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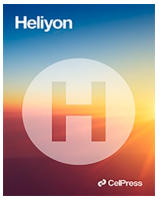

Research article

PemB, a type III secretion effector in *Pseudomonas aeruginosa,* affects *Caenorhabditis elegans* life span

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ABSTRACT

Pseudomonas aeruginosa is one of the leading nosocomial opportunistic pathogens causing acute and chronic infections. Among its main virulent factors is the Type III secretion system (T3SS) which enhances disease severity by delivering effectors to the host in a highly regulated manner. Despite its importance for virulence, only six T3SS-dependent effectors have been discovered so far. Previously, we identified two new potential effectors using a machine-learning algorithm approach. Here we demonstrate that one of these effectors, PemB, is indeed virulent. Using a live *Caenorhabditis elegans* infection model, we demonstrate this effector damages the integrity of the intestine barrier leading to the death of the host. Implementing a high-throughput assay using *Saccharomyces cerevisiae,* we identified several candidate proteins that interact with PemB. One of them, EFT1, has an ortholog in *C. elegans* (*eef-2*) and is also an essential gene and a well-known target utilized by different pathogens to induce toxicity to the worm. Accordingly, we found that by silencing the *eef-2* gene in *C. elegans*, PemB could no longer induce its toxic effect. The current study further uncovers the complex machinery assisting *P. aeruginosa* virulence and may provide novel insight how to manage infection associated with this hard-to-treat pathogen.

Declarations of interest: none.

1. Introduction

P. aeruginosa is a non-fermentative, aerobic, Gram-negative rod that can live in aquatic soil, and plant environments and can infect a verity of animal hosts including mammals, insects and nematodes [1–4]. In humans, *P. aeruginosa* is one of the leading opportunistic pathogens in nosocomial infections including infections of immunocompromised patients, burn victims, urinary tract infections, and chronic lung infections in cystic fibrosis patients [1–4]. With minimal nutrition requirements, *P. aeruginosa* is able to utilize a wide

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range of different organic compounds for growth, which contributes to its broad ecological adaptability and distribution [4].

One of the known virulence factors of *P. aeruginosa* is the Type III Secretion System (T3SS), a multi-protein complex that allows pathogens to modulate eukaryotic host cell response during infection by directly delivering exo-proteins (effectors) from the bacteria into the host [5]. T3SS enhances the severity of acute pneumonia, keratitis, bacteremia, peritonitis, burn infections, and gut-derived sepsis in neutropenia. Among the pathogens that have been shown to have T3SS in addition to *P. aeruginosa* are *Escherichia coli, Salmonella enterica, Shigella flexneri* and *Aeromonas salmonicida* [5,6].

Unlike the other pathogens that encode a multitude of effectors, thus far only six T3SS effector molecules have been identified and extensively studied in *P. aeruginosa* to date. This minimal number is in contrast to the wide variety of hosts *P. aeruginosa* can infect [6]. To this end, we hypothesized that there are additional unknown effectors yet to be discovered. In our previous work, we developed a machine-learning algorithm, which enables in-silico identification of new potential T3SS effectors in *P. aeruginosa*. Using this method we identified two new potential T3SS effectors – PemA and PemB [7]. However, although PemA and PemB translocated in a T3SS-dependent manner to yeast and mammalian HeLa cells, they did not induce toxicity [7].

Here, we demonstrate that PemB is a bona-fide effector which enhances PA14 toxicity in *C. elegans* infection. Furthermore, we provide insights into its pathogenic mechanisms.

2. Material and methods

2.1. Bacterial strains maintenance

The bacterial strains and plasmids used in this study are listed in Table S2. All bacterial strains were grown in LB (Luria-Bertani broth, Difco) at 37 °C.

Antibiotic concentrations used in this study are indicated in the following sections. Primers used in this study are listed in Table S3. Δ*toxA* mutant was prepared for this study as the protocol previously described [8]. Δ3tox strain was used instead of the PA14 WT strain for further experiments, to assure that observed phenotypes are related to the novel studied PemB effector and to reduce the possible competition in secretion between PemB and the major known effectors.

2.2. C. elegans maintenance

The following *C. elegans* strains were used in this study: N2, MD701: *Plim-7:: ced-1::gfp V* and CF2185: *ced-3(n1286) IV*. Strains were maintained on Nematode Growth Media (NGM) agar plates seeded with OP50 bacteria at 20 °C using the standard *C. elegans* methods [9]. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

2.3. Life span assay

Bacterial PA14 strains were grown overnight in LB with 100 μg/mL Gentamicin (Gm). 120 μL of overnight culture was spread on 3.5 cm diameter NGM plates. Each tested strain was seeded on six plates and the plates were incubated at 37 °C overnight. For each lifespan, 120 Day 1 nematodes were distributed between the 6 plates. Using a light microscope, the nematodes were scored as live or dead twice a day. SPSS software was used for statistical analysis and to determine means and percentiles. In all cases, p-values were calculated using the Log-rank (Mantel-Cox) method. Related lifespans were performed concurrently to minimize variability. Animals that ruptured or crawled off the plates were included in the lifespan analysis as censored worms.

2.4. RNAi treatment

Bacteria expressing dsRNA were cultured overnight in LB containing Tetracycline 12.5 μg/ml and Ampicillin 100 μg/ml and seeded on NGM plates containing 2 mM IPTG and 0.05 mg/ml Carbenicillin. *C. elegans* synchronized eggs were grown on the RNAi bacteria until day-1 of adulthood, as previously described [10], then transferred to NGM plates supplemented with 100 μg/ml Gentamycin and seeded with different PA14 bacteria strains for life span assay.

