EprS, an autotransporter serine protease, plays an important role in various pathogenic phenotypes of *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* possesses an arsenal of both cell-associated (flagella, pili, alginate, etc.) and extracellular (exotoxin A, proteases, type III secretion effectors, etc.) virulence factors. Among them, secreted proteases that damage host tissues are considered to play an important role in the pathogenesis of *P. aeruginosa* infections. We previously reported that EprS, an autotransporter protease of *P. aeruginosa*, induces host inflammatory responses through protease-activated receptors. However, little is known about the role of EprS as a virulence factor of *P. aeruginosa*. In this study, to investigate whether EprS participates in the pathogenicity of *P. aeruginosa*, we characterized various pathogenic phenotypes of the wild-type PAO1 strain and its *eprS*-disrupted mutant. The growth assays demonstrated that the growth of the *eprS* mutant was somewhat lower than that of the wild-type strain in a minimal medium containing BSA as the sole carbon and nitrogen source. Thus, these results indicate that *eprS* would have a role in the growth of *P. aeruginosa* in the presence of limited nutrients, such as a medium containing proteinaceous materials as a sole nutrient source. Furthermore, disruption of *eprS* resulted in a decreased production of elastase, pigments, autoinducers and surfactants, and a reduction of swimming and swarming motilities. In addition, the *eprS* mutant exhibited a reduction in the ability to associate with A549 cells and an attenuation of virulence in leucopenic mice as compared with the wild-type strain. Collectively, these results suggest that EprS exerts pleiotropic effects on various pathogenic phenotypes of *P. aeruginosa*.

INTRODUCTION

*Pseudomonas aeruginosa* is a Gram-negative bacterium found in almost every ecological niche, including soil, water and plants, which is also a major cause of both community-acquired and hospital-acquired infections (Blanc et al., 1998; Driscoll et al., 2007; Lyczak et al., 2000). *P. aeruginosa* is inherently resistant to various commonly used antibiotics, due to a large arsenal of intrinsic resistance mechanisms such as a reduction in outer membrane permeability, the existence of multidrug efflux pump systems, and the production of antibiotic-inactivating enzymes (Breidenstein et al., 2011; Hancock & Speert, 2000). Hence, the treatment of *P. aeruginosa* infections is often problematic.

The pathogenesis of *P. aeruginosa* infections is obviously multifactorial as suggested by the large number of both cell-associated and extracellular virulence factors, and the broad spectrum of diseases due to the bacterium (Gellatly & Hancock, 2013; Gooderham & Hancock, 2009). Cell-associated virulence factors include flagella, type IV pili and alginate; extracellular virulence factors include exotoxin A, pyocyanin, haemolysins, proteases and type III secretion effectors. The complete *P. aeruginosa* infection appears to be composed of three distinct stages: first, bacterial adherence and colonization; second, local invasion; and third, disseminated systemic diseases. After an initial colonization phase, mostly dependent on cell-associated virulence factors, the infectious process advances either to an acute infection or to a chronic infection characterized by different expression levels of extracellular virulence determinants (Coggan & Wolfgang, 2012; Okkotsu et al., 2014). Many of the extracellular virulence determinants required for tissue invasion and dissemination are controlled by the Las, Rhl and PQS quorum-sensing (QS) systems that induce the expression of various virulence factors.

Abbreviations: AHSL, acylhomoserine lactone; i.p., intraperitoneal; PAR, protease-activated receptor; QS, quorum-sensing.
in response to high cell densities or other external stimuli like iron limitation (Coggan & Wolfgang, 2012; Nadal Jimenez et al., 2012).

Although *P. aeruginosa* produces various virulence factors as described above, in particular, secreted proteases that damage host tissues are considered to be major virulence factors produced by this pathogen. Several *P. aeruginosa* proteases have been isolated and shown to be involved in pathogenesis. Of the proteases analysed, alkaline protease (AprA) (Kharazmi, 1991), elastase (LasA and LasB) (Preston et al., 1997; Suter, 1994), protease IV (PrpL) (Engel et al., 1998; O’Callaghan et al., 1996), small protease (PasP) (Marquart et al., 2005; Tang et al., 2009), immunomodulating metalloprotease (ImpA) (Bardoel et al., 2012), aminopeptidase (Luckett et al., 2012), large extracellular protease (LeprA) (Kida et al., 2008, 2011) and extracellular serine protease (EprS) (Kida et al., 2013) have been characterized extensively. We previously reported that EprS, an autotransporter protease of *P. aeruginosa*, induces host inflammatory responses through protease-activated receptors (PARs) (Kida et al., 2013). However, little is known about the role of EprS as a virulence factor of *P. aeruginosa*. In this study, we aimed to investigate whether EprS is involved in the pathogenicity of *P. aeruginosa*. Our results indicate that disruption of *eprS* results in a decreased production of elastase, pigments, autoinducers and surfactants, and a reduction of swimming and swarming motilities. Furthermore, the isogenic *eprS* mutant showed a decrease in the ability to associate with A549 cells and an attenuation of virulence in leucopenic mice as compared with the wild-type strain. Herein, we report that EprS exerts pleiotropic effects on various pathogenic phenotypes of *P. aeruginosa*.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table S1 (available in the online Supplementary Material). All bacterial strains were grown in Luria–Bertani (LB) medium (LB-Miller; Nacalai Tesque) unless otherwise noted. Vogel–Bonner minimal medium (VBMM) was used for selection of transconjugants from conjugation mixtures (Schweizer, 1992). VBMM is VB medium (Vogel & Bonner, 1956) containing 0.3 % trisodium citrate and is selective for *P. aeruginosa* since *Escherichia coli* cannot utilize citrate. Antibiotics were used in selection media at the following concentrations: ampicillin, 100 µg ml⁻¹; gentamicin, 100 µg ml⁻¹; carbenicillin, 500 µg ml⁻¹; kanamycin, 1 mg ml⁻¹; kanamycin, 100 µg ml⁻¹ (P. aeruginosa); ampicillin, 100 µg ml⁻¹ (E. coli); carbenicillin, 500 µg ml⁻¹ (P. aeruginosa); and gentamicin, 100 µg ml⁻¹ (P. aeruginosa).

