J Infect Chemother (2012) 18:332–340
DOI 10.1007/s10156-011-0340-0

ORIGINAL ARTICLE

Complementation of the *exoS* gene in the *pvdE* pyoverdine synthesis gene-deficient mutant of *Pseudomonas aeruginosa* results in recovery of the *pvdE* gene-mediated penetration through the intestinal epithelial cell barrier but not the *pvdE*-mediated virulence in silkworms

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Received: 16 September 2011 / Accepted: 24 October 2011 / Published online: 12 November 2011

Abstract Translocation of endogenous *Pseudomonas aeruginosa* from the colonized intestinal tract is an important pathogenic phenomenon. Comparative genome hybridization analysis of high virulent and low virulent strains allowed us to identify bacterial genes that are associated with bacterial translocation from gut in infected hosts. Here we focused on the *pvdE* pyoverdine synthesis gene among the identified bacterial genes, showing that the *pvdE* gene is required for bacterial penetration through epithelial cell monolayers and for bacterial translocation from gut to hemolymph in infected silkworms. We next revealed that mRNA expression level of the *exoS* gene in a *pvdE*-deficient mutant (Δ*pvdE*) after incubation with Caco-2 cells was greatly reduced as compared with that in the wild-type strain. The *pvdE*- and *exoS*-complemented Δ*pvdE* strains (Δ*pvdE/pvdE* and Δ*pvdE/exoS*) showed recovery of the ability of bacterial penetration through Caco-2 cell monolayers and of the ability of bacterial translocation from gut to hemolymph in infected silkworms. However, there were differences between the ability of Δ*pvdE/pvdE* and Δ*pvdE/exoS* to kill silkworms after intestinal infection and to replicate in hemolymph following direct injection into the hemolymph: Δ*pvdE/pvdE* could kill silkworms after intestinal infection and could replicate in hemolymph to levels similar to those of the wild-type strain, but Δ*pvdE/exoS* could not. Taken together, our results suggest that the virulence of the wild-strain mediated by the *pvdE* gene is the result of the ability to both penetrate through the intestinal epithelial cell barrier depending on ExoS and to replicate in hemolymph independently of ExoS.

Keywords *Pseudomonas aeruginosa* · *pvdE* pyoverdine synthesis gene · *exoS* gene · Intestinal epithelial cell barrier · Virulence to silkworms

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is a leading cause of infection-related mortality among hospitalized patients who are ill or individuals with compromised immune systems. The lungs are considered to be one of the major sites of *P. aeruginosa* infection in ill patients; however, even in the absence of established extraintestinal infection and bacteremia, the presence of highly virulent strains of *P. aeruginosa* within the intestinal tract alone could be a main source of sepsis and death among immune-compromised patients [1–3]. In addition, the lethal effect of intestinal *P. aeruginosa* is dependent on its ability to adhere to and disrupt the intestinal epithelial barrier [4]. Translocation of *P. aeruginosa* through the colonized intestinal tract, a process whereby indigenous intestinal *P. aeruginosa* is relocated extraluminally, is considered an important pathogenic phenomenon. Recently, we revealed that infection of polarized intestinal epithelial cells with *P. aeruginosa* PAO1 disrupted intact tight junctions (TJs) by altering the distribution of TJ proteins ZO-1 and occludin through the binding of a type III effector, ExoS, to an Na/K-ATPase regulator, FXYD3 [5]. However, the disruption of TJs by ExoS will be one aspect to

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systematically understanding the precise molecular mechanism by which *P. aeruginosa* is translocated to the colonized intestinal tract.

To systematically elucidate the precise molecular mechanism by which *P. aeruginosa* is translocated to the colonized intestinal tract, we performed comparative genomic hybridization (CGH) to detect gain or loss of DNA sequences [6–8] in a *P. aeruginosa* clinical strain that shows higher virulence in silkworms following midgut injection. To evaluate the virulence of *P. aeruginosa*, we have been exploiting silkworms because the silkworm-infection model was previously established and the silkworm has certain advantages; for example, it is large enough to inject bacterial culture into the midgut and hemolymph, and it is killed by injection of the opportunistic pathogen *P. aeruginosa* because it lacks acquired immunity [9–11]. In our recent study, CGH analysis allowed us to identify 61 genes that were specifically detected in the strain exhibiting high virulence [5]. In this study, from among these 61 genes, we focused our attention on the *pvdE* gene (PA2397), which is essential for pyoverdine (PVD) production [12]. Here, we demonstrate that the *pvdE* gene is associated with the ability of *P. aeruginosa* to penetrate through the intestinal epithelial cell barrier by inducing expression of the *exoS* gene, as the contribution of the *pvdE* gene to the penetration ability of *P. aeruginosa* is unknown at present.