2.5. Intestine integrity (smurf) assay

Synchronized eggs of wild type animals were grown on NGM plates seeded with OP50/RNAi bacteria until day-1 of adulthood. On day-1 of adulthood the animals were transferred to NGM plates containing 100 μg/ml Gentamycin seeded with different PA14 bacteria strains until day-3 of adulthood. At that point, animals were washed once with M9 buffer and shaked for 4 h at room temperature in a blue dye solution (Erioglaucine disodium salt, 10 % V/W). The nematodes were then transferred to an OP50 plate for 20 min to remove excess dye and then placed on an agarose slide with Levamisole (Sigma-Aldrich) and pictures were taken using LMD6 microscope (Leica).

2.6. Apoptotic cell staining in C. elegans

Synchronized eggs of wild type or *Plim-7::ced-1::gfp C. elegans* were grown on NGM plates seeded with OP50/RNAi bacteria until day-1 of adulthood. On day-1 of adulthood animals were transferred to NGM plates containing 100 μg/ml Gentamycin seeded with

different PA14 bacteria strains until day-3 of adulthood. For Propidium iodide staining, animals were washed once with M9 buffer and were shaked for 5 h at room temperature with 10 μg/ml PI staining solution (Rhenium). Before documentation under the microscope, the nematodes were transferred to an OP50 plate to remove excess dye and then placed on an agarose slide with Levamisole (Sigma-Aldrich). Images were taken with a CCD digital camera using a Nikon 90i fluorescence microscope. For CED-1GFP engulfed cell analysis, animals were directly placed on an agarose slide with Levamisole (Sigma-Aldrich). Images were taken with a CCD digital camera using a Nikon 90i fluorescence microscope.

2.7. Protein fragment complementation assay (PCA)assay in S. cerevisiae

To construct *S. cerevisiae* strains expressing *pemB* fused to complement protein F3, we synthesized the product using Genewiz, Hylabs. The PCA was performed as described previously [11]. Strains were mated on YPD, and diploids were selected on YPD supplemented with clonNAT and -Leu plates. SD supplemented with noble agar (Difco), and methotrexate (MTX; Bioshop Canada) was used for the final selection steps. Drugs were added to the following final concentrations: clonNAT (100 μg/ml, Werner Bioagents); MTX (200 μg/ml (prepared from a 10 mg/ml methotrexate in DMSO stock solution, Bioshop, Canada). Positive yeast colonies were analyzed using the "Balony" software [11].

2.8. Quantitative RT-PCR analysis for eef-2

Animals were raised on control RNAi or *eef-2* RNAi at 20 °C from eggs until day 1 of adulthood. On day 1, animals were collected for RNA extraction. RNA extraction, purification, and reverse transcription were carried out using standard protocols. Real-time PCR was performed using Maxima SYBR (Fermentas) in a StepOnePlus instrument. Transcript levels were analyzed by the ΔΔCT method. Transcript levels of *ama-1* were used for normalization. Each sample was run in triplicates. 3 independent biological samples were analyzed. Statistical analysis was performed using one sample *t*-test. Error bars represent SD. For *ama-1* and *eef-2* primers, see Table S3.

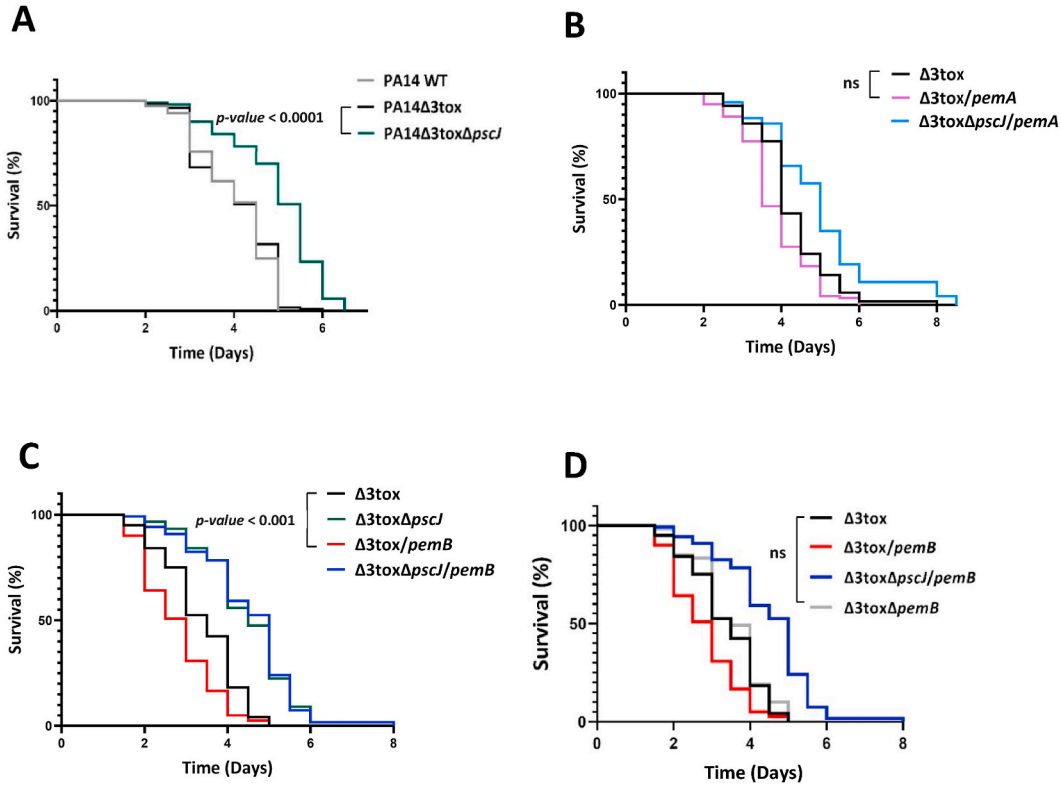

Fig. 1. The impact of T3SS and PemA/PemB effectors on *C. elegans* lifespan. (A) N2 WT worms were exposed to three different strains of PA14: WT, Δ3tox, and Δ3toxΔ*pscJ*. (B) Overexpression of PemA. (C) Overexpression of PemB.)D(deletion of PemB. The Y-axis represents the percentage of worms alive, while the X-axis shows the lifespan in days. Each experiment was repeated at least three times, and representative data from one experiment are shown. The statistical analysis was performed using the Log-rank (Mantel-Cox) method. p-values are presented on graphs. Ns = not significant.

