

The Apparent Quorum-Sensing Inhibitory Activity of Pyrogallol Is a Side Effect of Peroxide Production

Tom Defoirdt, Gde Sasmita Julyantoro Pande, Kartik Baruah, Peter Bossier

Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Ghent, Belgium

There currently is more and more interest in the use of natural products, such as tea polyphenols, as therapeutic agents. The polyphenol compound pyrogallol has been reported before to inhibit quorum-sensing-regulated bioluminescence in *Vibrio harveyi*. Here, we report that the addition of $10 \text{ mg} \cdot \text{liter}^{-1}$ pyrogallol protects both brine shrimp (*Artemia franciscana*) and giant river prawn (*Macrobrachium rosenbergii*) larvae from pathogenic *Vibrio harveyi*, whereas the compound showed relatively low toxicity (therapeutic index of 10). We further demonstrate that the apparent quorum-sensing-disrupting activity is a side effect of the peroxide-producing activity of this compound rather than true quorum-sensing inhibition. Our results emphasize that verification of minor toxic effects by using sensitive methods and the use of appropriate controls are essential when characterizing compounds as being able to disrupt quorum sensing.

Polyphenols are a large group of compounds found in plants, including coffee and tea (1). They have been reported to have both antioxidant (2) and prooxidant activities (3). The polyphenol compound pyrogallol (1,2,3-trihydroxybenzene) has been reported to have antibacterial activity against many bacteria, including vibrios (4). More recently, the compound has been reported to inhibit quorum-sensing-regulated bioluminescence at subinhibitory concentrations (i.e., concentrations that did not affect growth) in a *Vibrio harveyi* HAI-1 receptor mutant (50% inhibitory concentration [IC_{50}] of $2 \mu\text{M}$ [$\sim 0.25 \text{ mg} \cdot \text{liter}^{-1}$]) (5). However, the effect of pyrogallol on the bioluminescence of a constitutively luminescent strain (which would allow verification that the bioluminescence inhibition is really caused by interference with its regulation) has not been studied, nor has the compound been reported to affect any other quorum-sensing-regulated phenotype in the bacterium.

Impact of pyrogallol on the virulence of *Vibrio harveyi* toward brine shrimp larvae. We previously showed that the virulence of *Vibrio harveyi* BB120 (= ATCC BAA-1116; recently reclassified as *Vibrio campbellii* [6]) in our model system with gnotobiotic brine shrimp larvae is regulated by quorum sensing (7). Using this model system, we investigated whether pyrogallol could protect challenged larvae from the pathogen and found that when added to the culture water at $10 \text{ mg} \cdot \text{liter}^{-1}$ or more, the compound significantly increased the survival of challenged larvae

TABLE 1 Percent survival of brine shrimp larvae with and without pyrogallol after 2 days of challenge with *Vibrio harveyi* BB120

Treatment	% Shrimp survival (avg \pm SD) ^a
Control	87 \pm 8 A
Pyrogallol $50 \text{ mg} \cdot \text{liter}^{-1}$	90 \pm 7 A
Pyrogallol $100 \text{ mg} \cdot \text{liter}^{-1}$	75 \pm 7 A
Pyrogallol $500 \text{ mg} \cdot \text{liter}^{-1}$	0 \pm 0 B
BB120	40 \pm 5 C
BB120 + pyrogallol $1 \text{ mg} \cdot \text{liter}^{-1}$	43 \pm 8 C
BB120 + pyrogallol $5 \text{ mg} \cdot \text{liter}^{-1}$	38 \pm 12 C
BB120 + pyrogallol $10 \text{ mg} \cdot \text{liter}^{-1}$	87 \pm 3 A
BB120 + pyrogallol $50 \text{ mg} \cdot \text{liter}^{-1}$	87 \pm 10 A

^a Results are from three replicates. Values with different letters are significantly different ($P < 0.01$).

