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Dithiothreitol Attenuates the Pathogenic Interaction Between *Pseudomonas aeruginosa* and *Drosophila melanogaster*

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Abstract Infection of *Drosophila melanogaster* adults with *Pseudomonas aeruginosa* (PA14) can kill the flies within 48 h. We found that the virulence of PA14 was significantly attenuated when treated with a reducing agent, dithiothreitol (DTT), prior to infection. Infection with DTT-treated PA14 elevated Metchnikowin expression at 22 h post-infection and the virulence of DTT-treated PA14 was not attenuated in *Dif* and *Relish* mutants. These results suggest that DTT pre-treatment of PA14 can aggravate certain virulence factors that may be required to paralyze fly immune responses, triggering Metchnikowin expression via Dif and Relish activations.

Key words: Redox, *Pseudomonas*, *Drosophila*, virulence, dithiothreitol, immune response

The studies on the signal transduction cascades underlying innate immunity in *Drosophila melanogaster* have revealed remarkable similarities to those in mammals [12, 17]. In both flies and mammals, Toll family receptors and their associated kinases are required to signal the presence of microbes, and Rel family transactivators are responsible for the synthesis of inducible effector proteins such as antimicrobial peptides in flies and cytokines and tumor necrosis factors in mammals [7]. A characteristic feature of the activation of Rel family transactivators, for instance, mammalian NF-κB, is the posttranslational mechanism involving the dissociation of the inhibitory protein, IκB [1, 26]. Agents known to induce NF-κB activation include various cytokines, phorbol esters, and viral transactivators. Recent studies have proposed that the formation of reactive oxygen species (ROS) or the intracellular redox levels may be the common denominator of the diverse NF-κB-activating signals. This integrative role of ROS has been suggested, since NF-κB can be activated by hydrogen peroxide (H_2O_2) and activation by various stimuli is commonly inhibited by antioxidants [21, 25].

In addition to these relatively recent discoveries of their regulatory roles in the immune pathways, ROS have long been proposed as having important functions in killing infectious agents like bacteria during immune responses. It is most likely that ROS or the presence of exogenously provided redox-active antibiotics could directly interfere with bacterial factors by damaging their structures or indirectly interfere by altering redox potentials within the bacterial cells [30]. To overcome these kinds of stressful conditions that the hosts may provide, bacterial pathogens have evolved a battery of redox or stress response-related proteins as virulence factors, for example, anti-oxidant defense enzymes [2, 29], and the periplasmic function proteins including extracytoplasmic function (ECF) sigma factors [3, 11].

Pseudomonas aeruginosa is a well-known opportunistic pathogen that primarily causes fatal infections in immuno-compromised patients such as cancer patients under chemo- or radiation therapies, patients with severe burns or cystic fibrosis, and patients receiving bone marrow transplantation. This Gram-negative bacterium is extremely widespread in nature, existing even in polluted environments [22], and pathogenically interacts with diversified non-mammalian counterparts including plants [23], nematodes [27], insects [4, 14], slime molds [5], and mycelial fungi [13]. By exploiting the interactions between this multi-host pathogen and non-mammalian hosts, many of its virulence factors have been newly identified, including DegP and DsbA which are proposed as playing important roles in redox-related stress responses [9, 20, 31].

To elucidate the impacts of conserved redox-related mechanisms on the interface between bacterial virulence factors and host immune responses, this study has utilized the pathogenic interaction between *P. aeruginosa* and *D. melanogaster* and tested for the effects of the several redox-active chemicals and enzymes on their interaction. The antagonistic effect of a strong reducing agent,

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dithiothreitol (DTT) on *P. aeruginosa*-induced killing and immune responses of *D. melanogaster* is also described. We propose that DTT may antagonize certain virulence factors that are required to paralyze the concerted involvement of Rel factors, Dif and Relish and an antimicrobial peptide, Metchnikowin, in the immune responses against *P. aeruginosa* infection.