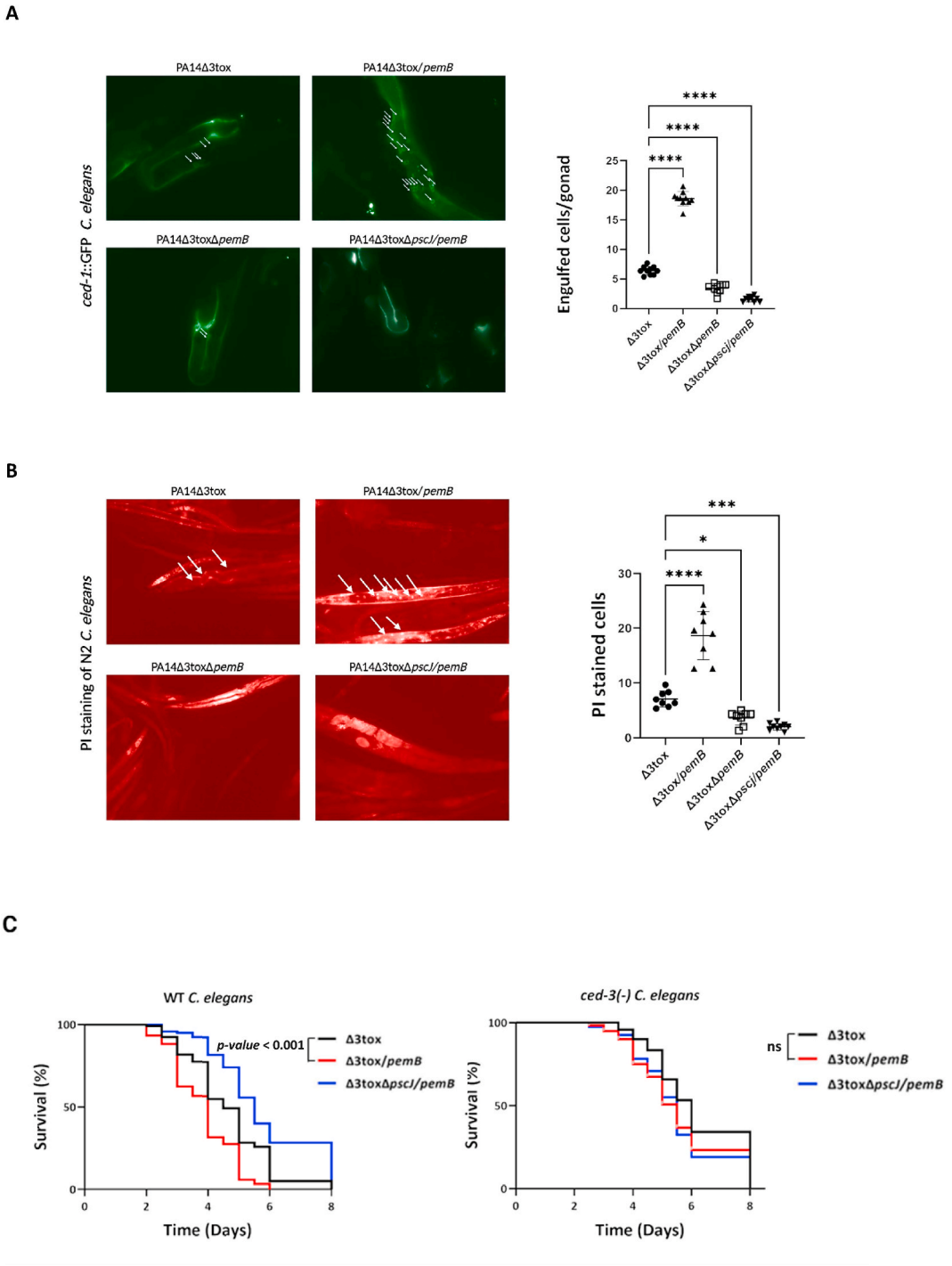

Fig. 2. PemB induces programmed cell death in *C. elegans*. (A) White arrows indicate cells undergoing engulfment in *C. elegans* infected by different PA14 strains. Quantification of cell corpses per gonad (****) represent p-value<0.0001. Statistical analysis was performed using the ordinary one-way ANOVA method. Data represent the average of at least three biological replicates. (B) Representative images of propidium iodide (PI) fluorescence staining (red) of dead cells (marked by nuclear signal, white arrows) in *C. elegans* exposed to *P. aeruginosa* strains. Quantification of PI experiment indicating number of cell death by compromised membrane (****) represent p-value<0.0001. Statistical analysis was performed using one-way ANOVA. Data represent the average of at least three biological replicates (C) Survival of WT *C. elegans* exposed to three different *P. aeruginosa* strains and survival of *ced-3(−) C. elegans* exposed to three different *P. aeruginosa* strains. Y-axis shows the percentage of worms alive, and X-axis shows the lifespan in days. Each experiment was repeated at least three times, and data from one representative experiment is shown. Statistical analysis was performed using the Log-rank (Mantel-Cox) method. Ns = not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. The T3SS contributes to PA14 toxicity in C. elegans

In a previous study, we identified two effectors, PemB and PemA, which translocate to a host cell in a T3SS-dependent manner but were not toxic to yeast and mammalian HeLa cells [7]. To further elucidate the biological activity of these effectors, we assessed their toxicity using a *C. elegans* infection model.

