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Quorum-sensing effector pyocyanin but not farnesol & acyl homoserine lactone exhibit antibacterial activity

Sir,

Quorum sensing (QS) is a mechanism of cell-to-cell communication in microbes, involving cell-density-dependent production of small signalling molecules that regulate a wide array of traits including cell morphology, motility, expression of virulence factors and drug resistance[1-4]. Amongst bacteria, the major QS signals are linear/cyclic oligopeptides (in Gram-positive bacteria), acyl homoserine lactones (in Gram-negative bacteria), furanone derivatives (in both) and others such as quinolone, phenazines, indole and cis-2-decenoic acid in selected species[1,2]. In fungi, QS signals have been well characterized for *Candida* spp., and primarily include farnesol and tyrosol that reciprocally regulate the yeast-to-hyphal transition[3]. A lipoxygenase-derived mediator and pantothenic acid derivative have also been described in *Aspergillus flavus* and *Cryptococcus neoformans,* respectively[3,4].

Over the past several years, QS-based interference has emerged as an important strategy against microbial infections[1-3]. This therapeutic approach involves the use of certain natural or synthetic compounds[1-3,5], and even repurposed drugs[6], to inhibit the QS and thereby the associated virulence mechanisms. Alternatively, many of the QS effector molecules are important mediators of both intra- and interspecies microbial communication[1-3], and themselves display antimicrobial or antibiofilm activity against unrelated species[7-16]. These include farnesol, which is a sesquiterpene alcohol produced by *Candida* spp. and is also a component of many essential oils[3]; long-chain (C_{10} to C_{14}) acyl homoserine lactones are synthesized by Gram-negative bacteria[1,2] and pyocyanin, a blue-green N-methyl-1-hydroxyphenazine pigment that is a terminal signalling factor in the quorum-sensing network of *Pseudomonas aeruginosa*[7]. While farnesol reportedly exhibits antimicrobial function by compromising the cell membrane integrity, biofilm formation or virulence-factor production[9-12], long-chain acyl homoserine lactones may disrupt the bacterial cell wall and virulence gene expression[13-15], and pyocyanin interferes in the electron transport chain during respiration, leading to the production of toxic oxygen radicals[7,8,16]. Although the inhibitory activity of these agents has been explored in the literature against many Gram-positive and Gram-negative bacterial pathogens[7-16], majority of the studies have tested one or few strains only, especially the standard strains. Considering the emergent drug resistance crisis, the present work aimed to evaluate the antimicrobial potential of these three compounds against culture-type strains and multiple clinical isolates of four common nosocomial pathogens that are frequently antibiotic resistant: *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*.

The study was undertaken in the department of Microbial Biotechnology, Panjab University, Chandigarh, India, from January 2016 to May 2017. *S. epidermidis* (ATCC 35984), *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603) were used as the culture-type strains. Clinical isolates of *S. epidermidis* (n=14), *S. aureus* (n=10), *E. coli* (n=3) and *K. pneumoniae* (n=11) (Table I) were from our laboratory collection and had been obtained from catheter-related bloodstream infections in a previous study[17]. The bacterial identification was performed by biochemical testing and subsequently confirmed by mass spectrometry (MALDI Biotyper, Bruker Daltonics, Bremen, Germany). The strains were preserved in the brain–heart infusion broth (Hi-Media, Mumbai) with 15 per cent glycerol (SRL Pvt, Ltd., Mumbai) at −20°C. Antibiotic susceptibility testing was performed as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[18,19] and

Table I. Antibiotic susceptibility of *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* culture-type strains and clinical isolates used

Bacterial species	Antibiotic susceptibility						
	Cefotaxime (30 µg)	Imipenem (10 µg)	Oxacillin[#]	Amikacin (30 µg)	Ciprofloxacin (5 µg)	Chloramphenicol (30 µg)	Vancomycin[#]
S. epidermidis							
CI-6[*]	S	ND	S	S	S	S	S
ATCC 12228	S	ND	S	R	R	S	S
ATCC 35984, CI-1, CI-2, CI-3, CI-5, CI-7, CI-10, CI-12	R	ND	R	S	S	S	S
CI-4, CI-8	R	ND	R	S	I	S	S
CI-9, CI-13, CI-14	R	ND	R	S	R	S	S
CI-11	R	ND	R	S	R	R	S
S. aureus							
ATCC 29213, ATCC 25923, CI-1, CI-5	S	ND	S	S	S	S	S
CI-9[*]	S	ND	S	S	I	S	S
CI-10[*]	S	ND	S	S	R	S	S
CI-8	S	ND	S	S	I	R	S
CI-2, CI-3	R	ND	R	S	I	S	S
CI-4, CI-7	R	ND	R	S	R	S	S
CI-6	R	ND	R	S	R	R	S
E. coli							
ATCC 25922	S	S	ND	S	S	S	ND
CI-1[*], CI-2, CI-3[*]	R	S	ND	S	R	S	ND
K. pneumoniae							
CI-2, CI-4	S	S	ND	S	S	S	ND
ATCC 700603	I	S	ND	S	S	I	ND
CI-9[*]	R	S	ND	S	I	S	ND
CI-10	R	S	ND	S	R	I	ND
CI-6, CI-11	R	S	ND	R	R	S	ND
CI-3	R	S	ND	R	R	I	ND
CI-7, CI-8	R	R	ND	R	S	S	ND
CI-1, CI-5	R	R	ND	R	R	R	ND