MATERIALS AND METHODS

Bacterial strains and Culture Conditions

Escherichia coli DH5α and *Pseudomonas aeruginosa* UCBPP-PA14 were grown at 37°C using Luria-Bertani (LB) medium as described elsewhere [16].

Drosophila melanogaster Stocks and Culture Conditions

D. melanogaster Oregon R was grown at 25°C using corn meal-dextrose medium [0.93% agar, 6.24% dry yeast, 4.08% corn meal, 8.62% dextrose, 0.1% methyl paraben, 0.45% (v/v) propionic acid]. Loss-of-function homozygous mutants for *Relish* (*e Relish*^{E20}) and *Dif* (*y w DD1; cn bw Dif*^{1}) were used [10, 24].

D. melanogaster Infection and Mortality Assay

Infection of flies was performed by pricking 3- to 6-day-old adult flies in the dorsal thorax with a 10-μm needle (Ernest F Fullam) dipped halfway into a 10 mM $MgSO_4$-diluted bacterial suspension containing 10^7 cfu/ml from an early stationary phase (OD_{600}=3.0) culture. We consistently introduced 50-200 bacterial cells per fly (i.e. 5-20 nl). Fly mortality was monitored for up to 72 h post-infection. Flies that died within 12 h post-infection (less than 5%) were not included in the mortality determination. Mortality studies were repeated at least five times. Similar results were obtained each time, and the mortality percentage was calculated based on the total of five independent experiments. For viable bacterial determination, infected flies were collected at various time intervals, ground in LB, and plated on LB agar plates. To assess the growth *in vitro*, cells were inoculated into fresh LB with the initial number being ~10^4 cfu/ml. Cells reached the maximal OD_{600} of ~6, implying ~5-log proliferation within 15 h *in vitro*.

DTT Treatment of *P. aeruginosa*

DTT pre-treatment was a 1 h incubation of bacterial cells in the presence of 100 mM DTT, followed by centrifugation to discard the DTT solution. The cell pellet was resuspended in the same volume of 10 mM $MgSO_4$, washed once, and used for infection experiments.

RNA Isolation and Northern Analysis

Appropriately infected male flies were collected at the indicated time intervals for total RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen). Total RNA aliquots (20 μg) were separated on 1.2% agarose/formaldehyde denaturing gel, and transferred to a Hybond-XL nylon membrane (Amersham-Pharmacia). The PCR products corresponding to the internal regions of the *AttA*, *Drs*, *Dpt*, *Mtk*, *sp4*, and *rp49* were cloned into pCR2.1-TOPO vector (Invitrogen). Inserts were digested with *Eco*RI, gel-purified using a Gel Extraction kit (Qiagen), and labeled using a Rediprime II random priming labeling kit (Amersham-Pharmacia). A labeled *rp49* probe was used to normalize RNA expression levels by a phospho-image analyzer (Fuji).

RESULTS

P. aeruginosa Virulence is Attenuated by DTT Pre-Treatment in *D. melanogaster* Model

We tested for the effect of several oxidants and reductants on the growth and the virulence of *P. aeruginosa* UCBPP-PA14 (PA14) strain by pre-treating bacteria with the chemicals before injecting the bacteria into a fly, *D. melanogaster* Oregon R (data not shown). It was found that dithiothreitol (DTT) pre-treatment caused the dramatic decrease in fly mortality accompanied by delayed fly killing (Fig. 1).

Proliferations of DTT-treated PA14 were measured to examine whether this treatment restricted the growth of PA14 *in vivo* (in flies) and *in vitro* (in LB). The number of viable bacteria in PA14-infected flies proliferated rapidly by increasing approximately 4 logs within 24 h post-infection (Fig. 2B). In contrast, the growth of 100 mM

