Effects of null mutation of the heat-shock gene *htpG* on the production of virulence factors by *Pseudomonas aeruginosa*

Anna M Grudniak^{*,1}, Barbara Klecha¹ & Krystyna I Wolska¹

¹Department of Bacterial Genetics, Institute of Microbiology, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland

* Author for correspondence: Tel.: +48 22 554 1322; Fax: +48 22 554 1402; grudam@biol.uw.edu.pl

Aim: *Pseudomonas aeruginosa* is one of the most clinically important opportunistic pathogen in humans. The aim of the project was to study effects of HtpG on the selected virulence factors responsible for pathogenesis and biofilm formation of *P. aeruginosa*. **Methodology:** By characterizing a *htpG* null mutant of *P. aeruginosa*, we have identified the role of HtpG in the production of selected factors. **Results:** We showed that $\Delta htpG$ mutant affects many physiological processes containing: decreased activity of the LasA protease, reduction of biofilm formation, decreased motility, and diminished amount of rhamnolipids and pyoverdine/pyocyanin. These defects were most evident when the $\Delta htpG$ strain was cultured at 42°C. **Conclusion:** Our findings demonstrate the unexplored role of HtpG in the pathogenicity of *P. aeruginosa*, and indicate potential targets for antibacterial therapeutics.

First draft submitted: 13 June 2017; Accepted for publication: 5 September 2017; Published online: 4 December 2017

Keywords: HtpG • *Pseudomonas aeruginosa* • virulence

The heat-shock response is one of the best described cellular adaptations to stress conditions. Exposure of cells to high temperature and other environmental stresses induces the synthesis of a group of proteins named heat-shock proteins (HSPs) [1]. HSPs can bind other proteins and influence their folding, oligomeric assembly, stabilization, transport across membranes and degradation [2]. HSPs interact with partially folded or misfolded polypeptides and assist in their correct folding. They also catalyze the disaggregation of aggregated proteins and mediate their proteolytic degradation.

HSPs have been classified into several groups according to their molecular weight: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and sHsps [3]. Some well-characterized machineries of HSPs are DnaK/DnaJ/GrpE and GroEL/GroES, which play essential roles in many physiological processes [4,5].

Proteins belonging to the Hsp90 family are evolutionarily well conserved. Hsp90s comprise 1–2% of all cytosolic proteins in bacterial and eukaryotic cells, and are synthesized under both physiological and stress conditions [6,7]. Hsp90s act as dimers in the open conformation and contain three conserved domains: an N-terminal domain, middle domain and carboxy-terminal domain. The N-terminal domain includes a nucleotide-binding site and an ATP-binding domain [8,9]. The bacterial Hsp90 homolog HtpG, first described in *Escherichia coli*, is encoded by the *htpG* gene. Null mutation of this gene is not lethal to bacterial cells and only results in impaired growth at high temperatures. HtpG acts as a chaperone that enables the optimal folding of newly synthesized proteins under stress conditions. The HtpG-mediated folding mechanism does not involve cochaperones and the identity of proteins acting as substrates in this process remains unknown [7,10]. In *E. coli*, HtpG interacts with the DnaK/DnaJ/GrpE chaperone complex [11].

Pseudomonas aeruginosa is a motile Gram-negative bacterium that is widespread in the natural environment [12]. This bacterium is one of the most clinically important opportunistic pathogens of humans and animals [13]. It causes acute infections in immunocompromised individuals, that is, urinary tract infections, eye infections, bacteremia and sepsis, and serious skin infections in burns patients [14]. This bacterium is considered the primary etiological agent of pneumonia in individuals with cystic fibrosis (CF) [15], and infections with *P. aeruginosa* are the predominant cause of death in patients with this condition. The pathogenesis of *P. aeruginosa* is dependent on biofilm formation and the production and secretion of multiple virulence factors: cytotoxins, adhesins, proteases (elastase, alkaline protease, LasA protease), hemolysins and alginates [16]. These bacteria often produce effector proteins and pigments such as the blue–green pigment pyocyanin, pyoverdine, redox-active phenazine, plus the biosurfactant rhamnolipid. The virulence factors of *P. aeruginosa* form part of the extracellular matrix and can disrupt or control host cellular pathways [17].

Bacterial cells forming biofilm exhibit a phenotype that is significantly different from that of planktonic bacteria. This includes a substantial increase in antibiotic and biocide resistance that makes complete eradication difficult. *P. aeruginosa* has the ability to develop incredibly robust biofilms on seemingly uninhabitable surfaces. Moreover, this bacterium possesses various mechanisms that reduce the effectiveness of antimicrobial chemotherapy, which make infections notoriously hard to treat [18,19]. Its resistance mechanisms include systems mediating active efflux (tetracycline, chloramphenicol), degradation (β -lactams) or modification (aminoglycosides) of antibiotics, plus reduced accumulation of these compounds due to membrane impermeability and mutation of the cellular targets [20].

