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Virulence of an exotoxin A-deficient strain of *Pseudomonas aeruginosa* toward the silkworm, *Bombyx mori*

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ABSTRACT

We studied the contribution of exotoxin A to the virulence of *Pseudomonas aeruginosa* against the silkworm, *Bombyx mori*. First, an exotoxin A-deficient mutant strain (PAO1*toxA*) was created, and its virulence compared with that of the parental PAO1 strain. In a short-term mortality assay, the mutant harboring pBBR1MCS2 did not kill *B. mori* until 120 h after inoculation and complementation of the corresponding gene *in trans* restored the strain's virulence. Next, to ascertain whether or not it lost all virulence, PAO1*toxA* (pBBR1MCS2, pGFP) was used in a long-term mortality assay. *B. mori* inoculated with the mutant strain did not die until early in the 5th instar (240 h after inoculation). However, 50% of the inoculated *B. mori* died late in the 5th instar or in the early pupal stage (408 h after inoculation). All had died by the pupal stage (600 h after inoculation). The mutant strain was isolated from dead larvae and cocoons. The bacterial population of PAO1*toxA* in hemolymph reached 4.77×10^7 cfu/ml. These results indicated that exotoxin A acts as a virulence factor in *B. mori* and that other virulence factor(s) are involved during the late stages of infection.

1. Introduction

Pseudomonas aeruginosa is an important opportunistic human pathogen causing a wide range of acute and chronic infections. *P. aeruginosa* rarely causes infections in healthy hosts, but it causes a variety of diseases in individuals predisposed to infections as a result of severe burns, wounds, corneal or urinary tract injury, or an immunocompromised state [1,2].

P. aeruginosa also infects amoeba, nematodes, plants, and insects. These organisms are often utilized as hosts in models of *P. aeruginosa* infection instead of mammals [3–8]. In particular, a number of studies using insects to examine microorganisms pathogenic to humans have been reported [9]. The substitution of insects for mammals as models for the study of opportunistic pathogens has a number of advantages. Insects can be used in large numbers and are easily manipulated, resulting in minimal time and cost in their maintenance. Although insect hosts are not useful or applicable to the study of all microbial pathogens, their substitution for mammalian hosts in appropriate situations can reduce mammalian suffering.

To use insect hosts as a substitute for mammals, research that classifies virulence factors into host-specific or common factors is required. The pathogenesis of *P. aeruginosa* involves the production of a number of virulence factors. It has been demonstrated that some virulence-associated genes, such as *rpoN* and *algD*, are host-specific [3,10–13]. On the other hand, phospholipase C and exotoxin A are required for virulence in multiple hosts, including mice, *Arabidopsis*, and *Caenorhabditis elegans* [14,15]. Mutation of the *toxA* gene resulted in diminished pathogenicity in both mice and *Arabidopsis* [14]. In a *C. elegans* model, a *toxA* mutant showed moderately reduced virulence under slow-killing conditions [15].

The silkworm *Bombyx mori* is a good model with which to study the interaction between insects and opportunistic bacterial pathogens, since it is one of the best characterized insect species, both phenotypically and genotypically. Our previous reports suggested that some virulence-associated factors in *P. aeruginosa* were important to the virulence against both mammalian hosts and *B. mori* [16,17]. Although pyocyanin is involved in the pathogenicity of *P. aeruginosa* in a number of hosts, this is not the case in *B. mori* [18].

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Exotoxin A inhibits protein synthesis through ADP-ribosylation of elongation factor 2 [19]. In mammals, it is an important virulence factor in septicemia [20], and corneal [21] and lung [22] infections. The contribution of this virulence factor to acute and chronic lung infections has also been studied [23–25].

Hossain *et al.* showed that exotoxin A is toxic to *B. mori* [26]. In a preliminary test, we reconfirmed the toxicity and found that the median lethal dose (LD_{50}) was 67.1 ng/larva 48 h after injection. In addition, *P. aeruginosa* is virulent to *B. mori*. However, these results are not sufficient to prove that exotoxin A acts as a virulence factor in *B. mori*. In other words, these results show interaction between a toxic protein and a host, but not interaction between a pathogenic microorganism and a host. For example, although purified pyocyanin was toxic to *B. mori*, pyocyanin-deficient *P. aeruginosa* did not show reduced virulence, and pyocyanin was not detected in the hemolymph of infected *B. mori* [18]. These results indicate that pyocyanin is not a real virulence factor in *B. mori*. Similarly, to prove that exotoxin A acts as virulence factor in *B. mori*, it is necessary to evaluate the virulence of an exotoxin A-deficient mutant. In this study, we created such a mutant and investigated the contribution of exotoxin A to the virulence of *P. aeruginosa* against *B. mori*.

2. Results

2.1. Mutagenesis of the toxA gene of P. aeruginosa PAO1 and complementation by a plasmid

Mutagenesis of the *toxA* gene encoding exotoxin A of *P. aeruginosa* PAO1 was carried out, and the mutant strain was designated PAO1*toxA* (Fig. 1A). Insertion of the 1.7-kb Ω*aac* cassette into the mutant was confirmed by Southern hybridization (Fig. 1B). The 1.8-kb *toxA* probe hybridized to 3.1-kb *Pst*I fragments in the PAO1 strain, whereas the former fragment was increased to 4.9-kb in strain PAO1*toxA*.

