The *Pseudomonas aeruginosa* dnaK gene is involved in bacterial translocation across the intestinal epithelial cell barrier

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**Abstract**

*Pseudomonas aeruginosa* can penetrate through polarized epithelial cell monolayers produced by the human adenocarcinoma cell line Caco-2. We previously identified genes associated with bacterial translocation through Caco-2 cell monolayers by analysing transposon insertion mutants with dramatically reduced penetration activity relative to that of the wild-type *P. aeruginosa* PAO1 strain. In this study, we focused on the *dnaK* mutant because the association between this gene and penetration activity is unknown. Inactivation of *dnaK* caused significant repression of bacterial penetration through Caco-2 cell monolayers, with decreased swimming, swarming and twitching motilities; bacterial adherence; and fly mortality rate; as well as dramatic repression of type III effector secretion and production of elastase and exotoxin A. However, type IV pilus protein PilA expression was not affected. These results suggest that *dnaK* is associated with bacterial motility and adherence, which are mediated by flagella and pilis, and with toxin secretion, which plays a key role in the penetration of *P. aeruginosa* through Caco-2 cell monolayers. Inactivation of *P. aeruginosa* *dnaK* function may interfere with bacterial translocation and prevent septicaemia caused by *P. aeruginosa*.

**INTRODUCTION**

*Pseudomonas aeruginosa* is an opportunistic pathogen and a cause of infection-related mortality among individuals with compromised immune systems. Gut-derived sepsis in individuals with compromised immune systems frequently arises from penetration of *P. aeruginosa* strains through the intestinal barrier [1–6].

As described previously [7], the human adenocarcinoma cell line Caco-2, although isolated from an adult human colon, expresses several markers characteristic of normal small intestinal villus cells and is considered highly analogous to enterocytes of the foetal colon. Using Caco-2 cells, bacterial adherence, invasion and penetration have been studied in several bacterial species, such as *Salmonella*, *Vibrio cholerae*, *Escherichia coli*, *Klebsiella pneumoniae* and *P. aeruginosa*.

We recently identified critical factors responsible for *P. aeruginosa* penetration of polarized epithelial cell monolayers produced by Caco-2 cells (a standard model of the human small intestine mucosa) by analysing transposon insertion mutants that demonstrated a dramatic reduction in penetration activity relative to the *P. aeruginosa* PAO1 strain [8]. Depending on the genes affected, mutations can be grouped into the five following classes: flagellin-associated genes, pilis-associated genes, heat-shock protein genes, glycolytic pathway-related genes and biosynthesis-related genes. As described previously, among these mutants, we first focused on the *serA* mutant and found that inactivation of *serA* caused significant repression of bacterial penetration of Caco-2 cell monolayers, with decreased swimming and swarming motilities, bacterial adherence and fly mortality rate, as well as repression of ExoS secretion; however, twitching motility was not affected [8]. In the present study, we focused on the *dnaK* mutant. As previously described [9], DnaK (also known as heat-shock protein 70) is a bacterial molecular chaperone that plays key roles in the folding and refolding of denatured and aggregated proteins in cooperation with cochaperones DnaJ and GrpE. DnaK contributes to diverse cellular functions, including stress responses, cell division [10], motility [11] and pathogenesis [12]. Recently, it was reported that *P. aeruginosa* DnaK co-localized with the flagellum protein and nitrite reductase at the cell surface of *P. aeruginosa*, suggesting that DnaK serves as a stabilizer of the flagellum protein and nitrite reductase as a guide from the cytoplasmic to the outer membrane during flagellum assembly [13]. However, the association between this gene and penetration activity remains unknown. In the current study, we investigated whether *dnaK* is a key factor mediating *P. aeruginosa* penetration through Caco-2 cell monolayers. Furthermore, to evaluate whether *dnaK* is...
associated with PAO1 virulence, we used the insect infection model *Drosophila melanogaster*, which has been shown to be useful for evaluating the virulence traits of *P. aeruginosa* [8]. Here, we suggest that inactivation of *P. aeruginosa* *dnaK* function may interfere with bacterial translocation and prevent septicemia caused by *P. aeruginosa*.