The aim of the project was to study effects of encoded Hsp90 homolog on the selected virulence factors responsible for pathogenesis and biofilm formation of *P. aeruginosa*. By characterizing an *htpG*-null mutant of *P. aeruginosa*, we have identified the role of HtpG in the production of mentioned factors. Our findings indicate potential cellular targets for new antibacterial therapeutics.

Materials & methods

Bacterial strains, culture & media

P. aeruginosa PAO1-CECT4122 was obtained from the Spanish Type Culture Collection. Cultures were grown in tryptone soy yeast extract broth (BTL, Warsaw, Poland) or lysogeny broth (LB; Sigma-Aldrich, Poznań, Poland). *Staphylococcus aureus* ATCC29213 was obtained from the American Type Culture Collec-

tion, *E. coli* MC1061 Δ *htpG* was constructed in our laboratory by standard methodology using a classical gene replacement method in agreement with Grudniak *et al.* [21]. To receive an *htpG* null mutation, we inactivated the gene by inserting a *kan* cassette (1.4 kb) within the coding region of *htpG* by standard methodology [21]. The upstream regions of the target gene were amplified from *E. coli* MC1061 genomic DNA using primer pairs: – HG2-L, 5'-AGCCTGAAGGGCATGACGTGGAAGCCAGC-3' and HG2-R, 5'-TGACCTGTTAGGATCCTCTCCTGCTTACCTTCAACAC-3'; and downstream regions using primer pairs: – HG3-L, 5'-AGGATCCTAACAGGTCAACTGACTCCTTCATCGACC-3' and HG3-R, 5'-AGCCTGAAGGGCCGACGACGACGATAT-3' (BamHI restriction sites underlined). The Taq DNA polymerase (Qiagen, Manchester, UK) with high fidelity was used in the PCR. A kanamycin cassette (1.4-kb fragment) from plasmid pKRP11 (Km^r, Ap^r) was excised by digestion with BamHI. The two obtained by PCR products were digested with BamHI and ligated with the *kan* cassette and this fragment was cloned into plasmid pJET/1.2 (CloneJET™ PCR Cloning Kit – Fermentas). This product was then excised from the construct pJET/1.2 *htpG::kan* using BglI and introduced into pDS132 (Cm^r, ori R6K, *sacB*). This vector contains the *sacB* gene from *Bacillus subtilis*, encoding levansucrase, which is often used for counter selection. Levansucrase transforms sucrose into polymeric levans, which confers sucrose sensitivity. The construct pDS132 *htpG::kan* was then cloned into *E. coli* MC1061 and colonies Km-resistant were selected. Transformants were grown under kanamycin pressure in conditions nonpermissive for plasmid replication. The cells with plasmid DNA inserted into the genome were capable to survive under these conditions. Obtained cointegrate was then resolved by spontaneous recombination of the allele pair resulting in cells with either a wild-type (wt) or a mutant allele in the chromosome. PCR and DNA sequencing was used to verify the final construction in the *E. coli* genome [21]. The Δ *htpG* deletion was transferred to *P. aeruginosa* PAO1 by P1 transduction. The preparation of P1 lysates and transduction were carried out according to Miller [22]. Deletion mutants were initially selected on agar plates containing kanamycin (50 µg/ml; Sigma-Aldrich). The presence of the appropriate deletion was confirmed by PCR analysis and amplicon sequencing (Genomed, Warsaw, Poland), and the temperature-sensitive growth phenotype was verified. In this report, the strain *P. aeruginosa* PAO1 Δ *htpG* will be referred to as *P. aeruginosa* Δ *htpG*. Plates of (0.6%) M8 agar [23] or (1.5%) LB agar medium were used to test motility.

LasA staphylolytic assay

LasA protease activity was measured by determining the ability of stationary-phase *P. aeruginosa* wt and Δ *htpG* culture supernatants to lyse boiled *S. aureus* cells. The two *P. aeruginosa* strains were cultured at 30°C for 4 h or growth at 42°C for 2 h and then 2 h at 30°C. The cultures were then centrifuged (10 min at 11,100 r.p.m.) and the cell-free supernatants collected. Meanwhile, 30 ml of an overnight culture of *S. aureus* was boiled for 10 min and then centrifuged for 10 min at 11,100 r.p.m. The cell pellet was resuspended in 10 mM Na₂PO₄ (pH 4.5) to give an A₆₀₀ of approximately 0.8. Then, 100-µl aliquots of the *P. aeruginosa* supernatants were mixed with 900 µl of the prepared *S. aureus* cell suspension, and the A₆₀₀ values were measured after 0, 10, 20, 30, 40 and 50 min at room temperature. LasA protease activity was determined as A₆₀₀ per mg protein total in supernatant [24]. Protein concentration was measured using a Pierce BCA assay (Sigma-Aldrich).

