Inactivation of the *hmgA* gene of *Pseudomonas aeruginosa* leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection

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Clinical isolates of *Pseudomonas aeruginosa* that hyperproduce a dark-brown pigment are quite often found in the lungs of chronically infected patients, suggesting that they may have an adaptive advantage in chronic infections. We have screened a library of random transposon insertions in *P. aeruginosa*. Transposon insertions resulting in the hyperproduction of a dark-brown pigment were found to be located in the *hmgA* gene, which putatively encodes the enzyme homogentisate-1,2-dioxygenase. Complementation studies indicate that *hmgA* disruption is responsible for the hyperproduction of pyomelanin in both laboratory and clinical isolates. A relationship between *hmgA* disruption and adaptation to chronic infection was explored and our results show that the inactivation of *hmgA* produces a slight reduction of killing ability in an acute murine model of lung infection. On the other hand, it also confers decreased clearance and increased persistence in chronic lung infections. Whether pyomelanin production is the cause of the increased adaptation to chronicity or just a side effect of *hmgA* inactivation is a question to be studied in future; however, this adaptation is consistent with the higher resistance to oxidative stress conferred *in vitro* by the pyomelanin pigment. Our results clearly demonstrate that *hmgA* inactivation leads to a better adaptation to chronic infection, and strongly suggest that this mechanism may be exploited in naturally occurring *P. aeruginosa* strains.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a major opportunistic pathogen that severely affects immunocompromised patients and burn victims, and causes lung infections in patients with chronic underlying diseases such as cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary disease (COPD) (Bodey et al., 1983; Doring et al., 1987; Gilligan, 1991). Due to its extraordinary metabolic plasticity *P. aeruginosa* is capable of adapting to many different environments (Morrison & Wenzel, 1984; Stover et al., 2000) and infecting very different types of hosts, including plants, insects and mammals (Fick, 1993; Oliver et al., 2000). Adaptation to new hosts may involve a high capacity for genetic change, thus producing new variants (Oliver et al., 2000). In this respect, the most extensively studied model is the chronic respiratory infection in CF patients, known to be driven by an intense genetic adaptation process (Smith et al., 2006). Several mutations favour adaptation to chronic lung infection, including those leading to the hyperproduction of alginate (mucoid phenotype), reduced growth rate (small colony variant, SCV, phenotype), increased biofilm formation capacity, reduced expression of exoenzymes and increased antibiotic resistance (Driffield et al., 2008; Govan & Deretic, 1996; Haussler et al., 2003; Oliver et al., 2000; Pritt et al., 2007; Smith et al., 2006).

Interestingly, the isolation of *P. aeruginosa* strains producing a brown pigment from CF and COPD patients is not an infrequent event: in one study about 5% of patients presented pyomelanogenic isolates (A. Oliver, unpublished results). Thus, the rationale was that these pyomelanin-hyperproducer strains could have an adaptive advantage in chronic infections (the pyomelanin itself being the cause of this advantage, or simply an unrelated epiphenomenon).

Several micro-organisms have been reported to produce pyomelanins (Yabuuchi & Ohyama, 1972), including *P.
**METHODS**

**Bacteria, plasmids and media.** The *P. aeruginosa* strain used to construct the ISlacZhab insertion library was the laboratory strain PAO1 (obtained from J. L. Martinez, Centro Nacional de Biotecnología, CSIC, Madrid). *Escherichia coli* strain SM10pir(pIT2) was used as the transposon donor (Jacobs et al., 2003). *P. aeruginosa* strain PA14 and its *hmgA*::MAR2xT7 derivative were kindly provided by Dr Nicole T. Liberati (Harvard Medical School; Liberati et al., 2006). The wild-type *hmgA* gene from PA14 was amplified by using oligonucleotides hmgAF1 (5’-GGGCCCTTGAGGATATCGG-3’) and hmgARI (5’-AGGCGACCCAGCTACGAGTG-3’) as primers of a PCR. The PCR product was cloned directly into the Smal-digested and T-tailed pBBR1MCS-3 plasmid vector (Kovach et al., 1993). The construction was verified by sequencing and the resulting plasmid was termed pBBR1-HmgA. Plasmids pBBR1-HmgA and pBBR1MCS-3 were transferred by electroporation into *E. coli* S17-1 and transferred to the corresponding *P. aeruginosa* strain by conjugation, as described by Zhang et al. (1993). Growth of *P. aeruginosa* was performed in Luria–Bertani (LB) broth and on LB agar plates. Tetracycline and gentamicin were added, when necessary, to final concentrations of 15 and 15 μg ml⁻¹, respectively. *E. coli* strains were routinely cultured in LB (Sambrook & Russell, 2001) and, when appropriate, supplemented with tetracycline to a final concentration of 20 μg ml⁻¹.