TABLE 2 Percent survival of giant river prawn (*Macrobrachium rosenbergii*) larvae after 5 and 8 days of challenge with *Vibrio harveyi* BB120 with and without pyrogallol

Treatment	% Prawn survival (avg \pm SD) on day ^a :	
	5	8
Control	97 \pm 3 A	85 \pm 5 a
BB120	70 \pm 5 C	46 \pm 5 b
BB120 + pyrogallol $10 \text{ mg} \cdot \text{liter}^{-1}$	86 \pm 5 B	77 \pm 8 a

^a Results are from five replicates. Values with different letters are significantly different ($P < 0.01$).

vae (Table 1). Furthermore, pyrogallol showed relatively low toxicity to brine shrimp larvae, as there was no significant negative effect on the survival of nonchallenged larvae for concentrations up to $100 \text{ mg} \cdot \text{liter}^{-1}$ (Table 1).

Impact of pyrogallol on the virulence of *Vibrio harveyi* toward giant river prawn larvae. Because of these promising results, we went further to investigate the effect of pyrogallol in a commercial crustacean species. We had previously found that the virulence of *Vibrio harveyi* to larvae of the giant river prawn *Macrobrachium rosenbergii* is regulated by quorum sensing (G. S. J. Pande, F. M. I. Natrah, P. Sorgeloos, P. Bossier, and T. Defoirdt, submitted for publication). Using the same challenge assay, we found that the addition of $10 \text{ mg} \cdot \text{liter}^{-1}$ pyrogallol to the culture water also resulted in significantly increased survival of challenged giant river prawn larvae (Table 2).

Impact of pyrogallol on bioluminescence of *Vibrio harveyi*. *Vibrio harveyi* quorum sensing has been reported to control different virulence-related phenotypes, including biofilm formation

Received 25 February 2013 Returned for modification 14 March 2013

Accepted 25 March 2013

Published ahead of print 1 April 2013

Address correspondence to Tom Defoirdt, Tom.Defoirdt@UGent.be.

