Honey-sensitive *Pseudomonas aeruginosa* mutants are impaired in catalase A

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The antimicrobial power of honey seems to be ascribable to several factors, including oxidative and osmotic stress. The aim of this study was to find genetic determinants involved in the response to honey stress in the opportunistic pathogen *Pseudomonas aeruginosa*, chosen as model micro-organism. A library of transposon mutants of *P. aeruginosa* PAO1 was constructed and only four mutants unable to grow in presence of fir honeydew honey were selected. All four mutants were impaired in the major H$_2$O$_2$-scavenging enzyme catalase A (KatA). The knockout of *katA* gene caused sensitivity, as expected, not only to hydrogen peroxide but also to different types of honey including Manuka GMO 220 honey. Genetic complementation, as well as the addition of PAO1 supernatant containing extracellular catalase, restored tolerance to honey stress in all the mutants. As *P. aeruginosa* PAO1 catalase KatA copes with H$_2$O$_2$ stress, it is conceivable that the antimicrobial activity of honey is, at least partially, due to the presence of hydrogen peroxide in honey or the ability of honey to induce production of hydrogen peroxide. The *katA*-deficient mutants could be used as tester micro-organisms to compare the power of different types of natural and curative honeys in eliciting oxidative stress mediated by hydrogen peroxide.

INTRODUCTION

The antimicrobial properties of honeys have been known for millennia, as ancient Egyptians and Greeks used honey to treat infections (Fidaleo *et al*., 2011). Honey is a sweet substance produced as a food source by honey bees (Codex standard for honey, 2001). Floral honeys are produced from the nectar and secretions of different plants (Uthurry *et al*., 2011) whereas honeydew honeys (HD honey) are produced from the excretions of plant-sucking insects such as aphids (Astwood *et al*., 1998). Both honeys of different floral origin and HD honey have been investigated for their potential as natural antimicrobials (Vatansever *et al*., 2011). However, honeys have complex chemical compositions that vary depending on the floral or the honeydew source, the climate and the harvesting conditions (Hayashi *et al*., 2014), thus making it difficult to identify the main antibacterial ingredients.

Current evidence suggests that several factors may contribute to the antimicrobial properties of honey, such as oxidative stress, osmolarity, acidity, methylglyoxal (MGO) and bee-derived defensin-1 (Bogdanov, 1997; Brudzynski & Sjaarda, 2015; Kwakman *et al*., 2010). For instance, MGO has been identified as one of the major antimicrobial compounds in Manuka honey (Mavric *et al*., 2008), one of the most investigated and utilized curative honeys. The antimicrobial activity of honey of different botanical origins seems to be also ascribable to proteins and the glycoprotein defensin-1, footprint of the innate immune system operating in plants and insects (Brudzynski & Sjaarda, 2015). It is also known that a correlation exists between the level of endogenous hydrogen peroxide and the antimicrobial activity of honey (Brudzynski & Lannigan, 2012). Hydrogen peroxide is mainly produced during glucose oxidation catalysed by the bee enzyme glucose oxidase (GOX), which is introduced during nectar harvesting by bees (White *et al*., 1963). GOX is a regular honey component but its content varies significantly amongst different honeys (Bucekova *et al*., 2014). Furthermore, there is a positive correlation between the level of GOX in honey and the level of hydrogen peroxide in diluted honey. However, the peroxide level in honey can also be affected by catalases of pollen origin that efficiently hydrolyse H$_2$O$_2$ to oxygen and water. On the other hand, bioactive molecules such as catechins and melanoids, protein-polyphenolic-carbohydrate complexes, may enhance the oxidizing action of hydrogen peroxide (Brudzynski & Miotto, 2011).