The level of exotoxin A production is shown in Fig. 1C. No production of the exotoxin by the *toxA* mutant strain (PAO1*toxA*) was detected in the LB broth. On the other hand, production was restored when PAO1*toxA* was complemented with the plasmid carrying the *toxA* gene (pToxA). The complemented strain produced more exotoxin A than the parental strain.

To visualize bacterial cells in *B. mori* and cocoons, pGFP was created. The fluorescent signal emitted by *P. aeruginosa* (pGFP) was confirmed *in vitro* (data not shown). *P. aeruginosa* strains harboring pGFP were used in the long-term mortality assay described below.

2.2. Bacterial growth of P. aeruginosa strains

The growth of *P. aeruginosa* strains in LB broth was monitored (Fig. 2). A difference in growth from 2 to 11 h after inoculation was found between the group without pGFP and the group harboring it. The OD values of PAO1 and PAO1*toxA* were significantly higher than those of other strains between 20 and 24 h after inoculation. No significant difference was observed between the other combinations.

2.3. Short-term mortality assay with the toxA mutant

The exotoxin A-deficient strain was compared to the parental PAO1 strain in terms of virulence against *B. mori* (Fig. 3). PAO1*toxA* (pToxA) was inoculated to confirm the restoration of virulence by gene complementation. At all doses used, PAO1*toxA* (pBBR1MCS2) did not cause larval death until 120 h. On the other hand, PAO1*toxA* (pToxA) restored virulence. The mortality within 120 h was 100, 100, and 73.3% at doses of 10^6, 10^5 and 10^4 cells, respectively. Notably, inoculation with 10^6 cells caused 100% mortality within 24 h PAO1*toxA* (pToxA) was more virulent than the wild type strain at all doses tested.

2.4. Long-term mortality assay with the toxA mutant

To ascertain whether or not the *toxA* mutant lost all virulence, a long-term mortality assay was carried out. In this experiment, *P. aeruginosa* strains harboring pGFP were used to visualize the bacterial cells inoculated into *B. mori*. As shown in Table 1, the virulence decreased in the order PAO1*toxA* (pToxA, pGFP) > PAO1 (pBBR1MCS2, pGFP) > PAO1*toxA* (pBBR1MCS2, pGFP) up to 96 h. This order was the same result as in the short-term mortality assay described above (Fig. 3). All larvae inoculated with PAO1 (pBBR1MCS2, pGFP) and PAO1*toxA* (pToxA, pGFP) died within 144 h. In contrast, PAO1*toxA* (pBBR1MCS2, pGFP) did not show any lethal effect until 240 h. However, 408 h after inoculation, 50% of *B. mori* died at the late stage of the 5th-larval instar or early pupal stage (Table 1). Although some larvae could spin cocoons, the shapes of the cocoons were distorted and the pupae in the cocoons were dead (Fig. 4C). Finally, all *B. mori* inoculated with PAO1*toxA* (pBBR1MCS, pGFP) died before emergence (Table 2).

Table 2 shows the CFU of *P. aeruginosa* recovered from the *B. mori* hemolymph. Although no colony of PAO1*toxA* (pBBR1MCS2, pGFP) grew from the larval hemolymph collected 18 h after inoculation, colonies were detected at 42 h.

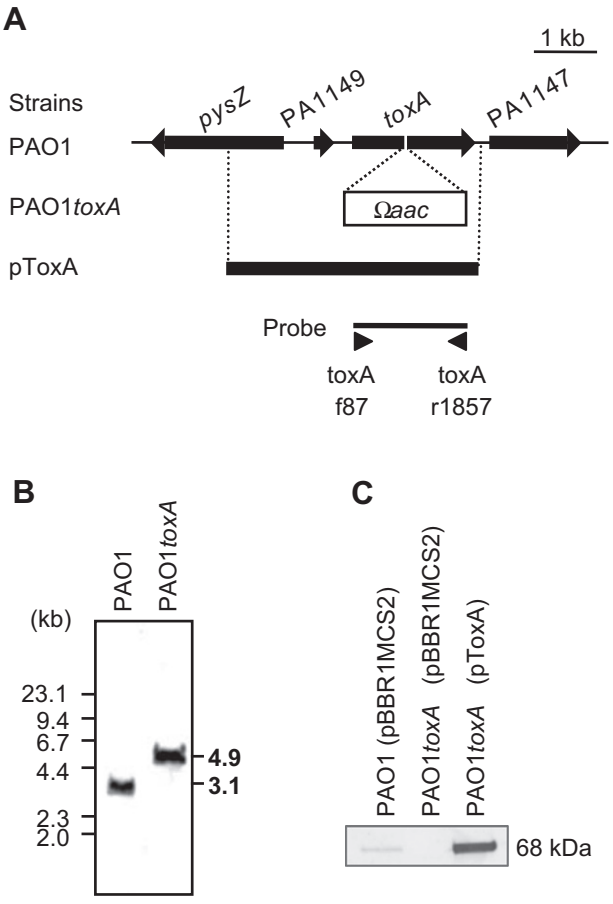

Fig. 1. Construction of the *toxA* mutant strain used in this study. A schematic map of the *toxA* region in *P. aeruginosa* PAO1 (A). In PAO1*toxA*, *toxA* is inactivated by insertion of the Ω*aac* cassette as described in Materials and Methods. The plasmid pToxA was constructed to complement the *toxA* gene. The primer positions are shown by triangles. Confirmation that *toxA* was disrupted in PAO1*toxA* was carried out by Southern hybridization (B). Probes used in Southern hybridization are also shown in (A). Western blot analysis to detect exotoxin A (C).