**METHODS**

**Bacterial strains**

*Pseudomonas aeruginosa* strain PAO1 is regarded as a laboratory strain of *P. aeruginosa* and a whole genome sequence is available [14]; *P. aeruginosa* strain PAO1 is one of our laboratory stock strains [8] and was used as a standard strain that penetrates epithelial cell monolayers [7]. *E. coli* DH5α cells (genotype: *deor*, *endA1*, *gyrA96*, *hsdR17* (rK, mB), *phoA*, *recA1*, *relA1*, supE44, *thi-1*, *DlacZYA-argF*) U169, *φ80lacZAM15, F, A*) were purchased from TOYOBO (Osaka, Japan) and used as a control that does not penetrate epithelial cell monolayers [7, 8].

The PAO1Tn::*dnaK* (Δ*dnaK*) mutant and PAO1Tn::*pilB* mutant (Δ*pilB*) were prepared in our laboratory as described in detail previously [8]: the *dnaK* locus tag: PA4761; homologue in database: heat-shock protein DnaK; GenBank accession no. NP_253216.1) genes were specified through an analysis of transposon insertion sites using TOYOBO (Osaka, Japan) and used as a control that does not penetrate epithelial cell monolayers [7, 8].

The PAO1Tn::*dnaK* (Δ*dnaK*) mutant and PAO1Tn::*pilB* mutant (Δ*pilB*) were prepared in our laboratory as described in detail previously [8]: the *dnaK* locus tag: PA4761; homologue in database: heat-shock protein DnaK; GenBank accession no. NP_253449.1) and *pilB* locus tag: PA4526; homologue in database: type 4 fimbrial biogenesis protein PilB; GenBank accession no. NP_253216.1) genes were specified through an analysis of transposon insertion sites using a two-round arbitrary PCR protocol. To construct the plasmid used for *dnaK* complementation, a 2002 bp *HindIII*-*XbaI* fragment carrying the *P. aeruginosa* *dnaK* ORF was amplified by PCR using the 5-PA-*dnaK*-Hind3-pro-5349200 (5′-GAGAAAGCTTAATTCGACGCGACGA CCCCCA-3′) and 3-PA-*dnaK*-Xba1-end-5347198 (5′-AGAG TCTAGATTCTTGTTGGTCCTTGAACCT-3′) primers. The insert corresponds to nucleotides 5 347 198–5 349 200 in the PAO1 genome sequence (www.pseudomonas.com). The *HindIII*-*XbaI* fragment was cloned into the *HindIII*-*XbaI* site of *pUCP19* [15], and the resultant plasmid was designated pUCP19-*dnaK*. The pUCP19-*dnaK* plasmid was electroporated into PAO1Tn::*dnaK*, and the resulting transformant was designated PAO1Tn::*dnaK* (pUCP19-*dnaK*).

**Penetration assay**

We performed a penetration assay using Caco-2 cell monolayers on Transwell (Corning, NY, USA) plates at a m.o.i. of 100 as described previously [8]. The assay was performed in triplicate and the results expressed as the mean±SD. *P. aeruginosa* PAO1 and *E. coli* DH5α were used as positive and negative controls, respectively.

**Secretion assay of type III effectors**

Secretion of the type III effector ExoS can be induced *in vitro* by removing calcium from the medium [16]. A secretion assay was performed as described previously [16] with slight modifications. Bacteria were diluted 1:300 in high-salt lysogeny broth (LB; medium containing 200 mM NaCl, 10 mM MgCl₂ and 0.5 mM CaCl₂) and grown for 2 h, and then EGTA (5 mM, final concentration) was added to induce secretion. The cultures were allowed to grow for an additional 2 h and the bacteria were pelleted by centrifugation. Supernatant proteins were precipitated with 10% (final concentration) trichloroacetic acid. Pellets were washed three times with acetone and resuspended in PBS. After determining the protein concentration with a Bicinchoninic Acid Protein Assay Reagent kit (Thermo Fisher Scientific, Waltham, MA, USA), samples (8 μg protein) were separated by SDS-PAGE, transferred to Amersham Protran 0.45 μm nitrocellulose (GE Healthcare, Little Chalfont, UK), and probed with a rabbit anti-ExoS polyclonal antibody. The anti-ExoS polyclonal antibody was prepared as described previously [17].