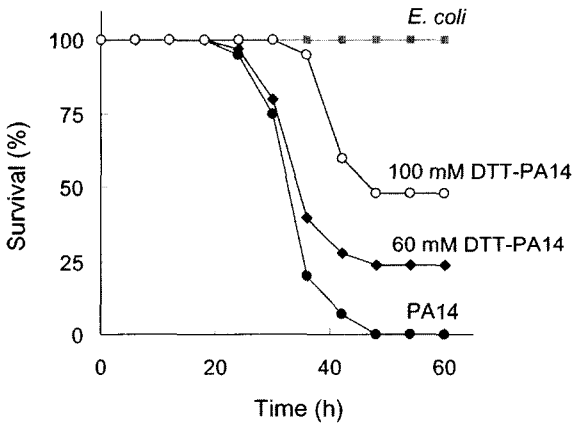

Fig. 1. DTT pre-treatment effect on *P. aeruginosa* virulence in *D. melanogaster*.

Survival rates of adult Oregon R flies were determined following infection with PA14 cells that had been pre-treated with water (●), 60 mM DTT (◆), or 100 mM DTT (○) as described in Materials and Methods. Five hundred flies were infected with a needle dipped in each bacterial suspension and incubated at 25°C and the survival was measured over time. No deaths were observed of flies injured with a needle dipped in either 10 mM $MgSO_4$ or *Escherichia coli* (■).

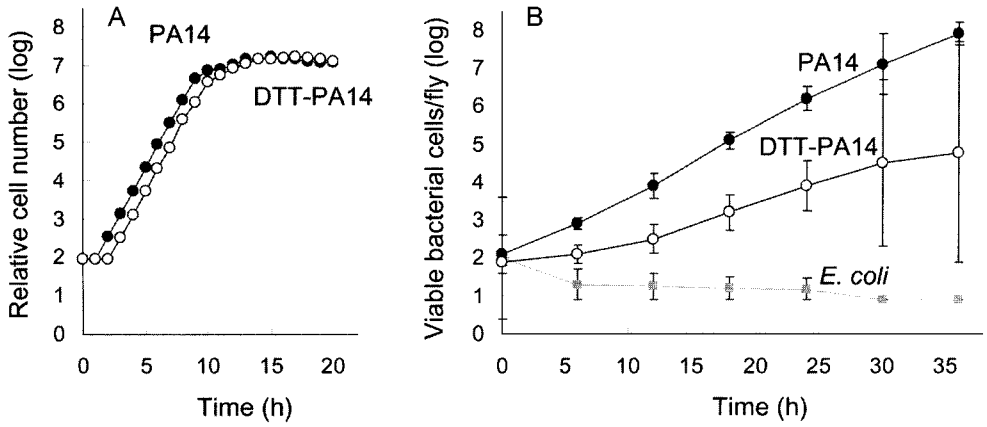

Fig. 2. DTT pre-treatment effect on *P. aeruginosa* proliferation in *D. melanogaster.*
(A) Growth of PA14 in LB after DTT pre-treatment was determined by viable counts and OD_{600} measurements at logarithmic scale. One OD_{600} unit corresponds to ~3×10^8 cfu/ml, slightly varying according to the growth phases. PA14 cells treated with either 100 mM DTT or water were used to inoculate LB (~10^4 cfu/ml). The relative number of cells were log(cell number/ml) minus 2 to be set as ~2 at the zero time point for the comparison with the numbers in B. Symbols: ●, PA14; ○, DTT-treated PA14. (B) Growth of PA14 in flies after DTT pre-treatment was determined by viable counts at logarithmic scale. Homogenates of batches of six flies were made at various time points after infection. Homogenates were plated on LB agar to determine viable bacterial cell counts. *E. coli* infection was included as a nongrowing control in the flies. The means of six flies per time point ± standard deviation are shown. Three independent experiments gave similar results. Symbols: ●, PA14; ○, DTT-treated PA14; ■, *E. coli.*

DTT-treated PA14 in flies was restricted over time, with no significant bacterial proliferation observed in fly tissues for the first 12 h post-infection. Even at 24 h post-infection, DTT-treated PA14 titers were significantly lower than those of untreated PA14, which is in contrast to the proliferation *in vitro* (Fig. 2A). The growth *in vitro* was affected by DTT-treatment, resulting in a slightly longer growth lag period (2 h 12 min vs. 1 h 20 min). However, no differences were observed in the specific growth rate (although much higher than the growth rate *in vivo*) and in the capability to reach the maximal saturation level. Therefore, the growth impairment of DTT-treated PA14 *in vivo* is specific to host environments, although it might be related to the longer growth lag by the DTT pre-treatment as verified *in vivo* as well as *in vitro*.