Materials and methods

Bacterial strains

Pseudomonas aeruginosa PAO1 is one of our laboratory stock strains. The Δ*pvdE* strain was constructed using a suicide vector pEX18Tc as described previously [5]. An upstream sequence of the *pvdE* gene was amplified with primers PA2397FF (5′-CCGGATCCCCATGGTCGCCCAATCCACC-3′) and PA2397FR (5′-GATGAAAAAGGTTGCTTCCCGGATGTACTAGG-3′). A downstream sequence of the *pvdE* gene was amplified with primers PA2397BF (5′-GGGAAGCAACCTTTTTCATCCGGTTTTCCGTGGG-3′) and PA2397BR (5′-GGTCTAGAGGCCGCTGACGTCCAGTTC-3′). These two flanking sequences were fused by means of the fusion polymerase chain reaction (PCR) method as described previously [5]. The fused gene construct was cloned into pEX18Tc (Δ*pvdE*-pEX18Tc). Δ*exoS*-pEX18Tc was mobilized into PAO1 by conjugation. From the resulting transconjugants, Δ*exoS* was selected as described previously [5].

Δ*pvdE/pvdE* was constructed by using the pME6032 plasmid as described previously [5]. A 2.1-kb fragment containing the *pvdE* gene with its native promoter (PA2397; region 2653435–2655084 of the PAO1 chromosome) was amplified by PCR with primers 5′-GAGAGAATTCCACCAAGGACTCCAGTACCG-3′ and 5′-TGAACCTAGGTCACGCCGTCTCGGTTTCCTTG-3′. The PCR product was cloned into pME6032. The resulting plasmid was transformed into the Δ*pvdE* strain. The Δ*pvdE/exoS* strain was constructed as follows: a 1.7-kb fragment containing the *exoS* gene with its native promoter (PA3841; region 4302981–4304502 of the PAO1 chromosome) was amplified by PCR with primers 5′-GTGGGAATTCGGCGTGTTCCGAGTCACTGG-3′ and 5′-AGAGGGATCCTCAGGCCAGATCAAGGCCGCGCATCCTCAG-3′. The PCR product was cloned into pME6032. The resulting plasmid was transformed into the Δ*pvdE* strain.

The *Escherichia coli* K-12 W3110 strain is a standard strain used in the genome analysis project in Japan (http://ecoli.aist-nara.ac.jp/GB5/search.jsp), and was used as a negative control that does not show the ability of penetration of epithelial cell monolayers.

PVD production assay

PVD was assessed by means of *P. aeruginosa* growing in iron-limited succinate minimal medium as described previously [13].

Bacterial infection model involving silkworms

Injection of a bacterial culture [10^5 colony-forming units (CFU) per silkworm] into the midgut and hemolymph of silkworms (Hu-Yo × Tukuba-Ne) at the fifth-instar stage was carried out as described previously [5, 10, 14]. As a negative control, saline was used. The infected silkworms were maintained without food, and survival was monitored for 5 days.

The numbers of bacteria in the hemolymph at 6 and 24 h after injection of the bacterial culture (10^5 CFU per silkworm) into the midguts of three silkworms were determined by spreading 100 µl hemolymph fluid onto Luria–Bertani (LB) agar. The results are expressed as mean ± SD.

Viability in the hemolymph after direct injection of a bacterial culture (10^7–10^1 CFU/silkworm) into the hemolymph was compared by injecting bacteria into the hemolymph of ten silkworms.

Penetration assay

We carried out the penetration assay using MDCK epithelial cell monolayers and Caco-2 intestinal epithelial cell monolayers at a multiplicity of infection (m.o.i.) of 100 using the same system as that described previously [5, 15].