**Abbreviations:** GOX, glucose oxidase; HD, honey dew; MGO, methylglyoxal; ROS, reactive oxygen species.

Four supplementary figures are available with the online Supplementary Material.
Whereas many studies have investigated the honey multimodal mechanism of action, only a few have investigated the response of bacteria to honey from a molecular point of view and most of them were transcriptional and/or proteomic studies performed using Manuka honey. These studies mostly showed a change in the expression of genes involved in stress response both in *Escherichia coli* and in *Staphylococcus aureus* (Blair et al., 2009; Jenkins et al., 2014; Packer et al., 2012). Until now, no honey-sensitive bacterial mutants, to the best of our knowledge, have been selected and studied. Nevertheless, the knowledge of the molecular mechanism(s) responsible for bacterial honey sensitivity may help in selecting honeys endowed with better curative features. In this study *Pseudomonas aeruginosa* was chosen as model micro-organism because of the clinical relevance of this opportunistic human pathogen. Besides its ability to infect immune-compromised patients, i.e. cystic fibrosis and AIDS patients, and to colonize burn wounds, pressure ulcers and diabetic wounds (Gellatly & Hancock, 2013), *P. aeruginosa* is difficult to control with antibiotics or disinfectants (Lambert, 2002). To identify the *P. aeruginosa* PAO1 genetic determinants involved in the response to honey, we constructed a transposon library and screened it for honey-sensitive mutants. The identification of genes or regulatory elements conferring susceptibility to honey could shed some light on the type(s) of stress that honey induces in bacteria.

**METHODS**

**Bacteria, plasmids and media.** The bacteria strains and the plasmids used in this work are listed in Table 1. *E. coli* JM109 was used for DNA cloning procedures. *E. coli* S17Apir was used as transposon donor to construct the transposon library of *P. aeruginosa* PAO1.

**Table 1. Strains and plasmids used in this work**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type strain</td>
<td>Stover et al. (2000)</td>
</tr>
<tr>
<td>MN08</td>
<td>PAO1 derivative Gm&lt;sup&gt;R&lt;/sup&gt; katA&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>MN08pMBkatA</td>
<td>MN08 derivative carrying pMBkatA</td>
<td>This study</td>
</tr>
<tr>
<td>MN27</td>
<td>PAO1 derivative Gm&lt;sup&gt;R&lt;/sup&gt; katA&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>H18</td>
<td>PAO1 derivative Gm&lt;sup&gt;R&lt;/sup&gt; katA&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>33/16</td>
<td>PAO1 derivative Gm&lt;sup&gt;R&lt;/sup&gt; katA&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17Apir</td>
<td>Δ(tara-leu) araD lacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1 Apir phage lysogen</td>
<td>Herrera et al. (1990)</td>
</tr>
<tr>
<td>JM109</td>
<td>recA1 hsd17 thi Δ(lac-proAB) (F&lt;sup&gt;+&lt;/sup&gt; traD36 proAB lacI&lt;sup&gt;2&lt;/sup&gt; ZAM15)</td>
<td>Yanish Peron et al. (1985)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBT20</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; Gm&lt;sup&gt;R&lt;/sup&gt;; Mariner transposon plasmid</td>
<td>Kulasakera et al. (2005)</td>
</tr>
<tr>
<td>pJB3Km1</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; cloning vector</td>
<td>Blatny et al. (1997)</td>
</tr>
<tr>
<td>pMB</td>
<td>Like pJB3Km1, ColE1</td>
<td>This study</td>
</tr>
<tr>
<td>pMBkatA</td>
<td>Like pMB encoding katA with its own promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>

Unless otherwise indicated, *P. aeruginosa* and *E. coli* were routinely grown in Luria–Bertani (LB) medium. Cultures in liquid medium were inoculated in order to obtain an OD<sub>600</sub>~0.1 and were incubated under shaking and aerobic conditions at 37°C. For growth curves, the OD<sub>600</sub> was monitored at 30 min intervals. When necessary, the bacterial concentration was determined by viable count technique and expressed as c.f.u. ml<sup>−1</sup>.

**Antibacterial activity of honeys.** Commercial batches of seven different types of honey were used: acacia honey, chestnut honey, linden honey, orange tree honey, eucalyptus honey, fir HD honey and Manuka 220 MGO honey. A spot assay (Shin et al., 2008) was adapted to check the antibacterial activity of the honeys. Briefly, cells were grown overnight in LB medium at 37°C to OD<sub>600</sub> ~ 5, corresponding to approximately 5×10<sup>8</sup> c.f.u. ml<sup>−1</sup>. Droplets (approximately 2 µl) of five 10-fold serial dilutions in LB broth were spotted by means of replica plater on LB agar and freshly prepared LB agar containing 5, 10, 15 or 20% honeys. Plates were incubated for 24 h at 37°C.

