acyl homoserine lactone synthesis enzymes, upstream of lasI or rhlII, in *Pseudomonas aeruginosa*. *Pulm Pharmacol Ther* 22(6):483–486. doi:10.1016/j.pupt.2009.04.004
- Hardman JG, Limbard LE (eds) (2002) Goodman & Gilman's the pharmacological basis of therapeutics, 10th edn. McGraw-Hill, New York