Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6436-1

ENVIRONMENTAL BIOTECHNOLOGY

Deciphering the role of coumarin as a novel quorum sensing inhibitor suppressing virulence phenotypes in bacterial pathogens

José A. Gutiérrez-Barranquero · F. Jerry Reen · Ronan R. McCarthy · Fergal O'Gara

Received: 10 November 2014 / Revised: 6 January 2015 / Accepted: 25 January 2015

Abstract The rapid unchecked rise in antibiotic resistance over the last few decades has led to an increased focus on the need for alternative therapeutic strategies for the treatment and clinical management of microbial infections. In particular, small molecules that can suppress microbial virulence systems independent of any impact on growth are receiving increased attention. Quorum sensing (QS) is a cell-to-cell signalling communication system that controls the virulence behaviour of a broad spectrum of bacterial pathogens. QS systems have been proposed as an effective target, particularly as they control biofilm formation in pathogens, a key driver of antibiotic ineffectiveness. In this study, we identified coumarin, a natural plant phenolic compound, as a novel QS inhibitor, with potent anti-virulence activity in a broad spectrum of pathogens. Using a range of biosensor systems, coumarin was active against short, medium and long chain *N*-acyl-homoserine lactones, independent of any effect on growth. To determine if this suppression was linked to anti-virulence activity, key virulence systems were studied in the nosocomial pathogen *Pseudomonas aeruginosa*. Consistent with suppression of QS, coumarin inhibited biofilm, the production of phenazines and swarming motility in this organism potentially linked to reduced expression of the *rhlI* and *pqsA* quorum sensing genes. Furthermore, coumarin significantly inhibited biofilm formation and protease activity in other bacterial pathogens and inhibited bioluminescence in *Aliivibrio fischeri*. In light of these findings, coumarin would appear to have potential as a novel quorum sensing inhibitor with a broad spectrum of action.

Keywords Coumarin · Natural compound · Quorum sensing inhibition · Anti-biofilm · Bacterial pathogens

Introduction

The emergence of multidrug-resistant bacteria over the last few decades has become a global health issue for medical professionals and public health systems throughout the world (Tanwar et al. 2014). Once the cornerstone of medical intervention to treat microbial infections, the indiscriminate use of antibiotics has contributed to the evolution of broad spectrum resistance mechanisms (Fernández and Hancock 2012). In most cases, the selective pressure underpinning the emergence of resistant strains has arisen due to the mode of action of antibiotics, i.e. inhibiting bacterial growth. In the last decade, there has been a sharp decline in the development of new classes of antibiotics, with the result that resistance is continuing to increase unchecked. Furthermore, the realisation that microbial communities exist predominantly as multicellular antibiotic-refractive aggregates called biofilms in situ has further emphasised the challenge facing antibiotic therapies in the clinic. Therefore, there is an urgent need for the development of alternative therapies that (1) can target these multidrug-resistant isolates in the biofilm state and (2) are less likely to select for resistant strains (Cegelski et al. 2008; Cooper and Shlaes 2011; LaSarre and Federle 2013).

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6436-1) contains supplementary material, which is available to authorized users.

J. A. Gutiérrez-Barranquero · F. J. Reen · R. R. McCarthy · F. O'Gara (✉)
BIOMERIT Research Centre, School of Microbiology, University College Cork, National University of Ireland, Cork, Ireland
e-mail: f.ogara@ucc.ie

F. O'Gara
School of Biomedical Sciences, Curtin University, Perth WA 6845, Australia

Published online: 12 February 2015

One of the new generation of promising targets to emerge with the capacity to fulfil both criteria is the bacterial quorum sensing system (QS) (Atkinson and Williams 2009; Njoroge and Sperandio 2009). QS is a population-density-dependent cell-cell communication system affording bacteria the capacity for multicellular control of gene expression linked to virulence and pathogenesis. A broad spectrum of QS systems have been reported, with Gram-negative and Gram-positive bacteria favouring distinct signalling components. Gram-negative bacteria use a spectrum of signalling molecules as autoinducers for cell-cell communication, the principal among these being the *N*-acyl-homoserine lactones (AHLs). Gram-positive bacteria typically employ autoinducing peptides (AIPs) for this role. QS systems are central to the regulation of the biofilm mode of growth and the expression of virulence phenotypes (Ng and Bassler 2009; Schertzer et al. 2009). Biofilm formation is a major virulence factor of clinical human pathogens, and this mode of growth can lead to bacteria being 10–1000-fold more resistant to conventional antibiotics (Høiby et al. 2011). As it has been previously reported, QS systems are not directly involved in essential cellular processes (Rasmussen and Givskov 2006). Thus, molecules that inhibit the QS system, and by extension biofilm formation, instead of bacterial growth, commonly called quorum quenching (QQ) molecules or quorum sensing inhibitors (QSi) have been suggested as a powerful strategy for disease control (Kalia 2013; Rasmussen and Givskov 2006).

Since the first published studies on QQ (Dong et al. 2000; Manefield et al. 1999), several studies have been undertaken to discover novel QSi isolated from different organisms and different environments (Kalia 2013; Kalia and Purohit 2011; Koh et al. 2013), while new QSi have also been synthesised based on the structure of natural known QS inhibitors (Kim et al. 2008). In this sense, the plant kingdom has been described as a rich source of potent molecules to develop new drugs for medicine (Hanson 2003; Koh et al. 2013; Savoia 2012), and some plant extracts have been shown to have QS inhibition activity (Choo et al. 2006; Chu et al. 2013; Vattem et al. 2007). Some of the most promising bioactive molecules found in plants are from the coumarin family, a large structurally diverse family of plant phenolic compounds characterised by their pharmacological properties (Venugopala et al. 2013). The most important medical applications of these compounds have been related to their antitumour (Devji et al. 2011; Jamier et al. 2014; Vanamala et al. 2006), anti-inflammatory (Bucolo et al. 2009; Fylaktakidou et al. 2004) and anticoagulant activities (Golfakhrabadi et al. 2014; Payá et al. 1992). Several studies have been conducted analysing the role of some of these compounds against bacterial pathogens (Fig. 1). Some coumarin derivatives have been described for their antimicrobial activities (Ojala et al. 2000; de Souza et al. 2005), for their inhibition of virulence phenotypes such as biofilm and motility (Lee et al. 2014; Zeng et al. 2008), and in the case of 2 furanocoumarins (dihydroxybergamottin and bergamottin) for their inhibition of the AI-1 and AI-2 QS systems (Girennavar et al. 2008). Therefore, we selected coumarin, the prototype molecule of the family, to analyse its ability to inhibit QS signalling in a range of pathogenic organisms, focusing particularly on the classical AHL-based system of Gram-negative bacteria. We also extended this analysis to assess the capacity for coumarin to inhibit different virulence phenotypes in bacterial pathogens and to link these inhibition effects with its possible QS inhibition mode of action. The growth-independent QQ activity of coumarin was found to be particularly potent towards the respiratory pathogen *Pseudomonas aeruginosa* and extended to a range of Gram-negative organisms. The anti-biofilm activity of coumarin towards this pathogen is highly significant in light of the global morbidity and mortality associated with respiratory infection, particularly the chronic biofilm infections that are characteristic of a range of respiratory diseases.

Materials and methods

Bacterial strains and culture conditions The bacterial strains used in the present study are summarised in Table 1. Luria-Bertani (LB, agar or broth) was routinely used to grow *Edwardsiella tarda*, *Escherichia coli* MUH, *P. aeruginosa* PA14, *Salmonella typhimurium* C5369, *Staphylococcus aureus* NCDO949 and *Stenotrophomonas maltophilia*. *Listeria monocytogenes* and *Aliivibrio fischeri* MJ11 were grown on brain-heart infusion (BHI, agar or broth) and LB salt medium (agar or broth), respectively. All of the strains used in this study were grown at 37 °C, with the exception of *A. fischeri* MJ11, which was routinely grown at 23 °C. Culture media were supplemented with the appropriate antibiotics when necessary (carbenicillin 200 μg ml^{-1}, gentamicin 30 μg ml^{-1} and tetracycline 25 μg ml^{-1}). Coumarin was purchased from Sigma-Aldrich (UK) and was dissolved in methanol. Different AHLs used in this work were purchased from Sigma-Aldrich (UK) and were dissolved in DMSO.