**Preparation of inocula for infection.** Each *P. aeruginosa* strain was cultured in LB medium to exponential phase at 37 °C with rotary shaking at 150 r.p.m. (AT-12R shaker; Thomas). The culture was centrifuged at 10 000 g for 5 min and the bacterial pellet was washed twice with saline (0.9 % NaCl). The pellet was resuspended in an adequate volume of saline, and the optical density was adjusted to 0.60 nm adjusted to give the approximate desired inocula (OD₆₀₀ of 1 × 5 × 10⁷ cf.u. ml⁻¹). The inocula were verified by serial 10-fold dilutions of the suspensions and plating on cetrimide agar (Nissui Pharmaceutical).

**Cell culture.** A human type II alveolar epithelial cell line, A549 (RIKEN BioResource Center), was cultured in DMEM (Dulbecco's modified Eagle's medium; Nissui Pharmaceutical) supplemented with 10 % heat-inactivated FBS [endotoxin contents, <0.1 ng (ml serum)⁻¹]; Gibco), 4 mM l-glutamine, 0.35 % d-glucose and 0.37 % sodium bicarbonate. The cell cultures were maintained at 37 °C in a 5 % CO₂ atmosphere in air.

**Gene replacement and in vivo marker excision in *P. aeruginosa* PAO1.** Allele replacement of *eprS* was performed by a modified Schweizer method (Schweizer, 1992). The primers for PCR are listed in Table S2. To prepare the vector for cloning by an In-Fusion cloning kit (Takara), pYK6 (Kida et al., 2013), pUC18Not with a 4.0 kb fragment containing *eprS*, was amplified by PCR using primers ∆*eprS*-F and ∆*eprS*-R. The resultant fragment contains the ORF of *eprS* (2988 bp) with a deletion from 91 to 1881 bp. A 1.0 kb fragment containing a selectable marker gentamicin-resistance gene (Gen') flanked by Flp recombinase target (FRT) sites was amplified from pUC18T-mini-Tn7T-Gm (Choi & Schweizer, 2006) by using primers FRTGM-F and FRTGM-R. PCR was performed with a PrimeSTAR GXL DNA polymerase (Takara) according to the manufacturer’s protocol. The PCR-amplified FRT/Gen’ cassette was cloned into the PCRamplified pYK6 fragment with an In-Fusion cloning kit according to the manufacturer’s protocol. The resultant plasmid was designated pYK6-FRT/Gm. The ∆*eprS*: FRT/Gen’ fragment was then subcloned into the NotI site of the suicide vector pYK1-T, which has the oriT for conjugative transfer and the counter-selectable marker sacB, producing plasmid pYK8. This plasmid was used for allelic exchange and conjugated from *E. coli* S17-1Δpir into *P. aeruginosa* PAO1 on LB agar using filters.

Merodiploid single-crossover mutants were selected from the conjugation mixture by plating on VBMM agar containing 100 µg gentamicin ml⁻¹. Purified single-crossover mutants were cultured overnight in LB broth without antibiotics. This culture was then serially diluted in saline and plated on LB agar containing 100 µg gentamicin ml⁻¹ and 7 % sucrose to select against the sacB marker present on the pYK8 vector and, hence, select for strains that had undergone a second homologous recombination event resulting in loss of the pYK8 vector. This was confirmed by the loss of the vector-encoded carbenicillin resistance. In addition, the double-crossover mutants were confirmed by PCR using primers pBAD/PA3535-F and pBAD/PA3535-R (data not shown).

Deletion of the chromosomally integrated Gen’ marker by Flp recombinase-catalysed excision was achieved by conjugally transferring the Flp-expressing pFLP2 (Hoang et al., 1998) from *E. coli* S17-1Δpir into *P. aeruginosa* PAO1Δ*eprS*: FRT/Gen’ at 37 °C. The mating mixtures were then serially diluted in saline and plated on VBMM agar containing 500 µg carbenicillin ml⁻¹ and incubated at 37 °C until single colonies were discernible. Twenty single colonies were screened for gentamicin sensitivity on VBMM agar supplemented with or without 100 µg gentamicin ml⁻¹. Plasmid pFLP2 was cured by generously streaking cells from four patches containing gentamicin-sensitive colonies on VBMM agar containing 7 % sucrose, followed by incubation at 37 °C. Loss of pFLP2 was tested by patching 20 sucrose-resistant colonies on VBMM agar supplemented with or without 500 µg carbenicillin ml⁻¹. The mutants were then verified by PCR using primers pBAD/PA3535-F and pBAD/PA3535-R (data not shown).