**Detection of exotoxin A**

Caco-2 cells were seeded and grown in a 25 cm² tissue culture flask (AGC Techno Glass, Shizuoka, Japan). Bacteria were grown to mid-log growth phase in LB and introduced at an m.o.i. of 100 bacteria per Caco-2 cell, and the infections were allowed to proceed for 6 h at 37 °C and 5% CO₂. The bacteria were pelleted by centrifugation, and the supernatant proteins (5 μg) and exotoxin A standard (1.2 μg) from *P. aeruginosa* (List Biological Laboratories, Campbell, CA, USA) were separated by SDS-PAGE, transferred to Amersham Protran 0.45 μm nitrocellulose, and probed with a goat anti-exotoxin A (List Biological Laboratories) antibody. Western blotting was repeated three times, and bands were quantified with ImageJ software (ver. 1.45i) (NIH, Bethesda, MD, USA).

**Elastase activity assay**

The LasB elastase activity assay was performed as described previously [18] with slight modifications. Caco-2 cells were seeded and grown in 12-well tissue culture plates (AGC Techno Glass). Bacteria were grown to mid-log growth phase in LB and introduced at an m.o.i. of 10 bacteria per Caco-2 cell, and the infections were allowed to proceed for 6 h at 37 °C and 5% CO₂. LasB elastase activity in the culture supernatants of the test strains was determined by the elastin Congo red (ECR) assay as previously described [18]. Briefly, 0.75 ml of buffered (0.1 M Tris-HCl, [pH 8.0], 1 mM CaCl₂) ECR (Elastin Products, Owensville, MO, USA) at a concentration of 10 mg ml⁻¹ was mixed with 0.25 ml of culture supernatants that had been collected by centrifugation and filtered through a 0.2 μm filter. The mixture was incubated at 37 °C with shaking for 18 h. Any red colour in the supernatant due to the cleavage of ECR was detected at 492 nm after centrifugation of the reaction mixture at 3000 g for 10 min. As a mock control, a mixture containing 0.75 ml buffered ECR and 0.25 ml LB was used.

**Detection of PilA pilus protein**

Detection of PilA pilus protein was performed as described previously [19] with slight modifications. To isolate surface pili, a portion (100 μl) of a 2 ml LB culture grown with shaking for 18 h at 37 °C was plated onto LB agar and grown at
37 °C for 24 h. After growth, bacteria were scraped from the agar surface and resuspended in 6 ml of PBS to an OD₆₀₀ of 20. Cells were vortexed for 30 min to remove surface pili. The suspension was centrifuged at room temperature (20,000 g for 10 min), and the supernatant was collected and centrifuged (20,000 g for 10 min) to remove cellular debris. The resulting supernatant was incubated overnight at 4 °C in 100 mM MgCl₂ to precipitate the pili. The precipitate was collected by centrifugation at 4 °C (20,000 g, 20 min), and the pellet was resuspended in SDS Sample Buffer (Nacalai Tesque, Kyoto, Japan). Samples (10 µl) were separated by SDS-PAGE, transferred to Amersham Protran 0.45 µm nitrocellulose, and probed with a rabbit anti-PilA polyclonal antibody. The anti-PilA polyclonal antibody was prepared as described previously [20]. To isolate intracellular pili, a portion (100 µl) of a 2 ml LB culture grown with shaking for 18 h at 37 °C was plated onto LB agar and grown at 37 °C for 24 h. After growth, bacteria were scraped from the agar surface and resuspended in 6 ml of PBS to an OD₆₀₀ of 20. Cells were vortexed for 30 min to remove surface pili. The suspension was centrifuged at room temperature (20,000 g for 10 min), the supernatant was removed, and the cells were resuspended in SDS Sample Buffer. To detect cell-associated pili, 15 µg of total cell-associated protein was separated by SDS-PAGE and probed with an anti-PilA polyclonal antibody as described above.

**Bacterial adherence to Caco-2 cells**

Bacterial adherence to Caco-2 cells was performed as described previously [8]. Caco-2 cells were seeded and grown on sterile glass coverslips in four-well tissue culture plates (Nunc Lab-Tek II Chamber Slide System, Thermo Fisher Scientific). For the adherence assays, bacteria were grown to mid-log growth phase in LB and collected by centrifugation and diluted in Hank’s Balanced Salt Solution (HBSS) containing supplements (1 mM CaCl₂, 2 mM MgCl₂ and 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]). Bacteria were introduced at an m.o.i. of 100 bacteria per Caco-2 cell, and the infection was allowed to proceed for 1 h at 37 °C and 5 % CO₂. The Caco-2 cells were washed four times with 1 ml of HBSS containing supplements to remove unattached bacteria. Co-cultures were fixed with 2.5 % glutaraldehyde in PBS overnight at 4 °C and then in 5 % formaldehyde, 5 % glacial acetic acid and 70 % methanol for 1 h, followed by staining with Giemsa stain for 10 min. Coverslips were mounted on glass slides and viewed by light microscopy at ×1000 magnification. Eukaryotic cells and associated bacteria from six fields, representing different regions of the coverslip, were enumerated and reported as the number of bacteria per Caco-2 cell.