Quorum quenching biosensor assay The following quorum quenching reporter strains were used to identify quorum quenching ability of coumarin: *Serratia marcescens* SP15 for the detection of short AHLs (Slater et al. 2003), *Chromobacterium violaceum* DSM30191 for the detection of medium AHLs (McClean et al. 1997) and *Agrobacterium tumefaciens* NTL4 for the detection of long AHLs (contains a plasmid pZLR4 carrying a *traG*::*lacZ* reporter fusion; Farrand et al. 1996; Yin et al. 2012). Different amounts of coumarin (150, 125, 100, 75, 50 and 25 μg) were inoculated on a TLC Silica Gel 60 (Millipore, UK) and overlaid with soft LB agar (0.5 % agar) inoculated with the different biosensors strains at an OD 600 nm of 0.5. Methanol was used as a control. The

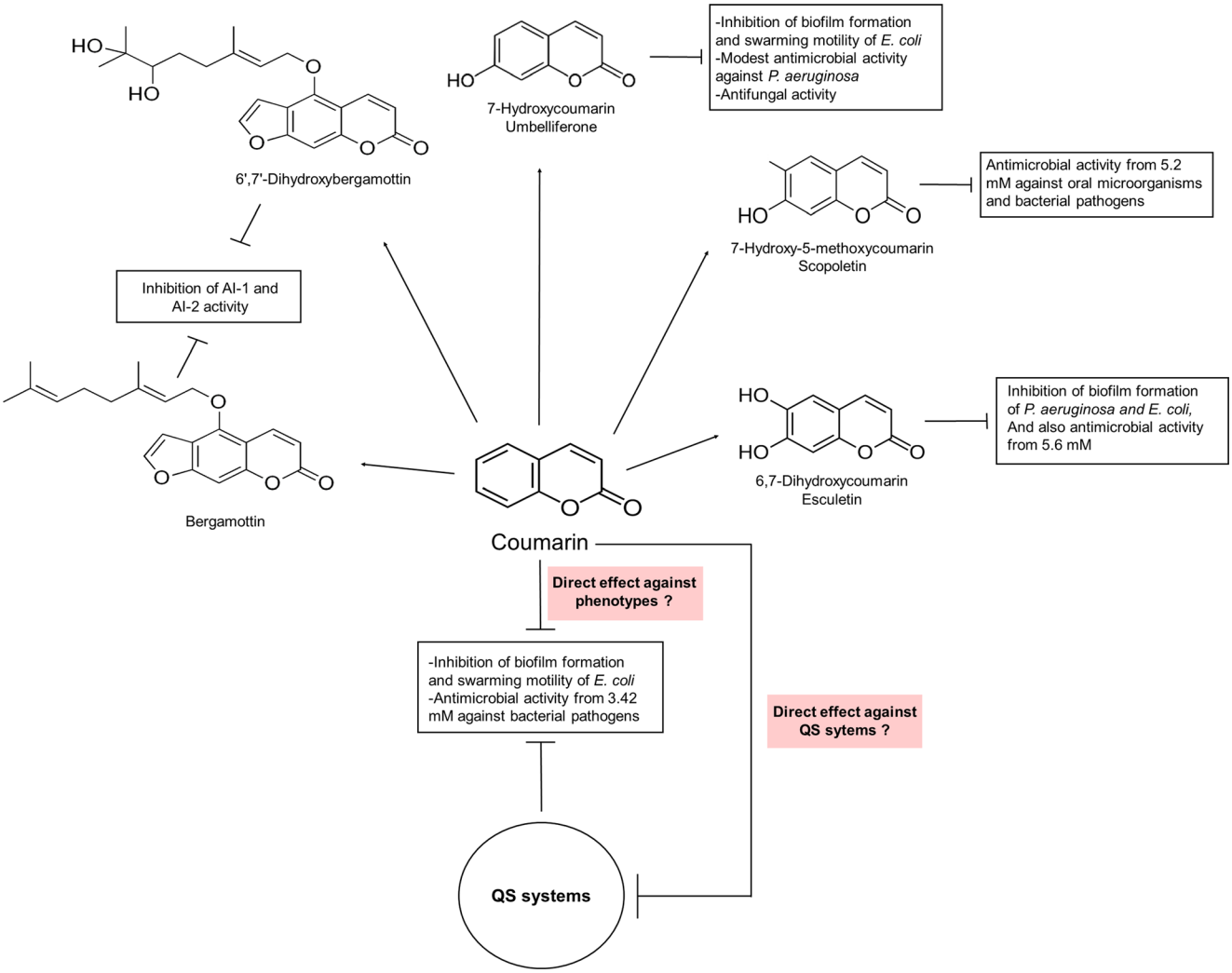

Fig. 1 Chemical structures of the prototype molecule of the coumarin family, coumarin and some derivative molecules

previous plating protocol was used for the different biosensor strains with the exception that soft LB agar for *A. tumefaciens* NTL4 was supplemented with 60 μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and 10 and 40 μM of 3OC10, 3OC12 and 3OC14 homoserine lactones (Sigma-Aldrich, UK), as this strain does not produce its own AHL.

Range of quorum quenching activity against different N-acyl homoserine lactones To elucidate the range of quorum quenching AHL specificity of coumarin, the reporter strains commonly used to detect quorum sensing activity were used. *S. marcescens* SP19 (Poulter et al. 2010) was used to detect the QQ activity of coumarin against short chain AHLs (C4-HSL and C6-HSL). *C. violaceum* CV026 was used to detect the QQ activity of coumarin against medium chain AHLs (C6-HSL and 3OC8-HSL) (McClean et al. 1997). *A. tumefaciens* NTL4, the same strain used in the quorum quenching biosensor assay, was used to detect QQ activity against long chain AHLs. LB agar plates inoculated with *S. marcescens* SP19 and *C. violaceum* CV026 at an OD 600 nm of 0.5 were prepared. Wells were made in the agar plates, and 20 μl of a 200 μM concentration of C4-HSL, C6-HSL and 3OC8-HSL were added. One hundred and 125 μg of coumarin were also added. To determine the effect of coumarin against long AHLs, a different protocol was used. In 96-well microtitre plates (Starstedt, UK), the wells were filled with LB agar supplemented with 60 μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, with the biosensor strain *A. tumefaciens* NTL4 at an OD 600 nm of 0.5, with 20 μl of a 50 and 100 μM concentration of the different long AHLs (3OC10-HSL, 3OC12-HSL and 3OC14-HSL) and with 100 and 125 μg of coumarin. Methanol was used as control in both protocols.

β-Galactosidase assay P. aeruginosa PAO1 transformants containing relevant promoter fusions of the quorum sensing genes *lasI*, *rhlI* and *pqsA* (Table 2) were analysed. Overnight cultures were diluted to OD 600 nm 0.02 in 20 ml LB in the

Table 1 Bacterial strains used in this study

Strains	Relevant characteristics	Reference/source
Gram negative		
Burkholderia cepacia NCTC10743	Opportunistic human pathogen	NCTC
Edwardsiella tarda	Fish pathogen and opportunistic human pathogen	UCC/Biomerit collection
Escherichia coli MUH76317	Multidrug-resistant pathogen	MUH
Pseudomonas aeruginosa PA14, DSM19882	Opportunistic human pathogen	DSMZ
Salmonella typhimurium C5369	food-borne pathogen, causes listeriosis	UCC/Biomerit collection
Stenotrophomonas maltophilia	Uncommon pathogen in humans	A. van der Sar, Vumc, Amsterdam
Vibrio anguillarum	Salmonid fish pathogen	UCC/Biomerit collection
*Allivibrio fischeri*MJ11	Isolated from *Monocentris japonica*, bioluminescent strain, symbiont	Ruby 1996
Gram positive		
Listeria monocytogenes LO28	Serotype 1/2c	C. Hill lab, UCC
Staphylococcus aureus NCDO949	Serotype 3	NCDO

NCTC National Collection of Type Cultures, Culture Collection of Public Health England; *MUH* Mercy University Hospital, Cork, Ireland; *DSMZ* DSMZ-German Collection of Microorganisms and Cell Cultures, Leibniz Institute; *NCDO* National Collection of Dairy Organisms, Reading, UK

presence and absence of coumarin (1.36 mM) grown at 37 °C with shaking at 150 rpm. Beta-galactosidase activity was measured at different times: 2, 4, 6, 8.5 and 24 h, as described previously by Miller (1992).

Biofilm formation and attachment Biofilm formation assays were performed as described previously (O'Toole and Kolter 1998) with minor modifications. *P. aeruginosa* PA14 was inoculated from a fresh overnight culture into 500 μl of LB broth in 24-well microtitre plates (Starstedt, UK) at an OD 600 nm of 0.05. Different concentrations of coumarin were tested: 10.0, 5.0, 2.0, 1.71, 1.36 and 1.0 mM; LB broth with methanol was used as control. These concentrations of coumarin were equivalent in coumarin content to those spotted on the TLC plates (e.g. 150, 125, 100 and 75 μg), in a final volume of 500 μl. All biofilms were incubated at 37 °C for 24 h statically. Attachment assays were conducted as described previously (McCarthy et al. 2014) with minor modifications. PA14 was inoculated into 500 μl of LB broth in 24-well microtitre plates (Starstedt, UK) at an OD 600 nm of 0.25. Different concentrations of coumarin were tested: 1.36 and 1.71 mM. Cultures were incubated at 37 °C for 3 h statically. Biofilm and attachment were measured by solubilisation of crystal violet with 96 % ethanol and quantified at 570 nm.

In vitro biofilm formation of PA14 Sterile slides were placed into 50 ml Falcon tubes containing 15 ml of LB broth, with different concentrations of coumarin (1.36 and 1.71 mM). Overnight cultures of *P. aeruginosa* PA14 were used to inoculate 15 ml of LB at an OD 600 nm of 0.05. Tubes were incubated for 24 h at 37 °C without shaking. Slides were gently recovered from the tubes and were washed with sterile distilled water. A simple staining with crystal violet was used to visualise the biofilm formation at the liquid-air interface.

Growth analysis of PA14 Growth curves of *P. aeruginosa* PA14 were carried out in LB broth at 37 °C using different concentrations of coumarin (2.0, 1.71, 1.36 and 1.0 mM; LB broth with methanol was used as control). PA14 was inoculated from a fresh overnight culture at an OD 600 nm of 0.05. Ten-fold serial dilutions in sterile phosphate buffer were carried out every 2 h. Aliquots (100 μl) of each 10-fold serial dilution were plated onto LB agar. The plates were incubated

Table 2 *Pseudomonas aeruginosa* transformants used for promoter expression analysis

P. aeruginosa strains	Relevant characteristics	Source
PAO1 pME3853::P*lasI*	PAO1 wild type with 174 bp *lasI* upstream fragment and translational *lasI*::*lacZ* fusion in pME6010	Pessi et al. (2001)
PAO1 pMP220::P*rhlI*	PAO1 wild type with *rhlI-lacZ* promoter fusion in pMP220	Baysse et al. (2005)
PAO1 pLP0996::P*pqsA*	PAO1 wild type with *pqsA-lacZ* promoter fusion in pLP170	McGrath et al. (2004)

at 37 °C for 24 h. The number of colony forming units (CFU) per ml were estimated from LB counts.