**Construction and screening of the insertional library.** Transposon insertions in the PAO1 chromosome were generated by following the protocol of Jacobs et al. (2003). Briefly, *E. coli* strain SM10pir(pIT2), containing the transposon ISlacZhab, was mated with *P. aeruginosa* PAO1. Mutagenized cells were selected by plating on LB agar containing tetracycline (60 μg ml⁻¹) to select insertions and chloramphenicol (10 μg ml⁻¹) for counterselection against the *E. coli* donor strain. After incubation for 2 days at 30 °C, about 18000 tetracycline-resistant colonies were picked and inoculated into 96-well plates, each well containing 200 μl of freezing medium (LB broth plus 6% glycerol) supplemented with tetracycline. Plates were incubated for 48 h at 37 °C, frozen and stored at −80 °C. Mutants producing a dark-brown pigment after incubation were isolated. Transposon insertion locations were determined by PCR and sequencing, as described by Jacobs et al. (2003).

**Characterization of natural pyomelanogenic strains.** Two pyomelanogenic *P. aeruginosa* clinical isolates, recovered from the sputum samples of two different patients, were obtained from the collection of the Microbiology Department of the Son Dureta Hospital. Plasmids pBBR1-HmgA and pBBR1MCS-3 were transferred from *E. coli* S17-1 to the corresponding strains of *P. aeruginosa*. PCR amplification of the *hmgA* genes was performed using the primers described above.

**Murine model of acute respiratory infection.** The murine model of acute lung infection was established by following previously described protocols (Mena et al., 2007). Female C57BL/6 J mice, 20–25 g in weight (Harlan Interfauna Ibérica), were used. Before inoculation, the mice were anaesthetized by intraperitoneal injection of ketamine (100 mg kg⁻¹, Pfizer) and xylazine (10 mg kg⁻¹, Sigma-Aldrich). A vertical midline neck incision was then performed to expose the trachea of the mice, and 20 μl of 2× saline-washed culture, containing approx. 1×10⁶ exponentially growing cells of PA14, the PA14 *hmgA*::MAR2xT7 mutant, or a 1:1 mixture of both strains, was transtracheally inoculated. Mouse survival was monitored daily over a 7 day period in groups of 16 mice (from two independent experiments) for each of the strains. In the competition experiments, a group of eight mice inoculated with the 1:1 mixture was sacrificed 48 h after inoculation; the lungs were aseptically extracted and homogenized in 2 ml 0.9% saline solution using an IKA Ultra-Turrax T-25 disperser. Serial 10-fold dilutions were plated in duplicate on Müller–Hinton agar (MHA). PA14 and the PA14 *hmgA*::MAR2xT7 mutant colonies were differentiated by their green or brown pigment respectively, and the competition index (CI) was then calculated as the mutant/wild-type ratio. Mouse survival was monitored daily over a 7 day period in groups of 16 mice (from two independent experiments) for each of the strains. The competition experiments, a group of eight mice inoculated with the 1:1 mixture was sacrificed 48 h after inoculation; the lungs were aseptically extracted and homogenized in 2 ml 0.9% saline solution using an IKA Ultra-Turrax T-25 disperser. Serial 10-fold dilutions were plated in duplicate on Müller–Hinton agar (MHA). PA14 and the PA14 *hmgA*::MAR2xT7 mutant colonies were differentiated by their green or brown pigment respectively, and the competition index (CI) was then calculated as the mutant/wild-type ratio. In order to rule out the possibility of loss of the phenotype during *in vitro* growth, a representative number (>10) of randomly selected colonies from each of the competition experiments were streaked on MHA with 20 μg gentamicin ml⁻¹ (MHA-Gm), to verify the presence of the resistance marker of the PA14 *hmgA*::MAR2xT7 mutant. All animal experiments were approved by the Animal Ethics Committees of the University of the Balearic Islands.