**Motility assays**

Swarming, swimming and twitching motility assays were performed as described previously [8]. Swarming-motility plates (0.5 % agar) and swim-motility plates (0.3 % agar) consisted of M8 media supplemented with 1 mM MgSO₄, 0.2 % glucose and 0.5 % casamino acids. LB overnight cultures (2 µl) were inoculated onto the surface of the swarm and swim plates and incubated for 12 h at 37 °C and for 14 h at 30 °C for the swarm and swim plates, respectively. The major axes of swarming and swimming were measured. Twitch-motility plates (1.5 % agar) were comprised of M8 media supplemented with 1 mM MgSO₄, 0.2 % glucose and 0.5 % casamino acids. Twitch plates were dried and strains were stab-inoculated with a toothpick to the bottom of the Petri dish using an LB agar plate that was grown overnight. After incubation for 24 h at 37 °C, the resulting zones of twitching motility were visualized by carefully removing the agar and staining bacteria that adhered to the polystyrene Petri dish with 1 % crystal violet for 10 min at room temperature. This was followed by brief rinsing with tap water to remove unbound dye. The major axes of twitching were measured.

**Staining of bacterial flagella**

Staining of bacterial flagella was performed as described previously [20]. Surface colonies grown on an LB agar plate were suspended directly in distilled water on the slides. After drying the slides, the slides were stained with Leifson stain (1 % tannic acid, 0.5 % sodium chloride, 0.3 % paraarabinol acetate, 0.1 % pararosaniline hydrochloride and 32 % ethanol) for 10 min and observed at ×1000 magnification after washing.

**Fly survival experiments**

Fly survival was evaluated as described previously [8]. Drosophila melanogaster flies (7–10 days old) were purchased from Sumika Technoservice Corporation (Takarazuka, Japan). A total of 24–30 flies in a plastic container were fed a 5 % sucrose suspension containing LB overnight cultures of each P. aeruginosa strain on a sterile paper disc. The flies were reared on the bacterium-sucrose mixture for the duration of the experiment. Flies were maintained at 25 °C and survival was monitored for 25 h.

**Statistical analysis**

Statistical analysis, except for fly survival analysis, was performed using a two-tailed Welch’s t-test. For fly survival analysis, the log-rank test was applied to evaluate differences between survival curves. All statistical analysis was conducted using EZR statistical software 1.28 [21].

**RESULTS AND DISCUSSION**

**Penetration activity of P. aeruginosa strains**

As described in detail previously [8], we identified 21 genes associated with bacterial translocation through Caco-2 cell monolayers. Of these 21 genes, we focused on dnaK to determine how DnaK contributes to P. aeruginosa’s ability to penetrate the intestinal epithelial cell barrier. First, there was no significant difference between the growth of the wild-type strain and PAO1Tn::dnaK mutant (P > 0.05) or between the growth of the PAO1Tn::dnaK mutant and complementary PAO1Tn::dnaK (pUCP19-dnaK) strain (P > 0.05) at all time-points tested after incubation in LB (Fig. 1a). However, the growth of the PAO1Tn::dnaK
mutant was always slightly weaker than that of the wild-type strain and the colony size of the PAO1Tn::dnaK mutant was consistently smaller than that of the wild-type strain. We performed penetration assays using PAO1, PAO1Tn::dnaK and complementary PAO1Tn::dnaK (pUC19::dnaK) strains. We infected Caco-2 cell monolayers with these variants to determine whether the dnaK gene is necessary for epithelial monolayer penetration in this strain. As shown in Fig. 1(b), penetration of the PAO1Tn::dnaK strain through Caco-2 cell monolayers showed a 99.995% reduction compared to the PAO1 strain at 6 h after infection; this difference was significant (P<0.05). The decrease in the ability of PAO1Tn::dnaK to penetrate the monolayers was largely restored in the PAO1Tn::dnaK (pUC19::dnaK) complemented strain (Fig. 1b; P<0.05).