To check the possible presence of bacterial contaminants in the used honey batches, we prepared LB agar plates containing 5, 10, 15 or 20% honey. At the highest honey concentrations tested and depending on the honey type, 10–100 small colonies were observed only after 48–72 h of incubation. It was thus considered unnecessary to filter the honey before use.

**Construction of the transposon library.** Transposon insertions in the PAO1 chromosome were generated by following the protocol of Kulasakera et al. (2005). Briefly, cultures of the donor strain *E. coli* S17Apir, carrying pBT20 containing the transposon Tn Mariner, and the recipient, *P. aeruginosa* PAO1, were centrifuged and resuspended in LB to a final OD<sub>600</sub> of 40 and 20, respectively. A total of 10 µl droplets of 1:1 conjugation mix were spotted on LB agar and incubated at 37°C for 2 h. *P. aeruginosa* PAO1 transconjugants were then selected on minimal medium M9 supplemented with 10 mM glucose and gentamicin 100 µg ml<sup>−1</sup>. Single colonies were inoculated into 96-well plates, each well containing 200 µl LB supplemented with gentamicin. After 48 h of incubation at 37°C, the recombinant clones were screened by replica plating on LB agar containing 10% fir HD honey.
Identification of the knockout genes. The DNA sequences flanking the transposon insertions were determined using arbitrary PCR (O'Toole & Kolter, 1998). Briefly, 100 ng of purified chromosomal DNA, obtained using Genomic DNA purification KIT (Promega), was amplified with the random-tagged primer Rnd1-ARB0 Pa5'-GGCCACCGTCGAC TAGTACNNNNNNNNNN-3' paired with Rnd1-Tm20 primer 5'-CAGGGCCTGATAATGGTTGG-3'. A nested PCR was then performed on the first PCR amplification product, using Rnd2-AR5 5'-GGCCACCGTCGACTAGTAC-3' and Rnd2-BT20 5'-ACAGGGAAAACGAGCTTCTAGAG-3' as the primers. Amplification products were then sequenced (Primm Biotech) using BT20Tm5 5'-ACAGGGAAAACGAGCTTCTAGAG-3' primer. Insertion sites were identified by BLASTN search, against the annotated sequence of the P. aeruginosa PAO1 genome, using the software provided by NCBI.

Catalase qualitative assay and H2O2 MIC determination. A simple qualitative test was used to assay the presence of catalase activity. 100 µl samples of overnight LB grown cultures or filtered spent broth were mixed with 100 µl of 1 M H2O2. Effervescence, due to O2 production, was read as a positive reaction.

To evaluate the MIC of H2O2 of P. aeruginosa PAO1 and its derivatives, we grew bacteria in LB at 37 °C and diluted them 100-fold in order to obtain a cellular concentration of 10^5 c.f.u. ml^-1. Twofold dilutions of H2O2, from 1 M to 3.9 × 10^-3 M were added to bacterial samples. After 24 h of incubation at 37 °C, the series of dilutions were observed for microbial growth. The MIC value corresponds to the lowest concentration of H2O2 that prevented visible growth.

Evaluation of the effect of osmotic stress and antioxidant treatments. The effects of osmotic stress or of antioxidant treatments on bacterial viability were evaluated by spot assays. Bacteria were grown overnight at 37 °C in LB broth, serially diluted and spotted on the appropriate medium. LB agar containing 32, 34, 36 or 40 % sucrose was used to evaluate the response to osmotic stress. LB agar containing 5, 10, 15 or 20 % honeys supplemented either with 0.5 mM sodium azide or with mannitol at different concentrations (200, 100, 50 or 10 mM) was used to evaluate the effect of antioxidant compounds. Plates were incubated for 24 h at 37 °C and the spots were compared with those grown on LB agar plates.

Genetic complementation. An approximately 1830 bp Mlu fragment containing katA ORF and its upstream promoter region was PCR amplified from P. aeruginosa PAO1 genome with primers KatAFW (5'- ATGGGAAGAGAAGACCCGCCTGACC-3') and KatARW (5'-TCAGTGCGCTGAGCGGGCGG-3') and was cloned following standard procedures (Sambrook et al., 1989) in Mlu site of pMB vector to obtain pMBkatA (Fig S1, available in the online Supplementary Material).