For complementation, a wild-type copy of *eprS* with the endogenous promoter region was cloned into a broad-host-range vector, pBBR1MCs-2 (Kovach et al., 1995). To prepare the vector for cloning by an In-Fusion cloning kit (Takara), pBBR1MCs-2 was amplified by PCR using primers pBBR1-F and pBBR1-R. A 3.5 kb fragment containing *eprS* and its endogenous promoter region was amplified from pYK6 (Kida et al., 2013) by PCR using primers Up/eprS-F and eprS-R.
followed by insertion into the PCR-amplified pBBR1MCS-2 as described above. The resultant plasmid was named pBBR1-eprS WT. To generate Ser308 to Ala308 point mutations of the EprS protein, pBBR1-eprS WT was mutated by PCR (Imai et al., 1991) using primers eprS/S308A-S and eprS/S308A-AS. The resultant plasmid was designated pBBR1-eprS S308A. These plasmids were used for complementation and conjugated into E. coli S17-1 pir into P. aeruginosa PAO1ΔeprS on LB agar using filters. Cells were suspended in saline and plated on VBM4 agar containing 1 mg kanamycin ml⁻¹. After overnight incubation at 37 °C, kanamycin-resistant colonies were obtained. To identify the clones of P. aeruginosa PAO1ΔeprS containing the complement plasmid, the plasmid DNA was isolated from kanamycin-resistant strains and subjected to agarose gel electrophoresis (data not shown).

**Growth of P. aeruginosa in albumin medium.** The BSA (Sigma-Aldrich) used in this study was electrophoretically 98% pure (endotoxin contents, <0.1 ng (mg protein)⁻¹) and essentially γ-globulin free. A minimal medium containing BSA as the sole carbon and nitrogen source was prepared as follows: basal buffer [42 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.6 mM NaCl and 1 mM MgCl₂ (pH 7.0)] was supplemented with BSA (10 mg ml⁻¹). Each P. aeruginosa strain was cultured in LB medium to stationary phase at 37 °C with rotary shaking at 150 r.p.m. After 24 h of growth, the cultures were diluted 1:5000 in fresh AB medium. Of the diluted reporter cultures, 2 ml was mixed with 20 μl filtered culture supernatant of P. aeruginosa in polystyrene test tubes. The tubes were incubated for 5 h at 30 °C with rotary shaking at 150 r.p.m. Bioluminescence values were determined with a GloMax 96 microplate luminometer (Promega). The values were normalized against the OD₆₀₀ of each sample. The value obtained for P. aeruginosa PAO1 wild-type strain was set to 100 %, and other measurements were normalized accordingly. All experiments were performed in triplicate, and the mean ± SD was calculated.

**Motility assays**

**Swimming.** Swim plates [0.5 % agar (Nacalai Tesque), 1 % tryptone (Nacalai Tesque) and 0.5 % NaCl (pH 7.2)] were inoculated with bacteria using a sterile toothpick at the centre of the agar surface and incubated for 24 h at 25 °C (Déziel et al., 2001). Motility was then evaluated qualitatively and quantitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

**Swarming.** Swarming motility was assessed qualitatively on swarm plates [0.5 % agar, 0.6 % Na₂HPO₄, 0.3 % KH₂PO₄, 0.05 % NaCl, 0.015 % MgSO₄, 0.05 % potassium glutamate and 0.2 % glucose (pH 7.0)] (Tremblay & Déziel, 2008). Bacteria were point inoculated onto swarm plates using a sterile toothpick and incubated for 24 h at 30 °C.

**Twitching.** Twitch plates [1 % agar, 1 % tryptone (Nacalai Tesque), 0.5 % yeast extract and 1 % NaCl (pH 7.2)] were stab inoculated with bacteria using a sterile toothpick through a thin (approx. 3 mm) agar layer to the bottom of the Petri dish (Déziel et al., 2001). After incubation for 24 h at 37 °C, the twitching zone at the interface between agar and Petri dish was visualized by crystal violet (1 %, w/v, solution), staining, and the zone was assessed qualitatively and quantitatively.

**Drop collapse assay.** Bacteria were grown for 24 h at 37 °C in LB medium with rotary shaking at 150 r.p.m., followed by centrifugation and filtration of culture supernatant through a 0.20 μm membrane. The filtered supernatant was serially diluted in saline, and aliquots of 5 μl filtered culture supernatant of PAO1 wild-type strain was set to 100 %, and other measurements were normalized accordingly. All experiments were performed in triplicate, and the mean ± SD was calculated.

**Association, invasion and intracellular survival assays.** A549 cells were seeded at 1×10⁵ cells per well in a 48-well tissue culture plate and cultured to semi-confluency (2×10⁵ cells per well). Then, the monolayers were infected with P. aeruginosa strains at the indicated m.o.i. for 3 h at 37 °C in a 5 % CO₂ atmosphere in air. For the association assay, after 3 h of infection the monolayers were washed three times with PBS, and the cells were lysed with PBS containing 0.25 % (v/v) Triton X-100 for 15 min and plated on LB agar to count the number of bacteria associated with A549 cells (both adherent and intracellular bacteria).