Quantitative biofilm formation assay

Biofilm formation by *P. aeruginosa* wt or Δ *htpG* strains in the wells of microtiter plates after 6 or 24 h of incubation was evaluated by crystal violet staining. Cultures were grown overnight at 30°C with shaking and then diluted 1:100 in fresh medium. Aliquots of 250 µl of these cultures were dispensed into the wells of 24-well microtiter plates. Replicate plates were incubated at 30 or 42°C to allow growth for a few generations. Cultures were exposed to 42°C for the first 2 h of growth. Growth of the mutant was not affected by incubation at 42°C for 2 h. After 6 h (adhesion step) or 24 h (biofilm formation) of static incubation, bacterial cells in the wells were stained with crystal violet [21,25]. Staining was quantified by measuring the absorbance at 570 nm using a microtiter plate reader (TECAN Sunrise, Männedorf, Switzerland) [26].

Motility assays

The swimming and swarming motility of *P. aeruginosa* wt or Δ *htpG* strains was determined on plates of M8 medium solidified with Bacto-Agar (Difco Laboratories, MI, USA) at concentrations of 0.3 or 0.6%, respectively. The plates were dried overnight at room temperature and then inoculated using a sterile toothpick with individual colonies taken from fresh LB agar (1.5%) plates. Replicate plates were incubated at 30°C for 24 h or for 2 h at

42°C and then 22 h at 30°C. To estimate the swimming ability of *P. aeruginosa*, the diameter of each growth zone in the 0.3% agar plates was measured. In the case of swarming motility, the length of the longest branch of the observed dendritic structures in the 0.6% agar plates was measured [23]. Twitching motility was examined on LB solidified with 1% agar in glass Petri dishes. The plates were dried and single colonies were inoculated deep into the agar using a sterile toothpick to allow subsequent measurement of motility at the glass–agar interface. Replicate plates were incubated at 30°C for 24 h or for 2 h at 42°C and then 22 h at 30°C. The agar was then removed and the bottom of each glass plate was stained with 0.1% crystal violet for 5 min. The plates were rinsed with distilled water and the diameters of the stained twitching zones were measured [27]. Each motility assay variant was performed independently at least five-times.

Pigments production

Production of pyocyanin and pyoverdine was quantified as described previously [28]. Cultures of the *P. aeruginosa* wt and $\Delta htpG$ strains were grown overnight at 30°C with shaking and then diluted 1:100 in fresh LB medium supplemented with 0.45% glucose. Aliquots of 250 µl of these cultures were dispensed into the wells of 96-well microtiter plates. Replicate plates were then incubated at 30 or 42°C to allow growth for a few generations. After incubation for 40 h, 200-µl lots of the culture supernatants were transferred to fresh microtiter plates. The amount of pyoverdine in each sample was estimated by measuring fluorescence (λ_{excit} – 398 nm/ λ_{emiss} – 460 nm; TECAN Sunrise). The amount of pyocyanin was measured by absorbance at 312 nm (TECAN Sunrise). Each experiment was performed in triplicate.

Rhamnolipids production

Production of rhamnolipids by *P. aeruginosa* wt and $\Delta htpG$ strains was evaluated using the semiquantitative agar plate test described by Siegmund and Wagner [29]. The strains were cultured on plates containing cetyltrimethylammonium bromide-methylene blue with 1.6% agar (all reagents from Sigma-Aldrich). The plates were incubated at 30°C or for 2 h at 42°C and then at 30°C for a few generations. After 24 h incubation, positive colonies were surrounded by dark blue halos with a diameter proportional to the concentration of excreted rhamnolipids [29].

Statistical analysis

All experiments were carried out at minimum in triplicate. In a statistical analysis, we used Student's *t*-test (Statistica 13.1) and differences were considered significant when **p* < 0.05, ***p* < 0.01, ****p* < 0.001 were obtained.

Results

The analysis of mutants can provide valuable information about the cellular function of genes and their encoded proteins. To examine the functions of the *htpG* gene, which encodes an Hsp90 homolog, the *E. coli* $\Delta htpG$ -null mutant MC1061 was constructed by insertion of a *kan* cassette using a standard methodology [21]. The $\Delta htpG$ mutation was then transferred to *P. aeruginosa* strain PAO1 by transduction using bacteriophage P1. The transferred mutation was verified by PCR and amplicon sequencing (data not shown), and as anticipated the $\Delta htpG$ mutant showed classic temperature-sensitive growth. This *P. aeruginosa* $\Delta htpG$ strain was then used in a series of experiments to examine the influence of HtpG on physiological processes connected with pathogenesis.

We examined the effect of HtpG deprivation on the proteolytic activity, ability to form biofilm, motility and production of pigments and rhamnolipids. The experiments were performed at 30°C and also including 2 h incubation at 42°C as, the cellular demand for chaperones is greater at elevated temperature. This period at the raised temperature did not affect the growth of the $\Delta htpG$ mutant strain as determined by turbidity measurements (growth was not inhibited until 3-h incubation at 42°C).