Electrocompetent MN08 cells were prepared according to Choi by a rapid microcentrifuge-based method (Choi et al., 2006). Briefly, 6 ml of overnight LB grown cultures was centrifuged (12 000 × g, 1 min at room temperature), and the pellets were washed twice with 1 ml of 300 mM sucrose and suspended in a final volume of 100 µl of 300 mM sucrose. For electroporation, 500 µg of pMBkatA was mixed with 100 µl of electrocompetent cells and the mixture was transferred to an electroporation cuvette. After the pulse (25 µF; 200 Ω; 2.5 kV, Eppendorf 2510 Electroporator), 1 ml of room temperature LB medium was added and cells were transferred to a glass tube and shaken for 2 h at 37 °C. A volume of 50 µl was plated on LB supplemented with 200 µg carbencillin ml^-1.

Recombinant KatA expression was checked by SDS-PAGE. Cells were grown overnight at 37 °C in LB supplemented with 200 µg carbencillin ml^-1. Samples (1 ml) were collected and the cells were harvested by centrifugation (14 000 × g, 5 min), suspended in 200 µl of SDS gel loading buffer (50 mM Tris/HCl (pH 6.8), 100 mM diithiothreitol, 2 % SDS, 0.01 % bromphenol blue, 10 % glycerol and 0.1 % 2-mercaptoethanol), boiled for 10 min and centrifuged (14 000 × g, 5 min). Twenty microlitres of the supernatants was analysed by 10 % SDS-PAGE and Coomassie Blue staining (Sambrook et al., 1989).

Functional complementation. Since catalase A, as well as various metabolites, can be released from cells during the stationary phase (Hassett et al., 2000), a series of experiments, aimed at evaluating whether the sensitivity to H2O2 of katA-deficient mutants could be overcome by adding supernatants of KatA expressing and not expressing strains, were performed. Supernatants of 24 h cultures of the appropriate strain were filtered (0.45 µm, Sarsted) and catalase activity was checked by the qualitative assay described above. Then the spent broths were twofold diluted in fresh LB broth and used as the source of the soluble enzyme. This preparation was dispensed in 96-well microtitre plates and used to serially dilute cultures of the katA-deficient mutant. H2O2 was then added to cells to obtain different final concentrations (1, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.025, 0.012, 0.006 and 0.003 M). The cell growth (turbidity) was checked after 24 h of incubation at 37 °C.

The spent broths of the wild-type strain PAO1 or of the mutant derivatives were also tested for their ability to rescue honey tolerance in katA-deficient mutants. Serial dilutions of overnight cultures of PAO1 and katA derivatives were plated on LB agar containing 5 %, fir HD honey treated or untreated with the spent broths. After 24 h of incubation at 37 °C, growth spots were observed. Treated honey was prepared by mechanically mixing for 1 h at 37 °C, the appropriate amount of honey with 50 ml of spent broths, which was then filtered as described above, before adding 50 ml of fluid 3 % agar LB medium. In both experiments, heat-treated spent broths were used as a control. The filtered supernatants were heated at 121 °C for 15 min, checked for the absence of catalase activity and then used as described above.

RESULTS

Isolation and characterization of P. aeruginosa PAO1 transposon mutants sensitive to honey

Before screening the P. aeruginosa PAO1 transposon library for honey-sensitive mutants, we investigated the antimicrobial activity of seven different types of honey. Amongst them, three groups of honeys with different antimicrobial activity were identified. One group was not active against P. aeruginosa PAO1 or active only at the highest tested concentration (20 %). Acacia honey, orange tree honey and eucalyptus honey belong to this group (Fig 1). In the second group, Manuka honey, linden honey and chestnut honey showed a good antimicrobial activity at a concentration of 15 % (Fig 1). Fir HD honey was the most efficient in inhibiting cellular growth since its antibacterial activity was already detectable at the concentration of 10 % (Fig 1). Fir HD honey was thus chosen to select sensitive clones.

The transposon bank was constructed in the model microorganism P. aeruginosa PAO1, and trans-conjugant clones were selected on M9 medium supplemented with gentamicin 100 µg ml^-1. Clones resistant to gentamicin were then screened on LB agar supplemented with fir HD honey 10 % (Fig S2). Amongst approximately 3000 gentamicin-resistant clones, only four, named MN08, MN27, H18 and 33/16, were unable to grow in the presence of 10 % HD honey.