For the invasion assay, associated extracellular bacteria were killed by incubation for 2 h with DMEM containing 500 μg gentamicin ml⁻¹. The antibiotic was aspirated, and the cells were washed three times with PBS, before lysis with PBS containing 0.25 % (v/v) Triton X-100. Intracellular bacteria were enumerated by culturing serial dilutions of the lysate on LB agar overnight at 37 °C.

To determine whether P. aeruginosa could survive or multiply within A549 cells, the standard invasion assay was modified by further incubation of the infected monolayers for up to 96 h (intracellular survival assay). A549 cells were infected with PAO1 wild-type at an
m.o.i. of 50 or PAO1ΔeprS at an m.o.i. of 200. After the A549 cells were washed three times with PBS, DMEM containing 50 μg gentamicin ml⁻¹ was added to all wells for 2 h to kill the extracellular bacteria. One group of wells was used to quantify bacterial invasion (as described above) to provide a baseline for the number of intracellular organisms present at time zero. To eliminate bacteria released from the infected cells, DMEM containing 50 μg gentamicin ml⁻¹ was added to the other wells for a further 24 to 96 h of incubation. At the end of this second incubation step, the cells were washed and treated with an additional 500 μg gentamicin ml⁻¹ for 2 h. Surviving bacteria were enumerated as described above.

**Animals.** Four- to six-week-old male ICR mice, weighing 20–22 g (Kyudo) were used. Leucopenia was induced by treatment with a single intraperitoneal (i.p.) dose of cyclophosphamide (Sigma-Aldrich) of 250 mg kg⁻¹, given 4 days before bacterial challenge (Miyazaki et al., 1995). All experimental procedures were reviewed and approved by the Kurume University School of Medicine Institutional Animal Care and Use Committee. Experimental procedures were performed in compliance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

**LD₅₀ determinations.** Leucopenic ICR mice, in groups of 16, were challenged with a single i.p. injection of 0.2 ml of the inoculum of each P. aeruginosa strain prepared as described above. Four dilutions, containing different numbers of viable bacteria, were used to determine LD₅₀ values, and mortality was assessed daily for 7 days after infection. The LD₅₀ of the bacteria was calculated by probit analysis (Bliss, 1934) from survival rates of the mice after 7 days of infection.

**Statistical analysis.** Statistical assessments of the differences between means were performed using Student’s paired t-test for comparison of two groups, or one-way ANOVA with Tukey’s post hoc test for comparison of multiple groups. Data with P values of <0.05 were considered significant.

**RESULTS**

**Phenotypic characterization of PAO1ΔeprS**

We previously reported that LepA secreted via a two-partner secretion system contributes to virulence and growth of P. aeruginosa (Kida et al., 2011). The growth of the lepA deletion strain has been shown to decrease compared to that of the wild-type strain in the presence of limited nutrients (Kida et al., 2011). Although bacterial growth appears to be closely relevant to virulence, it is not known whether EprS participates in the proliferation of P. aeruginosa. Accordingly, we examined the growth of an eprS-deficient P. aeruginosa mutant in a minimal medium containing BSA as the sole carbon and nitrogen source. As shown by the results in Fig. 1(a), the growth of PAO1ΔeprS was somewhat lower than that of the wild-type PAO1 in albumin medium. Complementation of the eprS-disrupted strain with a wild-type copy of eprS in trans, but not with the empty vector control or proteolytic-activity-deficient eprS S308A, restored the growth to the wild-type level. In contrast to the results of growth assays in albumin medium, the growth of the eprS mutant was similar to that of the wild-type strain in LB medium (Fig. 1b). Furthermore, there was little or no difference in the growth between the PAO1 wild-type strain and the eprS mutant in VBMM containing sodium citrate and ammonium sodium hydrogenphosphate as carbon and nitrogen source, respectively (Fig. 1b). Thus, these results indicate that eprS would have a role in the growth of P. aeruginosa in the presence of limited nutrients such as a medium containing proteinaceous materials as the sole carbon and nitrogen source.

Next, we evaluated whether deletion of eprS influences the production of elastase, pyocyanin and pyoverdine, because the production of these molecules has been shown to contribute to the virulence of P. aeruginosa.
(Gellatly & Hancock, 2013; Nadal Jimenez et al., 2012). As shown by the results in Fig. 2(a), elastase activity in the culture supernatant of *P. aeruginosa* PAO1ΔeprS was significantly lower than that of the wild-type strain. Additionally, *P. aeruginosa* PAO1ΔeprS exhibited a significant decrease in the production of pyocyanin and pyoverdine as compared to the wild-type strain (Fig. 2b, c). These data suggest that eprS would contribute to the production of various virulence factors.

The production of elastase, pyocyanin and pyoverdine has been shown to be regulated by QS in *P. aeruginosa* (Nadal Jimenez et al., 2012). *P. aeruginosa* produces QS signal molecules termed autoinducers, such as AHSLs and alkyl quinolones (Nadal Jimenez et al., 2012). We thus tried to determine the autoinducer production of each *P. aeruginosa* strain using bioassays in which *V. harveyi* BB886 is a bioluminescence reporter strain for AHSLs. As shown in Fig. 2(d), *P. aeruginosa* PAO1 eprS-disrupted strain showed a significant reduction in the level of autoinducer production as compared with the wild-type. When the eprS-disrupted strain was complemented in trans with a wild-type copy of eprS, but not with the empty vector control or proteolytic activity-deficient eprS S308A, the autoinducer production was increased to almost the wild-type level. Therefore, these