We evaluated the activity of LasA in *P. aeruginosa* wt and $\Delta htpG$ strains at both low temperature (30°C) and restrictive temperature (42°C). LasA protease activity was measured by determining the ability of *P. aeruginosa* culture supernatants to lyse *S. aureus* cells (Figure 1). Mutation of *htpG* was found to reduce the staphylolytic activity of *P. aeruginosa* irrespectively to the cultivation temperature, however, the observed effect is not very pronounced. The results indicate that of HtpG protein affects synthesis, or stabilization of LasA protease.

The capacity to form biofilms is crucial for the virulence of *P. aeruginosa* [30]. We investigated the biofilm formation ability of the wt and $\Delta htpG$ mutant strains at both 30 and 42°C using a standard crystal violet staining assay. The initial step in biofilm formation is adhesion. This was examined after 6 h incubation and the results are presented in Figure 2. Adhesion of the $\Delta htpG$ mutant was significantly diminished in comparison to the wt strain

Figure 1. Effect of $\Delta htpG$ mutation on staphylolytic activity *Pseudomonas aeruginosa*. The values are calculated for 1 mg of total proteins in supernatants. The experiments were performed in triplicate. wt: Wild-type.

Figure 2. Quantification of biofilm formation on polystyrene plates by wild-type *Pseudomonas aeruginosa* and $\Delta htpG$ mutant after 6 and 24 h incubation. The mean values from three independent experiments are presented, with error bars indicating the standard deviation. wt: Wild-type.

Figure 3. Motility assay of *Pseudomonas aeruginosa* wild-type and $\Delta htpG$ mutant. Effect of $\Delta htpG$ mutation on motility assay: twitching (A) and swimming (B). Each value represents the mean from three experiments $\pm$ standard deviation. wt: Wild-type.

at both tested temperatures and the observed defect was strong at 42°C, the decrease was 68%. Thus, the $\Delta htpG$ mutation reduced early steps of biofilm formation by *P. aeruginosa*. The further steps of biofilm development (Figure 2) are inhibited by $\Delta htpG$ mutation at 30°C (by 15%) and 42°C (by 22%) in comparison to the wt strain but the effect was irrespective at temperature.

Using different motility assays, we examined the motility of the wt and $\Delta htpG$ mutant strains at both 30 and 42°C. Twitching motility was examined on LB solidified with 1% agar in glass Petri dishes. The diameters of the stained zones on the twitching motility assay plates were measured and the results are presented in Figure 3A. The presented results showed that twitching motility was significantly diminished (by 45%) in the $\Delta htpG$ mutant cultured at 42°C in comparison to these observed in wt strain of *P. aeruginosa* in the same temperature. We also assayed swimming motility of the *P. aeruginosa* strains by measuring the area of the growth zones (mm²) on the surface of plates of 0.3% M8 agar medium. From the results presented in Figure 3B, it is clear that swimming motility was significantly reduced in the $\Delta htpG$ mutant cultured at 42°C (by 31%) in comparison to wt strain.

Analysis of swarming motility was carried out by measuring the length of the longest branches of dendritic structures formed following growth of the *P. aeruginosa* strains on plates of 0.6% M8 agar medium. The representative

Figure 4. Swarming motility of wild-type *Pseudomonas aeruginosa* and $\Delta htpG$ mutant. Representative micrograph showing swarming motility of *P. aeruginosa* wild-type (A) and *P. aeruginosa* $\Delta htpG$ (B) at 30 and 42°C.

Table 1. Effect of $\Delta htpG$ mutation on amount of pyocyanin in *Pseudomonas aeruginosa* at 30 and 42°C.

Strain	Absorbance A ₃₁₂ ± SD	
	30°C	42°C
<i>P. aeruginosa</i> PAO1 wt	3.09 ± 0.1	3.19 ± 0.09
<i>P. aeruginosa</i> PAO1 $\Delta htpG$	2.83 ± 0.18***	2.54 ± 0.21***

Each value represents the mean from three experiments ± SD.
*** p < 0.001.
SD: Standard deviation; wt: Wild-type.

Table 2. Effect of $\Delta htpG$ mutation amount of pyoverdine in *Pseudomonas aeruginosa* at 30 and 42°C.

Strain	Fluorescence units × 10 ⁴ ± SD	
	30°C	42°C
<i>P. aeruginosa</i> PAO1 wt	3.17 ± 0.28***	2.49 ± 0.35***
<i>P. aeruginosa</i> PAO1 $\Delta htpG$	1.28 ± 0.27***	0.83 ± 0.35***

Each value represents the mean from three experiments ± SD.
*** p < 0.001.
SD: Standard deviation; wt: Wild-type.

results shown in Figure 4 demonstrate that neither the wt nor the $\Delta htpG$ strain produced these structures at the lower temperature (30°C). However, at the raised temperature (42°C), both strains produced dendritic structures with the same maximum branch length, but the $\Delta htpG$ mutant made significantly less complex structures than the wt strain.