First, we examined whether the T3SS contributes to PA14 pathogenicity in *C. elegans*. To this end, we assessed the life span of *C. elegans* upon infection with PA14 WT, PA14Δ3tox - lacking the three major effectors ExoU, ExoT and ExoY, and PA14Δ3toxΔ*pscJ* – which has no active T3SS due to a deficiency in the basal substructure of the secretion apparatus. Whereas the lifespan of nematodes

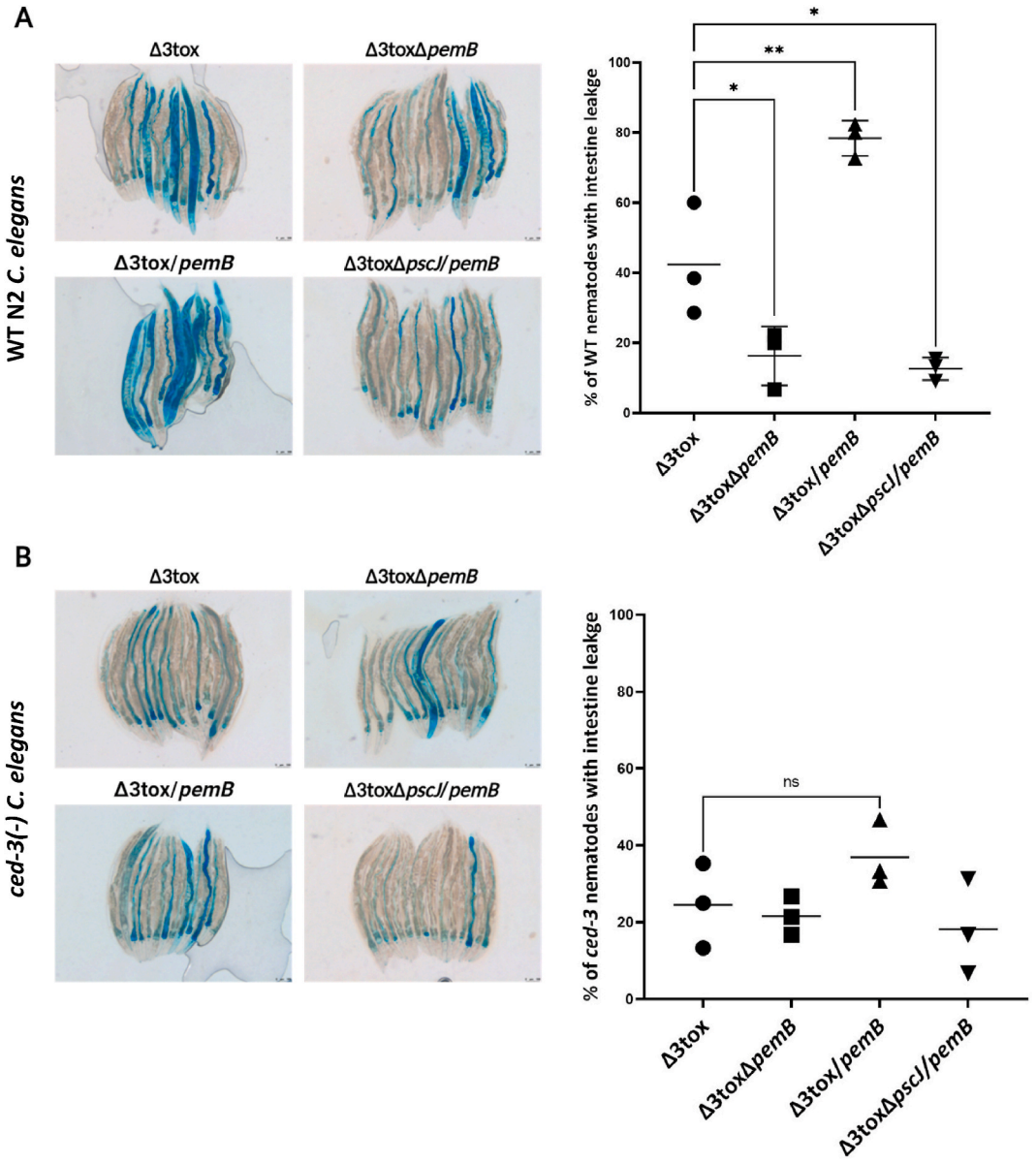

Fig. 3. PemB overexpression impairs *C. elegans* intestine integrity via CED-3. Representative images and quantification of intestinal blue food leakage in day 3 wild-type nematodes (A) and *ced-3* mutant nematodes (B) exposed to different *P. aeruginosa* strains, including PA14Δ3tox, PA14Δ3toxΔ*pemB*, PA14Δ3tox overexpressing PemB, and PA14Δ3toxΔ*pscJ* overexpressing PemB. Graph presents the percentage of nematodes with intestine leakage. The asterisk (*) indicates a significant difference with p-value <0.01. Ns = not significant. Statistical analysis was preformed using one-way ANOVA. Data represent the average of at least three biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

grown on WT and Δ3tox strains displayed a similar trend, the lifespan of nematodes grown on Δ3toxΔpscJ was significantly longer (Fig. 1A). The reduced toxicity of the Δ3toxΔpscJ strain indicates that the T3SS machinery plays an important role in PA14 infection kinetics in our *C. elegans*. Moreover, based on the comparable toxicity of the PA14 WT and PA14Δ3tox strains it can be inferred that the three major effectors are not essential for virulence in *C. elegans* or that the efficacy of other toxins conceals the impact of the major three effectors.Furthermore, the fact that Δ3toxΔpscJ strain is less virulent suggests that there likely are other still unknown T3SS effectors involved in the virulence of PA14 against *C. elegans*.