DTT Pre-Treatment Effect on Antimicrobial Peptide Expressions

We measured the level of accumulation of mRNA encoding the antimicrobial peptides such as Diptericin, Attacin, Metchnikowin, and Drosomycin following PA14 infection either with or without DTT pre-treatment (Fig. 3). Injury alone increased the transcription of all the four antimicrobial peptides, although the levels were lowered at 22 h post-injury. The steady state mRNA levels of Diptericin and Attacin were rapidly elevated (~4 fold at 1 h post-infection), whereas those of Drosomycin and Metchnikowin were relatively delayed (less than two-fold at 1 h post-infection). However, at 22 h post-injury, the mRNA levels of all four antimicrobial peptides were lower than those at 6 h, implying the recovery from the wound and a gradual decrease to basal levels.

When PA14 was administered, the levels at 1 h and 6 h were no higher than those of the controls. However, there was some RNA degradation at 22 h, which might reflect apparent fly death after ~20 h post-infection. The transcription levels at 22 h remained high, due probably to the presence of PA14 cells grown in flies. The levels of Diptericin and Attacin at 22 h were significantly higher than those at 6 h, whereas the levels of Drosomycin and Metchnikowin at 22 h were no higher, but lower than those at 6 h.

The flies infected with DTT-treated PA14 showed similar patterns and levels of expression compared with the flies infected with untreated PA14 except for the unexpected lower expression of Attacin at 6 h and higher expression of Metchnikowin at 22 h. Whereas pricking and untreated-PA14 infection gave ~3.5- and ~4.5-fold higher levels of Metchnikowin at 22 h, respectively, compared with that in the non-infected control, infection with DTT-treated PA14 gave a ~6.2-fold higher level at 22 h (Fig. 3B). This higher-level expression of Metchnikowin by DTT-attenuated PA14 implies that the Metchnikowin expression might be one of the targets for PA14 virulence factors.

DTT Attenuation Requires Host Factors, Dif and Relish

To test whether or not DTT pre-treatment affects the PA14-induced immune signaling cascade to Rel transactivators in the *P. aeruginosa*–*D. melanogaster* interaction, the Rel protein-deficient mutant flies were infected with DTT-treated PA14. PA14-induced killing of *Relish* mutants ($Relish^{E20}$) began ~4 h earlier compared to that of Oregon R (Fig. 4A). On the other hand, the survival of *Relish* flies