The production of pyocyanin by the wt and mutant *P. aeruginosa* strains grown at 30 or 42°C was evaluated by measuring absorbance at 312 nm (Table 1). Pyocyanin production was diminished in the $\Delta htpG$ mutant at both tested temperatures, but the defect was greater at 42°C; levels were diminished by 20% of those observed in the wt strain.

We quantified the amount of pyoverdine in wt and mutant *P. aeruginosa* strains grown at 30 or 42°C using a fluorescence assay (Table 2). Pyoverdine levels were substantially reduced in cells of both strains grown at the higher temperature. Production by the $\Delta htpG$ mutant was diminished by 60 and 67% compared with the wt strain at 30 and 42°C, respectively. These results indicated that HtpG plays a crucial role in the synthesis or stabilization of the studied pigment.

Colonies producing extracellular rhamnolipids on cetyltrimethylammonium bromide plates containing crystal violet are surrounded by dark blue halos with a diameter proportional to the rhamnolipid concentration. The production of rhamnolipids by the *P. aeruginosa* wt and $\Delta htpG$ mutant strains grown at 30 or 42°C was evaluated using this trait (Table 3). We observed decreased rhamnolipid production by the $\Delta htpG$ mutant, particularly at 42°C the level was reduced by 30% of that of the wt.

Table 3. Effect of $\Delta htpG$ mutation on rhamnolipid content in *Pseudomonas aeruginosa* at 30 and 42°C.

Strain	Zone of rhamnolipid production cm ² ± SD	
	30°C	42°C
<i>P. aeruginosa</i> PAO1 wt	1.32 ± 0.22	1.32 ± 0.19
<i>P. aeruginosa</i> PAO1 $\Delta htpG$	1.22 ± 0.21***	0.92 ± 0.18***

Each value represents the mean from three experiments ± SD.
 *** p < 0.001.
 SD: Standard deviation; wt: Wild-type.

Discussion

HSPs are molecular chaperones that play crucial roles in many cellular functions, especially under stress conditions. These proteins are essential in both eukaryotic and prokaryotic cells. The Hsp90 is a highly conserved molecular chaperone that participates in protein folding, signal transduction and receptor maturation [31]. The bacterial protein HtpG (a homolog of Hsp90) is essential for correct protein folding, the reactivation of heat-denatured proteins and the translocation of polypeptides across membranes during periods of environmental stress.

In this study, we have used a $\Delta htpG$ null mutant to examine the role of the encoded protein in physiological processes of *P. aeruginosa*. Our results clearly demonstrate that HtpG is important in the production and activity of virulence factors, especially when cells are exposed to environmental stresses.

Staphylolytic activity is the ability to lyse *S. aureus* cells, which is associated with the function of the protease LasA in *P. aeruginosa*. LasA is a 20-kDa zinc metalloendopeptidase, which is a member of the β -lytic endopeptidase family. This protease plays a crucial role in pathogenesis, especially during acute infections. The $\Delta htpG$ mutant displayed decreased activity or synthesis of protease LasA, which indicates that HtpG influences these processes. Jagger *et al.* demonstrated that proteases produced by *P. aeruginosa* are very important in the colonization of pulmonary tracts, especially in patient with CF [32]. The involvement of molecular chaperones in proteolysis is well recognized [33]. Defective proteolysis following puromycin treatment has also been demonstrated in *groEL*, *dnaK* and *dnaJ* mutants [33]. In our previous investigation, we found that HtpG was necessary for the degradation of abnormal canavanine-containing proteins in *E. coli* [21].

Biofilm production by *P. aeruginosa* increases its resistance to antibiotics and biocides in comparison with planktonic cells. This greatly complicates the treatment of *P. aeruginosa* infections, especially in individuals with CF [20]. Biofilm formation, which is determined by the processes of adhesion, cellular aggregation, bacterial motility and the production of extracellular factors (e.g., pigments and biosurfactants) was found to be greatly diminished by the loss of HtpG. All of these processes were adversely affected in the $\Delta htpG$ mutant strain, especially adhesion to surfaces. The adhesion phase was reduced in the mutant at both tested temperatures, but the defect was greater at 42°C (by 68%), which may be connected with decreased motility is very important for its pathogenicity moreover that biofilm. The further steps of biofilm formation were reduced in the mutant $\Delta htpG$ at 30°C (by 15%) and 42°C (by 22%) in comparison to the wt strain.