Antimicrobial activity Aliquots (1 ml) from an overnight fresh culture of *P. aeruginosa* PA14 were washed three times and resuspended in 1 ml of sterile distilled water. The OD 600 nm was measured, and the inoculum was prepared at an OD 600 nm of 0.1. Subsequently, a cotton swab was used to spread the inoculum on the complete surface of the agar plate. Sterile filter paper discs of 6 mm of diameter were used to determine the antimicrobial activity of coumarin. Different amounts of coumarin were inoculated onto the filter paper discs (150, 125, 100 and 75 μg) and were then placed on LB agar plates that were previously inoculated with PA14 as described above.

Phenazine production Overnight cultures of *P. aeruginosa* PA14 were inoculated into 5 ml of LB broth with different concentrations of coumarin (2.0, 1.71, 1.36 and 1.0 mM; LB broth with methanol was used as control) at an OD 600 nm of 0.05. Cultures were incubated at 37 °C for 24 h at 180 rpm. The total volume of the culture was then centrifuged at 4000*g* for 10 min. Three millilitre of chloroform was added to the supernatant and vortexted. The bottom phase was then recovered following centrifugation at 4000*g* for 5 min, and 2 ml of 0.2 M HCl was added. The samples were then vortexed for 20 s and allowed to settle, and the absorbance of the pink layer upper phase was measured at 570 nm.

Motility assays Swimming and swarming motility of *P. aeruginosa* PA14 in the presence of coumarin were assayed. For swim plates, LB 0.3 % (*w*/*v*) agar was used. For swarm plates, 0.8 % (*w*/*v*) nutrient broth Eiken, 0.5 % (*w*/*v*) glucose and 0.6 % (*w*/*v*) Eiken agar was used. Different concentrations of coumarin were tested in both types of motility (1.36 and 1.71 mM; methanol was used as control). The bacterial cells were gently inoculated using a toothpick at the centre of the agar surface, and the plates were incubated at 37 °C for 24 h.

Bioluminescence of Aliivibrio fischeri *A. fischeri* MJ11 strain was grown in LB salt agar at 23 °C for 24 h in the presence of different concentrations of coumarin (2.0, 1.71, 1.36 and 1.0 mM; LB salt agar with methanol was used as control). The results were based on the loss of luminescence in darkness.

Biofilm formation in different bacterial strains The same protocol described above for PA14 was used with minor modifications. Different culture media and times were tested for the different bacterial strains (Table 1). The 1.36 and 1.71 mM concentrations of coumarin were used. LB broth was used for *P. aeruginosa* PA14 and *S. maltophilia* for 24 h; LB salt for *A. fischeri* MJ11 for 24 h; BHI broth for *L. monocytogenes* for 24 h; TSB for *E. coli* MUH, *S. typhimurium* C5369 and *V. anguillarum* for 24 h, and TSB for 48 h was used for *E. tarda* and *S. aureus* NCDO949.

Determination of protease activity Overnight broth cultures of the different strains used in this study (Table 1) were prepared at an OD 600 nm of 1.0. Aliquots (5 μl) were placed onto different agar media supplemented with 1 % (*w*/*v*) skimmed milk with different concentrations of coumarin (1.71 and 1.36 mM; methanol was used as control). TSA+ 2 % (*w*/*v*) of NaCl was used for *A. fischeri* MJ11, TSA was used for *S. aureus* NCDO949 and *S. maltophilia*, BHI agar was used for *L. monocytogenes*, and LB agar was used for the rest of the strains. Protease activity was evaluated by measuring the zone of clearing around the inoculum spot after 72 h.

Statistical analysis Three independent biological replicates were performed for all experiments. Statistical analysis was performed using two-tailed paired Student's *t* test. Differences were considered significant when the *p* value was ≤0.05.

Results

Coumarin blocks the AHL-QS system Members of the coumarin family of plant phenolic compounds have previously been shown to possess promising pharmacological properties, with evidence of interference with the AI-1 and AI-2 QS systems. However, the structurally distinct coumarin molecule itself has not received attention in this regard. Therefore, to establish whether coumarin can target the classical AHL-based QS signal molecules, a range of QS inhibition experiments were performed. In the first approach, the effect of coumarin against the QQ biosensor reporter strains (*S. marcescens* SP15, *C. violaceum* DSM30191 and *A. tumefaciens* NTL4) was analysed (Fig. 2). Coumarin exhibited a strong QS inhibition activity against all three biosensors; the first evidence that this compound has QQ activity. Both SP15 and DSM30191 displayed pigmentation inhibition at 150, 125 and 100 μg, suggesting that coumarin could be blocking short and medium chain AHL-QS signalling. Inhibition was also observed with the long chain AHL biosensor, *A. tumefaciens* NTL4, which (unlike the other two biosensors) requires the addition of exogenous AHLs for activation. Pigmentation inhibition was detected in the presence of three different long chain AHLs (10 and 40 μM) upon addition of 150, 125 and 100 μg of coumarin to the medium. The greatest inhibition was at the lowest concentration of AHL (10 μM) for 3OC10-HSL. Importantly, to confirm that the reduction of pigment production was related to the inhibition of the QS system, and not any growth defect, re-isolation of the biosensor strains from the inhibition spots resulted in characteristic

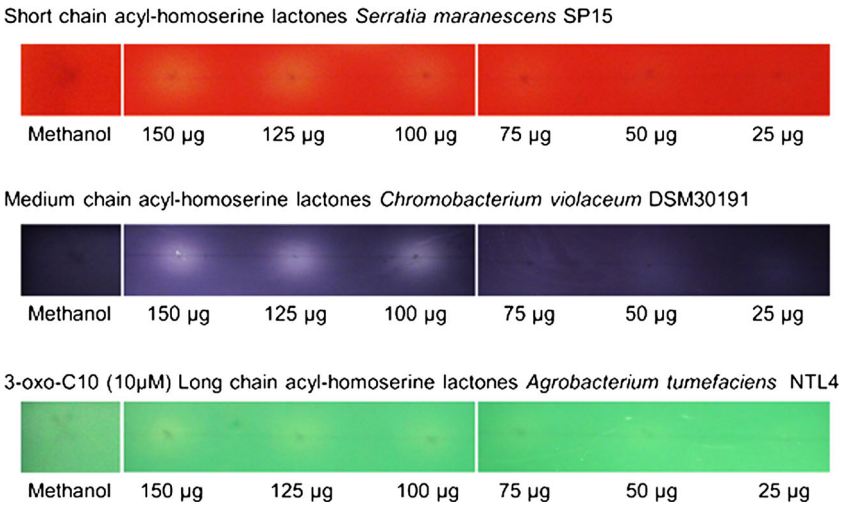

Fig. 2 Quorum quenching activity of coumarin. Different amounts of coumarin were spotted onto a TLC plate and then overlaid with the three different quorum quenching biosensor strains: *S. marcescens* SP15 for the detection of short AHLs, *C. violaceum* DSM30191 for the detection of medium AHLs and *A. tumefaciens* NTL4 for the detection of long AHLs. Pigmentation inhibition indicates that coumarin had a quorum sensing inhibition activity

pigment production after 24 h of growth in the absence of coumarin (data not shown).

In order to determine whether the activity of coumarin was AHL specific, or broad spectrum, the QS biosensor reporter strains (*C. violaceum* CV026, *S. marcescens* SP19 and *A. tumefaciens* NTL4) were used. Coumarin at 100 and 125 µg was able to inhibit QS system-associated pigment production on all three biosensor strains (Fig. 3). Coumarin at both concentrations displayed a similar inhibition effect against the different AHLs tested (C4-HSL, C6-HSL and 3OC8-HSL). As with the previous result using *Agrobacterium* to test the long AHLs (Fig. 2), coumarin showed the highest inhibition activity against 3OC10-HSL. These result suggested that coumarin could have a broad spectrum of action against AHL-QS signal molecules.

Quorum sensing systems are repressed in the presence of coumarin Having established that coumarin was capable of interfering with QS in all three classical biosensor strains, the next step was to test the activity in a QS active pathogen. For this, the serious nosocomial pathogen *P. aeruginosa* was selected encoding the LasIR and RhlIR AHL-based signalling systems. In addition, *P. aeruginosa* also encodes an alkyl quinolone signalling system (*Pseudomonas* quinolone signal) that interfaces with both AHL systems in a QS hierarchical structure that is characteristic of many pathogenic bacteria. Time course expression analysis of the promoters of the *pqsA* (Fig. 4a) and *rhlI* (Fig. 4b) genes revealed that coumarin (at 1.36 mM) decreases the expression of both. The expression of the *pqsA* promoter was decreased in early and late log phase (Fig. 4a), while promoter expression of the *rhlI* gene was decreased by coumarin at the stationary phase (Fig. 4b). Interestingly, expression of the *lasI* promoter was unaffected in the presence of coumarin, suggesting that some specificity exists in its interaction with the QS sensory proteins (Fig. 4c).