**Murine model of chronic lung infection.** The murine model of chronic lung infection using *P. aeruginosa*-laden agarose beads was established by following previously described protocols (Macia et al., 2006; Mulcahy et al., 2008). Briefly, for the preparation of the agarose beads, *P. aeruginosa* strains were grown to late-exponential phase, washed and mixed at a 1:1 ratio with 2% agarose in PBS (pH 7.4). The mixture was added to heavy mineral oil equilibrated at 55 °C, stirred for 6 min at room temperature and cooled for 10 min. The resulting agarose beads were washed with 0.5 and 0.25% deoxycholic acid (sodium salt) in PBS once and then three times with PBS alone. Serial 1/10 dilutions of homogenized bead slurry aliquots were plated on MHA for a quantification of the bacterial content. Female C57BL/6 J mice, 20–25 g in weight (Harlan Ibérica), were used. The animals were specific pathogen free, and sterile water and food were provided ad libitum. Before inoculation, the mice were anaesthetized by intraperitoneal injection of ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹). A vertical midline neck incision was then made to expose each mouse’s trachea, and 20 μl of the bead preparation containing approximately 1×10⁶ agarose-embedded cells was transtracheally inoculated. Mouse survival was monitored daily over a 7 day period in groups of 42 mice (from two independent experiments) for each of the strains. At 7 days post-infection animals were sacrificed. Lungs were aseptically extracted and homogenized in 2 ml 0.9% saline solution using an IKA Ultra-Turrax T-25 disperser. Serial 10-fold dilutions were plated in duplicate on MHA to calculate the bacterial load on each mouse. The bacteria were identified as *P. aeruginosa* using their characteristic colony morphology and biochemical tests. A vertical midline neck incision was then made to expose each mouse’s trachea, and 20 μl of the bead preparation containing approximately 1×10⁶ agarose-embedded cells was transtracheally inoculated. Mouse survival was monitored daily over a 7 day period in groups of 42 mice (from two independent experiments) for each of the strains. At 7 days post-infection animals were sacrificed. Lungs were aseptically extracted and homogenized in 2 ml 0.9% saline solution using an IKA Ultra-Turrax T-25 disperser. Serial 10-fold dilutions were plated in duplicate on MHA to calculate the bacterial load on each mouse. The bacteria were identified as *P. aeruginosa* using their characteristic colony morphology and biochemical tests. Lungs were aseptically extracted and homogenized in 2 ml 0.9% saline solution using an IKA Ultra-Turrax T-25 disperser. Serial 10-fold dilutions were plated in duplicate on MHA to calculate the bacterial load on each mouse. The bacteria were identified as *P. aeruginosa* using their characteristic colony morphology and biochemical tests.
Effect of hmgA inactivation and pyomelanin on peroxide resistance. Five independent exponential-phase cultures of the wild-type, the hmgA variant and the complemented mutant grown in LB medium to OD$_{600}$ 0.5 were incubated at room temperature in the presence of 25 mM or 50 mM hydrogen peroxide (H$_2$O$_2$) for 15, 30, 45 and 60 min. Viable-cell counts were determined before and after treatment in both treated and untreated strains. The protective effect against peroxide of the supernatant from the pyomelanogenic strain was tested on the wild-type non-producer strain. PA14 cells obtained from 48 h cultures in LB were washed and resuspended in the sterilized (by filtration) supernatant from the PA14 hmgA::MAR2xT7 pyomelanogenic mutant grown previously for 48 h in LB broth. As a control, identical experiments were performed using the supernatant obtained from the wild-type PA14 strain in the same conditions. The data were analysed using two-tailed unpaired t-tests. P-values of 0.05 were considered statistically significant.

Effect of pyomelanin-containing medium on antibiotic resistance. The minimal inhibitory concentrations (MICs) for ciprofloxacin, tobramycin, imipenem and cefazidime were determined for the hmgA mutant and wild-type strain PA14. Approximately $10^5$ cells from overnight cultures were inoculated into tubes containing 10 ml LB broth and incubated at 37°C with vigorous shaking until the mid-exponential phase of growth (approx. $10^6$ cells ml$^{-1}$) was reached. Then $2 \times 10^6$ to $4 \times 10^6$ cells from these cultures were inoculated into each microdilution well of a microwell plate ($1 \times 10^4$ to $2 \times 10^5$ c.f.u. ml$^{-1}$) and incubated for 24 h. The MIC was defined as the lowest concentration of antibiotic at which no growth could be observed. The same experiment was repeated with the wild-type strain PA14 resuspended in the pyomelanin-containing supernatant from a 48 h culture of the mutant hmgA::MAR2xT7.