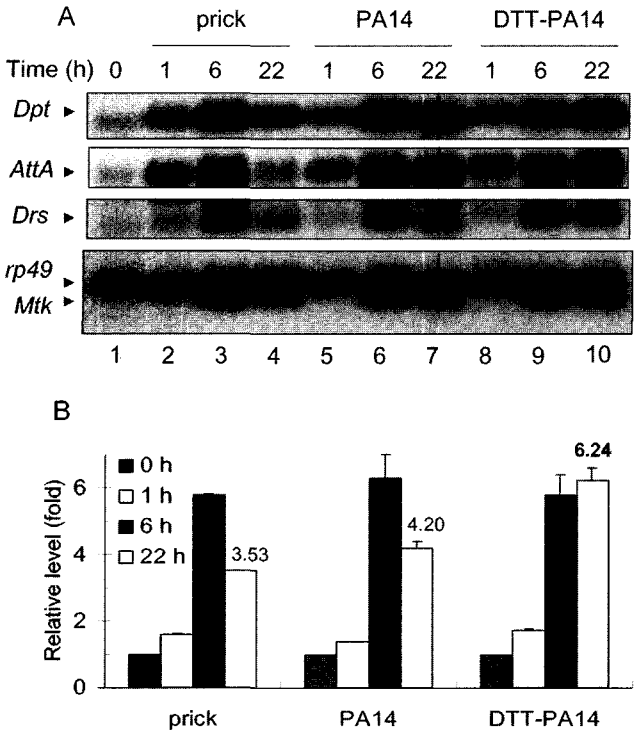

Fig. 3. Transcriptional profiles of four antimicrobial peptide genes following infection with DTT-treated *P. aeruginosa*.
(A) Northern blot analysis using total RNA extracted at different time intervals (1, 6, and 10 h) after pricking 30 flies with nothing (prick), PA14, and DTT-treated PA14 (PA14+DTT). Injection of 100 mM DTT gave the same results as pricking. Noninfection control is represented by 0 h. The same RNA blot was hybridized sequentially using the probes for antimicrobial peptide genes as indicated on the left: *AttA*, *Attacin A*; *Dpt*, *Diptericin*; *Drs*, *Drosomycin*; *Mtk*, *Metchnikowin*. The *rp49* was used as the loading control. (B) The signals of *Mtk* transcriptions in A were quantified using a phospho-imager system. The values of the *Mtk* signal were normalized with the corresponding values of the *rp49* signal. The levels of expression in noninfected wild-type flies were standardized as 1.0 and the results are given as a relative level (i.e. fold induction). Three independent experiments gave equivalent results, and the standard deviations are shown for each bar.

was not affected by DTT attenuation of PA14. *Dif* mutants (*Dif*^{1}) were more susceptible to PA14 infection (~2 h acceleration) than the isogenic wild-type, cn bw (*y w DD1; cn bw*) that was slightly more resistant (~2 h delay) than Oregon R. No DTT attenuation was observed in *Dif* flies either, as in *Relish* flies. These results lead to the hypothesis that Dif (Toll pathway) and Relish (Imd pathway) are important in the full resistance to infection with PA14 as well as DTT-attenuated PA14.

DISCUSSION

Mechanical injury is inevitable when the flies are artificially infected with bacteria that are not natural pathogens, and injury itself can trigger the fly immune responses. A high amount of highly immunogenic bacteria should be applied to infection to overcome the injury effect. In this present study, the infection conditions for pathogenic *P. aeruginosa* were optimized by introducing 50–200 cells of the early stationary phase. The outcome was that the fly deaths began only after ~20 h post-infection, and this came after the injury-triggered antimicrobial peptide synthesis had sufficiently diminished. In contrast to previous results that used different *P. aeruginosa* strains and probably different initial cell growth phase and numbers, where at most 15% of infected flies seemed to survive at the similar time points [6, 8, 28], it can be seen that more than 80% of infected flies were alive. This fact can be used to assess the *in vivo* interaction between the growing pathogens and