Coumarin inhibits biofilm formation of P. aeruginosa PA14 without inhibiting growth and attachment As QS is central to the control of biofilm and virulence in pathogenic bacteria, the ability of coumarin to interfere with these phenotypes in *P. aeruginosa* was examined. Addition of coumarin led to a significant decrease in biofilm formation of *P. aeruginosa* PA14 using the standard static biofilm formation assay. From the different concentrations tested (from 1.0 to 10 mM), the minimal concentration of coumarin that had an effect in biofilm formation inhibition was 1.36 mM (Fig. 5a). Subsequently, an in vitro biofilm formation assay (Fig. 5b) on slides confirmed this inhibition, enabling visualisation of the attached cells and supporting the previous results obtained by the static biofilm assay. Nevertheless, the presence of coumarin did not alter the cell attachment at any of the concentrations tested (Fig. S1a), suggesting that mature biofilm formation and not initial attachment was the target of the compound. In addition, bacterial growth curves were performed using different concentrations of coumarin (1.0, 1.36, 1.71 and 2.0 mM), to test if coumarin had an effect on bacterial growth (Fig. S1b). The cultures showed typical bacterial growth curves including the different phases (lag, exponential and stationary) during 10 h. No significant differences were observed under the different concentrations of coumarin. In addition, an antimicrobial activity assay on LB agar plates using coumarin from 1.0 to 2.0 mM revealed that coumarin did not show antimicrobial activity against *P. aeruginosa* PA14 at these concentrations (Fig. S2a). Therefore, coumarin suppresses biofilm formation in *P. aeruginosa* in a growth independent manner.

Coumarin is capable of inhibiting additional QS-regulated virulence phenotypes in P. aeruginosa Phenazines have been shown to act as an important virulence factor against bacteria, fungi and mammals (Lau et al. 2004; Price-Whelan et al. 2006). Importantly, phenazine production is under the control of QS in *P. aeruginosa*. Consistent with the QSi activity of

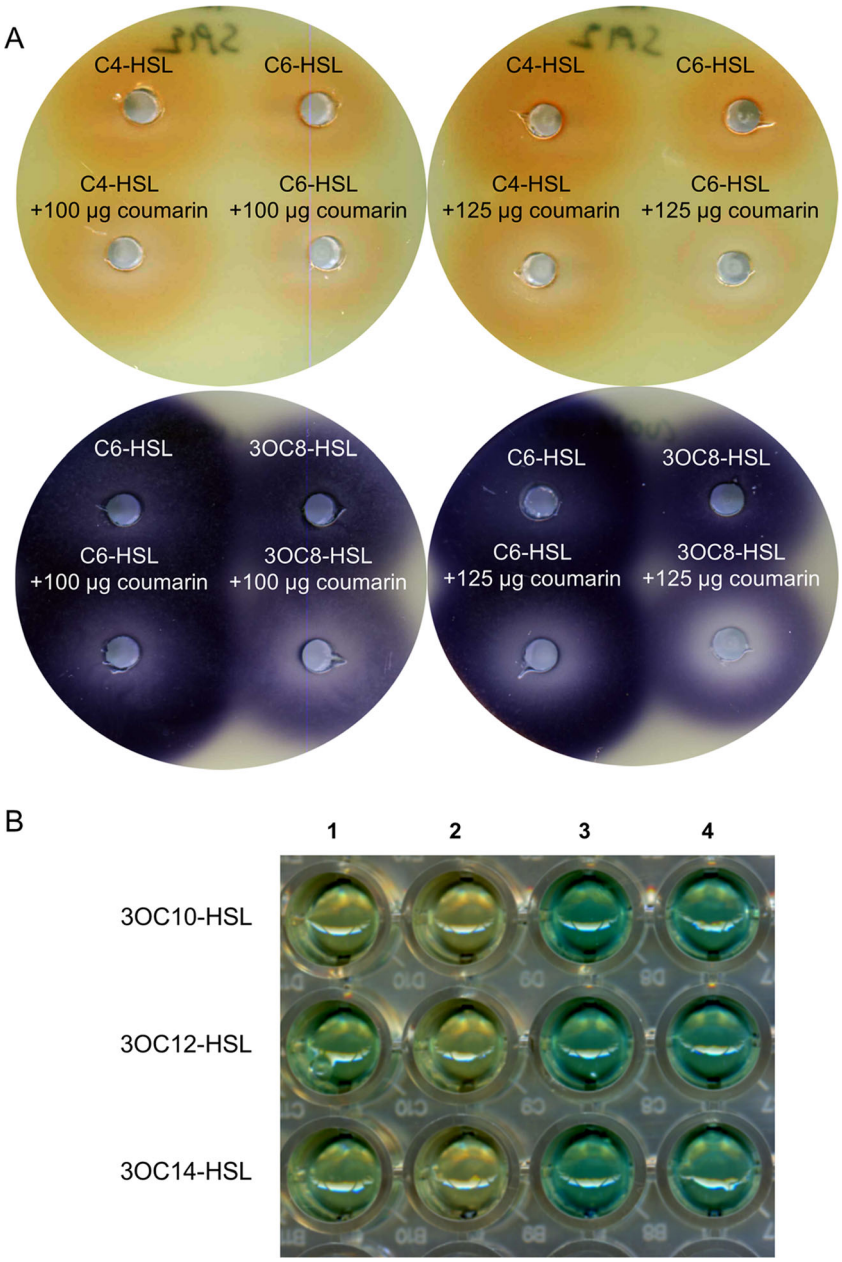

Fig. 3 Coumarin presented a broad spectrum of action against different AHLs. **a** The quorum sensing biosensor strains, *S. marcescens* SP19 and *C. violaceum* CV026, were used to detect coumarin activity against short and medium AHLs. **b** *A. tumefaciens* NTL4 was used to detect the specific activity of coumarin against long AHLs. *1*, 20 µl of a 100 µM of AHL+ 100 µg of coumarin; *2*, 20 µl of a 100 µM of AHL+125 µg of coumarin; *3*, 20 µl of a 100 µM of AHL; *4*, 20 µl of a 100 µM of AHL+methanol

coumarin, phenazine production was inhibited by coumarin in a concentration-dependent manner in *P. aeruginosa* PA14 at 24 h (Fig. 6). Significant differences were observed between the control with methanol and each coumarin concentration tested.

Another multicellular virulence marker in *P. aeruginosa*, swarming motility has also been described as affecting biofilm architecture in this species (Shrout et al. 2006). Swarming motility of *P. aeruginosa* PA14 with and without different concentrations of coumarin (Fig. 7a) was evaluated by measuring the length of dendrites from the centre (Fig. 7b). The dendrite length of cells grown with 1.36 and 1.71 mM of coumarin were 56.6±2.80 and 32.6±2.51 mm respectively. Significant differences were observed when compared with the control in swarm plates with methanol (69.3±6.8 mm). Thus, the swarming motility of *P. aeruginosa* PA14 was decreased by the addition of coumarin. However, swimming motility was unaffected by the presence of coumarin (Fig. S2b). Taken together, coumarin exhibits bioactivity towards distinct QS-associated virulence systems in *P. aeruginosa*.

Coumarin exhibits broad spectrum species-dependent inhibition of QS-associated phenotypes The AHL-based QS system is widespread among Gram-negative bacteria. Therefore, the analysis of coumarin QSi activity was extended to a range of phylogenetically distinct bacteria. The QS paradigm was first

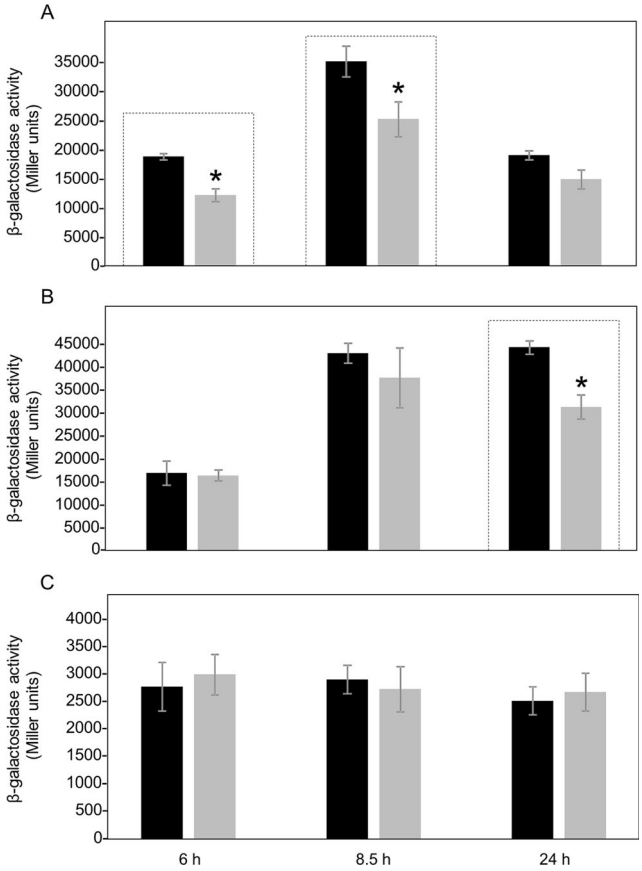

Fig. 4 QS is repressed in response to coumarin. **a** Promoter activity of the *pqsA* gene was significantly reduced in the presence of 1.36 mM of coumarin. The expression of the *pqsA* promoter was decreased in early and late log phase. **b** Promoter activity of the *rhlI* quorum sensing gene was significantly reduced by the addition of coumarin at 1.36 mM. The expression of the *rhlI* promoter was decreased at the stationary phase. **c** Promoter activity of the *lasI* quorum sensing gene was unaffected in the presence of coumarin. All experiments were performed in triplicate. *Black bars*: control treated with methanol and *grey bars*: 1.36 mM of coumarin. Significant differences with respect to the control were represented by *asterisk*

reported in bioluminescent *A. fischeri*, a phenotype that is regulated under the LuxI/R QS system. Consistent with the QSi activity of coumarin, bioluminescence was reduced in all concentrations assayed (from 2.0 to 1.0 mM) (Fig. 8a), without any impact on the bacterial growth observed directly on agar plates.

