The apparent quorum sensing inhibitory activity of pyrogallol is a side effect of peroxide production

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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 mg.l\(^{-1}\) 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 emphasise that verification of minor toxic effects by using sensitive methods and the use of appropriate controls are essential when characterising compounds as being able to disrupt quorum sensing.
Polyphenols are a large group of compounds found in plants, coffee and tea (1). They have been reported to have both antioxidant (2) and pro-oxidant 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 (IC_{50} = 2 µM ~ 0.25 mg.l^{-1}) (5). However, the effect of pyrogallol on the bioluminescence of a constitutively luminescent strain (which would allow to verify 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 towards 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 mg.l^{-1} or more, the compound significantly increased the survival of challenged larvae (Table 1). Further, pyrogallol showed relatively low toxicity to brine shrimp larvae as there was no significant negative effect on survival of non-challenged larvae for concentrations up to 100 mg.l^{-1} (Table 1).

Impact of pyrogallol on the virulence of Vibrio harveyi towards 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
Impact of pyrogallol on bioluminescence of Vibrio harveyi. Vibrio harveyi quorum sensing has been reported to control different virulence-related phenotypes, including biofilm formation (9) and metalloprotease production (10-12). 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 growth of wild type Vibrio harveyi at 10 mg.l\(^{-1}\) (i.e. the lowest dose that protected the shrimp) (Figure 1A), it was found to significantly decrease quorum sensing-regulated bioluminescence under the same conditions (Figure 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 bioluminescence of strain JAF548 pAKlux\(_1\), in which bioluminescence is independent of the quorum sensing system (13). We found that the luminescence in this strain was also inhibited by pyrogallol, similar to what was observed with wild type Vibrio harveyi (Figure 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 auto-oxidise in aqueous solutions, resulting in the release of H\(_2\)O\(_2\) (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 neutralise the effect of pyrogallol and found that the enzyme
indeed neutralised the bioluminescence inhibitory effect of pyrogallol, both in wild type
Vibrio harveyi (where bioluminescence is regulated by quorum sensing) and in strain
JAF548 pAKlux1 (in which bioluminescence is independent of quorum sensing) (Figure 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 our previous test, the addition of 10 mg.l⁻¹
pyrogallol resulted in a significantly increased survival of challenged larvae (Table 3).
However, this protective effect was not observed when we also added 10 mg.l⁻¹ catalase to
the culture water, and survival of the larvae was not significantly different from that of
untreated 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 6h of incubation, the time point at which maximal H₂O₂ 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 4). 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 similar decrease in bioluminescence as observed in the wild type strain
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 neutralising 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 auto-oxidation of the compound. During the past decade, multiple reports have been published describing compounds as quorum sensing inhibitors at so called subinhibitory concentrations (14-21). Our results emphasise that it is essential to verify the effect on viability by using sensitive methods (e.g. under non-optimal 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 independent of quorum sensing) in order to confirm that such kind of 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 in more harsh conditions as occurred in the brine shrimp culture water.
ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Percentage survival of brine shrimp larvae with and without pyrogallol (average ± standard deviation of three replicates), after 2 days of challenge with *Vibrio harveyi* BB120.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>87 ± 8^A</td>
</tr>
<tr>
<td>Pyrogallol 50 mg.l⁻¹</td>
<td>90 ± 7^A</td>
</tr>
<tr>
<td>Pyrogallol 100 mg.l⁻¹</td>
<td>75 ± 7^A</td>
</tr>
<tr>
<td>Pyrogallol 500 mg.l⁻¹</td>
<td>0 ± 0^B</td>
</tr>
<tr>
<td>BB120</td>
<td>40 ± 5^C</td>
</tr>
<tr>
<td>BB120 + pyrogallol 1 mg.l⁻¹</td>
<td>43 ± 8^C</td>
</tr>
<tr>
<td>BB120 + pyrogallol 5 mg.l⁻¹</td>
<td>38 ± 12^C</td>
</tr>
<tr>
<td>BB120 + pyrogallol 10 mg.l⁻¹</td>
<td>87 ± 3^A</td>
</tr>
<tr>
<td>BB120 + pyrogallol 50 mg.l⁻¹</td>
<td>87 ± 10^A</td>
</tr>
</tbody>
</table>

^1Treatments with a different superscript letter are significantly different (P < 0.01)
Table 2. Percentage survival of giant river prawn (*Macrobrachium rosenbergii*) larvae (average ± standard deviation of five replicates), after 5 and 8 days of challenge with *Vibrio harveyi* BB120.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
<td>Day 8</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>97 ± 3A</td>
<td>85 ± 5a</td>
<td></td>
</tr>
<tr>
<td>BB120</td>
<td>70 ± 5C</td>
<td>46 ± 5b</td>
<td></td>
</tr>
<tr>
<td>BB120 + pyrogallol 10 mg/l</td>
<td>86 ± 5B</td>
<td>77 ± 8a</td>
<td></td>
</tr>
</tbody>
</table>

Treatments with a different superscript letter are significantly different (P < 0.01)
Table 3. Percentage survival of brine shrimp larvae after 2 days of challenge with *Vibrio harveyi* BB120 (average ± standard deviation of three replicate shrimp cultures) and BB120 density in the culture water after 6h of incubation (average ± standard deviation of two replicate plate counts on Luria-Bertani medium containing 35 g.l⁻¹ synthetic sea salt).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Brine shrimp survival (%)</th>
<th>BB120 levels in water (x 10⁵ CFU.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83 ± 3²</td>
<td></td>
</tr>
<tr>
<td>BB120</td>
<td>28 ± 10⁸</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>BB120 + pyrogallol 10 mg.l⁻¹</td>
<td>80 ± 9⁴</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>BB120 + pyrogallol 10 mg.l⁻¹ + catalase</td>
<td>45 ± 13⁸</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>BB120 + pyrogallol 10 mg.l⁻¹ + catalase (boiled)</td>
<td>80 ± 5⁴</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

¹Treatments with a different superscript letter are significantly different (P < 0.01)
Figure legends

Figure 1. (A) Growth of wild type *Vibrio harveyi* BB120 in Luria-Bertani medium containing 35 g.l⁻¹ synthetic sea salt (LB₃₅), with and without pyrogallol (added at 5, 10, 20 and 50 mg.l⁻¹). (B) Bioluminescence of *Vibrio harveyi* under the same conditions, with and without pyrogallol. Error bars represent the standard deviation 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 deviation of 4 replicates.

Figure 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.l⁻¹ synthetic sea salt, with and without pyrogallol (10 mg.l⁻¹), and with or without catalase from bovine liver (10 mg.l⁻¹). Error bars represent the standard deviation of four replicates.