3.2. Overexpression of PemB in PA14 causes a toxic effect in C. elegans

The finding that there are more T3SS effectors beyond the three major effectors ExoU, ExoT and ExoY involved in the virulence of PA14 against *C. elegans* prompted us to investigate the recently identified effectors PemA and PemB [7]. To this end, we determined the survival of nematodes exposed to PA14 strains over-expressing each of these effectors. Our results demonstrated that PemB over-expressing PA14 significantly shortened the life span of *C. elegans* (Fig. 1C), whereas PemA overexpressing PA14 did not induce any additional toxicity (Fig. 1B). As expected, the increased toxicity of PemB overexpressing PA14 was completely dependent on the presence of a functional T3SS (Fig. 1C).Deletion of *pemB* did not affect *C. elegans* lifespan, and maintained a similar phenotype as the control PA14Δ3tox strain (Fig. 1D) despite the observed promoter activity and confirmed expression of PemB (Fig. 1S)

These results suggest that the novel T3SS effector PemB induces toxicity in the *C. elegans* infection model and that PemB toxicity is dependent on a functional T3SS.

3.3. PemB toxicity is dependent on the CED-3 caspase in the host nematode

Some of the major effectors of *P. aeruginosa* induce cell death in the host by activating apoptosis [12–16]. Thus, we hypothesized that PemB may also engage the apoptosis machinery to impact the survival of *C. elegans*. To this end, we followed apoptosis induction in the *C. elegans* germline and intestine upon infection by the different PA14 strains. Specifically, to track the engulfment and clearance of dead germ cells within the gonads, we used a *C. elegans* strain expressing a GFP tagged CED-1 transmembrane receptor that mediates cell corpse engulfment [17]. We found that whereas PA14Δ3tox infection induced more germ cell apoptosis compared to the mildly pathogenic OP50 *E. Coli* strain, nearly twice as much apoptotic cells were observed in the gonads of *C. elegans* that were exposed to a PA14 strain overexpressing PemB (Fig. 2A). PemB-mediated increased germ cell apoptosis was dependent on the presence of an intact T3SS (Fig. 2A). Strikingly, T3SS-dependent PemB-induced cell death was also observed in the intestine of the animals, as assessed by propidium iodide (PI) staining, which detects loss of membrane integrity (Fig. 2B). In both assays, a *pemB* (Δ3toxΔ*pemB*) mutant showed a significant decrease in germ cell apoptosis and intestinal cell death compared to the Δ3tox strain (Fig. 2A and B), indicating that native expression of PemB actively influences worm physiology.

Finally, we examined whether PemB virulence in *C. elegans* was dependent on its ability to induce apoptosis in the host. We found that PemB toxicity was curtailed in *ced-3 C. elegans* mutants lacking the main *ced-3* caspase gene, which is critical for programmed cell death (PCD) in *C. elegans* (Fig. 2C). Altogether, this suggests that PemB's ability to activate caspases in *C. elegans* is critical for its toxicity.

Interestingly, the inactivation of T3SS demonstrated no benefits in *ced-3(−) C. elegans* mutants, at least in the Δ3tox strain, indicating that T3SS and its effectors rely on and activate the CED-3 caspase to exert toxicity.

3.4. PemB impairs the C. elegans intestine barrier

P. aeruginosa invades *C. elegans* via its intestine [18,19]. To investigate the direct impact of PemB on the intestine, we examined the integrity of the intestine using the Smurf assay [20,21] upon feeding the nematodes with different PA14 strains. This non-invasive assay assesses intestinal barrier function by feeding the animals with a non-absorbable blue food dye and monitoring its leakage from the intestine lumen into the body cavity. Strikingly, 78 % of *C. elegans* grown on the PemB-overexpressing strain had an impaired intestine, resulting in dye leakage, whereas only 42 % of *C. elegans* grown on the Δ3tox strain exhibited this phenotype (Fig. 3A). The T3SS was required for PemB-induced intestinal leakage, as minimal intestinal leakage was observed in nematodes treated with PemB-overexpressing Δ3toxΔ*pscJ* strains. The deletion of PemB (Δ3toxΔ*pemB*) resulted in 16 % of *C. elegans* displaying intestinal leakage (Fig. 3A). Notably, PemB failed to induce intestine leakage in *ced-3(−) C. elegans* mutants, indicating that the CED-3 caspase is required for PemB-mediated effects on the intestine (Fig. 3B). These results reaffirm that T3SS and its effectors require the CED-3 caspase for their toxic effects on the *C. elegans* intestine, as blocking the T3SS does not provide additional benefits in *ced-3(−) C. elegans* mutants, consistent with the observed effects on nematode lifespan.

3.5. Identifying PemB potential protein interactions using a protein-fragment complementation assay (PCA)

To decipher the mechanism of action of PemB we attempted to identify potential proteins that interact with this protein. To this end, we performed a yeast two-hybrid screen using *Saccharomyces cerevisiae* as a model organism [11]. We reasoned that given the high degree of conservation in the core functions of both *S. cerevisiae* and *C. elegans* we could identify potential yeast *C. elegans* orthologs that interact with PemB using this approach. The criteria for a protein to have a reliable interaction with another protein using the PCA method involves physical interaction, reconstitution of the reporter activity, and meeting the threshold of colony intensity. The data from the experiment is analyzed by filtering false positives, assessing data quality, and comparing with existing interactions to obtain a