**Type III effector secretion**

The ADP-ribosylating domain of the type III effector ExoS was previously found to be associated with the permeability properties of polarized airway epithelial cells with intact tight junctions; strains expressing type III effectors altered the distribution of the tight junction proteins, zonula occludens-1 and occludin, and could transmigrate across polarized airway epithelial monolayers. These effects on epithelial permeability were associated with the ADP-ribosylating domain of ExoS, as bacteria expressing plasmids lacking expression of ExoS GAP activity showed increased permeation of bacteria across polarized airway epithelial cells [22]. Next, we investigated the influence of the dnaK mutation on ExoS expression.

As shown in Fig. 2(a), secretion of ExoS in the culture supernatant was not detected in the PAO1Tn::dnaK mutant compared to in the wild-type strain. Complementation of dnaK in the PAO1Tn::dnaK mutant resulted in the recovery of ExoS secretion compared to in the PAO1Tn::dnaK mutant, although the expression level of recovery was lower than that in the wild-type strain. In addition, secretion of ExoT in the culture supernatant was not observed in the PAO1Tn::dnaK mutant compared to in the wild-type strain, and complementation of dnaK in the PAO1Tn::dnaK mutant resulted in recovery of ExoT secretion compared to in the PAO1Tn::dnaK mutant.

For the type III effector ExoS, the dnaK mutation resulted in drastic repression of ExoS secretion in the current study. This result agrees with published results; two heat-shock proteins, DnaK and HtpG, were found to be required for

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**Fig. 1.** Penetration activity of *P. aeruginosa* strains. Penetration activity was determined as described previously [8]. (a) Comparison of growth between the wild-type strain (WT), PAO1Tn::dnaK mutant (ΔdnaK), and PAO1Tn::dnaK (pUC19::dnaK) complementary strain (+dnaK). Viable cells of WT, ΔdnaK and +dnaK at 0, 1, 3, 6, 18 and 24 h after incubation in LB were compared. The assay was performed in triplicate and the results are expressed as the mean±SD. A significant difference was not observed between WT and ΔdnaK (P>0.05) or between ΔdnaK and +dnaK (P>0.05) at all time-points tested. (b) Penetration activity of WT, ΔdnaK and +dnaK. WT, ΔdnaK and +dnaK were evaluated by inoculation onto the apical surfaces of Caco-2 cell monolayers at an m.o.i. of 100 and the number of bacteria in the basolateral media were counted at 6 h after infection. The assay was performed in triplicate and the results are expressed as the mean±SD. E. coli DH5α were used as a negative control. Significant differences were observed between WT and ΔdnaK (*: P<0.05), ΔdnaK and +dnaK (**: P<0.05), and WT and DH5α (*: P<0.05). Significant difference was not observed between WT and +dnaK (P>0.05).

**Fig. 2.** Western blot analysis to detect secretion of type III effectors in culture supernatant proteins (6 µg) of wild-type strain (WT); PAO1Tn::dnaK mutant (ΔdnaK) and PAO1Tn::dnaK (pUC19::dnaK) complementary strain (+dnaK) under TTSS (type III secretion system)-inducing conditions. (a) Western blot analysis to detect secretion of ExoS. The arrow shows the presence of ExoS with a deduced molecular weight of 48 kDa. (b) Western blot analysis to detect secretion of ExoT. The arrow shows the presence of ExoT with a deduced molecular weight of 48 kDa.
the expression or secretion of ExoS, and the loss of these genes may cause protein misfolding and affect the assembly or function of the T3SS apparatus [23]. However, in transposon mutant screening to identify a mutant showing a larger reduction in penetration ability, the exoS mutant was not detected [8]. In addition, Soong et al. previously reported that the exoS mutation caused a reduction in the bacterial transmigrational ability of the parental strain PAK through 16HBE cell monolayers, but the degree of reduction was low at approximately 50% [22]. Together, these results suggest that dnaK is associated with the regulation of ExoS secretion, but the contribution of ExoS to penetration ability mediated by DnaK may also be lower than that of bacterial motilities including swarming, swimming and twitching.