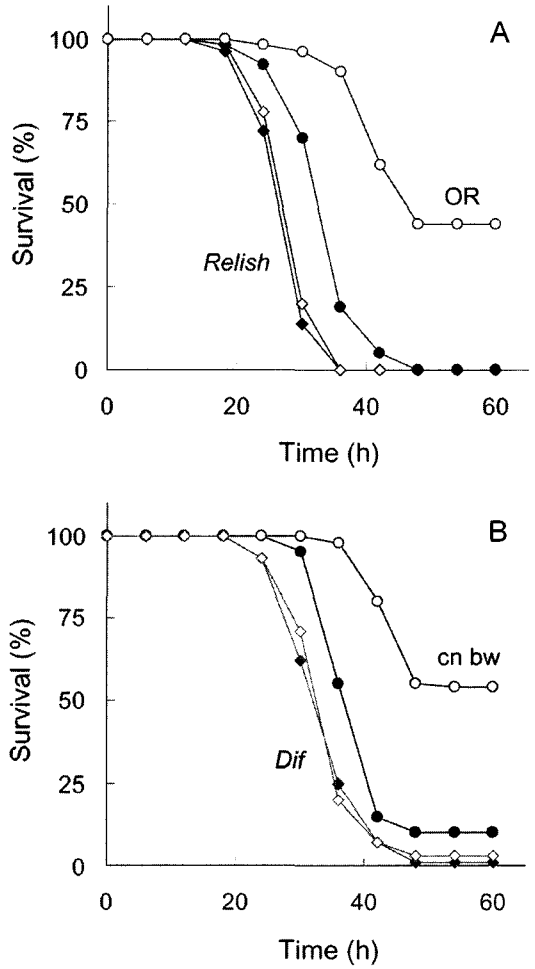

Fig. 4. Susceptibility of Rel transactivator mutants to DTT-treated *P. aeruginosa*.
Bacteria were introduced into flies as in Fig. 1. Rel transactivator mutants (*Relish* and *Dif*) of *D. melanogaster* were used. cn bw represents *y w DD1; cn bw*, which is isogenic to *Dif*^{1} [24]. Survival rates were determined up to 60 h after infection with PA14 cells that had been pre-treated with (empty) or without (filled) 100 mM DTT. The values are the averages of five replicate experiments, each with 20 flies. Symbols in A: ◆ and ◇, *Relish*; ● and ○, Oregon R (OR). Symbols in B: ◆ and ◇, *Dif*; ● and ○, cn bw.

immunity factors. Furthermore, this new condition indicates that some flies survive DTT-treated PA14 infection even after 72 h post-infection, whereas no bacterial cells were recovered at all. These observations of the percentage of mortality (%) give a new indicator of virulence potential in addition to the survival curve itself. We suggest that the percentage of mortality can be used as a new measurement of the PA14 virulence in this new infection condition, since many of the less virulent mutants relevant to mammalian pathogenesis have lower (less than 50%) mortality in *D. melanogaster* [18].

Among the antimicrobial peptides, Metchnikowin expression was decreased not slightly but significantly due to *P. aeruginosa* infection, assuming that the paralyzing effect of the virulent PA14 on the fly defense responses was impaired by DTT-affected signaling, eliciting the induction of Metchnikowin at the later phase of infection. This finding indicates that the virulence attenuation by DTT pre-treatment could be associated with a counterstrategy of *P. aeruginosa* to evade the *D. melanogaster* immune responses, and that Metchnikowin might play a role in restricting *P. aeruginosa* infection.

Our findings suggest that PA14 virulence factors target at least one of the components of the Toll pathway, Dif, which is in accordance with the recent observation by Lau *et al.* [18]. In support of this notion, our results also show that the expression of Metchnikowin is substantially reduced at the later stage. Metchnikowin is the only known antimicrobial peptide whose expression is regulated by either Toll or Imd pathways [19]. In addition to this, Metchnikowin might be the target of PA14 virulence factors which suggests that there is yet an unknown mechanism(s) that governs Metchnikowin expression, probably through Dif or Relish to defend this bacterium. Additional demonstration of the importance of Dif and Relish and of Metchnikowin in the defense of *D. melanogaster* against *P. aeruginosa*, remains to be discovered.

Since DTT-treatment reduced the PA14 virulence, some virulence factors can be affected by DTT pre-treatment. By reducing disulfide bonds or transferring electrons [15], DTT affects the redox properties of surface proteins or associated metals or the secretion of proteins, which are important in virulence mechanisms. The slight attenuation of the initial growth, or some qualitative changes that DTT pre-treatment may provide in the host environment, could attenuate survival in the host environment. This is because the balance between infecting bacteria and host immunity is the actual determinant of whether pathogenesis will be successful or not. The discovery of paralyzing mechanisms in Dif and Relish-related signals will be facilitated initially by finding the target molecules in *P. aeruginosa* for DTT modulation, or isolating *P. aeruginosa* mutants whose virulence is not affected by DTT pre-treatment.

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