The transposon insertions did not alter the bacterial growth of the selected clones with respect to the wild-type strain (Fig S3). All PAO1 derivatives showed the same growth
rate and reached a cellular concentration ranging from 5×10^9 to 8×10^9 c.f.u. ml⁻¹ after 24 h of growth at 37 °C in LB medium.

To identify the genes disrupted by the transposon insertions in the different clones, we performed arbitrary primed PCR followed by nested PCR reactions and we sequenced the amplified DNAs. Computer-assisted analysis of the sequences of four clones, MN08, MN27, H18 and 33/16, showed that, in all the mutants, the transposon was inserted in the katA gene (PA4236), codifying for the soluble catalase A, the most important H₂O₂ detoxifying enzyme in P. aeruginosa {Choi et al., 2007; Shin et al., 2008}. In each mutant, the gentamicin cassette was inserted in a different position within the katA gene and in both orientations (Fig. 2).

MIC of H₂O₂ was determined for each transposon mutant (Table 2). As expected, katA-deficient clones were more sensitive to hydrogen peroxide than PAO1 strain, showing MIC values approximately 100-fold lower with respect to PAO1 strain.

The selected mutants were also assayed for their sensitivity to honeys other than HD honey. P. aeruginosa mutants were spotted on LB agar plates each containing a different type of honey at 5, 10, 15 or 20 %. Similar results were obtained for all the mutants. The results for one of the mutants (MN08) are shown in Fig. 3. In particular, katA− mutants were sensitive to honeys belonging to the first group (acacia, orange tree and eucalyptus honey), the least active ones, only at the highest concentrations tested. The other honeys were active also at the lowest concentrations.

**Fig. 1.** Antimicrobial activity of different honeys against *P. aeruginosa* PAO1. LB agar plates containing 5, 10, 15 or 20 % honey were inoculated with decreasing concentrations of bacterial cells (c.f.u. per spot) and the growth was checked after 24 h at 37 °C.

**Table 1.** MIC values of different honeys for *P. aeruginosa* PAO1.

<table>
<thead>
<tr>
<th>Honey Type</th>
<th>5 %</th>
<th>10 %</th>
<th>15 %</th>
<th>20 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia honey</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
</tr>
<tr>
<td>Orange tree honey</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
</tr>
<tr>
<td>Eucalyptus honey</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
</tr>
<tr>
<td>Manuka 250 MGO honey</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
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</tr>
<tr>
<td>Linden honey</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
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</tr>
<tr>
<td>Chestnut honey</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
</tr>
<tr>
<td>Fir honeydew honey</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
</tr>
<tr>
<td>LB agar</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
<td>10^9 ± 10^9</td>
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</table>

**Complementation of *katA* knockout mutant**

As all of the mutants had insertions in the *katA* gene and had similar phenotypes, only one mutant strain was chosen for complementation. The plasmid pMBkatA was used to transform MN08. SDS-PAGE analysis of MN08(pMBkatA) cell extracts showed the presence of a polypeptide, not detectable in extracts of MN08, whose size (60 kDa) was consistent with that of KatA (55.6 kDa) (data not shown). The catalase qualitative assay showed that genetic complementation had successfully occurred since effervescence was observed following exposure to H₂O₂ of MN08(pMBkatA) but not following exposure to H₂O₂ of MN08 cells. H₂O₂ MIC value for MN08(pMBkatA) was 1.0±0.0 M (Table 2), higher than that of PAO1 wild-type strain (625±250 mM). The genetic complementation also restored the tolerance to fir HD honey as the growth of MN08(pMBkatA) in the presence of HD honey was comparable to that of PAO1 strain (Fig. 4). Given that KatA enzyme can be released in the extracellular environment upon cell lysis occurring during the stationary phase {Hassett et al., 2000}, the supernatants of *P. aeruginosa* PAO1 cultures were used as a source of the soluble enzyme. The addition of the PAO1 supernatants restored the growth of MN08, representative of the *katA*-deficient mutants, in the presence of 5 % fir HD honey (Fig. 5a), as well as the tolerance to increasing concentrations of H₂O₂ up to levels comparable to those of PAO1 (Fig. 5b). Similar results were obtained when the spent broths of MN08 (pMBkatA) were used (data not shown). In contrast, the spent broths of all the mutants were unable to restore both...
honey and H$_2$O$_2$ tolerance; in Fig. 5, the results obtained with the spent broth of H18, chosen as representative, are shown. The tolerance phenotype was not recovered when either the PAO1 (Fig. 5) or the MN08(pMBkatA) (data not shown) spent broths were heat inactivated. Similar results were obtained when all the possible combinations of supernatants and mutants were tested (data not shown).