The adhesion of Gram-negative bacteria to surfaces is largely dependent on the presence of cellular appendages such as flagella, pili and fimbriae [34,35]. Functional flagella enable the bacterium to swim and this ability is often required for surface colonization. Motility is an important virulence determinant of *P. aeruginosa* [34,36]. Besides swimming, *P. aeruginosa* cells can also move by swarming and twitching [23,37]. Twitching is dependent on pilus retraction and independent of the presence of flagella. Pili also bind to the epithelial cell membrane and are crucial to the adhesion phase of colonization. Thus, pili-mediated adhesion and twitching motility are essential for *P. aeruginosa* virulence. Twitching motility was diminished in the $\Delta htpG$ mutant strain at both temperatures, but the greatest defect was at 42°C, with a reduction of 45% in comparison to the wt. Flagella besides their function in swimming motility participate (with pili) in tethering bacteria to host epithelial cells by binding to common membrane components. Thus, flagella also are essential to the adhesion step of colonization and biofilm formation. In addition, flagella are highly immunogenic during the initial phase of infection. Swimming motility was diminished in the $\Delta htpG$ mutant strain at both temperatures, but the greatest defect was at 42°C, with a reduction of 31% in comparison to the wt.

P. aeruginosa has the ability to produce pigmented secondary metabolites including pyocyanin, pyoverdine and phenazine. Pyocyanin is a 1-hydroxy-5-methylphenazine with antimicrobial properties. It is a blue compound that can oxidize or reduce other molecules present in the infection zone [38]. Pyocyanin is recognized as a virulence factor of *P. aeruginosa* strains that have strong cytotoxicity [39]. Amount of pyocyanin in *P. aeruginosa* was diminished, by

20% in tested mutant strain especially at 42°C. Another pigment studied, pyoverdine was discovered more than 120 years ago in 1892 [40]. It is a chromophore that fluoresces green under ultraviolet radiation [41]. Pyoverdine is synthesized in the cytoplasm of *P. aeruginosa* as a complex formation, together with a siderophore. Both compounds are involved in iron binding and cellular uptake when the iron concentration in the environment is low [42]. Pyoverdine also regulates the secretion of two other *P. aeruginosa* virulence factors: exotoxin A and an endoprotease [40]. Pyoverdine is a crucial virulence factor that is necessary for pathogenesis in many models of infection. Our results showed that the amount of pyoverdine in mutant $\Delta htpG$ strain was diminished in both tested temperatures.

Biosurfactants are surface-active biomolecules produced by microorganisms. They include glycolipids, lipopolysaccharides, oligosaccharides and lipopeptides [43]. Rhamnolipids are one class of glycolipid produced by *P. aeruginosa*. Different substrates are used by bacteria to produce rhamnolipids, and the efficiency of their utilization depends on the cell number [44]. Rhamnolipids are key virulence factors of *P. aeruginosa* that have antimicrobial properties. They seem to be especially important in lung infections of ventilator-dependent patients, and in cases of CF and corneitis. Rhamnolipids appear to be crucial in each phase of biofilm development, where they reportedly create fluid-filled channels for the passage of water and oxygen. These compounds are particularly important in swarming motility [45–47]. Rhamnolipids can cause lysis of many cells of the human immune system [14,45]. They are also very important for bacterial biofilm formation due to their ability to create channels for the movement of water and oxygen [48]. In addition, rhamnolipids can eliminate other bacterial cells present in multispecies biofilms [49]. We found that the production of rhamnolipids was reduced by approximately 30% in the $\Delta htpG$ mutant at 42°C, when the cellular requirement for molecular chaperones is increased. A reduced ability to produce rhamnolipids in the $\Delta htpG$ mutant and this may have influenced biofilm formation. In influencing these processes, HtpG may cooperate with other proteins and participate in the export of polypeptides to the periplasm or outside the cell. This function of HtpG was confirmed in *E. coli* in the translocation of alkaline phosphatase and β -lactamase [21].

Conclusion

We have demonstrated that the Hsp90 homolog – HtpG – is involved in many physiological processes in *P. aeruginosa* cells, including those that are important for virulence, such as motility, biofilm formation, proteolytic activity and pigment and biosurfactant production. Many virulence factors of *P. aeruginosa* are extracellular and it is likely that HtpG plays a role in their secretion. To our best knowledge, our results constitute the first demonstration of HtpG chaperon involvement in the pathogenicity of *P. aeruginosa*.

Summary points

***Pseudomonas aeruginosa* is one of the most clinically important opportunistic pathogen**

- The pathogenesis of *P. aeruginosa* is dependent on biofilm formation and the production of multiple virulence factors.
- Bacteria forming biofilm exhibit a different phenotype that includes a substantial increase in antibiotic and biocide resistance.
- *P. aeruginosa* possesses various mechanisms that reduce the effectiveness of antimicrobial chemotherapy.
- HtpG is important to form a complexes with other bacterial proteins and is involved in the protection of cells against the environmental stress.

HtpG is important in the production & activity of many virulence factors of *P. aeruginosa*

- *P. aeruginosa* $\Delta htpG$ activity of the LasA protease is decreased.
- Biofilm formation by $\Delta htpG$ mutant strain is reduced especially on the first step of adhesion.
- Null mutation of *htpG* gene affects swimming and twitching motility of *P. aeruginosa* PAO1.
- Production of pyoverdine/pyocyanin and biosurfactant is decreased in mutant strain.