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doi:10.1128/AAC.00401-13

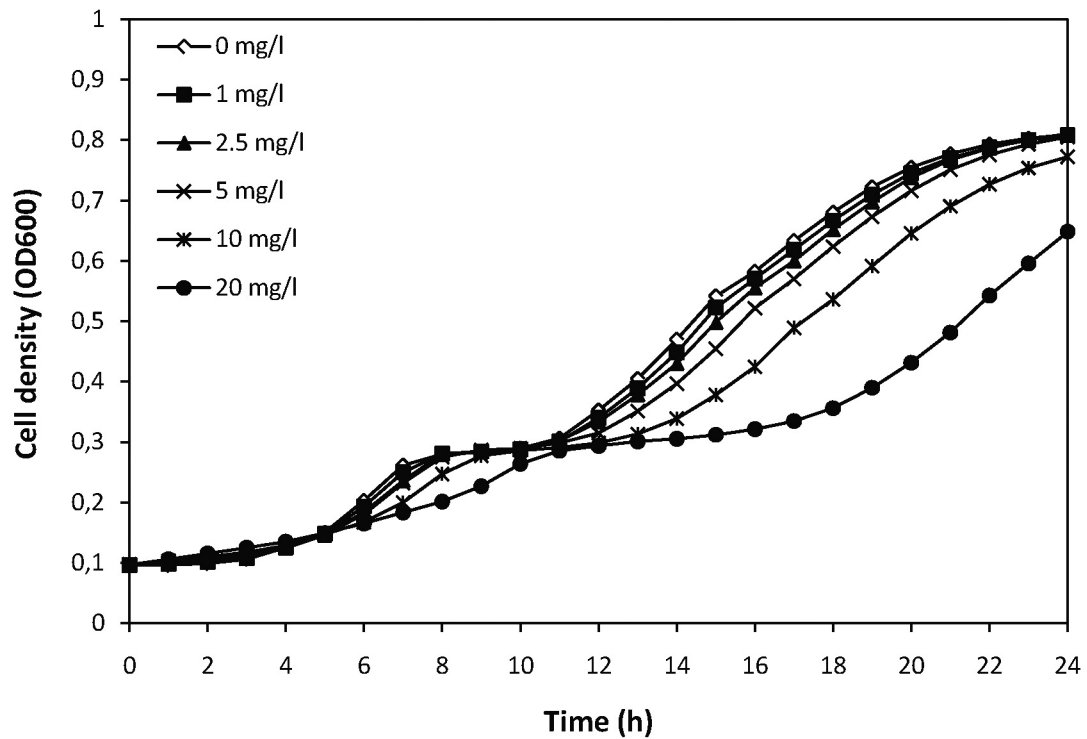
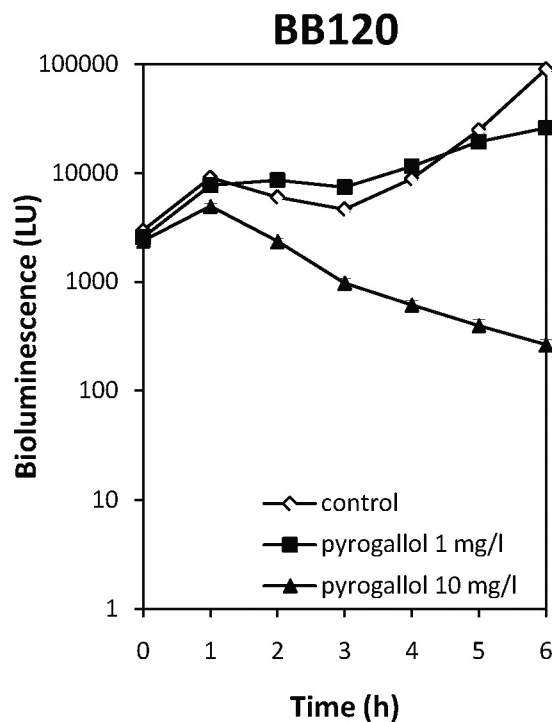
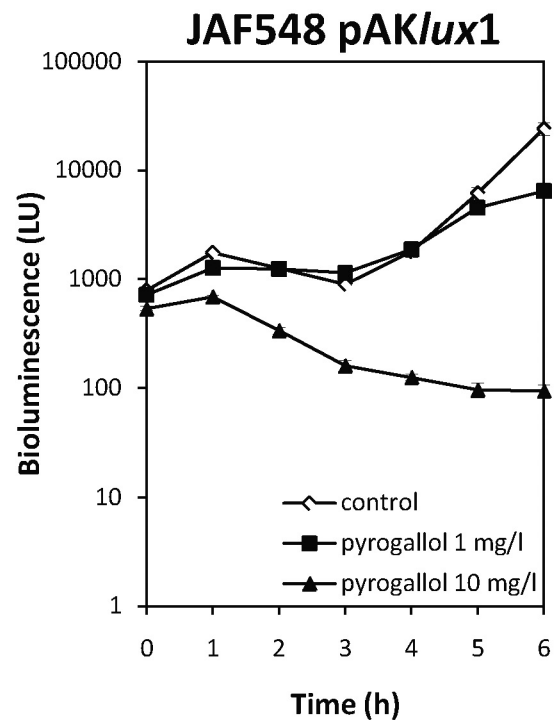
A**B****C**

FIG 1 (A) Growth of wild-type *Vibrio harveyi* BB120 in Luria-Bertani medium containing $35 \text{ g} \cdot \text{liter}^{-1}$ synthetic sea salt (LB_{35}) with and without pyrogallol. (B) Bioluminescence of *Vibrio harveyi* under the same conditions with and without pyrogallol. Error bars represent the standard deviations of 4 replicates. (C) Bioluminescence of *Vibrio harveyi* strain JAF548 pAKlux1 (in which luminescence is independent of quorum sensing) with and without pyrogallol. Error bars represent the standard deviations of 4 replicates. LU, light units.