As with *P. aeruginosa*, biofilm formation is under the control of QS in many pathogens. Therefore, the effect of coumarin on the biofilm formation on different opportunistic human pathogens, fish pathogens and symbiotic bacteria was analysed (Table 1). Of the 10 bacterial strains analysed, biofilm was significantly reduced in five of them (*P. aeruginosa* PA14, *E. coli* MUH, *V. anguillarum*, *E. tarda* and *S. aureus* NCDO949) (Fig. 8b). Interestingly, of these, only two possess homologues of AHL genes, with others encoding AI-2 or even AIP systems. *E. coli* utilises the AI-2 QS signal molecule to control the biofilm formation (González Barrios et al. 2006), while *S. aureus*, a Gram-positive pathogen, uses AIP signalling. Therefore, the QSi activity of coumarin may extend beyond the classical AHL system, although we cannot rule out an AHL-responsive sensor protein in these organisms.

The impact of coumarin on QS-associated protease activity was investigated in the same 10 bacterial strains (Table 1). Five of the bacterial strains tested were positive for protease activity: *P. aeruginosa* PA14, *V. anguillarum*, *S. maltophilia*, *B. cepacia* and *S. aureus*. *P. aeruginosa* PA14 and *V. anguillarum* displayed the highest protease activity, which was unaffected by the addition of coumarin, as was the case for *S. aureus* (data not shown). In contrast, coumarin was able to inhibit protease activity in *S. maltophilia* and *B. cepacia* (Fig. 8c), suggesting that the control of protease activity by QS may be species dependent.

Discussion

The emergence of multidrug-resistant bacteria has become a major global concern, with predictions of the perfect storm receiving increased attention in the face of sharp declines in alternative drug development (Cooper and Shlaes 2011; Gould 2009). Research efforts have shifted from 'killing' compounds to the search for new anti-virulence anti-biofilm compounds, providing an effective alternative to controlling bacterial infections (Kalia 2013; LaSarre and Federle 2013; Rasmussen and Givskov 2006). In this study, we describe a new member of a growing class of next generation compounds which exploit a key virulence switch in bacterial pathogens termed quorum sensing.

Coumarin is a natural molecule derived from plants, whose pharmacological safety has been established. Exposure to coumarin (from food or cosmetics products) does not pose any risk for human health, although high doses have been associated with liver toxicity in rodents (Felter et al. 2006; Lake 1999). Furthermore, coumarin has been recently shown to possess anti-biofilm properties against *E. coli* (Lee et al. 2014). Taken together with the fact that the biofilm mode of growth is under the control of QS systems in Gram-negative bacteria (Solano et al. 2014), these findings suggested that coumarin could have potential as an effective QSi anti-virulence compound. Therefore, to establish whether or not coumarin had QSi activity, the ability of the pure compound to inhibit AHL-QS systems was investigated using classical AHL QQ biosensor reporter strains. Coumarin was able to inhibit pigment production on all three QQ biosensor reporter strains and subsequently displayed a broad range of inhibition activity of different AHLs (Figs. 2 and 3). These data suggest that coumarin may inhibit native AHL-QS systems in bacterial pathogens and by extension, therefore, could be an effective

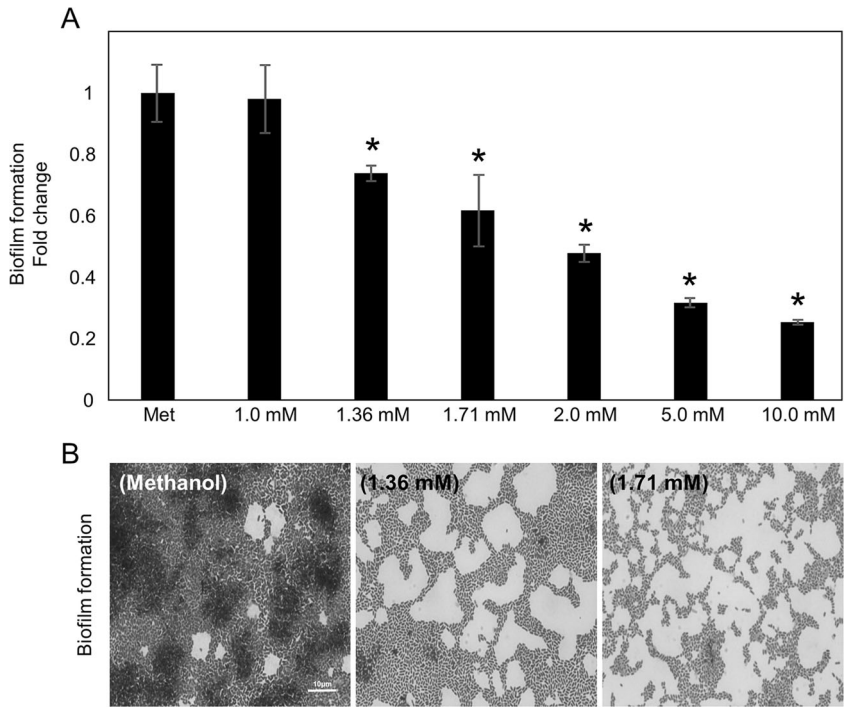

Fig. 5 Reduction of biofilm formation in *P. aeruginosa* at 24 h by the addition of different concentrations of coumarin. Significant differences with respect to the control are represented by an *asterisk*

inhibitor of virulence traits in these organisms. Importantly, all QSi effects were growth independent, and concentrations were below the MIC for this compound (de Souza et al. 2005).

In this study, coumarin was able to inhibit biofilm formation (Fig. 5), phenazine production (Fig. 6) and swarming motility (Fig. 7) without impairing *P. aeruginosa* growth capacities (Fig. S1b). These constitute three of the major QS-regulated virulence phenotypes in *P. aeruginosa* and other pathogens (Daniels et al. 2004; De Kievit 2009; Köhler et al. 2000). Biofilm formation presents a major challenge to the clinical management of infection, with chronic and medical device infections proving particularly refractive to conventional therapeutics. Initiated by the formation of microcolonies on a surface, maturation and subsequent dispersal underpin the persistence of a broad spectrum of serious pathogens. Both phenazine production and swarming motility are also multicellular phenotypes which are central to the pathophysiology of infection. It is worth noting that both phenotypes are intrinsically linked to biofilm formation in *P. aeruginosa*. Swarming motility has been reported to contribute to the early stages of *P. aeruginosa* biofilm formation (Shrout et al. 2006), playing an essential role in the surface colonisation contributing to the formation of the biofilms (Verstraeten et al. 2008). The role of phenazines in the formation of mature biofilms has also been described (Ramos et al. 2010). Together, modulation of both virulence systems may contribute to the suppression of biofilm formation by coumarin.

In order to gain an insight into the possible mode of QS inhibition by coumarin, *P. aeruginosa* was used as a model organism. *P. aeruginosa* encodes three different QS systems:

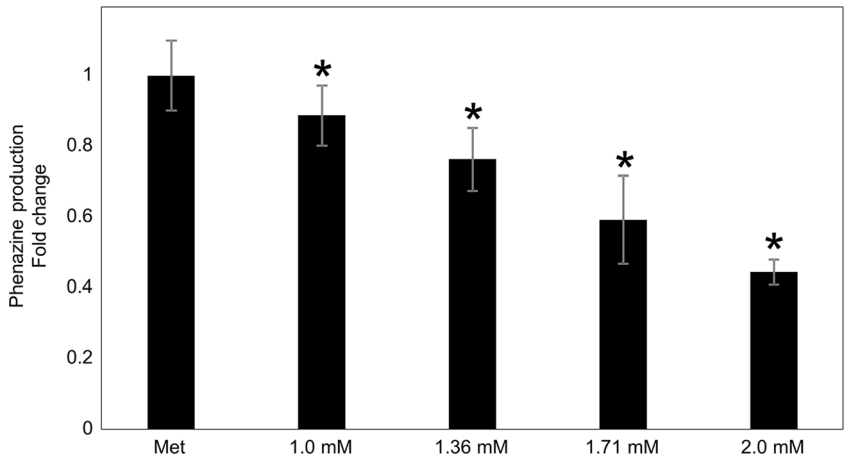

Fig. 6 Coumarin displays an inhibition of phenazine production. Phenazine production was calculated with respect to the methanol control, and significant differences are represented by an *asterisk*

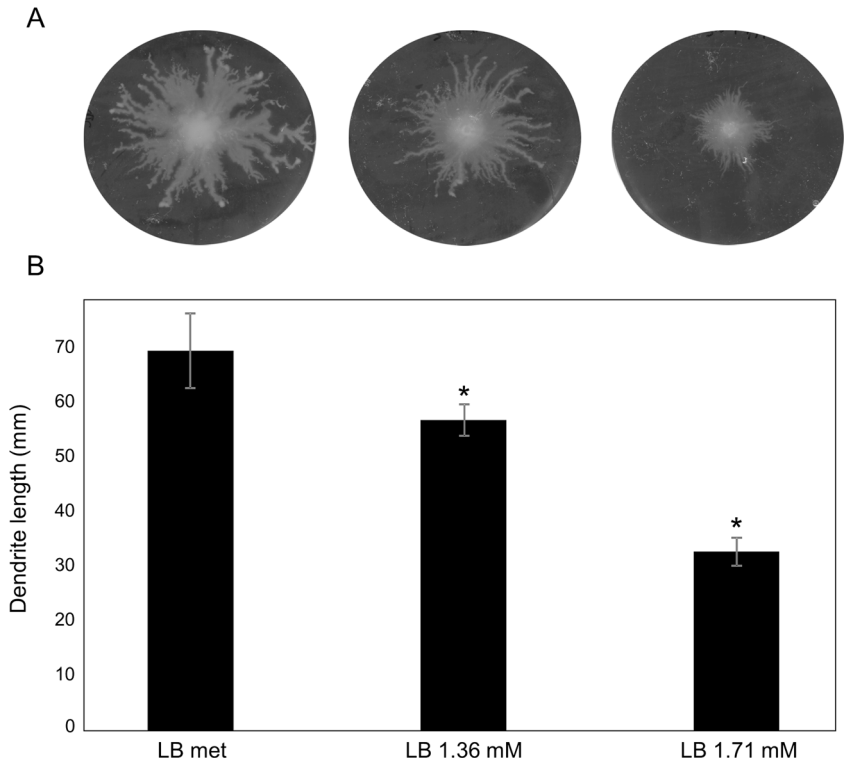

Fig. 7 Impact of coumarin on swarming motility. **a** Coumarin inhibited swarming motility of *Pseudomonas aeruginosa* PA14; from the left to the right: swarm plate+methanol, swarm plate+ 1.36 mM of coumarin and swarm plate+1.71 mM of coumarin. **b** Swarming motility was measured by the length of dendrites from the centre of the plate. Significant differences with respect to the control are represented by an *asterisk*