Conclusion

- Null mutation of the *htpG* gene, encoding a chaperone Hsp90 homolog, affects many physiological processes connected with the pathogenesis of *P. aeruginosa*.
- Our findings demonstrate a previously unrecognized role of HtpG in the virulence and pathogenicity of *P. aeruginosa*, and indicate potential targets for antibacterial therapeutics.

Future perspective

The HtpG protein is envisioned as probable target for developing drugs against *P. aeruginosa* and other pathogens especially by inhibition of biofilm formation.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Open access

This work is licensed under the Attribution-NonCommercial-NoDerivatives 4.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>

References

Papers of special note have been highlighted as: ●● of considerable interest

- Guisbert E, Herman C, Lu CZ, Gross CA. A chaperone network controls the heat shock response in *E. coli*. *Genes Dev.* 18(22), 2812–2821 (2004).
- An important article describing heat-shock response in *Escherichia coli*.
- Muga A, Moro F. Thermal adaptation of heat shock proteins. *Curr. Protein Pept. Sci.* 9(6), 552–566 (2008).
- Parsek MR, Greenberg EP. Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol.* 13(1), 27–33 (2005).
- Craig EA, Gambill BD, Nelson RJ. Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol. Rev.* 57(2), 402–414 (1993).
- Houry WA, Frishman D, Eckerskorn C, Lottspeich F, Hartl FU. Identification of *in vivo* substrates of the chaperonin GroEL. *Nature* 402(6758), 147–154 (1999).
- Chen B, Zhong D, Monteiro A. Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms. *BMC Genomics* 7, 156 (2006).
- Justifies the relevance of evolution of the Hsp90 family of genes across all kingdoms of organism.
- Buchner J. Bacterial Hsp90 – desperately seeking clients. *Mol. Microbiol.* 76(3), 540–544 (2010).
- Mason CA, Dunner J, Indra P, Colangelo T. Heat-induced expression and chemically induced expression of the *Escherichia coli* stress protein HtpG are affected by the growth environment. *Appl. Environ. Microbiol.* 65(8), 3433–3440 (1999).
- Neckers L, Mollapour M, Tsutsumi S. The complex dance of the molecular chaperone Hsp90. *Trends Biochem. Sci.* 34(5), 223–226 (2009).
- Bukau B, Horwich AL. The Hsp70 and Hsp60 chaperone machines. *Cell* 92(3), 351–366 (1998).
- Doyle SM, Hoskins JR, Wickner S. Collaboration between the ClpB AAA+ remodeling protein and the DnaK chaperone system. *Proc. Natl Acad. Sci. USA* 104(27), 11138–11144 (2007).
- Banat IM, Franzetti A, Gandolfi I *et al.* Microbial biosurfactants production, applications and future potential. *Appl. Microbiol. Biotechnol.* 87(2), 427–444 (2010).
- An important article describing biosurfactants production by *Pseudomonas*.
- Ridgway HF, Safarik J, Phipps D, Carl P, Clark D. Identification and catabolic activity of well-derived gasoline-degrading bacteria from a contaminated aquifer. *Appl. Environ. Microbiol.* 56(11), 3565–3575 (1990).
- Van Gennip M, Christensen LD, Alhede M *et al.* Inactivation of the *rhlA* gene in *Pseudomonas aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes. *APMIS* 117(7), 537–546 (2009).
- Bogiel T, Kwiecinska-Pirog J, Kozusko S, Gospodarek E. Incidence of alginate-coding gene in carbapenem-resistant *Pseudomonas aeruginosa* strains. *Med. Dosw. Mikrobiol.* 63(2), 145–153 (2011).
- Flemming HC, Wingender J. The biofilm matrix. *Nat. Rev. Microbiol.* 8(9), 623–633 (2010).
- Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* 61(1), 47–64 (1997).

●● **Another study that highlights the relevance of production surfactants and their commercial potential.**

18. Poole K. Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. *Trends Microbiol.* 20(5), 227–234 (2012).
19. Strateva T, Yordanov D. *Pseudomonas aeruginosa* – a phenomenon of bacterial resistance. *J. Med. Microbiol.* 58(Pt 9), 1133–1148 (2009).

●● **An important article describing *Pseudomonas aeruginosa* as a serious human pathogen.**