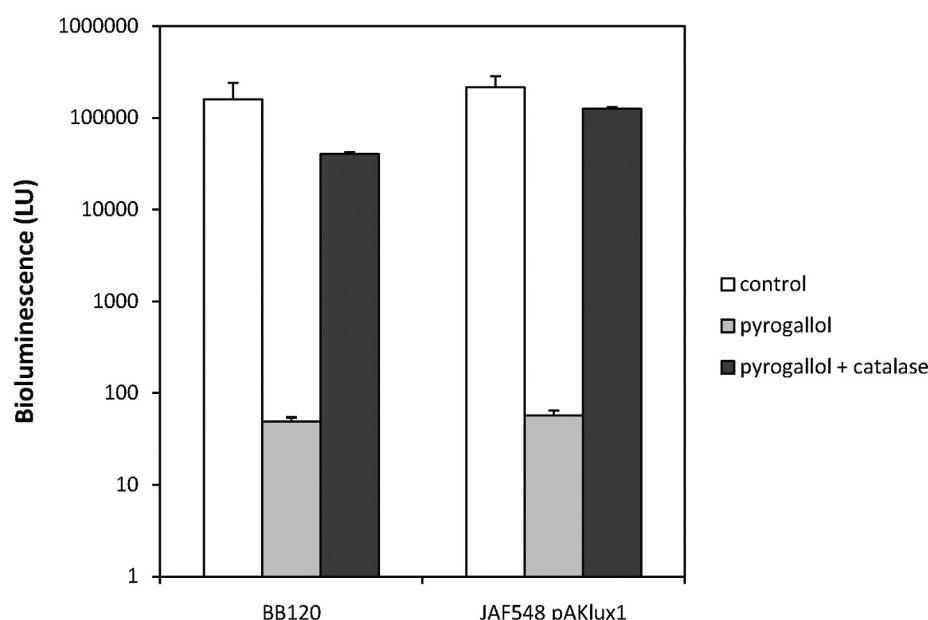


FIG 2 Bioluminescence of *Vibrio harveyi* wild-type BB120 and strain JAF548 pAKlux1 (in which luminescence is independent of quorum sensing) in Luria-Bertani medium containing 35 g · liter⁻¹ synthetic sea salt with and without pyrogallol (10 mg · liter⁻¹) and with or without catalase from bovine liver (10 mg · liter⁻¹). Error bars represent the standard deviations of four replicates.

(8) and metalloprotease production (9–11). Because pyrogallol did not have a significant effect on these phenotypes (data not shown), we aimed to further explore the mechanism by which pyrogallol protected the crustaceans from *Vibrio harveyi*. Although the compound had only a slight impact on the growth of wild-type *Vibrio harveyi* at 10 mg · liter⁻¹ (i.e., the lowest dose that protected the shrimp) (Fig. 1A), it was found to significantly decrease quorum-sensing-regulated bioluminescence under the same conditions (Fig. 1B). A similar decrease in bioluminescence was observed in double mutants that are only sensitive to one of the three signal molecules and mutants with a constitutively active quorum-sensing-signal transduction cascade (data not shown). In order to further investigate the possibility that the effect observed in the bioluminescence experiments was due to minor toxicity, we investigated the effect of the compounds on the bioluminescence of strain JAF548 pAKlux1, in which bioluminescence is independent of the quorum-sensing system (12). We found that the luminescence in this strain was also inhibited by pyrogallol, similar to what was observed with wild-type *Vibrio harveyi* (Fig. 1C). These data indicate that the apparent quorum-sensing inhibitory effect as observed in the wild type was a side effect of significant toxic activity of pyrogallol that was undetected in the growth assay.

Impact of catalase on the bioluminescence-inhibitory activity of pyrogallol. Because pyrogallol had been reported before to autooxidize in aqueous solutions, resulting in the release of H₂O₂ (3), we reasoned that this reactive compound might be responsible for the bioluminescence-inhibitory activity. In order to verify this, we investigated whether the addition of catalase could neutralize the effect of pyrogallol and found that the enzyme indeed neutralized the bioluminescence-inhibitory effect of pyrogallol, both in wild-type *Vibrio harveyi* (where bioluminescence is regu-

lated by quorum sensing) and in strain JAF548 pAKlux1 (in which bioluminescence is independent of quorum sensing) (Fig. 2).