RESULTS AND DISCUSSION

Isolation and genetic characterization of the pyomelanin hyperproducer mutants

Among the 18 000 PAO1 mutants screened for the desired phenotype, two mutants showed a dark-brown pigment production. Transposon insertion locations were determined using the two-stage semi-degenerate PCR and sequencing described by Jacobs et al. (2003). In both cases the insertion had occurred in the same position in the hmgA gene, which putatively encodes the enzyme homogentisate 1,2-dioxygenase. Since both mutants contained identical transposon insertions and showed an identical dark-brown phenotype, we took one of them for further analysis. The structure of the compounds present in the supernatant from this hyperproducer mutant (genetically uncharacterized at that time) was previously determined (Salgado & Blazquez, 2006). These results showed that the main pigment molecule was a derivative of homogentisic acid. It is interesting to note that this structure corresponds to the dehydrogenated (oxidized) form of homogentisic acid, the main precursor of melanin in other micro-organisms such as Pseudomonas putida, Vibrio cholerae, Burkholderia cenocepacia and Shewanella colwelliana (Arias-Barrau et al., 2004; Coon et al., 1994; Kotob et al., 1995; Kovach et al., 1995). Thus, the pigment produced by the hmgA mutant will be considered here as a pyomelanin.

As indicated below, for further studies we decided to use the more virulent strain PA14 and its pyomelanin hyperproducer PA14 hmgA::MAR2xT7 derivative (Liberati et al., 2006) instead of PAO1. Fig. 1(A) shows the phenotype of pyomelanin hyperproduction of PA14 hmgA::MAR2xT7 in comparison to that of the wild-type PA14. To verify that the inactivation of the hmgA gene was the only cause of pyomelanin hyperproduction, we performed a complementation study. Hyperproduction was abolished when plasmid pBBR1-HmgA, harbouring the wild-type hmgA gene, was introduced into the PA14 hmgA::MAR2xT7 mutant strain (Fig. 1B). Similarly, the introduction of plasmid pBBR1-HmgA into the hmgA-deficient strain PAO1 hmgA::ISlacZ/hah completely abolished the production of the pyomelanin pigment (data not shown).

hmgA, but not maiA or fahA, inactivation is the only cause of pyomelanin production

The enzyme homogentisate 1,2-dioxygenase is involved in the pathway of phenylalanine and tyrosine catabolism. This enzyme converts homogentisic acid (2,5-dihydroxyphenylacetate) into 4-maleylacetoacetate. In Pseudomonas putida, homogentisate is catabolized via a pathway that finally yields fumarate and acetoacetate (Fig. 2A). This pathway involves three enzymes: homogentisate dioxygenase, mal-
eylactoacetate isomerase and fumarylactoacetate hydro-lase. The genes encoding these enzymes appear to form a single transcriptional unit (Arias-Barrau et al., 2004). In the P. aeruginosa PA14 and PAO1 genomes, the genes hmgA, fahA and maiA (putatively encoding these three enzymes) seem to form an operon as well (Fig. 2B). In order to know if the inactivation of the other two genes of the operon, maiA and fahA, hyperproduces pyomelanin, we cultured (in both LB-agar plates and LB broth) the mutant derivatives maiA::MAR2xT7 and fahA::MAR2xT7 (Liberati et al., 2006). No visible pigment production was obtained, even after prolonged incubation for 72 h (data not shown). This result suggests that 4-maleylactoacetate or any other derivative in this pathway cannot originate pyomelanin pigments in P. aeruginosa PA14.

Together these results demonstrate that the inactivation of the hmgA gene is the only cause of the hyperproduction of the pyomelanin pigment in P. aeruginosa, and that neither of the other two genes (maiA and fahA) from the same transcriptional unit is related to pigment hyperproduction. Finally, our data suggest that, due to the lack of homogentisate 1,2-dioxygenase activity, pyomelanin formation in the hmgA mutant results from the accumulation, spontaneous oxidation and polymerization of homogentisic acid.