![Fig. 2. Production of extracellular products in *P. aeruginosa* PAO1 wild-type and the eprS mutant. (a) Elastase activity was measured as the A490 in an elastin–Congo red hydrolysis assay of the supernatant from a 16 h culture at 37 °C as described in Methods. Normalized elastase activity was expressed as the value of A490 divided by the cell density of each sample (OD600). (b) Pyocyanin production was measured as the A695 of the supernatant of a 36 h culture at 37 °C in King’s A medium. The value of A695 was normalized against the OD600 of the bacterial culture. (c) Pyoverdine production was measured as the A403 of the supernatant of a 24 h culture at 37 °C in King’s B medium. The value of A403 was normalized against the OD600 of the bacterial culture. Values represent the mean ± SD from triplicate determinations of a representative experiment. Similar results were obtained in three independent experiments. The difference between PAO1 wild-type and PAO1ΔeprS was significant with a *P* value of <0.01 by Student’s paired *t*-test. (d) Autoinducers were detected as bioluminescence in a bioassay of the supernatant from a 24 h culture at 37 °C as described in Methods. *V. harveyi* BB886, a bioluminescence reporter strain for AHSLs, was used in bioassays. Measured bioluminescence values were normalized against the OD600 of each sample. The value obtained for *P. aeruginosa* PAO1 wild-type strain was set to 100 %, and other measurements were normalized accordingly. Values represent the mean ± SD from triplicate determinations of a representative experiment. Similar results were obtained in three independent experiments. Comparisons of multiple groups were performed using one-way ANOVA with Tukey’s post hoc test. There were significant differences between the group of WT and ΔeprS, or the group of ΔeprS and ΔeprS complemented with a wild-type copy of eprS (*P*<0.01), while there were no significant differences among the groups of WT, WT complemented with the empty control vector and ΔeprS complemented with a wild-type copy of eprS, or the groups of ΔeprS, ΔeprS complemented with the empty control vector and ΔeprS complemented with the proteolytic activity-deficient eprS S308A. WT, PAO1 wild-type; ΔeprS, PAO1ΔeprS.](image-url)
results suggest that eprS would participate in the expression of QS-regulated virulence factors in *P. aeruginosa*.

**Disruption of eprS results in reduced swimming and swarming motilities of *P. aeruginosa***

*P. aeruginosa* is capable of performing three different types of cell motility: flagellum-mediated swimming, flagellum- and type IV pilus-mediated multicellular migration called swarming, and type IV pilus-mediated twitching (Burrows, 2012; Köhler *et al.*, 2000). The motilities of *P. aeruginosa* have been shown to participate in the pathogenicity of this bacterium (Arora *et al.*, 2005; Zolfaghar *et al.*, 2003). To assess whether the swimming, swarming and twitching motilities of *P. aeruginosa* are influenced by disruption of eprS, we compared the motilities of the PAO1 wild-type strain and the eprS mutant. As shown by the results in

![Fig. 3. Disruption of eprS results in reduced swimming and swarming motilities of *P. aeruginosa*.](image)

(a) Swimming motility on swim plates (0.3 % agar); (centre) swarming motility on swarm plates (0.5 % agar); (right) twitching motility at the agar/dish interface. In the case of swimming and swarming motilities the bacteria were inoculated on top of the agar, whereas for monitoring of the twitching motility the bacteria were stab inoculated with a toothpick through twitch plates (1 % agar) to the bottom of the Petri dish. (b) Diameter of the swimming zone. (c) Diameter of the twitching zone. Values represent the mean ± SD from triplicate determinations of a representative experiment. Similar results were obtained in three independent experiments. Comparisons of multiple groups were performed using one-way ANOVA with Tukey’s post hoc test. (b) There were significant differences between the group of WT and ΔeprS, or the group of ΔeprS and ΔeprS complemented with a wild-type copy of eprS (P<0.01), while there were no significant differences among the groups of WT, WT complemented with the empty control vector and ΔeprS complemented with a wild-type copy of eprS, or the groups of ΔeprS, ΔeprS complemented with the empty control vector and ΔeprS complemented with the proteolytic activity-deficient eprS S308A. (c) The differences between all the groups were not significant. WT, PAO1 wild-type; ΔeprS, PAO1ΔeprS.
Fig. 3(a), the eprS mutant was found to have reduced swimming (Fig. 3a, left) and swarming (Fig. 3a, centre) motilities compared to the wild-type strain. Furthermore, the swimming zone diameter of the eprS mutant was significantly smaller than that of the wild-type strain (Fig. 3b). The motilities were reconstituted by complementing the eprS mutant with a wild-type copy of eprS in trans but not by the empty vector control or proteolytic activity-deficient eprS S308A. In contrast to the results of swimming and swarming assays, the twitching motility ability of the eprS mutant was comparable to that of the wild-type strain (Fig. 3a, right). In practice, there was no significant difference in twitching motility between the wild-type strain and the eprS mutant (Fig. 3c). Therefore, these results indicate that eprS has a role in the swimming and swarming motilities of P. aeruginosa.