Impact of catalase on the protective effect of pyrogallol in the brine shrimp-*Vibrio harveyi* challenge system. In a last challenge test, we investigated whether the addition of catalase to the brine shrimp culture water could also nullify the protective effect seen in the previous challenge tests. Consistent with the results of our previous test, the addition of 10 mg · liter⁻¹ pyrogallol resulted in significantly increased survival of challenged larvae (Table 3). However, this protective effect was not observed when we also added 10 mg · liter⁻¹ catalase to the culture water, and the survival of the larvae was not significantly different from that of untreated

TABLE 3 Percent survival of brine shrimp larvae after challenge with *Vibrio harveyi* BB120 and BB120 density in the culture water after incubation with or without pyrogallol and catalase

Treatment	Mean value ± SD for ^a :	
	Brine shrimp survival (%) ^b	BB120 levels in water (×10 ⁵ CFU · ml ⁻¹)
Control	83 ± 3 A	
BB120	28 ± 10 B	1.4 ± 0.5
BB120 + pyrogallol 10 mg · liter ⁻¹	80 ± 9 A	0.0 ± 0.0
BB120 + pyrogallol 10 mg · liter ⁻¹ + catalase	45 ± 13 B	2.2 ± 0.2
BB120 + pyrogallol 10 mg · liter ⁻¹ + catalase (boiled)	80 ± 5 A	Not tested

^a Percent survival of brine shrimp larvae after 2 days of challenge with *Vibrio harveyi* BB120 (results from three replicate shrimp cultures) and BB120 density in the culture water after 6 h of incubation (results from two replicate plate counts on Luria-Bertani medium containing 35 g · liter⁻¹ synthetic sea salt) are shown.

^b Values with a different letter are significantly different ($P < 0.01$).

challenged larvae. Importantly, when we added the same dose of boiled catalase, the survival of the larvae was again significantly higher, indicating that the catalase needed to be active in order to nullify the effect of pyrogallol. Furthermore, we plated the brine shrimp culture water after 6 h of incubation, the time point at which maximal H_2O_2 levels have been reported to be released from pyrogallol in aqueous solution (3). We could not detect a single colony on plates covered with culture water from the pyrogallol treatment (Table 3). However, the plate counts of the treatment receiving both pyrogallol and catalase were similar to those of the treatment without pyrogallol.

Conclusions. Our data revealed that the apparent quorum-sensing-disrupting effect of pyrogallol as observed in previously published bioluminescence experiments is a side effect of the peroxide-producing activity of this compound rather than true quorum-sensing inhibition. Indeed, a decrease in bioluminescence similar to that observed in the wild-type strain (in which bioluminescence is regulated by quorum sensing) was observed in an engineered strain in which bioluminescence is independent of quorum sensing, and this effect could be nullified by adding catalase to the medium, thereby neutralizing the peroxide that is produced. Also, the protective effect offered by adding pyrogallol to the brine shrimp culture water could be nullified by adding catalase but not by adding heat-inactivated catalase, again confirming that the effect of pyrogallol could be attributed to peroxide production resulting from the autooxidation of the compound. During the past decade, multiple reports have been published describing compounds as quorum-sensing inhibitors at so-called subinhibitory concentrations (13–20). Our results emphasize that it is essential to verify the effect on viability by using sensitive methods (e.g., under nonoptimal growth conditions) and to include appropriate controls (e.g., tests on the same phenotype, but in a strain that is engineered in such a way to express the phenotype independently of quorum sensing) in order to confirm that such compounds really do interfere with quorum sensing. Tests to evaluate the impact of putative quorum-sensing-disrupting compounds on viability are usually performed under optimal conditions for bacterial growth, and such tests might simply miss subtle toxic and/or stressful activities of the compounds that are responsible for the apparent quorum-sensing-disrupting effect. Indeed, although pyrogallol had only a slight effect on growth in nutrient-rich broth, this “minor” toxic effect could fully account for the apparent quorum-sensing inhibitory activity observed under the same conditions. Furthermore, the compound completely inactivated all *Vibrio harveyi* cells under harsher conditions, as occurred in the brine shrimp culture water.

ACKNOWLEDGMENTS

This work was funded by the Research Foundation of Flanders (FWO-Vlaanderen; project number 1.5.013.12N) and by the Directorate General of Higher Education of Indonesia through a doctoral scholarship to Gde Sasmita Julyantoro Pande. Tom Defoirdt is a postdoctoral fellow of FWO-Vlaanderen.

We thank Gilles Brackman for performing LuxR DNA binding assays.

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