Characterization of the molecular basis of pyomelanin hyperproduction in clinical isolates of P. aeruginosa

The results presented raised the following question: is hmgA inactivation the molecular basis for pyomelanin production in clinical isolates? To answer this, isolates from a series of CF patients were analysed for the production of the dark pigment. Two pyomelanin-hyperproducing P. aeruginosa clinical isolates, recovered from the sputum samples of two CF patients, were isolated and studied. The plasmid pBBR1-HmgA, harbouring the wild-type hmgA gene from the wild-type PA14 strain, was introduced into the two pyomelanogenic isolates and transconjugants were obtained in the two strains. The introduction of the wild-type hmgA gene completely abolished the pigment production in these strains. PCR amplification of the hmgA gene and its promoter region, using primers hmgAF1 and hmgAR1, rendered a positive result (a band of the expected size) in one of the strains only, suggesting that this strain has a minor modification in the hmgA gene. The PCR-amplified hmgA gene was sequenced to verify whether a mutation was present. Although some polymorphisms were found, none of them produced amino acid changes in the deduced protein, suggesting that mutations in other regions, such as transcriptional regulators, may be responsible for the observed phenotype. No PCR band was obtained in the other strain. As for the second strain, the use of hmgA internal primers and PCR controls demonstrated that the hmgA gene is absent in this strain and that the PCR was not inhibited in this case (data not shown). Therefore, these results strongly suggest that in both cases the lack of homogentisate 1,2-dioxygenase is responsible for the pyomelanogenic phenotype.

Mortality in the murine acute lung infection model

Pyomelanogenic strains appear to be more commonly found among clinical strains isolated from chronic infections such as CF and COPD (A. Oliver, unpublished). Thus, once it was determined that the lack of hmgA activity is responsible for pyomelanin hyperproduction in both laboratory mutants and clinical isolates, we decided to study whether hmgA inactivation confers an adaptive advantage in chronic infections. During the final steps of the characterization of the gene responsible for pyomelanin hyperproduction in our insertional library of PAO1, the construction of an ordered and nonredundant library of
P. aeruginosa strain PA14 transposon insertion mutants was published by Liberati et al. (2006). We thought that since PA14 is a more virulent strain (Choi et al., 2002), it would probably be more appropriate than PAO1 for studying any differences in mortality, clearance and persistence in mouse models.

The genomic analysis of the PA14 genome had identified pathogenicity islands PAPI-1 and PAPI-2, unique to PA14 and absent in the PAO1 genome (He et al., 2004). The large set of ‘extra’ virulence factors encoded by these pathogenicity islands may provide some virulence and/or persistence characteristics to the PA14 strain, which are absent in PAO1. Thus, strain PA14 and its pyomelanogenic mutant hmgA::MAR2xT7 (Liberati et al., 2006) were used to perform the mouse studies in this work.

Fig. 3 shows accumulated lung infection mortality on a daily basis for both the wild-type PA14 and PA14 hmgA::MAR2xT7 mutant strains. Overall mouse mortality after 7 days of infection was similar for both strains, although the hmgA mutant tended to kill them at a slower pace. For instance, PA14 killed 94% of the infected mice by the second day, whereas the hmgA mutant killed only 62% (P=0.04). Likewise, PA14 hmgA::MAR2xT7 showed reduced fitness in the acute respiratory infection model, since it was outcompeted by the wild-type PA14 strain after 48 h of coinfection (CI 0.38, P<0.001) (data not shown). This result shows that the inactivation of hmgA is associated with reduced virulence in the acute infection model.

**hmgA disruption decreases clearance and increases persistence in the murine chronic lung infection model**

Mortality, clearance and persistence in the murine model of chronic lung infection were determined. As shown in Fig. 4, overall mortality, similarly to that observed in the acute lung infection model, was not significantly modified by hmgA disruption in the chronic lung infection model. Moreover, in agreement with the results of the acute infection model, the hmgA mutant initially showed reduced fitness in the chronic infection. The median bacterial load in the lungs after 24 h of infection was lower in the mutant (4.8×10^6 c.f.u. per lung) than in the wild-type strain (8.2×10^6) (data not shown). However, the mutant showed decreased clearance and increased persistence after 7 days of infection (Fig. 4): 52.4% of mice infected by PA14 hmgA::MAR2xT7 showed persisting colonization after 7 days, in contrast to only 23.8% of those infected by the wild-type PA14 (P=0.006). The median lung bacterial load at 7 days was similar in mice showing persisting colonization by PA14 hmgA::MAR2xT7 (2.3×10^9 c.f.u. per lung) or the wild-type PA14 strain (1.3×10^9), despite the fitness disadvantage of the hmgA mutant during the first stages of the infection. Therefore, it is clear that hmgA disruption conferred an adaptive advantage in chronic lung infection.

