The Psychrotrophic *Pseudomonas lundensis*, a Non-*aeruginosa* Pseudomonad, Has a Type III Secretion System of the Ysc Family, Which Is Transcriptionally Active at 37°C

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**ABSTRACT** The type III secretion system (T3SS) is a needle-like structure found in Gram-negative pathogens that directly delivers virulence factors like toxins and effector molecules into eukaryotic cells. The T3SS is classified into different families according to the type of effector and host. Of these, the Ysc family T3SS, found in *Yersinia* species and *Pseudomonas aeruginosa*, confers high virulence to bacteria against eukaryotic hosts. Here, we present the first identification and transcriptional analyses of a Ysc T3SS in a non-*aeruginosa* *Pseudomonas* species, *Pseudomonas lundensis*, an environmental psychrotrophic bacterium and important agent of frozen food spoilage. We have identified and sequenced isolates of *P. lundensis* from three very distinct ecological niches (Antarctic temporary meltwater pond, U.S. supermarket 1% pasteurized milk, and cystic fibrosis lungs) and compared these to previously reported food spoilage isolates in Europe. In this paper, we show that strains of *P. lundensis* isolated from these diverse environments with ambient temperatures ranging from below freezing to 37°C all possess a Ysc family T3SS secretion system and a T3S effector, ExoU. Using in vitro and in vivo transcriptomics, we show that the T3SS in *P. lundensis* is transcriptionally active, is expressed more highly at mammalian body temperature (37°C) than 4°C, and has even higher expression levels when colonizing a host environment (mouse intestine). Thus, this Ysc T3SS-expressing psychrotrophic Pseudomonad has an even greater range of growth niches than previously appreciated, including diseased human airways.

**IMPORTANCE** *P. lundensis* strains have been isolated from environments that are distinct and diverse in both nutrient availability and environmental pressures (cold food spoilage, Antarctic melt ponds, cystic fibrosis lungs). As a species, this bacterium can grow in diverse niches that markedly vary in available nutrients and temperature, and in our study, we show that these various strains share greater than 99% sequence similarity. In addition, all isolates studied here encoded complete homologs of the Ysc family T3SS seen in *P. aeruginosa*. Until recently, *P. aeruginosa* has remained as the only *Pseudomonas* species to have a characterized functional Ysc (Psc) family T3SS. With the identification of a complete Ysc T3SS in *P. lundensis* that is expressed at 37°C in vivo, it is intriguing to wonder whether this bacterium may indeed have some level of symbiotic activity, of yet unknown type, when consumed by a mammalian host.

**KEYWORDS** 2T.2.5.2, AU1044, Antarctica, M101, M105, *Pseudomonas fluorescens*, milk, spoilage
**Pseudomonas lundensis** is a psychrotrophic bacterium whose extent of distribution across diverse environments has not been fully appreciated. *P. lundensis* was first reported in 1986 as a prime spoilage bacterium in cold beef and pork (1) and has since been found growing in chilled meat, milk, and milk products (2–4). It has also been isolated from the feces of wild boar (5), Antarctic temporary meltwater ponds, and sputum samples in patients with cystic fibrosis (6), all of which demonstrate the wide diversity of environments in which this bacterium is able to persist and grow. *P. lundensis* has favorable growth temperatures between 0°C to 30°C, with optimal growth at 25°C (1). We can readily culture this bacterium at 37°C, indicating that the upper temperature limit for growth of this psychrotroph is at least 37°C. *P. lundensis* is a Gram-negative, polar flagellated bacterium belonging to the *Pseudomonas fragi* clade of the *Pseudomonas fluorescens* species complex (7). This bacterium exhibits surface-associated motility and is capable of forming stable biofilm structures across a wide temperature range (8). However, we report in this paper that *P. lundensis* also encodes a Ysc family type III secretion system (T3SS), similar to that seen in *P. aeruginosa* and *Yersinia* spp. T3SS is a molecular system that mediates cell-to-cell interactions between Gram-negative bacteria and eukaryotes. There are several families of T3SS, classified based on phylogenetic analysis, all varying in their effector and the hosts with which they interact (9). Depending on the bacterial species and host, the T3SS can sway the interaction outcomes from commensalism to symbiotic or mutualistic (e.g., Rsp family T3SS in *P. fluorescens* [10, 11]) or pathogenic (e.g., Ysc family, Inv-Mxi Spa family, and Hrc Hrp1/2 family T3SS in Gram-negative pathogens like *P. aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas putida* [9, 12, 13]).

In *P. aeruginosa*, the Ysc (Psc) family T3SS is one of many virulence characteristics in its arsenal. Although it is not essential for causing infection, the T3SS plays a very important role in interacting with a host and establishing chronic infections by dampening host defense and activating inflammasome signaling (14, 15). *P. aeruginosa* encodes up to four T3SS effector proteins that have known toxic activities (ExoS, ExoT, ExoY, and ExoU) of which ExoU-mediated cytotoxicity is the most potent (16, 17). However, these potent virulence characteristics are not unique to clinical isolates and can also be found in environmental isolates of *P. aeruginosa* (17), where T3SS confers a fitness advantage to the bacterium (18). To date, *P. aeruginosa* is the only *Pseudomonas* species in which a functional Ysc T3SS and its effector proteins have been reported.

In this current study, we have cultured, identified, and sequenced isolates of *P. lundensis* grown from three very distinct ecological niches (Antarctic temporary meltwater pond, U.S. supermarket pasteurized milk, and human cystic fibrosis lungs) and compared these to previously reported food spoilage isolates from Europe. The isolates described here all grow in very different environments in terms of nutritional availability, temperature, and environmental pressures. Yet strains with a complete genome assembly share >99% sequence similarity (draft genomes share >98% sequence identity). We report the identification of a complete Ysc T3SS complex in *P. lundensis*, as well as homologs of the ExoU effector protein and its chaperone SpcU. Like *P. aeruginosa*, all isolates of *P. lundensis* (environmental and clinical) encode a complete Ysc T3SS. The clinical isolate AU1044 expressed its T3SS in *in vitro* growth conditions at 37°C while having no expression at low temperature, 4°C. High T3SS expression is also seen in bacteria colonizing the intestinal tract of gnotobiotic mice, raising further questions about how this bacterium may interact with a mammalian host.