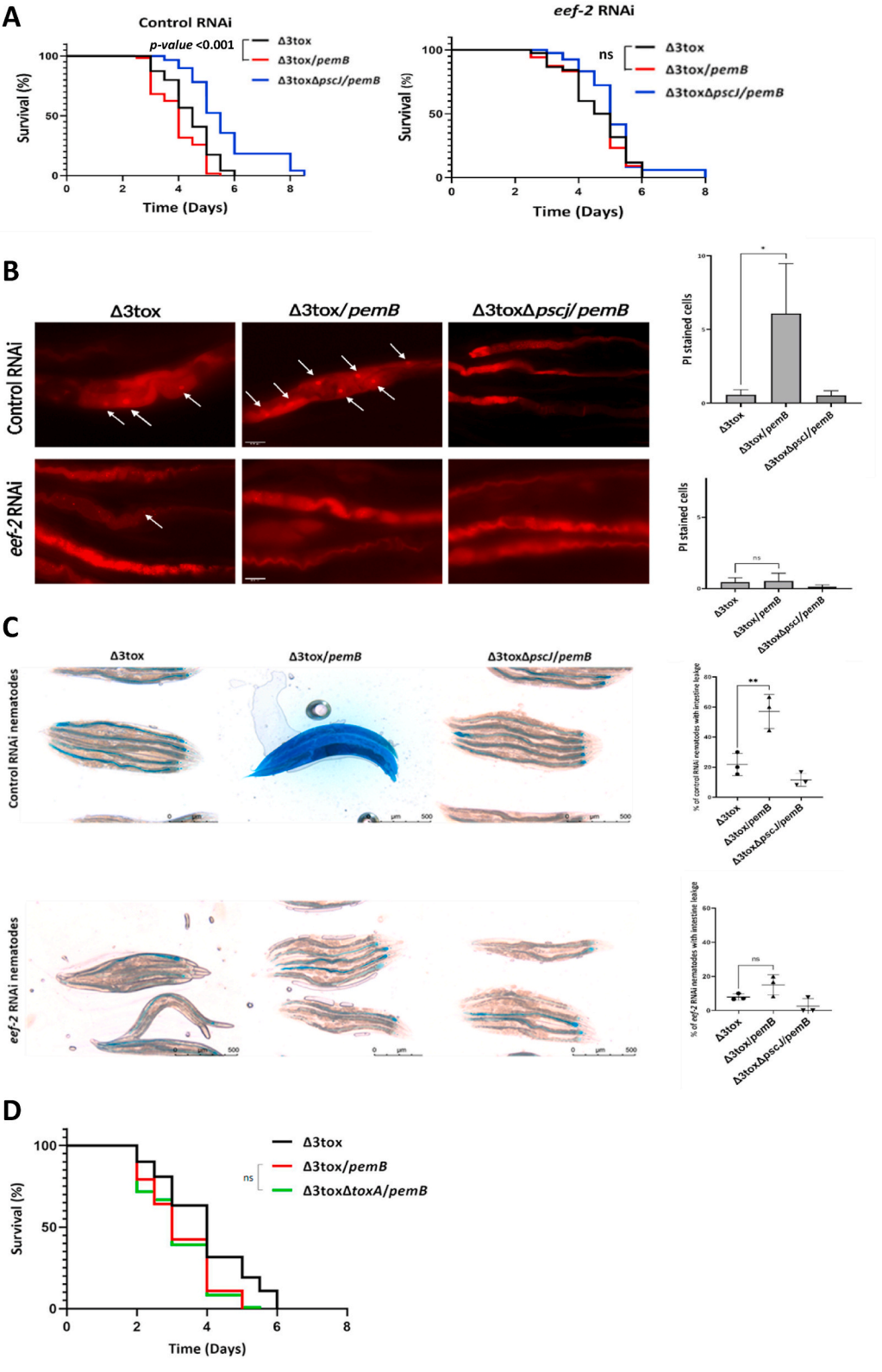

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Fig. 4. EEF-2 is essential for PemB toxic effect in *C. elegans*. (A) Survival of *C. elegans* treated with control or *eef-2* RNAi exposed to three different *P. aeruginosa strains*. The y-axis shows the fraction of worms alive and the x-axis shows the survival time in days. Each experiment was repeated at least three times, and representative data from one experiment are shown. Statistical analysis was performed using the Log-rank (Mantel-Cox) method (B) Representative propidium iodide labeling of dead cells within the intestine of *C. elegans* treated with control RNAi and *eef-2* RNAi exposed to different *P. aeruginosa* strains. PI positive dead cells are marked by white arrows. Quantification of the number of PI stained cells per intestine in worms pre-treated with control RNAi or with *eef-2* RNAi. (*) represent p-value = 0.0481, statistical analysis was preformed using one-way ANOVA. (C) Representative images of nematodes treated with control or eef-2 RNAi fed with different *P. aeruginosa* strains, including PA14Δ3tox, PA14Δ3tox overexpressing PemB, and PA14Δ3toxΔ*pscJ* overexpressing PemB, and soaked in blue food dye for 3 h on day 3. Quantitative representation of the percentage of nematodes with intestine leakage. The (**) represent p-value = 0.0012. ns = non-significant, statistical analysis was preformed using one-way ANOVA. method. (D) ToxA is not essential for PemB toxic effect. N2 WT worms were grown on different strains of PA14. Y-axis shows fraction of worms alive. X the x-axis shows the survival time in days. Each experiment was repeated at least three times and representative data from one experiment is shown. P-values were calculated using the Log-rank (Mantel-Cox) method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

list of real possible interactions. As described in Kirill Tarassov et al. [11], we analyzed our positive yeast colonies using the "Balony" software, and identified 18 most high-scoring candidates with possible protein interactions with PemB (Table S1). Thirteen of these candidates have orthologs in *C. elegans*, and we focused on one candidate, the EFT1 gene in yeast and its ortholog in *C. elegans*, *the eef-2* gene. We chose to focus on the EFT1 ortholog due to the fact that among the candidate proteins, the EFT1/EEF2 proteins are essential and important for viability in both in *S. cerevisiae* [22] and in *C. elegans* [22,23].Furthermore, EEF2 is a known target for *P. aeruginosa* virulence [24,25].