[*]The antibiotic susceptibility of these isolates has been reported previously in Singh *et al*[17], 2016. Cefotaxime, imipenem, amikacin, ciprofloxacin and chloramphenicol were tested by disk diffusion. [#]Vancomycin and oxacillin in staphylococci were tested by vancomycin agar screen test (brain heart infusion agar with 6 µg/ml vancomycin) and oxacillin agar screen test (Mueller Hinton agar with 4% NaCl and 6 µg/ml oxacillin), respectively. S, susceptible; I, intermediate; R, resistant; ND, not done (imipenem for *S. epidermidis* and *S. aureus*; vancomycin and oxacillin for *E. coli* and *K. pneumoniae*)

the zones of growth inhibition (ZOI) were interpreted accordingly.

The stock solutions of pyocyanin and N-(3-oxododecanoyl)-L-homoserine lactone (3-Oxo-C_{12}-HSL; Sigma-Aldrich, Bengaluru) were prepared in dimethyl sulfoxide (SRL Pvt., Ltd., Mumbai), and that of trans, trans-farnesol (Sigma-Aldrich, Bengaluru) was prepared in methanol (Merck Millipore, Bengaluru) as

Table II. Antibacterial activity of pyocyanin against culture-type strains of *S. epidermidis*, *S. aureus*, *E. coli* and *K. pneumoniae*, tested using disk-diffusion assay with pyocyanin content ranging from 5 to 30 µg

Pyocyanin disk content (µg)	ZOI (mm; mean±SD)			
	S. epidermidis (ATCC 35984)	*S. aureus* (ATCC 29213)	*E. coli* (ATCC 25922)	*K. pneumoniae* (ATCC 700603)
5	12±1	14±2	11±3	0
10	18±1	18±1	17±2	0
20	24±1	21±1	19±4	9±2
30	29±2	22±1	24±2	9±1

The results are represented as the diameters of the ZOI, rounded up to the nearest whole millimetres. ZOI, zones of growth inhibition; SD, standard deviation

per the manufacturer's instructions. The antimicrobial activity of these agents was determined by disk-diffusion assay, according to the CLSI guidelines given for antibiotics[18,19]. Disk contents ranging from 5 to 30 µg (pyocyanin) or up to 100 µg (farnesol and 3-Oxo-C_{12}-HSL) were used. The diameters of ZOI were measured up to the nearest whole millimetres. Control set-ups comprising dimethyl sulphoxide and methanol were set up in parallel to confirm that the observed antibacterial activity was indeed resulting from the test agent. The ZOIs of test compounds were interpreted for activity according to the method of Quinto and Santos[20], wherein the zone sizes <10, 10-13, 14-19 and >19 were considered as inactive, partially active, active and very active, respectively. In addition, the minimum inhibitory concentrations (MICs) of all the three compounds against the culture-type strains were determined by broth microdilution method[18,19]. The experiments were performed in triplicate and repeated thrice to confirm the observations. Statistical significance was determined using analysis of variance test for normal distribution. The data were represented as mean±standard deviation (SD).

Pyocyanin exhibited a significant, dose-dependent antibacterial activity against culture-type strains of *S. epidermidis*, *S. aureus* and *E. coli* by disk-diffusion assay (Table II). The MICs of pyocyanin were found to be four, eight and 32 µg/ml for *S. epidermidis*, *S. aureus* and *E. coli*, respectively, by broth microdilution method. Notably, pyocyanin was also observed to be highly active against all the tested clinical isolates of these three species (Table III), including the isolates resistant to antibiotic classes such as β-lactams, fluoroquinolone (ciprofloxacin) and phenicol (chloramphenicol), as well as the multidrug-resistant isolates such as methicillin-resistant staphylococci (Tables I and III). The average ZOI diameters in clinical isolates of *S. epidermidis*, *S. aureus* and *E. coli* were 29±3, 24±1 and 18±1 mm (mean±SD), respectively, after exposure to 30 µg pyocyanin by disk diffusion (Table III). A statistical comparison revealed that this inhibitory effect was highest against *S. epidermidis*, followed by *S. aureus* and then *E. coli* (P<0.001). It should be noted, however, that only a limited number of *E. coli* cultures were tested in the present study.

To assess if pyocyanin was auto-inhibitory for the producing species, its activity was also tested against a *P. aeruginosa* strain (ATCC 27583), which was found to be resistant to its action. These results thus affirm the role of pyocyanin in conferring a competitive advantage to *P. aeruginosa* during interspecies interaction in human infections and corroborate with some of the previous publications wherein *P. aeruginosa* cells or purified pyocyanin were reported to exhibit antagonistic behaviour against staphylococci and *E. coli*[7,8,16]. Other studies, however, indicate that the competitive advantage of *P. aeruginosa* over staphylococci is independent of pyocyanin[21,22] and rather mediated by certain polysaccharides[23].