**Disruption of eprS decreases the production of surfactants in P. aeruginosa**

*P. aeruginosa* is known to produce surfactants that are involved in its swimming motility behaviour, such as rhamnolipids and their precursors 3-[(3-hydroxyalkanoyloxy)-alkanoic acids (Déziel et al., 2003; Wilhelm et al., 2007). We then performed drop collapse assays using spent supernatant for each *P. aeruginosa* strain to assess semiquantitatively the production of surfactants in *P. aeruginosa*. As shown in Fig. 4, the wild-type spent supernatant did not form a bead until the eightfold-diluted sample, indicating drop collapse capability for a dilution of up to fourfold. In contrast, the eprS mutant spent supernatant formed beads at all concentrations tested, indicating no measurable drop collapse activity. Complementation of the eprS-disrupted strain with a wild-type copy of eprS in trans, but not with the empty vector control or proteolytic activity-deficient eprS S308A, restored the drop collapse activity to the wild-type level. Thus, these data indicate that the eprS mutant spent supernatant has less drop collapse activity than the wild-type, namely, disruption of eprS results in the decreased production of surfactants in *P. aeruginosa*.

**Interaction of P. aeruginosa PAO1 with A549 cells**

Clinical isolated strains of *P. aeruginosa* have been grouped into invasive and non-invasive (cytolytic) strains based on their interactions with non-phagocytic epithelial cells (Fleischig et al., 1997). Approximately 80% of *P. aeruginosa* clinical isolates exhibit invasive phenotypes (Stepińska & Trafny, 2008). Since adherence of bacteria to mammalian cells and their internalization are thought to be closely related to the pathogenicity of *P. aeruginosa*, we evaluated whether disruption of eprS influences the interaction of *P. aeruginosa* PAO1 with A549 cells. To examine the ability of *P. aeruginosa* to adhere with A549 cells, association assays were performed. As shown in Fig. 5(a), the PAO1ΔeprS strain exhibited significantly less association with A549 cells than the wild-type strain. When the eprS-disrupted strain was complemented in trans with a wild-type copy of eprS, but not with the empty vector control or proteolytic activity-deficient eprS S308A, the numbers of bacteria associated with A549 cells were restored to almost the wild-type level.

We next performed an invasion assay to count the numbers of intracellular *P. aeruginosa*. To achieve the same number of bacteria bound to A549 cells, a twofold higher m.o.i. was used for PAO1ΔeprS (with the exception of ΔeprS + eprS WT) than for the PAO1 wild-type based on the results of Fig. 5(a). As shown in Fig. 5(b), the eprS mutant was found to be less invasive than the wild-type strain.

**Fig. 4.** Disruption of eprS decreases the production of surfactants in *P. aeruginosa*. Drop collapse assays were performed using spent supernatant for each *P. aeruginosa* strain to assess semiquantitatively the production of surfactants by *P. aeruginosa*, as described in Methods. Samples were diluted in saline, spotted on the lid of a microtitre plate and assayed for bead formation. The dilutions are shown at the top of the panel. The results shown are representative of those for two different experiments. WT, PAO1 wild-type; ΔeprS, PAO1ΔeprS; + empty, complemented with pBBR1MCS-2; + eprS WT, complemented with pBBR1-eprS WT; + eprS S308A, complemented with pBBR1-eprS S308A.

**Fig. 5.** Interaction of *P. aeruginosa* PAO1 with A549 cells. In Fig. 5(a), the PAO1ΔeprS strain exhibited significantly less association with A549 cells than the wild-type strain. When the eprS-disrupted strain was complemented in trans with a wild-type copy of eprS, the numbers of bacteria associated with A549 cells were restored to almost the wild-type level. We next performed an invasion assay to count the numbers of intracellular *P. aeruginosa*. To achieve the same number of bacteria bound to A549 cells, a twofold higher m.o.i. was used for PAO1ΔeprS (with the exception of ΔeprS + eprS WT) than for the PAO1 wild-type based on the results of Fig. 5(a). As shown in Fig. 5(b), the eprS mutant was found to be less invasive than the wild-type strain.
Fig. 5. Disruption of eprS decreases the adherence and invasion abilities of P. aeruginosa. A549 cells were infected with each P. aeruginosa strain at the indicated m.o.i. for 3 h at 37 °C. Following the infection, c.f.u. counts per well were determined for (a, c) associated (both adherent and intracellular) or (b) solely intracellular (after treatment with gentamicin for 2 h) bacteria by culturing A549 cell lysates on LB agar. (d) A549 cells were infected with PAO1 wild-type at an m.o.i. of 50 or PAO1 ΔeprS at an m.o.i. of 200 to achieve the same number of bacteria invading A549 cells. Following the standard 2 h incubation with gentamicin, intracellular bacteria were quantified at 0, 24, 48, 72 and 96 h as described in Methods. Values represent the mean ± SD from triplicate determinations of a representative experiment. Similar results were obtained in three independent experiments. Comparisons of multiple groups were performed using one-way ANOVA with Tukey’s post hoc test. (a, b) There were significant differences between the group of WT and ΔeprS, or the group of ΔeprS and ΔeprS complemented with a wild-type copy of eprS (P<0.01), while there were no significant differences among the groups of WT, WT complemented with the empty control vector and ΔeprS complemented with a wild-type copy of eprS, or the groups of ΔeprS, ΔeprS complemented with the empty control vector and ΔeprS complemented with the proteolytic activity-deficient eprS S308A. (c) The differences between all the groups were not significant. (d) Comparisons of two groups were performed using Student’s paired t-test. The difference between PAO1 wild-type and PAO1 ΔeprS was not significant at each time point. WT, PAO1 wild-type; ΔeprS, PAO1 ΔeprS.
The eprS mutant as well as the wild-type strain associated with A549 cells (Fig. 5c). In addition, invasion efficiency of the eprS mutant was 3.2-fold lower than that of wild-type strain (Table 1). Invasion was restored by complementing the eprS mutant with a wild-type copy of eprS in trans but not by the empty vector control or proteolytic activity-deficient eprS S308A.