**RESULTS**

Isolation and characterization of *P. lundensis* strains from diverse environments. *Pseudomonas lundensis*, commonly reported as a psychrotrophic food spoilage bacterium (for example, in cold storage spoilage of pasteurized milk), can colonize the lungs of cystic fibrosis (CF) patients (6). Strain AU1044, the strain studied in this paper, is one such clinical isolate of *P. lundensis* and was isolated from a sputum sample of a CF patient in Columbia, Missouri, United States (6). To test if this clinical isolate was able
to grow in the known environmental niches of *P. lundensis*, AU1044 was cultured in milk and LB broth at ambient and cold temperatures, representing environments similar to that of spoiled foods. Under aerobic conditions, strain AU1044 was successfully grown in both milk media and LB broth and at temperatures ranging from 4°C to 37°C (data not shown). The generation times at room temperature through 37°C were similar, while the generation time at 4°C was significantly longer. Thus, the clinical isolate AU1044 can grow in cold storage food spoilage conditions similar to what has been reported for other isolates of *P. lundensis* (19). This was also true for the 11 other clinical isolates (AU11122, AU11136, AU11164, AU11235, AU7350, AU12597, AU12644, AU2390, AU10414, AU9518, and AU14641) that we reported earlier, which were isolated from cystic fibrosis patients from seven other sites across the United States (Ann Arbor, Michigan; Boston, Massachusetts; Chapel Hill, North Carolina; Cincinnati, Ohio; Hartford, Connecticut; Palo Alto, California, and St. Louis, Missouri) (6). Strain 2T.2.5.2 was isolated during the 5th Belarusian Antarctic Expedition (58th Russian Antarctic Expedition) in the area of the base Gora Vechernyaya from the temporary meltwater ponds (puddles) of the Lazurnaya Bay, Alasheev Bay, and Cosmonauts Sea. Strains M101 and M105 were isolated from 1% milkfat pasteurized milk, purchased from a grocery store in Ann Arbor, Michigan, in which the milk had been allowed to spoil after extended storage at 4°C. Strains 2T.2.5.2, M101, and M105 were all successfully cultured in milk media and LB broth and at temperatures ranging from 4°C to 37°C. Thus, the isolates from these extremely diverse environments (human lungs, spoiled milk, and Antarctic temporary meltwater ponds) all behaved similarly under a range of *in vitro* conditions.

**Genetic organization of *P. lundensis* T3SS.** We screened the genome of strain AU1044 using the Virulence Factors of Pathogenic Bacteria (VFDB; http://www.mgc.ac.cn/VFs/) database as a reference and identified a set of genes that were identified as Ysc family T3SS genes. A phylogenetic analysis of T3SS structural proteins PscS, PscT, and PscU from *P. lundensis* strains and their respective homologs showed that the T3SS in *P. lundensis* is most homologous to that of *P. aeruginosa* and falls among other species that carry Ysc family T3SS (Fig. 1A). A protein BLAST analysis was performed with the putative T3SS genes identified in AU1044 and 2T.2.5.2 (Fig. 1B; Table 1), setting the Psc family T3SS in *P. aeruginosa* PA14 as the reference. Results from this analysis further confirmed that *P. lundensis* has a Ysc family T3SS, and this system shares high protein sequence similarities with the Psc family T3SS in *P. aeruginosa*.

Our next step was to check if this T3SS gene locus is conserved across various strains of this bacterium or whether it is unique to clinical isolates or only this strain. To do this, we screened the genomes of several strains of *P. lundensis*, which were isolated from spoiled food sources (M101 and M105 from spoiled pasteurized milk in the United States; DSM6252 and MFPA15A12 from spoiled meat in Europe), cold environments (2T.2.5.2 from Antarctic temporary meltwater pond), and cystic fibrosis (AU10414 and AU11222). Strains AU1044, 2T.2.5.2, M101, and M105 are closed genomes that are annotated by the NCBI annotation pipeline, while the rest are present as draft genomes. Using the Psc family T3SS from PA14 as well as the newly identified T3SS genes from AU1044 as the reference, the 8 strains of *P. lundensis* were screened for similar genes, and BLAST results showed that all the strains of *P. lundensis* had a T3SS similar to strain AU1044. In all these clinical and environmental isolates, the identified T3SS genes were present as a single contiguous region and in the same order (Fig. 1C). Thus, we choose strain AU1044 as a representative strain of *P. lundensis* for the remainder of this study.

The genome of *P. lundensis* contains 38 genes belonging to Ysc/Psc family T3SS, clustered together as 5 consecutive operons and an effector-chaperone pair on the chromosome. *pscNOPSTU, popNpcs1234DR, pcrGVH-popBD, excCEBA*, and *exsD-pscBCDEFGHIJKL* operons are the core T3SS genes that code for structural, secretory, and regulatory proteins, while the effector-chaperone pair (*exoUspcU*) encodes a type III effector protein (ExoU) and its respective chaperone (SpcU). Because *P. lundensis* falls under the same genus as *P. aeruginosa*, we used the same nomenclature for the T3SS genes found in *P. lundensis* as the Psc family T3SS in *P. aeruginosa* as well as the...
corresponding unified Sct (secretion and cellular translocation) designation (20, 21) (Fig. 2; Table 1). The organization of the core T3SS genes in *P. lundensis* is clustered together as five consecutive operons and shares some similarities with the Psc family T3SS in *P. aeruginosa*. However, there are some striking differences, the key one being the presence of the type III effector *exoU* immediately downstream to the structural genes. In species such as *Yersinia pestis* and *Aeromonas hydrophilia* where the T3SS is carried on the plasmid, the type III effectors and their chaperones are clustered together in the same plasmid, interspersed with other genes (22). However, when carried on the chromosome, type III effectors are generally found scattered. In *P. aeruginosa*, the 4 cytotoxic effectors are