In comparison to the other species, *K. pneumoniae* was inhibited by pyocyanin to a lesser extent (Tables II and III). The average ZOI diameter in culture-type strain and clinical isolates was 11±3 mm after exposure to 30 µg pyocyanin. Increasing the dose of pyocyanin up to 50 or 100 µg did not improve the inhibition zone size. Furthermore, the different isolates of *K. pneumoniae* tested varied in their susceptibility to pyocyanin. The compound was inactive against four, partially active against six and active against two of the *K. pneumoniae* tested, as observed by disk diffusion. The MIC of pyocyanin against *K. pneumoniae* ATCC 700603 was found to be >64 µg/ml. Contrary to the generally accepted notion, this decreased sensitivity

Table III. Antibacterial activity of pyocyanin against clinical isolates of *S. epidermidis, S. aureus, E. coli* and *K. pneumoniae*, tested using disk-diffusion assay with pyocyanin content of 30 µg

Bacterial strain/ isolate	ZOI with 30-µg pyocyanin (mm; mean±SD)
S. epidermidis	
CI-1	31±1
CI-2	31±2
CI-3	28±1
CI-4	31±6
CI-5	31±1
CI-6	31±1
CI-7	29±1
CI-8	33±4
CI-9	25±5
CI-10	26±1
CI-11	27±1
CI-12	20±4
CI-13	28±2
CI-14	28±2
S. aureus	
CI-1	29±2
CI-2	22±1
CI-3	24±1
CI-4	25±2
CI-5	29±2
CI-6	26±1
CI-7	24±1
CI-8	23±1
CI-9	19±1
CI-10	24±4
E. coli	
CI-1	18±4
CI-2	19±1
CI-3	18±3
K. pneumoniae	
CI-1	10±0
CI-2	10±0
CI-3	10±0
CI-4	10±0
CI-5	18±1
CI-6	9±1
CI-7	10±1
CI-8	16±1
CI-9	10±1
CI-10	8±1
CI-11	9±1

The results are represented as the diameters of the ZOI, rounded up to the nearest whole millimetres

of *K. pneumoniae* was not solely the result of its Gram-negative nature, as all the *E. coli* isolates tested were observed to be sensitive to pyocyanin. Instead, it could have resulted from the thick capsule that *K. pneumoniae* harbours, which limits the cell permeability. Alternatively, an increased expression of enzymes such as catalase and superoxide dismutase, which inactivate the reactive oxygen intermediates, may contribute to the decreased pyocyanin efficacy[7]. This reduced susceptibility to pyocyanin may contribute to the ability of *K. pneumoniae* to co-exist with *P. aeruginosa* during mixed-species infections[24]. In contrast, *S. aureus*, being sensitive to pyocyanin and other respiratory inhibitors produced by *P. aeruginosa,* often exists in temporal or spatial segregation, or may form electron-deficient small-colony variants to counter these respiratory toxins during co-existance[25,26].

Neither 3-Oxo-C_{12}-HSL nor farnesol demonstrated any antibacterial activity against *S. epidermidis*, *S. aureus*, *E. coli* and *K. pneumoniae* even when tested up to a dose of 100 µg by disk diffusion (ZOI, 0 mm) or up to 64 µg/ml by broth microdilution in the present study. These results differ from many of the previous reports, which describe the antibacterial properties of farnesol and long-chain acyl homoserine lactones[11,13-15]. This is likely due to differences in the experimental protocol employed. In the present work, antimicrobial susceptibility testing was performed using the assay parameters recommended by CLSI for clinical testing so as to obtain results that are physiologically relevant. The earlier studies have followed variable methodologies, including the culture media, inoculum density and the method of antibacterial activity assessment[11,13-15]; these parameters are known to influence the results of susceptibility testing[18,19]. To validate our findings, we tested two additional culture-type strains (*S. epidermidis* ATCC 12228 and *S. aureus* ATCC 25923) used in earlier studies[13,14]. While pyocyanin (30 µg) exhibited antibacterial activity

against these strains (*S. epidermidis* ATCC 12228, 15±2 mm; *S. aureus* ATCC 25923, 24±2 mm), 3-Oxo-C_{12}-HSL and farnesol were found to be ineffective against both these strains as well (ZOI, 0 mm up to 100 µg disk content), under our tested conditions.

In conclusion, our results indicated that of the three quorum-sensing effectors tested, only pyocyanin exhibited a potent antibacterial activity. The inhibitory effect was the maximum against *S. epidermidis*, followed by *S. aureus* and *E. coli*, including their drug-resistant clinical isolates. *K. pneumoniae* was inhibited to a lesser extent, and the various isolates tested differed in their susceptibility to pyocyanin. In contrast, farnesol and 3-Oxo-C_{12}-HSL did not show any growth inhibitory activity against the four bacterial species under the tested conditions, suggesting that these quorum-sensing effectors do not have an antagonistic action, and would not be potentially antimicrobial.

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