3.6. Eef-2 is required for PemB toxic effect in C. elegans

Elongation factor 2 (eEF-2), is a key component of the protein synthesis machinery in eukaryotes in charge of promoting the GTP-dependent translocation of the ribosome. Recent discoveries emphasize the significance of controlling the elongation cycle, with eEF-2 playing a key role in facilitating the movement of peptidyl-tRNA during protein synthesis [23,26,27].

We sought to further investigate the mechanism underlying the toxic effects of PemB in *C. elegans* by examining its interaction with eEF-2. To this end, synchronized WT *C. elegans* eggs were grown on control or *eef-2* RNAi bacteria until day-1 of adulthood and then transferred onto a lawn of different *P. aeruginosa* strains, as previously described [10]. We tested the expression levels of *eef-2* using qRT-PCR to ensure RNAi efficacy (Fig. 2S). The adult RNAi-treated animals were exposed to PA14Δ3tox, PA14Δ3tox overexpressing PemB and PA14Δ3toxΔpscJ overexpressing PemB strains. Our results showed that upon *eef-2* gene silencing, the overexpression of PemB no longer had a significant toxic effect on *C. elegans* lifespan (Fig. 4A).

Next, we performed PI staining on *C. elegans* pre-treated with control RNAi or *eef-2* RNAi that were subsequently exposed to Δ3tox strains (Fig. 4B). We hypothesized that if PemB must bind EEF-2 to induce cell death in the host, less dead cells would be detected in the intestine of *eef-2*-silenced animals. Consistent with this hypothesis, whereas dead intestinal cells were detected in control RNAi-treated animals exposed to Δ3tox overexpressing PemB, PemB overexpressing PA14 did not induce apoptosis in the *eef-2* RNAi-treated animals (Fig. 4B).

Consistent with the lack of PemB-induced cell death, when infected with a PA14 strain overexpressing PemB, nearly 60 % of control RNAi-treated WT *C. elegans* animals had intestinal leakage, whereas less than 20 % of the *eef-2*-silenced animals displayed intestinal leakage upon a similar infection (Fig. 4C), These data suggest that the EEF-2 protein is an essential target for PemB to induce its toxic effects in *C. elegans*.

3.7. PemB effect on C. elegans lifespan is independent of ToxA

A common strategy employed by bacteria during infection is to halt host mRNA translation, thereby impeding the production of antimicrobial peptides and host immune effector molecules, thus enhancing bacterial survival [25,28–30]. In *C. elegans*, it has been observed that *P. aeruginosa* infection activates an effector-mediated immune pathway that inhibits translation elongation. This pathway is triggered by the secreted endocytic Exotoxin A, which eventually suppresses the expression of EEF-2 [25,29]. Building upon these observations, we hypothesized that PemB may interact with EEF-2 in conjunction with ToxA. To investigate this hypothesis and assess whether the absence of ToxA affects the toxic effect of PemB, we conducted a lifespan experiment utilizing a PA14 *toxA* mutant strain (Fig. 4D). Notably, similar PemB-mediated lifespan shortening was observed in *C. elegans* exposed to either PA14Δ3tox overexpressing PemB and PA14Δ3toxΔtoxA overexpressing PemB. Based on these results, we conclude that the toxic effect of PemB in *C. elegans* is independent of ToxA.

4. Discussion

Previously we described the identification of two novel T3SS effectors PemA and PemB in *P. aeruginosa,* but were not able to show their contribution to virulence in several model systems including yeast and mammalian cells [7]. In the current study we examined their virulence in a *P. aeruginosa* – *C. elegans* infection. This model is widely used to investigate host-pathogen interaction as the nematode nutrition is based on bacterial diet making it very easy to infect [2,31]. *C. elegans* has emerged as a valuable model organism for investigating the pathogenesis of *P. aeruginosa* and studying its virulence factors. Numerous studies have employed *C. elegans* as an

effective experimental system to elucidate the mechanisms underlying *P. aeruginosa* pathogenicity [2]. This model has been widely used especially in the context of virulence factor analysis [32–35].

Although a previous study demonstrated that the PA14 type III secretion system is not essential to virulence in *C. elegans* [36], this is not the case in our experimental infection model. Specifically, in our model, the T3SS machinery of *P. aeruginosa* plays a crucial role in the bacterial pathogenesis efficiency in *C. elegans,* as its' absence resulted in a significant extension of the nematodes' lifespan. This paradox may be explained by the different conditions in which the pathogenic bacteria have been propagated in the two studies, for example growth media composition, incubation temperature, worm life cycle stage etc. For example, previous studies have shown that the rate of PA14-mediated killing of *C. elegans* depends on the composition of the agar medium on which PA14 is grown [37] Therefore, the T3SS's contribution to PA14 pathogenicity in *C. elegans* may be context-dependent, varying under different growth conditions.

The similar lifespan trend between the wild-type strain and the Δ3tox strain suggested the involvement of additional T3SS effectors in the virulence of *P. aeruginosa* in the *C. elegans* model. Accordingly, our results revealed that PemB overexpression significantly reduced the lifespan of *C. elegans* in a T3SS-dependent manner (Fig. 1). *pemB* is a pseudomonas-specific gene, found in several different *P. aeruginosa* strains, both laboratory and clinical isolates. Moreover, ExsA binding site was identified in PemB, further linking it to the T3SS and highlighting its possible involvement in virulence [38–40]. Interestingly, PemB was recently reported [41] to have a pleckstrin homology-like domain (PHLD) that exhibits strong binding affinity for specific phosphoinositides, namely PI, PI(5)P, PI(3,4,5)P3, and PI(3,5)P2. The interaction of PemB with these phosphoinositides is similar to other effector proteins from different bacteria [42–44], highlighting the importance of lipid binding in the virulence mechanisms of pathogens. The strong binding affinity for phosphoinositides suggests that PemB may play a role in manipulating host cell machinery and establishing infection by binding also to the plasmatic membrane. Moreover, PemB tends to form higher-order oligomers in aqueous solution [41].The oligomerization tendency of PemB implies its involvement in complex protein-protein interactions and the formation of larger functional assemblies.