**FIG 1** Ysc family T3SS in *P. lundensis*. (A) The T3SS of *P. lundensis* is most homologous to that of *P. aeruginosa*. Phylogenetic tree depicting the similarity between T3SS among *Pseudomonas* species discussed in this paper. The tree was built on the predicted amino acid sequences of the T3SS structural proteins PscS/Ysc, PscT/YscT, PscU/PscT, and their T3SS homologues in the other *Pseudomonas* species. Branch length is on the top side of branch and labeled in blue; confidence intervals based on 1,000 bootstraps of the tree are on the underside of the branch and are labeled in red. (B) pBLAST of T3SS protein from an environmental and clinical strain of *P. lundensis*, 2T.2.5.2 and AU1044, respectively. The BLAST analysis was done against the Psc family protein from *P. aeruginosa* strain PA14. (C) Gene maps of the type III secretion system from *P. aeruginosa* strain PA14 and *P. lundensis* strains DSM6252, AU104, AU10414, M101, M105, MFPA15A1205, and 2T.2.5.2. DSM6252 is the type strain isolated from spoiled beef. AU1044, AU11122, and AU10414 are clinical isolates of *P. lundensis* from sputum samples of patients with cystic fibrosis. M101 and M105 are strains isolated from spoiled milk, MFPA15A1205 is a strain from a spoiled food source, and 2T.2.5.2 was isolated from an Antarctic temporary meltwater pond. In PA14, the *exoU* and *spcU* are not present immediately downstream to the core genes as seen in *P. lundensis*. The gene annotations seen for PA14 also apply to the *P. lundensis* strains.
| Functional name               | Sct name | Psc name | UniProtKB ID | Protein sequence length (aa) | GenPept accession no. | Protein sequence length (aa) | Alignment score (%) | Query cover (%) | E value |
|------------------------------|----------|----------|--------------|-----------------------------|-----------------------|-----------------------------|---------------------|----------------|---------|
| Export apparatus switch protein | sctU     | pscU     | Q02K1_PSEAB  | 340                         | AOZ1 2679             | 348                         | 79.472              | 97             | 1.04E-177 |
| Minor export apparatus protein | sctT     | pscT     | Q02K2_PSEAB  | 262                         | AOZ1 2680             | 262                         | 79.615              | 97             | 4.80E-127 |
| Minor export apparatus protein | sctS     | pscS     | Q02K3_PSEAB  | 88                          | AOZ1 5077             | 88                          | 79.545              | 99             | 4.53E-37  |
| Minor export apparatus protein | sctR     | pscR     | Q02K4_PSEAB  | 217                         | AOZ1 2681             | 219                         | 90.278              | 100            | 2.49E-137 |
| C-ring protein                | sctQ     | pscQ     | Q02K5_PSEAB  | 309                         | AOZ1 2682             | 308                         | 52.787              | 98             | 4.96E-85  |
| Needle length determinant protein | sctP   | pscP     | WP_010895580 | 369                         | AOZ1 5078             | 403                         | 55.385              | 98             | 2.32E-23  |
| Stalk                        | sctO     | pscO     | Q02K7_PSEAB  | 158                         | AOZ1 2683             | 155                         | 46                  | 76             | 1.47E-31  |
| ATPase                       | sctN     | pscN     | Q02K8_PSEAB  | 440                         | AOZ1 5079             | 440                         | 84.018              | 99             | <1E-200  |
| Gatekeeper                   | sctW     | popN     | Q02K9_PSEAB  | 288                         | AOZ1 2684             | 219                         | 68.198              | 97             | 6.53E-139 |
|                             | pcr1     | pcr1     | Q02K0_PSEAB  | 92                          | AOZ1 2685             | 91                          | 58.621              | 95             | 2.69E-38  |
|                             | pcr2     | pcr2     | Q02K1_PSEAB  | 123                         | AOZ1 2686             | 123                         | 61.475              | 99             | 1.35E-52  |
|                             | pcr3     | pcr3     | Q02K2_PSEAB  | 121                         | AOZ1 2687             | 121                         | 52.066              | 100            | 8.14E-36  |
|                             | pcr4     | pcr4     | Q02K3_PSEAB  | 109                         | AOZ1 2688             | 110                         | 56.19               | 84             | 1.52E-33  |
| Major export apparatus protein | sctV    | pcrD     | Q02K4_PSEAB  | 706                         | AOZ1 2689             | 707                         | 83.027              | 100            | <1E-200  |
|                             | pcrR     | pcrR     | Q02K5_PSEAB  | 144                         | prot_1660             | 144                         | 60.67               | 61             | 1E-34     |
| PcrV chaperone protein       | pcrG     | pcrG     | Q02K6_PSEAB  | 98                          | AOZ1 2690             | 94                          | 49.425              | 92             | 3.09E-17  |
| Tip protein                  | pcrV     | pcrV     | Q02K7_PSEAB  | 294                         | AOZ1 2691             | 314                         | 53.676              | 86             | 1.46E-85  |
| Translocon chaperone protein | pcrH     | pcrH     | Q02K8_PSEAB  | 164                         | AOZ1 2692             | 161                         | 76.582              | 95             | 1.85E-84  |
| Translocon protein           | popB     | popB     | Q02K9_PSEAB  | 390                         | AOZ1 2693             | 388                         | 59.74               | 98             | 5.77E-141 |
| Translocon protein           | popD     | popD     | Q02K0_PSEAB  | 295                         | AOZ1 5080             | 295                         | 52.881              | 100            | 5.69E-101 |
| Regulatory protein           | exsC     | exsC     | Q02K1_PSEAB  | 145                         | AOZ1 2694             | 147                         | 71.429              | 100            | 9.63E-65  |
| Regulatory protein           | exsE     | exsE     | Q02K2_PSEAB  | 81                          | AOZ1 2695             | 81                          | 46.914              | 97             | 1.62E-18  |
| Regulatory protein           | exsB     | exsB     | Q02K3_PSEAB  | 137                         | AOZ1 2696             | 134                         | 35.248              | 88             | 3.37E-20  |
| Regulatory protein           | exsA     | exsA     | Q02K4_PSEAB  | 278                         | AOZ1 2697             | 270                         | 75.646              | 100            | 1.40E-157 |
| Regulatory protein           | exsD     | exsD     | Q02K5_PSEAB  | 276                         | AOZ1 2698             | 277                         | 61.091              | 99             | 1.68E-121 |
| Secretin                     | sctC     | pscC     | Q02K6_PSEAB  | 600                         | AOZ1 5081             | 597                         | 69.631              | 95             | <1E-200  |
| Outer MS ring protein        | sctD     | pscD     | Q02K8_PSEAB  | 432                         | AOZ1 2700             | 442                         | 57.109              | 99             | 6.31E-169 |
| PscF-stabilizing protein     | sctF     | pscF     | Q02K9_PSEAB  | 67                          | AOZ1 2701             | 68                          | 46.875              | 94             | 8.30E-15  |
| Needle filament protein      | sctL     | pscL     | Q02K0_PSEAB  | 85                          | AOZ1 2702             | 84                          | 69.412              | 100            | 4.55E-38  |
| PscF chaperone protein       | sctF     | pscF     | Q02K1_PSEAB  | 115                         | AOZ1 5082             | 119                         | 58.407              | 94             | 2.05E-40  |
| Type III secretion protein   | sctH     | pscH     | Q02K2_PSEAB  | 142                         | AOZ1 2703             | 143                         | 42.857              | 89             | 2.40E-10  |
| Inner rod protein            | sctI     | pscI     | Q02K3_PSEAB  | 137                         | AOZ1 2704             | 137                         | 71.681              | 97             | 4.22E-55  |
| Inner MS ring protein        | sctJ     | pscJ     | Q02K4_PSEAB  | 248                         | prot_1679             | 237                         | 87.437              | 83             | 7.45E-131 |
| Accessory cytosolic protein  | sctK     | pscK     | Q02K5_PSEAB  | 206                         | AOZ1 2705             | 208                         | 54.902              | 98             | 2.18E-60  |
| Stator                       | sctL     | pscL     | Q02K6_PSEAB  | 231                         | AOZ1 2706             | 205                         | 66.832              | 98             | 5.80E-101 |
| Effector                     | exoU     | Q02F1_PSEAB  | 687                         | QPD07247.1             | 681                         | 61.919              | 97             | <1E-200  |
| Chaperone                    | spcU     | Q02F2_PSEAB  | 137                         | AOZ1 2707             | 136                         | 55.085              | 86             | 3.07E-42  |