Production of elastase and exotoxin A

As investigated in detail previously, Azqhani et al. reported that elastase disturbs the barrier function of epithelial monolayers, in part by changing the cell architecture and altering at least one protein of the zonula occludens [24]. Furthermore, transepithelial resistance of the Caco-2 cell monolayer infected with P. aeruginosa PAO1 decreased in a time-dependent manner, while transepithelial resistance values for the Caco-2 cell monolayer infected with exotoxin A mutants were significantly higher than those determined after infection with the parent strain [7]. Next, we investigated the influence of the dnaK mutation on the production of elastase and exotoxin A.

As shown in Fig. 3(a), LasB elastase activity in the Caco-2 culture supernatants showed a 53.3% reduction in the PAO1Tn::dnaK mutant compared to in the wild-type strain, and the difference was significant (P<0.05). Further, production of ToxA in the culture supernatant of the PAO1Tn::dnaK mutant was repressed compared to in the wild-type strain (Fig. 3b).

The dnaK mutant showed significant repression of elastase and exotoxin A secretion, but in the transposon mutant screening to identify a mutant showing a larger reduction in penetration ability, elastase and exotoxin A mutants were not detected [8]. These results suggest that dnaK is associated with elastase and exotoxin A secretion, but the contribution of elastase and exotoxin A to penetration ability mediated by DnaK may be lower than that of bacterial motilities, including swarming, swimming and twitching.

Motility assays

We previously reported that inactivation of serA results in significant repression of bacterial penetration through Caco-2 cell monolayers with decreased swimming and swarming motilities, while twitching motility was not affected [8]. Here, we evaluated the influence of the dnaK mutation on bacterial motilities.

As shown in Fig. 4(a), the swimming motility of the PAO1Tn::dnaK mutant was significantly reduced compared to the wild-type strain (P<0.05). In addition, complementation with dnaK in the PAO1Tn::dnaK mutant resulted in

![Graph](image1)

**Fig. 3.** Analysis to detect toxin secretion of wild-type strain (WT), PAO1Tn::dnaK mutant (ΔdnaK) and PAO1Tn::dnaK (pUCP19-dnaK) complementary strain (+dnaK). (a) LasB elastase activity in the Caco-2 culture supernatants of the test strains was determined by the ECR assay. The assay was performed in triplicate and the results are expressed as the mean±SD. A significant difference was observed between WT and ΔdnaK (**: P<0.05), and ΔdnaK and + dnaK (*: P<0.05). (b) Western blot analysis to detect exotoxin A in bacterial supernatant proteins (5 μg). A representative Western blot image is shown. Exotoxin A standard (1.2 μg) from P. aeruginosa was used as a positive control. The arrow shows the presence of exotoxin A with a deduced molecular weight of 69 kDa. Expression ratio of exotoxin A based on Western blot analyses of WT, ΔdnaK and + dnaK. Photos are representative examples. Western blotting was repeated three times and bands were quantified with ImageJ. Data are shown as the ratio of exotoxin A expression to that of WT and expressed as the mean±SD. A significant difference was observed for exotoxin A expression between WT and ΔdnaK (**: P<0.05), and ΔdnaK and + dnaK (*: P<0.05).

significant recovery of swimming motility (P<0.05) (Fig. 4a). Swimming motility in the PAO1Tn::dnaK mutant was also significantly reduced compared to in the wild-type strain (P<0.05). Complementation with dnaK in the PAO1Tn::dnaK mutant resulted in significant recovery of swimming motility (P<0.05) (Fig. 4b). Furthermore, twitching motility of the PAO1Tn::dnaK mutant was also
significantly reduced compared to the wild-type strain \((P<0.05)\) (Fig. 4c). Complementation with \(dnaK\) in the PAO1Tn::\(dnaK\) mutant also resulted in significant recovery of twitching motility \((P<0.05)\) (Fig. 4c).