LasI/R, RhlI/R which are the classical AHL-based systems and a species-specific *Pseudomonas* quinolone signal (PQS) system, all of which regulate biofilm and virulence phenotypes in this species (Bacalso et al. 2011; Bjarnsholt et al. 2010; Häussler and Becker 2008). These systems in the QS network of *P. aeruginosa* are arranged hierarchically and control the transcription of hundreds of genes (Dietrich et al. 2006; Wilder et al. 2011). All three QS systems have been shown to play a role in the formation of mature biofilms, with RhlI/R and PQS in particular associated with the latter stages of development (Davies 1998; Favre-Bonté et al. 2003; Yang et al. 2009). It was perhaps unsurprising, therefore, that reduced promoter activity of *rhlI* and *pqsA* was observed in the presence of coumarin. Phenazine production in *P. aeruginosa* is under the control of the PQS signalling system (Déziel et al. 2004). Therefore, the inhibition of phenazine production by coumarin (Fig. 6) is perhaps a result of the reduced expression of the *pqsA* promoter (Fig. 4a). Swarming motility in *P. aeruginosa* is modulated by RhlI/R-associated biosurfactant rhamnolipids (Caiazza et al. 2005; Ochsner et al. 1994), with the reduced expression of the *rhlI* promoter (Fig. 4b) offering a possible mechanism for its suppression. Transcriptional promoter activity of *lasI* was not influenced (Fig. 4c), suggesting that the activity of coumarin is linked to the interaction of the signal molecule with the LasR receptor and not the production of the signal per se.

In establishing coumarin as a QSi compound, the question remained as to its spectrum of activity. Importantly, while the AHL-based QS system exhibits a degree of conservation in Gram-negative bacteria, its circuitry and downstream targets can be quite diverse. Therefore, further experiments analysing the effect of coumarin on different quorum sensing regulated phenotypes in different bacterial species were undertaken. The first AHL molecule and its regulatory circuit were discovered in the bioluminescent marine bacterium *A. fischeri* (formerly *Vibrio fischeri*) (Ruby 1996). This bacterium is found in symbiosis with marine animals and has excellent bioluminescent properties, controlling itself through the LuxI/R QS system, homologous to the LasI/R and RhlI/R systems of *P. aeruginosa* (Ruby 1996; Rutherford and Bassler 2012). Coumarin was capable of inhibiting bioluminescence in *A. fischeri* MJ11 (Fig. 8a), supporting a role for coumarin against the LuxI/R QS system. Protease activity is another important virulence factor (Lebrun et al. 2009) that has been reported to be under the control of QS systems in different bacteria (Hentzer et al. 2003; Suppiger et al. 2013). Coumarin inhibited protease activity in *S. maltophilia* and *B. cepacia* (Fig. 8c).

Biofilm formation was investigated in a range of bacterial pathogens. Some but not all of the bacterial species tested showed inhibition of biofilm formation upon addition of coumarin (Fig. 8b). Coumarin was effective in reducing biofilm formation of *E. tarda* and *V. anguillarum*, both of which encode AHL-QS homologue genes (Milton et al. 1997; Morohoshi et al. 2004). These data suggest that the inhibition effect could be through blocking the AHL signalling system, at least in these species. Interestingly, coumarin also had an impact on biofilm formation in *E. coli* and *S. aureus*. It has

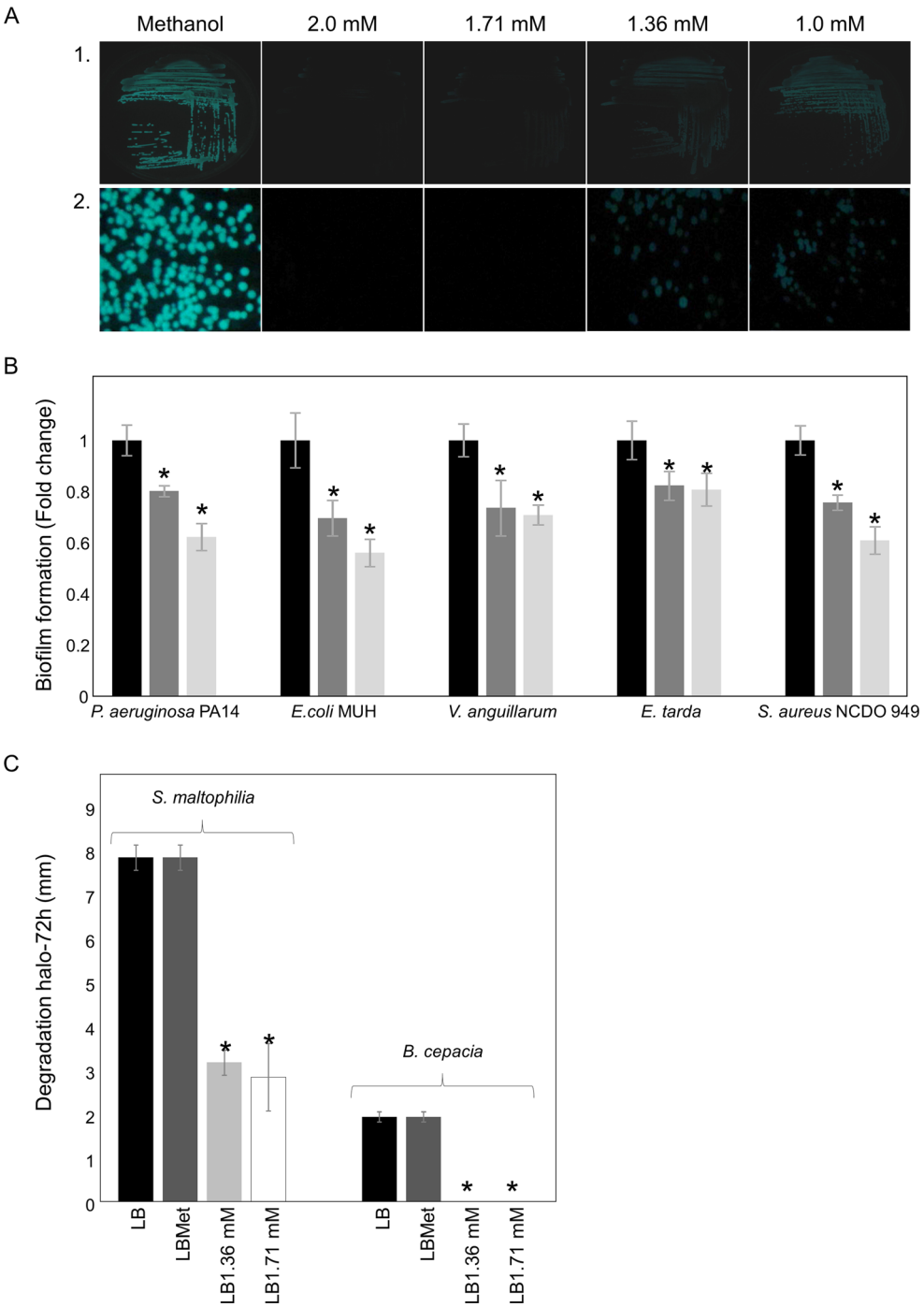

Fig. 8 Coumarin inhibits different QS-associated phenotypes in different bacterial strains. **a** Inhibition of bioluminescence in *Aliivibrio fischeri* by the addition of coumarin. *Aliivibrio fischeri* MJ11 was grown in LB salt at 23 °C at different concentrations of coumarin. *1* The bioluminescence of *Aliivibrio fischeri* was inhibited at all the concentrations of coumarin tested. *2* Image magnification where the decrease in bioluminescence was observed in isolated colonies. This experiment was carried out in triplicate. **b** Coumarin affected biofilm formation in different bacterial strains. Significant differences with respect to the control in each bacterial species were represented by an *asterisk. Black bar*: control treated with methanol; *dark grey bar*: 1.36 mM of coumarin; *light grey bar*: 1.71 mM of coumarin. **c** Coumarin reduces protease activity. Significant differences with respect to the control in each bacterial species are represented by an *asterisk*

previously been described that *E. coli* utilises a different QS signal molecule, the AI-2 to control the biofilm formation (González Barrios et al. 2006), and the anti-biofilm activity of coumarin towards *E. coli* was previously reported (Lee et al. 2014). The AI-2 signal molecule, produced by LuxS, is a communication signal used by both Gram-negative and Gram-positive bacteria (Surette et al. 1999). In this sense, our observation that coumarin also inhibited the biofilm formation in the Gram-positive pathogen *S. aureus* (Fig. 8b), a process regulated by the *agr* QS system (Periasamy et al. 2012; Yarwood et al. 2004), could support a role for coumarin as a universal QSi.