*Unified nomenclature for conserved proteins of the type III secretion apparatus. Sct, secretion and cellular translocation (20, 21). Type III secretion system genes in Pseudomonas.*
randomly distributed across the genome. In fact, in PA14, the ExoU effector and its chaperone SpcU are present in a pathogenicity island, PAPI-II (23). Screening AU1044 using a pathogenicity island prediction tool suggests that T3SS genes do not fall in pathogenicity islands (Fig. 3A). Screening other strains of *P. lundensis* (2T.2.5.2, M101, and M105) showed similar results (Fig. 3B to D) in that T3SS genes are not present in pathogenicity islands.

Another difference in T3SS gene organization in *P. lundensis* versus *P. aeruginosa* is the G+C content of this region. In *P. aeruginosa*, the G+C content of the T3SS is the same as that of the core genome (66.6%). In *P. lundensis* strains AU1044, 2T.2.5.2, M101, and M105, the G+C content of the T3SS is 61.75% ± 0.01% (standard deviation [SD]), which is different from the core genome of 58.67% ± 0.04% (SD). However, when using prediction tools (Alien_Hunter; https://www.sanger.ac.uk/tool/alien-hunter/), T3SS was not identified as a predicted region of horizontal gene transfer (HGT) (Fig. 3A). The distribution of insertion sequence (IS) elements in strain AU1044 also does not indicate any IS elements immediately upstream or downstream of the T3SS. A similar HGT prediction analysis was performed on the environmental and milk isolates of *P. lundensis* (Fig. 3B to D) and gave the same conclusion as for strain AU1044 in that T3SS genes do not appear in any predicted HGT regions.

**In vitro transcriptomics of the T3SS.** Our next objective was to investigate the transcriptional activity of the T3SS in *P. lundensis*. To do this, we set up an *in vitro* transcriptomics experiment. In *P. aeruginosa*, it is seen that temperature can be a T3SS-inducing condition where expression is seen at a human body temperature of 37°C but not at a lower temperature of 28°C (24). Hence, to investigate the transcriptional activity of T3SS in *P. lundensis* AU1044, the expression profiles of cells grown at different temperatures were compared. At the early stationary phase, *P. lundensis* AU1044 was
able to actively transcribe several of its T3SS genes at 37°C and 21°C (Fig. 4A; data not shown; Table 2). When looking at the transcriptome of the *P. lundensis* in the two temperatures, at 37°C, the T3SS is among the top highly expressed genes compared to their expression at 4°C (Fig. 4B). On the other hand, the expression of several of these genes was almost undetectable at 4°C (Fig. 4A).