**Staining of bacterial flagella**

As shown in Fig. 4(a, b), swimming and swarming motilities were significantly reduced in the PAO1Tn::\(dnaK\) mutant; thus, we predicted that bacterial flagella were absent in the

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**Fig. 4.** Motility assays. Motility assays were performed as described in detail previously [8]. (a) Swimming motility of the wild-type strain (WT), PAO1Tn::\(dnaK\) mutant (\(\Delta dnaK\)), PAO1Tn::\(dnaK\) (pUCP19-\(dnaK\)) complementary strain (+\(dnaK\)) and \(E. coli\) DH5\(\alpha\) (as a negative control). A representative image from the swimming motility assay is shown. The major axis of swimming is the longest length of the swimming area. The assay was performed in triplicate and the results are expressed as the mean±SD. Significant differences were observed between WT and \(\Delta dnaK\) (*: \(P<0.05\)), \(\Delta dnaK\) and +\(dnaK\) (#: \(P<0.05\)), and WT and DH5\(\alpha\) (*: \(P<0.05\)). (b) Swarming motility in the WT, \(\Delta dnaK\), +\(dnaK\) and \(E. coli\) DH5\(\alpha\) (as a negative control). A representative image of the swarming motility assay is shown. The major axis of swarming is the longest length of the swarming area. The assay was performed in triplicate and the results are expressed as the mean±SD. Significant differences were observed between WT and \(\Delta dnaK\) (*: \(P<0.05\)), \(\Delta dnaK\) and +\(dnaK\) (#: \(P<0.05\)), and WT and DH5\(\alpha\) (*: \(P<0.05\)). (c) Twitching motility in WT, \(\Delta dnaK\), +\(dnaK\) and \(E. coli\) DH5\(\alpha\) (as a negative control). A representative image of the twitching motility assay is shown. The major axis of twitching is the longest length of the twitching area. The assay was performed in triplicate and the results are expressed as the mean±SD. Significant differences were observed between WT and \(\Delta dnaK\) (*: \(P<0.05\)), \(\Delta dnaK\) and +\(dnaK\) (#: \(P<0.05\)), and WT and DH5\(\alpha\) (*: \(P<0.05\)).
PAO1Tn::dnaK mutant. To investigate whether bacterial flagella were lost in the PAO1Tn::dnaK mutant, we stained the bacterial flagella. As shown in Fig. 5, bacterial flagella were observed in the PAO1Tn::dnaK mutant, wild-type strain and PAO1Tn::dnaK (pUCP19-dnaK) complementary strain, but not the ΔflgE strain used as a negative control. As a result, despite the decreased swimming and swarming motilities, flagella were still present in the PAO1Tn::dnaK mutant. Similar results were recently reported for the motAB mutant of atypical enteropathogenic E. coli (αEPEC); the presence and function of flagella in the wild-type αEPEC strain and its isogenic mutant deficient in motAB were evaluated by negative staining and motility assays in semisolid agar (0.3%). Negative-staining analysis revealed that the motAB mutant strain produced flagella, whereas the isogenic motAB mutants were non-motile [25]. As described in the same study, the flagellar apparatus is associated with the MotA and MotB proteins, which form the stator, a membrane pore channel essential for generating the proton motive force required for flagellar motility [25]. Overall, we hypothesize that the dnaK mutation might impair the motAB gene in P. aeruginosa. Further studies are required to confirm this hypothesis.

**Detection of PilA pilus protein**

As shown in Fig. 4(c), twitching motility was significantly reduced in the PAO1Tn::dnaK mutant; we predicted that the PilA pilus protein was absent in the PAO1Tn::dnaK mutant. To investigate whether the PilA pilus protein on the cell surface was lost in the PAO1Tn::dnaK mutant, we conducted Western blot analysis for the PilA protein. As shown in Fig. 6, the PilA pilus protein on the cell surface was detected in the PAO1Tn::dnaK mutant, wild-type strain and PAO1Tn::dnaK (pUCP19-dnaK) complementary strain, but not the ΔpilB strain used as a negative control. However, the intracellular PilA protein was detected in all strains tested. Thus, despite decreased twitching motility, the PilA protein was still detected in the PAO1Tn::dnaK mutant.

**Inhibitory effect of dnaK mutation on Caco-2 adherence**

Adherence to Caco-2 cells by the PAO1Tn::dnaK mutant was significantly reduced by 69.8% compared to that of the wild-type strain (P<0.05) (Fig. 7). Furthermore, complementation with dnaK in the PAO1Tn::dnaK mutant resulted in the recovery of this phenotype (Fig. 7). Bacterial adherence to Caco-2 cells with E. coli DH5α also showed a significant 85.4% reduction compared to that of the wild-type strain, as previously reported [8] (P<0.05) (Fig. 7).