Inhibition of the QS cell-to-cell communication system is one of the most promising strategies to control bacterial diseases based on its ability to modulate virulence phenotypes in a broad spectrum of pathogenic bacteria. In this study, we have identified the QS inhibition mode of action of coumarin, a natural product isolated from a variety of plants. As we have described in this work, coumarin not only exhibits a potent inhibitory effect against AHL-QS systems but also displays

inhibition towards the species-specific PQS system in *P. aeruginosa*. Coumarin may have the potential for broad spectrum QSi activity, based on the inhibition of virulence phenotypes regulated by different QS systems in different bacterial species. Future work will focus on unravelling, at the molecular level, the mode of action of this promising molecule.

Acknowledgments This research was supported in part by grants awarded by the Science Foundation of Ireland (07/IN.1/B948, 12/TIDA/B2411, 12/TIDA/B2405, 09/RFP/BMT 2350, SSPC212/RC/2275); the Department of Agriculture, Fisheries and Food (FIRM/RSF/CoFoRD; DAFF11/F/009MabS); the Environmental Protection Agency (EPA 2008-PhD/S-2); the Irish Research Council for Science, Engineering and Technology (PD/2011/2414; RS/2010/2413); the European Commission (FP7-PEOPLE-2013-ITN, 607786; FP7-KBBE-2012-6, FP7-KBBE-2012-6, Marie Curie 256596); Marine Microbial Biodiversity, Bioinformatics and Biotechnology (MICRO B3)(OCEAN 2012 287589), Marine Microorganisms: Cultivation Methods for improving their Biotechnological Applications (MACUMBA)(CP-TP 311975), Increasing Value and Flow in the Marine Biodiscovery Pipeline (PharmaSea) (CP-TP 312184) and the Marine Institute (Beaufort award C2CRA 2007/082); Teagasc (Walsh Fellowship 2013) and the Health Research Board (HRA/2009/146).

References

Atkinson S, Williams P (2009) Quorum sensing and social networking in the microbial world. J R Soc Interface 6:959–978. doi:10.1098/rsif.2009.0203

Bacalso M, Xu T, Yeung K, Zheng D (2011) Biofilm formation of *Pseudomonas aeruginosa* PA14 required *lasI* and was stimulated by the Pseudomonas Quinolone Signal although Salicylic acid Inhibition is independent of the pqs pathway. J Exp Microbiol Immun 15:84–89

Baysse C, Cullinane M, Dénervaud V, Burrowes E, Dow JM, Morrissey JP, Tam L, Trevors JT, O'Gara F (2005) Modulation of quorum sensing in *Pseudomonas aeruginosa* through alteration of membrane properties. Microbiology 151:2529–2542. doi:10.1099/mic.0.28324-0

Bjarnsholt T, Jensen P, Jakobsen TH, Phipps R, Nielsen AK, Rybtke MT, Tolker-Nielsen T, Givskov M, Høiby N, Ciofu O (2010) Quorum sensing and virulence of *Pseudomonas aeruginosa* during lung infection of cystic fibrosis patients. PLoS One 5:e10115. doi:10.1371/journal.pone.0010115

Bucolo C, Ward KW, Mazzon E, Cuzzocrea S, Drago F (2009) Protective effects of a coumarin derivative in diabetic rats. Invest Ophthalmol Vis Sci 50:3846–5382. doi:10.1167/iovs. 08-3328

Caiazza NC, Shanks RMQ, O'Toole GA (2005) Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. J Bacteriol 187(21):7351–7361. doi:10.1128/JB.187.21.7351

Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ (2008) The biology and future prospects of antivirulence therapies. Nat Rev Microbiol 6(1):17–27. doi:10.1038/nrmicro1818

Choo JH, Rukayadi Y, Hwang JK (2006) Inhibition of bacterial quorum sensing by vanilla extract. Lett Appl Microbiol 42:637–641. doi:10.1111/j.1472-765X.2006.01928.x

Chu W, Zhou S, Jiang Y, Zhu W, Zhuang X, Fu J (2013) Effect of traditional chinese herbal medicine with antiquorum sensing activity on *Pseudomonas aeruginosa*. Evid Based Complement Alternat Med Volume 2013, Article ID 648257, 7 doi:10.1155/2013/648257

Cooper MA, Shlaes D (2011) Fix the antibiotics pipeline. Nature 472:32. doi:10.1038/472032a

Daniels R, Vanderleyden J, Michiels J (2004) Quorum sensing and swarming migration in bacteria. FEMS Microbiol Rev 28:261–289. doi:10.1016/j.femsre.2003.09.004

Davies DG (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298. doi:10.1126/science.280.5361.295

De Kievit TR (2009) Quorum sensing in *Pseudomonas aeruginosa* biofilms. Environ Microbiol 11:279–288. doi:10.1111/j.1462-2920.2008.01792.x

de Souza SM, Monache FD, Smânia A (2005) Antibacterial activity of coumarins. Z Naturforsch 60:693–700

Devji T, Reddy C, Woo C, Awale S, Kadota S, Carrico-Moniz D (2011) Pancreatic anticancer activity of a novel geranylgeranylated coumarin derivative. Bioorg Med Chem Lett 21:57705773. doi:10.1016/j.bmcl.2011.08.005

Déziel E, Lépine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG (2004) Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. Proc Natl Acad Sci USA 101:1339–1344. doi:10.1073/pnas.0307694100

Dietrich LEP, Price-Whelan A, Petersen A, Whiteley M, Newman DK (2006) The phenazinepyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Mol Microbiol 61:1308–1321. doi:10.1111/j.1365-2958.2006.05306.x

Dong YH, Xu JL, Li XZ, Zhang LH (2000) AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc Natl Acad Sci 97:3526–3531. doi:10.1073/pnas.97.7.3526

Farrand SK, Hwang I, Cook DM (1996) The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. J Bacteriol 178(14):4233–4247

Favre-Bonté S, Köhler T, Van Delden C (2003) Biofilm formation by *Pseudomonas aeruginosa*: role of the C4-HSL cell-to-cell signal and inhibition by azithromycin. J Antimicrob Chemother 52:598–604. doi:10.1093/jac/dkg397

Felter SP, Vassallo JD, Carlton BD, Daston GP (2006) A safety assessment of coumarin taking into account species-specificity of toxicokinetics. Food Chem Toxicol 44:462–475. doi:10.1016/j.fct.2005.08.019

Fernández L, Hancock REW (2012) Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev 25:661–681. doi:10.1128/CMR. 00043-12

Fylaktakidou KC, Hadjipavlou-Litina DJ, Litinas KE, Nicolaides DN (2004) Natural and synthetic coumarin derivatives with anti-inflammatory/antioxidant activities. Curr Pharm Des 10:3813–3833. doi:10.2174/1871520613666131224124445

Girennavar B, Cepeda ML, Soni KA, Vikram A, Jesudhasan P, Jayaprakasha GK, Pillai SD, Patil BS (2008) Grapefruit juice and its furocoumarins inhibits autoinducersignaling and biofilm formation in bacteria. Int J Food Microbiol 125:204–208. doi:10.1016/j.ijfoodmicro.2008.03.028

Golfakhrabadi F, Abdollahi M, Ardakani MRS, Saeidnia S, Akbarzadeh T, Ahmadabadi AN, Ebrahimi A, Yousefbeyk F, Hassanzadeh A, Khanavi M (2014) Anticoagulant activity of isolated coumarins (suberosin and suberenol) and toxicity evaluation of *Ferulago carduchorum* in rats. Pharm Biol 52:1335–1340. doi:10.3109/13880209.2014.892140

González Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE, Wood TK (2006) Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). J Bacteriol 188:305–316. doi:10.1128/JB.188.1.305-316.2006

Gould IM (2009) Antibiotic resistance: the perfect storm. Int J Antimicrob Agents 34:52–55. doi:10.1016/S0924-8579(09)70549-7

Hanson JR (2003) The classes of natural product and their isolation. In: Abel EW (ed) Natural products: the secondary metabolites. Royal Society of Chemistry, Cambridge, pp 1–34

Häussler S, Becker T (2008) The pseudomonas quinolone signal (PQS) balances life and death in *Pseudomonas aeruginosa* populations. PLoS Pathog 4:e1000166. doi:10.1371/journal.ppat.1000166

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Høiby N, Givskov M (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J 22:3803–3815. doi:10.1093/emboj/cdg366

Høiby N, Ciofu O, Johansen HK, Song Z, Moser C, Jensen P, Molin S, Givskov M, Tolker-Nielsen T, Bjarnsholt T (2011) The clinical impact of bacterial biofilms. Int J Oral Sci 3:55–65. doi:10.4248/IJOS11026

Jamier V, Marut W, Valente S, Chereau C, Chouzenoux S, Nicco C, Lemarechal H, Weill B, Kirsch G, Jacob C, Batteux F (2014) Chalcone-Coumarin derivatives as potential anti-cancer drugs: an in vitro and in vivo investigation. Anticancer Agents Med Chem 14(7):963–974. doi:10.2174/1871520613666131224124445

Kalia VC (2013) Quorum sensing inhibitors: an overview. Biotechnol Adv 31:224–245. doi:10.1016/j.biotechadv.2012.10.004

Kalia VC, Purohit HJ (2011) Quenching the quorum sensing system: potential antibacterial drug targets. Crit Rev Microbiol 37:121–140. doi:10.3109/1040841X.2010.532479

Kim C, Kim J, Park HY, Park HJ, Lee JH, Kim CK, Yoon J (2008) Furanone derivatives as quorum-sensing antagonists of *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 80:37–47. doi:10.1007/s00253-008-1474-6