Our experiments revealed that PemB triggers cell death in both the germline and intestine of *C. elegans*. Cell death induction *in C. elegans* is dependent on the expression of PemB and on the presence of the T3SS. Moreover, a complete deletion of *pemB*, presents lower cell death in both the germline and intestine of *C. elegans*, suggesting the impact of PemB in *C. elegans* infection (Fig. 2). Notably, both the induction of cell death and the pathogenicity of PemB are abolished in *C. elegans* mutants lacking the *ced-3* caspase gene, emphasizing the pivotal role of caspases in PemB-induced toxicity. Strikingly, caspase activation by PA14 T3SS has been demonstrated in HeLa cells, demonstrating that this is an evolutionary conserved cytotoxicity mechanism of the T3SS system [12] This finding reinforces the significance of T3SS in inducing cell death within a host and aligns with previous observations made in cell culture [16]. In *C. elegans ced-3*-induced apoptosis is an inherent part of the developmental program of the animal and of the germline, but is prohibited in somatic cells in the adult [45,46]. Our data suggests that CED-3-induced cell death does occur in the adult soma upon exposure to PemB overexpressing PA14. This may be classical caspase-mediated programmed cell death [47,48], an alternative *ced-3* dependent cell death mechanism or another non-canonical function of CED-3 [49–51].

We further investigated the impact of PemB on *C. elegans* intestine integrity (Fig. 3). Our results demonstrate that PemB overexpression leads to loss of intestinal barrier function, and this effect is dependent on both the T3SS and the presence of PemB. Deletion of PemB is sufficient to mitigate intestinal leakage in a significant manner. This result correlates with decreased cell-death observed in the *pemB* mutant (Fig. 2). Importantly, the observed effects on intestinal leakage were diminished in *C. elegans* mutants lacking the *ced-3* gene, highlighting the requirement of the CED-3 caspase for PemB-mediated effects on the intestine. In fact, targeting the intestinal barrier can be an attack strategy to avoid infiltrating the interstitial zone.

To gain further insights into the mechanism of action of PemB, we conducted a yeast two-hybrid screen to identify potential interacting proteins. The analysis revealed a high-scoring candidate, eEF-2. This protein is an essential for protein synthesis and is a known target for several bacterial toxins including ToxA from *P. aeruginosa*. It was reported that in PAO1, *toxA* inhibits translation in *C. elegans* by ADP ribosylation of EEF-2, specifically targeting the modified histidine known as the *eef-2* diphthamide moiety [25]. Interestingly, the crystal structure Cholix effector, produced mainly by *Vibrio cholerae*, is similar to the ToxA effector and is known to induce programmed cell death by targeting *eef-2* in mammalian cells [30]. In these cases, inhibition of translation in the host is detected by the host surveillance mechanisms and triggers protective defense responses [52,53]. Nevertheless, when silencing *eef-2*, we observed a decrease in apoptotic cells and less intestinal leakage (Fig. 4), suggesting that the *eef-2* gene plays a role in the toxicity of PemB. Accordingly, results from the lifespan analysis indicate that the activity of PemB is independent of ToxA, highlighting the complex interplay between PemB, EEF-2, and ToxA in modulating cellular toxicity.

P. aeruginosa is capable of inducing disease in a broad range of hosts, to which common strategies of microbial pathogenicity have co-evolved. EEF-2, a host protein that interacts with PemB and is important for its virulence, is conserved from yeast to mammals. Nevertheless, PemB toxicity was not detected in infected yeast or HeLa cells. This is in spite the fact that we demonstrate a physical interaction between PemB and the yeast EFT1 protein in yeast. This may be explained by inter-organismal differences downstream to the PemB-EFT1/EEF-2 interaction. Alternatively, the virulence may be executed at the organism level for example by manipulating the host immune response or affecting tissue organization and integrity and hence detected only in multi-cellular model organisms.

In conclusion, this study sheds new light on the virulence mechanisms of *P. aeruginosa*, particularly in the context of its effect on the *C. elegans* infection model. We have demonstrated the significance of the T3SS machinery in the bacterial pathogenesis efficiency in this model organism. We assigned a virulence function to the recently identified T3SS effector PemB, implicating it in caspase-dependent cell death and loss of intestinal barriers in *C. elegans*. Another interesting finding is the possible relationship between PemB, eEF-2 and CED-3 which should further be explored. Taken together, this study highlights the diversity of virulence factors that can impact infection outcomes and underscores the complexity of bacterial-host interactions and the multifaceted nature of toxicity mechanisms. Our findings may direct further studies to other model organisms, broadening the scope of its relevance and significance in diverse biological contexts. This may contribute to our fundamental understanding of host-microbe dynamics and exemplifies the

ongoing exploration of microbial virulence in shaping disease outcomes.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Shira Zelikman: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Reut Dudkevich:** Writing – original draft, Validation, Project administration, Methodology, Investigation. **Hadar Korenfeld-Tzemach:** Methodology, Investigation, Formal analysis. **Esther Shmidov:** Validation, Formal analysis, Data curation. **Mor Levi-Ferber:** Validation, Data curation. **Sivan Shoshani:** Writing – review & editing. **Shay Ben-Aroya:** Supervision, Resources. **Sivan Henis-Korenblit:** Writing – review & editing, Supervision, Resources, Conceptualization. **Ehud Banin:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29751.

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