There is a significant upregulation of T3SS genes at 37°C compared to 4°C (Fig. 4A). Some T3SS genes have >5-log₂ fold changes in expression, for example, the T3SS assembly regulatory gene *exsB* (25), ATPase *pscN* (26), chaperone and cytoplasmic regulator *pcrG* (27), effector *exoU*, T3SS negative regulator *exsE* (28), and negative regulators of exoenzyme secretion *popN* and *pcr2* (29). A few genes have a <1.5-log₂ fold change. These include ATPase regulator *pscl* (30), basal body component *pscU*, basal body rod
component psci (31), basal structure component pscJ (26), membrane component of the export apparatus pscT (32), secretion protein pscH, and inner membrane component pcrD (21). Different levels of upregulation were also seen in the expression of translocation proteins popB and popD, tip protein pcrV (33), and the needle protein pscF (34), all components of the injectosome necessary for exporting effectors into host cells. Thus, the in vitro transcriptomics data demonstrated that the T3SS in P. lundensis is transcriptionally active at 37°C.

In vivo transcriptomics of the T3SS. Our next objective was to study whether the host environment has an effect on the expression profile of T3SS at 37°C. Since P. lundensis is a common milk contaminant, the most common route of exposure to this bacterium would be through the consumption of milk. Therefore, we decided to study the expression profile of P. lundensis in the intestinal tract (by employing monocolonization of germfree mice) and compare it to the expression of bacterial cells cultured in vitro. P. lundensis was able to successfully colonize the gut of all the germfree mice after a single oral gavage (Fig. 4C) without any histological or clinical signs of disease (data not shown). The cecum and feces, collected after 14 days of inoculation, had an
average level of $10^5$ CFU/g. Cecal content (CC) collected from the lumen of the cecum had the highest level of colonization, with an average of $10^6$ CFU/g. The ileum had low-level colonization, while the remaining two samples had undetectable levels. Interestingly, no colonization was seen in the stomach, duodenum, and jejunum. We used the cecal contents of germfree mice colonized for 14 days with strain AU1044 as the samples for transcriptomics analysis. Compared to the expression profile seen in vitro at 37°C, several genes had higher expression in vivo, including T3SS genes (Fig. 4A and D). Several T3SS genes had either increased expression levels or no significant change in expression levels in vivo compared to their expression in vitro at 37°C. Some of the genes having higher expression in vivo include genes coding for export apparatus pscT, pscU, and pcrD; basal body components pscJ, pscI, and pscD; and outer membrane ring protein pscC. exsA also had significant upregulation of 4.9-log$_2$ fold change (Fig. 4A; Table 2). Other regulatory genes, such as exsD and exsB, also had greater than 3-log$_2$ fold change in expression. These data clearly indicate that P. lundensis can express its T3SS when colonizing a mammalian host.

### Table 2 Differential expression of T3SS genes in P. lundensis

| Locus tag     | Gene name | Gene expression data for: | 37°C vs 4°C | In vivo vs in vitro 37°C |
|---------------|-----------|---------------------------|-------------|-------------------------|
|               |           | Log$_2$ fold change | $P_{adj}$ | Log$_2$ fold change | $P_{adj}$ |
| AA042_08435   | pscU$^{a,b}$ | 1.00 | 6.21E-04 | 2.89 | 3.62E-03 |
| AA042_08440   | pscT$^{a,b}$ | 1.47 | 4.17E-04 | 3.59 | 2.86E-05 |
| AA042_08445   | pscS$^{a}$ | 4.36 | 8.98E-07 | 1.75 | 6.67E-01 |
| AA042_08450   | pscR$^{a,b}$ | 2.32 | 5.00E-07 | 2.97 | 1.76E-02 |
| AA042_08455   | pscQ$^{a}$ | 4.78 | 3.02E-47 | 2.41 | 3.19E-01 |
| AA042_08460   | pscP$^{a}$ | 3.87 | 2.02E-27 | 1.94 | 7.39E-01 |
| AA042_08465   | pscO$^{a}$ | 4.78 | 7.56E-27 | 2.19 | 9.99E-02 |
| AA042_08470   | pscN$^{a}$ | 6.51 | 1.35E-94 | 1.88 | 7.62E-01 |
| AA042_08475   | popN$^{a}$ | 6.02 | 4.88E-132 | 0.40 | 1.00E+00 |
| AA042_08480   | pcr1$^{a}$ | 6.39 | 5.32E-19 | 2.73 | 1.72E-01 |
| AA042_08485   | pcr2$^{a}$ | 5.53 | 2.90E-53 | 1.02 | 1.00E+00 |
| AA042_08490   | pcr3$^{a}$ | 4.28 | 2.02E-27 | 2.39 | 1.75E-01 |
| AA042_08495   | pcr4$^{a,b}$ | 2.33 | 7.99E-07 | 5.00E-02 |
| AA042_08500   | pcrD$^{a,b}$ | 1.68 | 8.56E-11 | 2.97 | 1.76E-02 |
| AA042_08505   | pcrR$^{a,b}$ | 2.69 | 1.34E-11 | 3.18 | 1.31E-03 |
| AA042_08510   | pcrG$^{a}$ | 6.39 | 5.32E-19 | 2.73 | 1.72E-01 |
| AA042_08515   | pcrV$^{a}$ | 6.39 | 5.32E-19 | 2.73 | 1.72E-01 |
| AA042_08520   | pcrH$^{a}$ | 4.16 | 7.19E-89 | 1.22 | 1.00E+00 |
| AA042_08525   | popB$^{a}$ | 5.36 | 2.24E-159 | 2.80 | 1.93E-01 |
| AA042_08530   | popD$^{a}$ | 4.94 | 1.05E-88 | 2.29 | 4.59E-01 |
| AA042_08535   | exsC$^{a}$ | 5.89 | 2.99E-163 | 1.70 | 5.27E-01 |
| AA042_08540   | exsE$^{a}$ | 6.59 | 4.70E-96 | 1.52 | 1.00E+00 |
| AA042_08545   | exsB$^{a,b}$ | 6.07 | 3.92E-52 | 3.31 | 2.25E-02 |
| AA042_08550   | exsA$^{a,b}$ | 1.66 | 4.00E-08 | 4.99 | 8.43E-06 |
| AA042_08555   | exsD$^{a,b}$ | 3.74 | 3.53E-88 | 3.30 | 3.78E-02 |
| AA042_08560   | pscB$^{a,b}$ | 3.21 | 5.01E-16 | 3.61 | 1.53E-02 |
| AA042_08565   | pscC$^{a,b}$ | 2.65 | 2.86E-24 | 3.37 | 2.37E-02 |
| AA042_08570   | pscD$^{a,b}$ | 6.93 | 4.38E-17 | 3.58 | 1.27E-02 |
| AA042_08575   | pscE$^{a,b}$ | 2.54 | 4.98E-07 | 3.62 | 2.87E-05 |
| AA042_08580   | pscF$^{a}$ | 2.08 | 1.03E-08 | 0.95 | 1.00E+00 |
| AA042_08585   | pscG$^{a}$ | 2.49 | 1.69E-18 | 2.07 | 2.13E-01 |
| AA042_08590   | pscH$^{a,b}$ | 1.66 | 2.47E-05 | 2.60 | 2.32E-02 |
| AA042_08595   | pscI$^{a,b}$ | 1.32 | 7.35E-03 | 3.48 | 3.46E-05 |
| AA042_08600   | pscJ$^{a,b}$ | 1.34 | 1.49E-05 | 4.11 | 5.55E-08 |
| AA042_08605   | pscK$^{a}$ | 2.16 | 7.36E-17 | 0.74 | 1.00E+00 |
| AA042_08610   | pscL$^{a,b}$ | 0.92 | 2.49E-02 | 3.93 | 7.70E-05 |
| AA042_08615   | exoU$^{a}$ | 4.94 | 8.66E-74 | 2.60 | 3.23E-01 |
| AA042_08620   | spcA$^{a}$ | 6.32 | 2.66E-169 | 1.40 | 1.00E+00 |