Koh CL, Sam CK, Yin WF, Tan LY, Krishnan T, Chong YM, Chan KG (2013) Plant-derived natural products as sources of anti-quorum sensing compounds. Sensors 13:6217–6228. doi:10.3390/s130506217

Köhler T, Curty LK, Barja F, Delden CV, Pechère JC (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J Bacteriol 182:5990–5996. doi:10.1128/JB.182.21.5990-5996.2000

Lake BG (1999) Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. Food Chem Toxicol 37:423–453. doi:10.1016/S0278-6915(99)00010-1

LaSarre B, Federle MJ (2013) Exploiting quorum sensing to confuse bacterial pathogens. Microbiol Mol Biol Rev 77:73–111. doi:10.1128/MMBR. 00046-12

Lau GW, Hassett DJ, Ran H, Kong F (2004) The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol Med 10:599–606. doi:10.1016/j.molmed.2004.10.002

Lebrun I, Marques-Porto R, Pereira AS, Pereira A, Perpetuo EA (2009) Bacterial toxins: an overview on bacterial proteases and their action as virulence factors. Mini Rev Med Chem 9:820–828. doi:10.2174/138955709788452603

Lee JH, Kim YG, Cho HS, Ryu SY, Cho MH, Lee J (2014) Coumarins reduce biofilm formation and the virulence of *Escherichia coli* O157:H7. Phytomedicine 21:1037–1042. doi:10.1016/j.phymed.2014.04.008

Manefield M, Nys R, Kumar N, Read R, Givskov M, Steinberg P, Kjelleberg S (1999) Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology 145:283–291. doi:10.1099/13500872-145-2-283

McCarthy RR, Mooij MJ, Reen FJ, Lesouhaitier O, O'Gara F (2014) A new regulator of pathogenicity (bvlR) is required for full virulence and tight microcolony formation in *Pseudomonas aeruginosa*. Microbiology 160:1488–1500. doi:10.1099/mic. 0.075291-0

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GSAB, Williams P (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acyl homoserine lactones. Microbiology 143:3703–3711. doi:10.1099/00221287-143-12-3703

McGrath S, Wade DS, Pesci EC (2004) Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the Pseudomonas quinolone signal (PQS). FEMS Microbiol Lett 230:27–34. doi:10.1016/S0378-1097(03)00849-8

Miller JH (1992) A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, New York

Milton DL, Hardman A, Camara M, Chhabra SR, Bycroft BW, Stewart GS, Williams P (1997) Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI*/*vanR* locus and identification of the autoinducer N-(3-Oxodecanoyl)-L-homoserine lactone. J Bacteriol 179:3004–3012

Morohoshi T, Inaba T, Kato N, Kanai K, Ikeda T (2004) Identification of quorum-sensing signal molecules and the LuxRI homologs in fish pathogen *Edwardsiella tarda*. J Biosci Bioeng 98:274–281. doi:10.1016/S1389-1723(04)00281-6

Ng WL, Bassler BL (2009) Bacterial quorum-sensing network architectures. Annu Rev Genet 43:197–222. doi:10.1146/annurev-genet-102108-134304

Njoroge J, Sperandio V (2009) Jamming bacterial communication: new approaches for the treatment of infectious diseases. EMBO Mol Med 1:201–210. doi:10.1002/emmm.200900032

O'Toole GA, Kolter R (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28:449–461. doi:10.1046/j.1365-2958.1998.00797.x

Ochsner UA, Fiechter A, Reisers J (1994) Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa rhlAB* genes encoding a rhamnosyl transferase involved in rhamnolipid biosurfactant synthesis. J Biol Chem 269:19787–19795

Ojala T, Remes S, Haansuu P, Vuorela H, Hiltunen R, Haahtela K, Vuorela P (2000) Antimicrobial activity of some coumarin containing herbal plants growing in Finland. J Ethnopharmacol 73:299–305. doi:10.1016/S0378-8741(00)00279-8

Payá M, Halliwell B, Hoult JR (1992) Interactions of a series of coumarins with reactive oxygen species. Scavenging of superoxide, hypochlorous acid and hydroxyl radicals. Biochem Pharmacol 44:205–214. doi:10.1016/0006-2952(92)90002-Z

Periasamy S, Joo HS, Duong AC, Bach T-HL, Tan VY, Chatterjee SS, Cheung GYC, Otto M (2012) How *Staphylococcus aureus* biofilms develop their characteristic structure. Proc Natl Acad Sci USA 109:1281–1286. doi:10.1073/pnas.1115006109

Pessi G, Williams F, Hindle Z, Heurlier K, Holden MT, Cámara M, Haas D, Williams P (2001) The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. J Bacteriol 183:6676–6683. doi:10.1128/JB.183.22.6676-6683.2001

Poulter S, Carlton TM, Su X, Spring DR, Salmond GPC (2010) Engineering of new prodigiosin-based biosensors of *Serratia* for facile detection of short-chain *N*-acyl homoserine lactone quorum-sensing molecules. Environ Microbiol Rep 2:322–328. doi:10.1111/j.1758-2229.2010.00140.x

Price-Whelan A, Dietrich LEP, Newman DK (2006) Rethinking "secondary" metabolism: physiological roles for phenazine antibiotics. Nat Chem Biol 2:71–78. doi:10.1038/nchembio764

Ramos I, Dietrich LEP, Price-Whelan A, Newman DK (2010) Phenazines affect biofilm formation by *Pseudomonas aeruginosa* in similar

ways at various scales. Res Microbiol 161:187–191. doi:10.1016/j.resmic.2010.01.003

Rasmussen TB, Givskov M (2006) Quorum sensing inhibitors: a bargain of effects. Microbiology 152:895–904. doi:10.1099/mic. 0.28601-0

Ruby EG (1996) Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. Annu Rev Microbiol 50:591–624. doi:10.1146/annurev.micro.50.1.591

Rutherford ST, Bassler BL (2012) Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med. doi:10.1101/cshperspect.a012427

Savoia D (2012) Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiol 7(8):979–990. doi:10.2217/fmb.12.68

Schertzer JW, Boulette ML, Whiteley M (2009) More than a signal: non-signaling properties of quorum sensing molecules. Trends Microbiol 17:189–195. doi:10.1016/j.tim.2009.02.001

Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR (2006) The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol Microbiol 62:1264–1277. doi:10.1111/j.1365-2958.2006.05421.x

Slater H, Crow M, Everson L, Salmond GPC (2003) Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. Mol Microbiol 47:303–320. doi:10.1046/j.1365-2958.2003.03295.x

Solano C, Echeverz M, Lasa I (2014) Biofilm dispersion and quorum sensing. Curr Opin Microbiol 18:96–104. doi:10.1016/j.mib.2014.02.008

Suppiger A, Schmid N, Aguilar C, Pessi G, Eberl L (2013) Two quorum sensing systems control biofilm formation and virulence in members of the *Burkholderia cepacia* complex. Virulence 4:400–409. doi:10.4161/viru.25338

Surette MG, Miller MB, Bassler BL (1999) Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: A new family of genes responsible for autoinducer production. Proc Natl Acad Sci USA 96:1639–1644. doi:10.1073/pnas.96.4.1639

Tanwar J, Das S, Fatima Z, Hameed S (2014) Multidrug resistance: an emerging crisis. Interdiscip Perspect Infect Dis Article ID 541340, 7 doi: 10.1155/2014/541340

Vanamala J, Leonardi T, Patil BS, Taddeo SS, Murphy ME, Pike LM, Chapkin RS, Lupton JR, Turner ND (2006) Suppression of colon carcinogenesis by bioactive compounds in grapefruit. Carcinogenesis 27:1257–1265. doi:10.1093/carcin/bgi318

Vattem DA, Mihalik K, Crixell SH, McLean RJC (2007) Dietary phytochemicals as quorum sensing inhibitors. Fitoterapia 78:302–310. doi:10.1016/j.fitote.2007.03.009

Venugopala KN, Rashmi V, Odhav B (2013) Review on natural coumarin lead compounds for their pharmacological activity. Biomed Res IntArticle ID 963248, 14 doi: 10.1155/2013/963248

Verstraeten N, Braeken K, Debkumari B, Fauvart M, Fransaer J, Vermant J, Michiels J (2008) Living on a surface: swarming and biofilm formation. Trends Microbiol 16:496–506. doi:10.1016/j.tim.2008.07.004

Wilder CN, Diggle SP, Schuster M (2011) Cooperation and cheating in *Pseudomonas aeruginosa*: the roles of the *las*, *rhl* and *pqs* quorum-sensing systems. ISME J 5:1332–1343. doi:10.1038/ismej.2011.13

Yang L, Nilsson M, Gjermansen M, Givskov M, Tolker-Nielsen T (2009) Pyoverdine and PQS mediated subpopulation interactions involved in *Pseudomonas aeruginosa* biofilm formation. Mol Microbiol 74(6):1380–1392. doi:10.1111/j.1365-2958.2009.06934.x

Yarwood JM, Bartels DJ, Volper EM, Greenberg EP (2004) Quorum sensing in *Staphylococcus aureus* biofilms. J Bacteriol 186:1838–1850. doi:10.1128/JB.186.6.1838-1850.2004

Yin WF, Purmal K, Chin S, Chan XY, Chan KG (2012) Long chain *N*-acyl homoserine lactone production by *Enterobacter* sp. isolated from human tongue surfaces. Sensors 12:14307–14314. doi:10.3390/s121114307

Zeng Z, Qian L, Cao L, Tan H, Huang Y, Xue X, Shen Y, Zhou S (2008) Virtual screening for novel quorum sensing inhibitors to eradicate biofilm formation of *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 79:119–126. doi:10.1007/s00253-008-1406-5