**Responses of the katA mutants to oxidative or osmotic stress**

In order to evaluate the possible presence and effect of ROS other than H$_2$O$_2$, we tested two antioxidants for their ability to counteract the honey-induced stress in bacterial cells.

### Table 2. Values of MIC for H$_2$O$_2$

MIC of H$_2$O$_2$ for *P. aeruginosa* PAO1 and mutant derivatives. The data are the means of at least three independent experiments±SD.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC±sd (mM)</th>
</tr>
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<tbody>
<tr>
<td>PAO1</td>
<td>625±250</td>
</tr>
<tr>
<td>MN08</td>
<td>6.25±0</td>
</tr>
<tr>
<td>MN08(pMBkatA)</td>
<td>1000±0</td>
</tr>
<tr>
<td>MN27</td>
<td>18.75±7.22</td>
</tr>
<tr>
<td>H18</td>
<td>9.38±3.61</td>
</tr>
<tr>
<td>33/16</td>
<td>6.09±4.58</td>
</tr>
</tbody>
</table>

Mannitol and sodium azide were chosen as it has been demonstrated that they could efficiently protect bacterial cells from oxidative stress ascribable to superoxide anion and hydroxyl radicals and to singlet oxygen, respectively (Huang *et al.*, 2012; Tavares *et al.*, 2011; Ishikawa *et al.*, 2010). Cells were grown in the presence of honey and of concentrations of mannitol that did not affect the growth or of sublethal concentrations of sodium azide. Mannitol, regardless of its concentration, did not influence the antimicrobial effect of HD honey even at the highest honey concentration tested (20%) (data not shown). On the contrary, sodium azide at 0.5 mM, which was the highest non-toxic concentration, not only failed to protect the cells from honey-induced stress but also even increased the toxic effect of HD honey 5% in both the KatA-expressing and non-expressing strains (Fig. 6).

As osmotic stress seems to contribute to antimicrobial activity of honeys (Lee *et al.*, 2005), the tolerance of PAO1 strain and its derivatives to hyperosmotic environment (sucrose at increasing concentrations) was assayed. PAO1 and all the katA$^{-}$ mutants showed the same sensitivity to osmotic stress (Fig. S4).

**DISCUSSION**

To identify bacterial genetic determinants involved in the response to honey stress, we screened a *P. aeruginosa* PAO1 transposon library.
The four mutants obtained were genetically distinct, all of them being impaired in the major H$_2$O$_2$-scavenging enzyme catalase A (KatA) and sensitive to the other honey types tested. In *P. aeruginosa*, there are three monofunctional catalase genes: katA, katB and katE. KatA is the major catalase and the most critical in H$_2$O$_2$ resistance. KatA seems to be constitutively expressed and not under the control of the master regulator OxyR (Ochsner *et al.*, 2000; Brown *et al.*, 1995); on the contrary, the resistance mediated by the H$_2$O$_2$-inducible KatB was only discernible when KatA expression was abolished (Lee *et al.*, 2005). KatA removes hydrogen peroxide by breakdown to harmless oxygen and water and, as expected in accordance with the lack of the functional enzyme, the katA$^-\$ mutants were found to be much more sensitive to H$_2$O$_2$ than the wild-type strain PAO1.

The complementation of the MN08 mutant by either plasmid-expressed or exogenous catalase-restored honey tolerance suggested that this enzyme plays a relevant role in coping with honey antibacterial activity. Nevertheless, a likely minor role of other antistress activities cannot be ruled out, as suggested by transcriptomic studies performed on different bacteria exposed to catalase-treated Manuka honey (Blair *et al.* 2009).

As katA-deficient mutants were sensitive to all the different types of honey tested in this work, it seems that all of them share hydrogen peroxide as antimicrobial compound, irrespective of the way in which H$_2$O$_2$ is generated.