$^a$Gene with significant change in expression at 37°C versus 4°C.

$^b$Gene with significant change in expression at in vivo versus in vitro 37°C.

$^c$P$_{adj}$, adjusted P value, generated though DESeq2 differential gene expression analysis.
DISCUSSION

In this paper, we have demonstrated the presence of a transcriptionally active Ysc family T3SS in a non-aeruginosa Pseudomonas species (NAPS), *P. lundensis*. Isolates from clinical samples and various food spoilage sources, as well as an environmental isolate from an Antarctic temporary meltwater pond, carry the Ysc family T3SS. *P. lundensis* also encodes an ExoU type III effector protein and its respective chaperone (SpcU), and genes for these proteins are present immediately downstream to the core T3SS genes. Our transcriptomics analysis demonstrates that under *in vitro* aerobic growth conditions, the T3SS in the clinical isolate AU1044 is expressed at 37°C, while the expression of the system is negligible at 4°C. When in the cecal lumen of monoclonized germfree mice, *P. lundensis* also transcribes its T3SS, and several genes were more highly expressed than at 37°C *in vitro*.

Most species in the *Pseudomonas fluorescens* species complex have an Hrp1 family T3SS. However, apart from the Hrp family, root-associated strains of *P. fluorescens* also carry an Rsp family T3SS (11), and *P. fluorescens* strain K113 has both the Hrp1 and SPI-1 family (*Salmonella* pathogenicity island) T3SS (35). Though *P. aeruginosa* is the only *Pseudomonas* species to have a well-characterized Ysc family T3SS, there have been previous reports of *P. fluorescens* complex species carrying individual genes that are highly homologues to those within the Ysc family; *P. baetica* has core Ysc family T3SS gene homologs (36); *P. weihenstephanensis* has ExoU homologs (37). A phylogenetic tree based on multilocus sequence analysis shows that the *P. fluorescens* group is distant from *P. aeruginosa* (7, 38). Though the functionality of the T3SS in these NAPS is not known, it raises interesting questions about the evolution of T3SS among the *Pseudomonas* species.

Among the strains studied here, out of the four known cytotoxic type III effectors in *P. aeruginosa*, *P. lundensis* only carries a homologue of the *exoU* gene. The T3SS genes found in *P. lundensis* all have high sequence similarity with those in *P. aeruginosa*. The occurrence of all four cytotoxic effector proteins in a single strain of *P. aeruginosa* strain is very rare. Most clinical isolates of *P. aeruginosa* code for either ExoU/ExoT or ExoS/ExoT. Out of the 4 effectors (ExoU, ExoS, ExoT, and ExoY), ExoU has the greatest impact on disease severity in humans (39). ExoU is a potent cytoxin with phospholipase A2 activity (16, 17, 39). Although only an *exoU* homologue was identified in *P. lundensis*, we also looked for genes with an upstream ExsA-activated promoter because all genes of the T3SS are regulated by the ExsA promoter, and the promoter-binding sequence is highly conserved across Ysc family T3SS (40). Preliminary analysis of scanning the genome for the conserved ExsA-binding motif (41) identified four putative type III effectors (DNA polymerase IV, TonB-dependent receptor, heme-binding protein, and response regulator transcription factor) (data not shown). However, it is important to note that ExsA has also been reported to regulate the expression of non-T3SS genes, including the metalloprotease *impA* and other secretory proteins in *P. aeruginosa* (42). Thus, it remains to be determined biochemically if there are additional T3SS effector proteins in *P. lundensis*, but there are some potential targets.