Since mammalian cells can effectively clear most intracellular bacteria, invasive bacterial pathogens are usually equipped with factors that enable them to evade the bactericidal effect of the host cells. Therefore, to examine whether an intracellular survival mechanism of P. aeruginosa is influenced by deletion of eprS, we compared the intracellular survival of the PAO1 wild-type strain and the eprS mutant. Similarly to the method described above, to achieve the same number of bacteria invaded A549 cells, a fourfold higher m.o.i. was used for PAO1-deprS than for the PAO1 wild-type. The A549 cells were lysed at various time points and plated to allow assessment of the number of intracellular bacteria. As shown in Fig. 5(d), intracellular survival of each strain decreased with a similar time-course. There was no significant difference in intracellular survival between the wild-type strain and the eprS mutant at each time point. The results indicate that the presence or absence of eprS does not affect an intracellular survival mechanism of P. aeruginosa. Taken together, these results suggest that eprS is involved in the adherence and invasion abilities of P. aeruginosa.

**Table 1. Invasion efficiency of each P. aeruginosa strain**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Invasion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>12.74</td>
</tr>
<tr>
<td>WT + empty</td>
<td>12.38</td>
</tr>
<tr>
<td>ΔeprS</td>
<td>3.98</td>
</tr>
<tr>
<td>ΔeprS + empty</td>
<td>4.19</td>
</tr>
<tr>
<td>ΔeprS + eprS WT</td>
<td>12.35</td>
</tr>
<tr>
<td>ΔeprS + eprS S308A</td>
<td>3.71</td>
</tr>
</tbody>
</table>

*Defined as percentage of associated bacteria that survive gentamicin treatment.

To examine whether eprS contributes to the in vivo virulence of P. aeruginosa, we compared the virulence of wild-type PAO1 and the eprS mutant using a mouse model of acute systemic infection by P. aeruginosa. The survival of mice, monitored after infection, is depicted in Fig. 6. The LD₅₀ of each P. aeruginosa strain in leucopenic mice was as follows: wild-type PAO1, 1.41 × 10⁴ c.f.u. per mouse; PAO1ΔeprS, 2.38 × 10⁵ c.f.u. per mouse. The results of these experiments demonstrated that the virulence of PAO1ΔeprS was attenuated compared to that of wild-type PAO1. Thus, these results indicate that EprS plays an important role in the in vivo virulence of P. aeruginosa.

**Fig. 6. Survival of leucopenic ICR mice infected intraperitoneally with different doses of wild-type P. aeruginosa PAO1 and the eprS mutant.** (a) Infection with 5 × 10³ to 5 × 10⁶ c.f.u. wild-type P. aeruginosa PAO1 per mouse. (b) Infection with 5 × 10³ to 5 × 10⁶ c.f.u. P. aeruginosa PAO1ΔeprS per mouse. Sixteen mice were used in each group.
DISCUSSION

In this study, we demonstrated that EprS participates in various phenotypic changes of *P. aeruginosa*. Overall, none of the mutant phenotypes could be complemented by expression of proteolytic activity-deficient eprS S308A, demonstrating that the phenotypes affected by the eprS mutation depend on the enzymically active protein. Thus, our results suggest that the proteolytic activity of EprS plays an important role in various cellular functions of *P. aeruginosa*.

We previously reported that EprS, an autotransporter protein of *P. aeruginosa*, is a serine protease (Kida et al., 2013). Serine proteases have serine as the active site residue and are classified into 13 clans in the MEROPS database (http://merops.sanger.ac.uk) (Rawlings et al., 2014). According to this classification, EprS belongs to family S8 of peptidases, also known as the subtilisin-like or subtilase family. The majority of subtilases are secreted, but several members are localized intracellularly (Siezen & Leunissen, 1997). In general, subtilases play a central role in protein maturation processes and precursor processing (Siezen et al., 2007). Intriguingly, the subtilisin-autotransporter SphB1 of *Bordetella pertussis* has been demonstrated to serve as a maturation protease for another protein secreted by the same organism (Coutte et al., 2001). Autotransporters are Sec-dependent proteins whose carboxy-terminal domains serve to translocate the amino-terminal ‘passenger’ domains across the outer membrane (Henderson et al., 2004). Mature SphB1 is released partly into the extracellular milieu, but is also retained at the cell surface, while the precursor form essentially exists in the outer membrane (Coutte et al., 2001, 2003). Thus, these early findings suggest that subtilisin-like autotransporter proteases would participate in protein maturation at the cell surface. We previously demonstrated that EprS is secreted into the extracellular milieu (Kida et al., 2013); however, it is still unknown whether mature EprS is also localized at the cell surface. Therefore, although the precise cellular localization of EprS remains poorly defined, EprS may function as a maturation protease in *P. aeruginosa*.