As regards the involvement of ROS other than H$_2$O$_2$, we observed that the antioxidant mannitol did not influence the antibacterial activity of HD honey, suggesting that,
under the conditions used, the possible presence of mannitol quenchable ROS (superoxide anion and hydroxyl radicals) was not relevant. On the contrary, sodium azide (singlet oxygen quencher) enhanced the antibacterial effect of HD honey exposure. Such potentiation of honey toxic effects on bacteria could be due to the arising of azidyl radicals following azide oxidation by hydroxyl radicals, which are known to be generated in honey in the presence of metal ions via Fenton reaction (Brudzynski & Lannigan, 2012a).

The antimicrobial properties of honeys have also been attributed to high sugar concentrations (Fidaleo et al., 2011). To screen the transposon bank, we used diluted honey, a condition not representative of osmotic stress. It is thus conceivable that the selection of mutants in genes directly involved in the response to osmotic stress was difficult. In our experiments, all the selected mutants were as sensitive as the wild-type strain PAO1 to osmotic stress although it has been reported that, in *P. aeruginosa* PA14, KatA is required for resistance to osmotic stress (Lee et al., 2005).

$\text{H}_2\text{O}_2$ is widely recognized as a relevant feature in medicinal honey (Brudzynski & Lannigan, 2012). Thus, a tool for a prelaminar screening of the potential oxidative ability of different types of honey, prior to more in depth investigation, might be useful. A *katA* mutant could be exploited for this purpose. The comparison between the degree of sensitivity of the wild-type strain PAO1 and that of a *katA* mutant to increasing concentrations of different types of honey may highlight the contribution of hydrogen peroxide to honey antimicrobial activity. If a *katA* mutant grows at high honey concentrations, then honey oxidative ability is low. On the other hand, if a *katA* mutant does not grow at low honey concentrations, the oxidative ability is high. Thus, a *katA* mutant, together with PAO1, could be used.

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**Fig. 5.** Functional complementation assays. (a) Decreasing concentrations (c.f.u. per spot) of *P. aeruginosa* PAO1 and of MN08 were spotted on LB supplemented with fir HD honey 5%. Honey was pre-treated or not pre-treated with PAO1 supernatant, heat-inactivated PAO1 supernatant or H18 supernatant. (b) Effect of PAO1 supernatant, heat-inactivated PAO1 supernatant or H18 supernatant on the sensitivity of *P. aeruginosa* PAO1 and of MN08 to increasing concentrations of $\text{H}_2\text{O}_2$.

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**Fig. 6.** Effect of sodium azide on honey toxicity. Decreasing concentrations (c.f.u. per spot) of the indicated strains were inoculated on LB agar and LB agar supplemented with 5% fir HD honey or with sodium azide 0.5 mM or both. The growth was checked after 24 h at 37°C.

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to evaluate the $\text{H}_2\text{O}_2$ mediated oxidative stress caused by different types of honey under different conditions. For instance, amongst the three types of honey, Manuka, linden and chestnut that were mildly effective against PAO1, Manuka honey appeared to be the one endowed with the highest oxidative capability (see Fig. 3, 10% honey).

In our experiments, HD honey has been found at least as effective as Manuka, which is considered a very promising curative honey (Robert et al., 2015; Carter et al., 2016). The antimicrobial power of Manuka honey has been widely documented (Blair et al., 2009; Robert et al., 2015), whereas only recently, a remarkable number of reports about HD honey have been published (Majtan et al., 2011; Bobis et al., 2008; Kacanikova et al., 2011). Both types of honey were found effective against P. aeruginosa (Blair et al., 2009; Kacanikova et al., 2011). Recently, the antimicrobial power of Manuka honey against multi-species biofilm has been compared with that of HD honey (Sojka et al., 2016), and also in this case, both honeys were able to significantly reduce the cell viability of this micro-organism. Our results are thus consistent with the above-mentioned observations.

In conclusion, although several studies have focused on the antibacterial activity of honey $\text{H}_2\text{O}_2$ (Brudzynski et al., 2011), to the best of our knowledge, this is the first report that highlights a direct correlation between impairment of bacterial catalase activity and sensitivity to honey. Thus, although we cannot rule out the contribution of other enzymes and/or compounds in hindering the honey antibacterial activity, it seems that, under our experimental conditions, catalase A has an important role in facing the stress induced in P. aeruginosa PAO1 by exposure to honey.

**REFERENCES**


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