*P. lundensis* grown aerobically at 4°C in LB broth was unable to express several T3SS genes. *P. lundensis* is primarily a psychrotrophic bacterium, and T3SS is conserved in all the strains studied here. *Our in vitro* growth set up at 4°C may not be an accurate representation of the various psychrotrophic niches that this bacterium inhabits. Though T3SS is a well-known virulence factor in *P. aeruginosa*, there are reports which show that *P. aeruginosa* uses T3SS to colonize and evade predation by protozoa by killing biofilm-associated amoebae. (18). Though it is unclear what role T3SS may play in the various niches that *P. lundensis* colonizes, it is intriguing to postulate that in *P. lundensis*, the T3SS may play a similar role in protecting the bacterium from environmental phagocytes.

At human body temperature, *P. lundensis* expresses its T3SS. The translocation pore of the T3SS needle complex, PopB/PopD, which can cause host cell injury independent of the effectors (43, 44) as well as the cytotoxic effector, ExoU, is expressed at human
body temperature. However, the various levels of expression seen across the system suggest that not all T3SS genes are transcribed at the same rate. The T3SS global transcription regulator exsA has a 1.6-log, fold change increase in expression at 37°C compared to growth at 4°C. The activity of ExsA protein, the global regulator of T3SS, is controlled by levels of anti-activator ExsD, anti-anti-activator ExsC, and secreted protein ExsE (41, 45). Upon induction of T3SS, ExsE gets secreted, thereby decreasing the cytosolic levels of the protein. This leads to increased affinity of ExsC and ExsD, resulting in the release of ExsA from ExsD. Our data show that the upregulation of exsA at 37°C is less than the genes involved in its regulation (exsD, exsC, and exsE). The PcrG protein acts as both a chaperone for the tip protein PcrV as well as a negative regulator of the effector export. PcrG forms a complex with PcrN, Pcr1, and chaperon protein Pcr2, and this complex docks of PcrD, effectively blocking the export of effectors (27). At 37°C, the negative regulators of the system, pcrG, pcrV, pcr1, and pcr2, are among the highly expressed genes. Interestingly, a similar expression pattern of the T3SS regulatory genes is reported in Vibrio parahaemolyticus (46). In both, upon increased growth temperature for P. lundensis and increased cytotoxic activity in V. parahaemolyticus, upregulation in the expression of exsA is relatively low compared to exsD, exsC, and exsE. Similarly, in both instances, there is an upregulation of genes that are known to block the export of effectors.

While direct studies remain to be performed with P. lundensis, the studies in V. parahaemolyticus provide support for the hypothesis that the T3SS of P. lundensis confers cytotoxic activity.

From our transcriptomics data, we see that the T3SS is expressed at high levels both during in vivo and in vitro growth conditions at 37°C but not at 4°C in vitro. The T3SS is expressed in vitro at 21°C at levels slightly lower than at 37°C but much higher than at 4°C (data not shown), so it remains to be determined whether there is a temperature “switch” for the upregulation of the T3SS or whether it generally increases between 4°C and 37°C. P. lundensis AU1044 isolated from the sputum samples of CF patients can successfully colonize and grow in the anaerobic environment of the gut of germfree mice as well as aerobically in vitro in LB broth. These niches present various environmental pressures to the bacterium like micro- and macronutrients, pH, and oxygen conditions as well as carbon sources, all suggesting that P. lundensis AU1044 isolates exhibit metabolic diversity. Comparative analysis of P. lundensis strains shows that CF isolate AU1044 shares 99% sequence similarity with strains isolated from food samples as well as Antarctic temporary meltwater ponds. In other preliminary studies, we have also been able to identify 16S rRNA gene sequences that include P. lundensis in data sets from patients with chronic obstructive pulmonary disease and interstitial pulmonary fibrosis but not in healthy human lungs. As a potent hydrocarbon degrader, the ability of P. lundensis to degrade aromatic hydrocarbons (47) could facilitate its colonization of the lungs because pulmonary surfactant is largely composed of phospholipid. Despite the expression of a T3SS in vivo, it was intriguing that germfree mice inoculated with P. lundensis did not show any historical evidence of inflammation or disease in the ileum, cecum, or colon after 14 days of colonization (n = 6; histological score, 0.0 ± 0.0; 0 to 12 scale as described in Materials and Methods). We did not investigate whether there are changes in host mucosal gene expression in these mice. Thus, further investigation is merited on the nature of the symbiotic interaction of P. lundensis with a mammalian host. There is one report of the oral administration of P. lundensis to healthy humans (as a negative control in a probiotic bacteria study) (48). In this study, the subjects did not have ill effects on their health after 2 weeks of oral administration of P. lundensis (at a concentration proposed to mimic the levels in 1,000 mL of milk). With the identification of a complete Ysc T3SS in P. lundensis that is expressed at 37°C in vivo, we wonder whether this bacterium has some level of interaction with the gastrointestinal tract after consumption by a mammalian host. It remains to be determined what environmental/evolutionary pressures act to retain the T3SS in P. lundensis during growth in the environment since mammalian hosts are not a primary environment for this bacterium.
**MATERIALS AND METHODS**

**Bacterial genomic sequences used for analysis.** Bacterial genomic sequences used include *Pseudomonas aeruginosa* UCBPP-PA14 (Assembly accession no. GCF_000014625.1) (reference strain), *Pseudomonas lundensis* AU1044 (GenBank accession no. CP017687) (reference strain), 2T.2.5.2 (GenBank accession no. NZ_CP062158.2), M101 (GenBank accession no. CP075177), M105 (GenBank accession no. CP075180), DSM6252 (GenBank accession no. NZ_JYKY00000000), AU11122 (GenBank accession no. LCYV00000000), and MFPA15A1205 (GenBank accession NZ_OBKZ00000000). AU1044 is deposited as DSM 103277 in the DSMZ culture collection in Germany.