**Association between dnaK and PAO1 virulence in flies**

The virulence of the PAO1Tn::dnaK mutant was significantly attenuated compared to that of the wild-type strain, and dnaK complementation in mutant bacteria resulted in a significantly increased mortality rate, although the virulence level of recovery was lower than that of the wild-type strain (Fig. 8). This suggests that dnaK is required for P. aeruginosa virulence in flies. Therefore, we hypothesized that the high mortality rate observed in flies following oral infection of the wild-type strain depends on the ability to efficiently penetrate the mid-gut barrier, which is dependent on dnaK.

As described previously, DnaK from E. coli is a molecular chaperone that assists in the correct folding and assembly of proteins and is involved in diverse cellular processes including DNA replication, UV mutagenesis, bacterial growth, RNA transcription and flagella synthesis, and can bind to nascent polypeptide chains and assist in protein refolding and degradation [10, 26]. Furthermore, quantitative

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Fig. 5. Staining of bacterial flagella. Flagella staining of the wild-type strain (WT), PAO1Tn::dnaK mutant (ΔdnaK), PAO1Tn::dnaK (pUCP19-dnaK) complementary strain (+dnaK) and PAO1Tn::flgE mutant (ΔflgE) was performed as described previously [8]. A total of seven fields were observed for each strain and a representative image is shown.
proteomics revealed that DnaK from *E. coli* could interact with ~700 mostly cytosolic proteins, including ~180 relatively aggregation-prone proteins that utilize DnaK extensively during and after initial folding [27]. For DnaK interactors identified by Calloni *et al.* [27], *serA*, *aroA*, *aceE*, *fruR*, *fliD* and *flgM*, which were detected in our previous study as bacterial genes associated with the penetration of *P. aeruginosa* through Caco-2 cell monolayers [8], were included, strongly suggesting that DnaK controls the function of *serA*, *aroA*, *aceE*, *fruR*, *fliD* and *flgM* by binding to and stabilizing these gene products directly. Particularly, we previously reported that inactivation of *serA* causes significant repression of bacterial penetration through Caco-2 cell monolayers with decreased swimming and swarming motilities, bacterial adherence and fly mortality rate, as well as the repression of ExoS secretion; however, twitching motility was not affected [8]. Characteristics except for the twitching motility of the *dnaK* mutant were very similar to those of the *serA* mutant, which supports the hypothesis that DnaK directly interacts with SerA and stabilizes SerA protein. However, decreased twitching motility was observed in the *dnaK* mutant but not in the *serA* mutant, and the degree of repression of bacterial penetration through Caco-2 cell monolayers was more than 100-fold higher with the *dnaK* mutant than with the *serA* mutant. This suggests that DnaK is associated with the penetration activity of *P. aeruginosa* through Caco-2 cell monolayers not only by binding to SerA, but also by interacting with other proteins. Further studies are required to clarify the other DnaK-binding proteins associated with the penetration activity of *P. aeruginosa* through Caco-2 cell monolayers.

Overall, *dnaK* regulated many cellular functions, including the ability to penetrate Caco-2 cells. Inactivation of the *P. aeruginosa* *dnaK* function may interfere with bacterial translocation; *serA* may also be involved, whose function we recently reported [8].

**Fig. 7.** Bacterial adherence to Caco-2 cells for wild-type strain (WT), PA01Tn::*dnaK* mutant (Δ*dnaK*), PA01Tn::*dnaK* (pUCP19-*dnaK*) complementary strain (+*dnaK*) and *E. coli* DH5α (as a negative control). Bacterial adherence was determined based on the number of adhered cells prepared with six replicates and the results are expressed as the means±SD. Significant differences were observed between WT and Δ*dnaK* (*: P<0.05), and WT and DH5α (*: P<0.05). Bacterial adherence of the +*dnaK* complementary strain was significantly restored compared to that of Δ*dnaK* (#: P<0.05).

**Fig. 8.** Fly survival experiments. Virulence of the wild-type strain (WT), PA01Tn::*dnaK* mutant (Δ*dnaK*), PA01Tn::*dnaK* (pUCP19-*dnaK*) complementary strain (+*dnaK*) and *E. coli* DH5α (as a negative control) was evaluated as described previously [8]. Significant differences, based on the log-rank test, were observed between WT and DH5α (*: P<0.05) and WT and Δ*dnaK* (*: P<0.05). Viability of the +*dnaK* complementary strain was significantly restored compared with that of Δ*dnaK* (#: P<0.05).

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**Conflicts of interest**
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