As shown in Fig. 2(d), disruption of eprS resulted in a decreased production of autoinducers in *P. aeruginosa*. In these experiments, we used *V. harveyi* BB886 as a bio-luminescence reporter strain for AHSLs. Because *V. harveyi* BB886 is a reporter strain for N-3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL) and N-3-hydroxybutyryl-homoserine lactone (3-hydroxy-C4-HSL), this strain has been shown to respond weakly to N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) and N-butyryl-homoserine lactone (C4-HSL), which are autoinducers produced by *P. aeruginosa* (Rickard et al., 2010). Therefore, our assay system is unlikely to be completely reliable in the evaluation of autoinducer production by *P. aeruginosa*. Further research will be required to determine, using a reporter strain for 3-oxo-C12-HSL and C4-HSL, whether EprS affects the production of autoinducers in *P. aeruginosa*.

Our data suggest that eprS is involved in the modulation of invasion ability in *P. aeruginosa* (Fig. 5). Also, disruption of eprS resulted in a reduced production of elastase, and a decrease of swimming and swarming motilities (Figs 2a and 3a,b). Indeed, deletion of elastase has been demonstrated to reduce the invasiveness of *P. aeruginosa* into epithelial cells (Cowell et al., 2003). Moreover, a reduction of flagellum-mediated motilities leads to a lowering of the invasion efficiency in *P. aeruginosa* (Fleiszeg et al., 2001). Therefore, these observations support the idea that a decreased production of elastase and a reduction of flagellum-mediated motilities, which are phenotypic changes represented by the eprS mutant, would be involved in a reduction of the invasiveness of *P. aeruginosa*.

We examined the contribution of eprS in the *in vivo* virulence of *P. aeruginosa* using a mouse model of leucopenia (Fig. 6). This model has been developed to study the pathogenesis of *P. aeruginosa* infection under immunosuppressed conditions and to evaluate the therapeutic agents (Cryz et al., 1983; Uezumi et al., 1992). Challenge of leucopenic mice by i.p. injection with *P. aeruginosa* was reported to cause sepsis by acute systemic infection, leading to the death of mice within 1 to 3 days after infection (Miyazaki et al., 1995; Uezumi et al., 1992). We previously demonstrated that EprS activates NF-κB-driven promoter through PAR-1, -2, or -4 and induces IL-8 production through PAR-2 in a human bronchiole epithelial cell line (Kida et al., 2013). Nevertheless, we did not investigate in this study whether EprS-induced PAR activation participates in the pathogenesis of *P. aeruginosa* infection using a mouse model of leucopenia. In fact, not only is the amount of EprS secreted from *P. aeruginosa* under *in vivo* conditions unknown, but so far it has not been elucidated whether EprS induces inflammatory responses through PARs under *in vivo* conditions. Additionally, the requirement levels of recombinant EprS for PAR activation under *in vitro* assay conditions have been shown to be above 800 ng ml$^{-1}$ (Kida et al., 2013). Because our previous data indicated that the amount of EprS secreted from *P. aeruginosa* is approximately 5–10 ng (ml culture supernatant)$^{-1}$ as estimated by Western blot analysis (Kida et al., 2013), the secreted amount of EprS seems to be apparently insufficient for PAR activation under *in vitro* assay conditions. Hence, it is feasible that EprS might play an important role in the *in vivo* phenotypic changes in *P. aeruginosa* rather than the activation of PARs under *in vivo* conditions. Further studies will be required to determine using PAR knockout mice whether EprS is able to induce inflammatory response through PARs under *in vivo* conditions.

Interestingly, SprP, a secreted and/or cell envelope subtilase of *P. aeruginosa*, in addition to EprS, has been revealed to affect various phenotypes of *P. aeruginosa*, including pyoverdine production, cell aggregation, biofilm formation, motility and anaerobic growth (Pelzer et al., 2014). Moreover, microarray analyses showed the downregulation of...
102 genes and upregulation of 116 genes in the sprP-disrupted mutant strain (Pelzer et al., 2014). Thus, it is conceivable that deletion of sprP leads to changes in the transcript levels of P. aeruginosa genes, thereby resulting in various phenotypic changes of this bacterium. Indeed, our recent quantitative real-time PCR analyses demonstrated that disruption of eprS results in a decreased expression of genes encoding secreted proteases, including alkaline protease, elastase and protease IV (unpublished data). Given these findings, it is possible that EprS may be involved in the regulation of transcriptional patterns relevant to various pathogenic phenotypic changes of P. aeruginosa. Further research will be needed to investigate the regulatory mechanisms of gene expression influenced by eprS.

In summary, we investigated whether EprS, an autotransporter protease of P. aeruginosa, participates in the pathogenicity of the bacterium. Our results suggest that disruption of eprS leads to a decrease in the production of elastase, pigments, autoinducers and surfactants, and a reduction of swimming and swarming motilities. In addition, the isogenic eprS mutant showed a reduction in the ability to associate with A549 cells and an attenuation of virulence in leukopemic mice as compared with the wild-type strain. Thus, these results suggest that EprS exerts pleiotropic effects on various pathogenic phenotypes of P. aeruginosa. Since the target molecules directly influenced by EprS remain to be clarified, whether conditional expression (cytoplasm, periplasm or outer membrane) of EprS in the eprS mutant, or recombinant EprS stimulation of the eprS mutant, affects various pathogenic phenotypes of P. aeruginosa is currently under investigation in our laboratory.

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Pleiotropic effects of EprS


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