**Effect of hmgA inactivation on resistance to H_2O_2 and antibiotics**

Protection from environmental stress has been suggested as a possible role of melanin production in micro-organisms, since the ability of free-living microbes to produce this pigment has been associated with increased survival in the environment. Melanin may protect bacteria and fungi from different types of stress, including toxic products such as H_2O_2, pesticides, heavy metals and antibiotics (Hoti & Balaraman, 1993; Hullo et al., 2001; Nosanchuk & Casadevall, 2006).

To test the possible role of hmgA disruption on H_2O_2 resistance, we estimated the viability of cultures of strains PA14, PA14 hmgA::MAR2xT7 and the complemented mutant PA14 hmgA::MAR2xT7(pBBR1-HmgA) subjected to H_2O_2 treatment (25 and 50 mM). Fig. 5(A) shows that...
hmgA disruption protects significantly against the effect of 50 mM peroxide after a 30, 45 or 60 min exposure. All three strains showed similar viability after exposure to 25 mM peroxide (data not shown).

Another possible advantage of pyomelanin hyperproduction is increased resistance to different antibiotics. This increased resistance could result in the selection of pyomelanin hyperproducer strains after prolonged treatments in chronically infected patients. To test this possibility, we measured the MICs of different currently used anti-
Pseudomonas antibiotics (ceftazidime, imipenem, ciprofloxacin and tobramycin) against the wild-type and the hmgA derivative of P. aeruginosa PA14. No MIC differences were found among the wild-type and mutants.

In conclusion, the results of this section indicate that inactivation of the hmgA gene provides protection against H₂O₂ but not antibiotics.

**Pyomelanin-containing supernatant is able to protect wild-type cells from H₂O₂**

To gain an insight into whether the production of the pyomelanin pigment is responsible for H₂O₂ protection, wild-type non-producer PA14 cells, obtained from 48 h cultures in LB, were washed and resuspended in filter-sterilized supernatant from the PA14 hmgA::MAR2xT7 pyomelanogenic mutant or, as control, its own supernatant (wild-type PA14), taken from cultures grown for 48 h in LB broth. Viable-cell counts determined before and after treatment showed that the supernatant from the mutant culture was able to protect the wild-type cells from the effect of H₂O₂ (Fig. 5B). This result suggests that the pyomelanin pigment may be the cause of such protection in P. aeruginosa. This interpretation is also supported by a previous report indicating that Burkholderia cepacia may derive protection against host-generated free-radicals via scavenging of these radicals by melanin (Zughaier et al., 1999). Our data may also explain why naturally occurring hyperproducer strains do not always take over the whole lung population when they appear in chronically infected patients, as non-producer strains may act as ‘cheaters’, and take advantage of the protection provided by hyperproducers without the associated cost (A. Oliver, unpublished).

Finally, as expected, the supernatant of the hyperproducer mutant did not provide protection against the four antibiotics tested (ceftazidime, imipenem, ciprofloxacin and tobramycin) (data not shown).

The results presented in this study demonstrate that: (i) inactivation of the hmgA gene in P. aeruginosa leads to the hyperproduction of pyomelanin; (ii) pyomelanogenic strains isolated from CF patients seem to lack HmgA activity, as complementation with the hmgA wild-type gene reverts the phenotype; (iii) an hmgA mutant strain is less virulent yet more persistent than the wild-type PA14. No MIC differences were found among the wild-type and mutants.

**Concluding remarks**

In conclusion, the results presented in this study demonstrate that: (i) inactivation of the hmgA gene in P. aeruginosa leads to the hyperproduction of pyomelanin; (ii) pyomelanogenic strains isolated from CF patients seem to lack HmgA activity, as complementation with the hmgA wild-type gene reverts the phenotype; (iii) an hmgA mutant strain is less virulent yet more persistent than the wild-type PA14.
in lung infection models; and (iv) \textit{hmgA} mutants show increased resistance to peroxide, probably due to the protective effect of the pyomelanin pigment itself. Despite our \textit{in vitro} results with peroxide suggesting that production of pyomelanin may be the cause of increased adaptation to chronicity, whether this production is the final production of pyomelanin may be the cause of increased capacity, reduced expression of exoenzymes and increased adaptive mutations for chronic lung infection already attempted.

The inactivation of \textit{hmgA} can now be included among the adaptive mutations for chronic lung infection already described in natural isolates, such as hyperproduction of alginate, reduced growth rate, increased biofilm formation capacity, reduced expression of exoenzymes and increased antibiotic resistance.

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