20. Hancock RE, Speert DP. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resist. Updat.* 3(4), 247–255 (2000).
21. Grudniak AM, Pawlak K, Bartosik K, Wolska KI. Physiological consequences of mutations in the *htpG* heat shock gene of *Escherichia coli*. *Mutat. Res.* 745–746, 1–5 (2013).
22. Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA (1972).
23. Kohler T, Curty LK, Barja F, Van Delden C, Pechere JC. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J. Bacteriol.* 182(21), 5990–5996 (2000).
24. Kong KF, Jayawardena SR, Indulkar SD *et al.* *Pseudomonas aeruginosa* AmpR is a global transcriptional factor that regulates expression of AmpC and PoxB beta-lactamases, proteases, quorum sensing, and other virulence factors. *Antimicrob. Agents Chemother.* 49(11), 4567–4575 (2005).
25. Smith K, Perez A, Ramage G, Lappin D, Gemmell CG, Lang S. Biofilm formation by Scottish clinical isolates of *Staphylococcus aureus*. *J. Med. Microbiol.* 57(Pt 8), 1018–1023 (2008).
26. Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual (3rd Edition)*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA (2001).
27. O'may C, Tufenkji N. The swarming motility of *Pseudomonas aeruginosa* is blocked by cranberry proanthocyanidins and other tannin-containing materials. *Appl. Environ. Microbiol.* 77(9), 3061–3067 (2011).
28. Sanchez P, Linares JF, Ruiz-Diez B *et al.* Fitness of *in vitro* selected *Pseudomonas aeruginosa* nalB and nfxB multidrug resistant mutants. *J. Antimicrob. Chemother.* 50(5), 657–664 (2002).
29. Siegmund I, Wagner F. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnology Techniques* 5(4), 265–268 (1991).

●● **Another important article describing detection of rhamnolipids excreted by *P. aeruginosa*.**

30. Donlan RM. Biofilms and device-associated infections. *Emerg. Infect. Dis.* 7(2), 277–281 (2001).
31. Arsene F, Tomoyasu T, Bukau B. The heat shock response of *Escherichia coli*. *Int. J. Food Microbiol.* 55(1–3), 3–9 (2000).
32. Jagger KS, Bahner DR, Warren RL. Protease phenotypes of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis. *J. Clin. Microbiol.* 17(1), 55–59 (1983).
33. Straus DB, Walter WA, Gross CA. *Escherichia coli* heat shock gene mutants are defective in proteolysis. *Genes Dev.* 2(12B), 1851–1858 (1988).
34. Kazmierczak BI, Schniederberend M, Jain R. Cross-regulation of *Pseudomonas* motility systems: the intimate relationship between flagella, pili and virulence. *Curr. Opin. Microbiol.* 28, 78–82 (2015).
35. Anyan ME, Amiri A, Harvey CW *et al.* Type IV pili interactions promote intercellular association and moderate swarming of *Pseudomonas aeruginosa*. *Proc. Natl Acad. Sci. USA* 111(50), 18013–18018 (2014).
36. O'toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* 30(2), 295–304 (1998).

●● **Justifies the relevance of twitching motility that is important for *P. aeruginosa* biofilm development.**

37. Conrad JC, Gibiansky ML, Jin F *et al.* Flagella and pili-mediated near-surface single-cell motility mechanisms in *P. aeruginosa*. *Biophys. J.* 100(7), 1608–1616 (2011).
38. Lau GW, Hassett DJ, Ran H, Kong F. The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol. Med.* 10(12), 599–606 (2004).
39. Denning GM, Iyer SS, Reszka KJ, O'malley Y, Rasmussen GT, Britigan BE. Phenazine-1-carboxylic acid, a secondary metabolite of *Pseudomonas aeruginosa*, alters expression of immunomodulatory proteins by human airway epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 285(3), L584–L592 (2003).
40. Schalk IJ, Guillon L. Pyoverdine biosynthesis and secretion in *Pseudomonas aeruginosa*: implications for metal homeostasis. *Environ. Microbiol.* 15(6), 1661–1673 (2013).
41. Meyer JM. Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. *Arch. Microbiol.* 174(3), 135–142 (2000).
42. Lamont IL, Martin LW. Identification and characterization of novel pyoverdine synthesis genes in *Pseudomonas aeruginosa*. *Microbiology* 149(Pt 4), 833–842 (2003).
43. Guerra-Santos L, Kappeli O, Fiechter A. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. *Appl. Environ. Microbiol.* 48(2), 301–305 (1984).

44. Wei Y-H, Cheng C-L, Chien C-C, Wan H-M. Enhanced di-rhamnolipid production with an indigenous isolate *Pseudomonas aeruginosa* J16. *Proc. Biochem.* 43(7), 769–774 (2008).
 45. Chrzanowski L, Lawniczak L, Czaczyk K. Why do microorganisms produce rhamnolipids? *World J. Microbiol. Biotechnol.* 28(2), 401–419 (2012).
 46. Eraqi WA, Yassin AS, Ali AE, Amin MA. Utilization of crude glycerol as a substrate for the production of rhamnolipid by *Pseudomonas aeruginosa*. *Biotechnol. Res. Int.* 2016, 3464509 (2016).
 47. Caiazza NC, Shanks RM, O'toole GA. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. *J. Bacteriol.* 187(21), 7351–7361 (2005).
 48. Davey ME, Caiazza NC, O'toole GA. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 185(3), 1027–1036 (2003).
 49. Espinosa-Urgel M. Resident parking only: rhamnolipids maintain fluid channels in biofilms. *J. Bacteriol.* 185(3), 699–700 (2003).
- **Another recent study that highlights the participation of rhamnolipids in fluid channels composition in biofilm.**