**In vitro growth.** The experiment was set up as shown in Fig. 5A using the AU1044 strain. Frozen overnight culture of AU1044 from frozen stock was set up in 20 mL of Luria Bertani (LB) broth (Thermo Fisher) at 37°C with shaking at 120 rpm. Twenty microliters of this culture were used to inoculate triplicates of 20 mL LB broth, incubated at 4°C, and 37°C with shaking at 120 rpm. Bacterial cells (average cell count of $5 \times 10^6$) were harvested once the culture reached an optical density (OD) of 2.1 at 600 nm. Cells were treated with Qiagen's RNAprotect bacteria reagent at a 2:1 ratio of RNAprotect for every volume of sample and then stored at $-80°C$ until RNA isolation.

**Gnotobiotic animal colonization experiment.** The experiment was set up as shown in Fig. 5A. All germfree mouse experiments were handled at the germfree animal facility at the University of Michigan. Ten- to 12-week-old, female germfree BALB/c mice underwent gastric colonization through oral gavage of $2.75 \times 10^9$ CFU/mL of *P. lundensis* AU1044 ($n = 6$). At 14 days postcolonization, the stomach, small intestine proximal and distal ends, cecal content, cecum, colon, and feces were collected for CFU plating and cecal contents for RNA isolation.

**RNA extraction and RNA sequencing.** In vitro samples from $-80°C$ were first thawed and then incubated with 200 μL of lysozyme (concentration of 1 mg/mL of Tris-EDTA [TE] buffer) for 10 min in a shaker. After cell lysis, RNA isolation was done using Qiagen's RNeasy minikit. RNA isolation from the cecal contents of colonized mice was performed using phenol-chloroform extraction followed by cleanup using RNeasy minikit as previously described (49). Isolated RNA samples were given for sequencing at the DNA sequencing core at the University of Michigan. Ribosomal depletion and library preparation were done by the core using the NEBNext depletion kit and the NEBNext Ultra II RNA library prep kit, respectively. In vitro samples were sequenced using Illumina NextSeq 500 and in vivo samples using NovaSeq 6000 both at 300 cycles with mid-sequencing output.

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**FIG 5** Schematic representation showing the outline for the experiments set up for *in vitro* and *in vivo* RNA transcriptomics (A) and the bioinformatics pipeline used to analyze the data (B).
RNA sequencing data analysis. The schematic representation of the analysis pipeline is shown in Fig. 5B. Raw RNA sequencing reads were quality trimmed using Trimmomatic 0.39 with LEADING and TRAILING set at 20 and MINLEN set at 100. TrimGalore (version 0.6.5) and Fastx-trimmer (version 0.1.14) tools were used to remove and trim out adapter reads from the 3’ end, respectively. The reads were aligned to the \textit{P. lundensis} strain AU1044 using Bowtie2 version 2.3.4.3 with default parameters for an end-to-end alignment. The resulting SAM files were converted to BAM files using SAMtools version 1.7. Count matrix was generated in Rstudio using the GenomicAlignments package. Fragments per kilobase of transcript per million mapped reads (FPKM) reads were generated, and differentially expressed genes were identified both using DESeq2. Genes were identified as differentially expressed if their adjusted \( P \) value \( (P_{\text{adj}}) \) was less than 0.05 at a log, fold change of \( >0 \) for the 37°C versus 4°C in vitro experiment and \( >1 \) for the in vivo versus 37°C in vitro experiment.

Genomic analysis. GC island prediction and pathogenicity island prediction were done using Alien_Hunter (50) and IslandViewer4 (51) at default parameters. Scanning for insertion sequence elements was done using ISEScan (52). The T3SS genes in AU1044 were identified using BLAST with \textit{Pseudomonas aeruginosa} UCBPP-PA14 strain as the reference. For generating the gene maps of T3SSs in different strains of \textit{P. lundensis}, the GFF files from NCBI and RAST annotations were downloaded for completely assembly and draft genomes, respectively. The maps were generated in RStudio using genoPlotR (53). The direction of the T3SS operons differs between the strains; however, we speculate that this might be due to a difference in annotations as the difference in the positive- and negative-strand genes extend to the genes upstream and downstream to the T3SS for \textit{P. lundensis} strains.

Histology and histological scoring. Intestinal tissue was fixed in 10% formalin for at least 24 h and then transferred to 70% ethanol. Tissue was processed, paraffin embedded, sectioned, and used to prepare hematoxylin and eosin (H&E)-stained slides. All section were analyzed at \( \times 4, \times 20, \) and \( \times 400 \) magnifications. Light microscopic evaluation of H&E-stained ileum, cecum, and colonic sections was performed using a previously established system (54). Slides were scored (0 [none] to 4 [extensive]) for mononuclear and neutrophilic inflammation, edema, and epithelial damage as defined below.

(i) Inflammation. For inflammation, 0 represents no inflammation; 1 represents minimal, multifocal mononuclear, or neutrophilic infiltration; 2 represents moderate, multifocal mononuclear, or neutrophilic infiltration (greater submucosal involvement); 3 represents severe multifocal to coalescing mononuclear or neutrophilic infiltration (greater submucosal with or without mural involvement); and 4 is the same as 3 but with abscesses or extensive transmural involvement.

(ii) Edema. For edema, 0 represents no edema; 1 represents mild, focal, or multifocal edema with minimal submucosal expansion (\(<2x\)); 2 represents moderate multifocal edema with moderate submucosal expansion (2 to \( 3x \)); 3 represents severe multifocal to coalescing edema with severe submucosal expansion (\( >3x \)), and 4 is the same as 3 but with diffuse submucosal expansion. 

(iii) Epithelial damage. For epithelial damage, 0 represents no epithelial damage; 1 represents mild multifocal, superficial damage (vacuolation, increased apoptosis, villus tip attenuation/necrosis); 2 represents moderate, multifocal superficial damage (same qualitative changes as above); 3 represents severe multifocal to coalescing mucosal damage with or without intraluminal aggregate of inflammatory cells and sloughed epithelium or ulcerated mucosa; and 4 is the same as 3 but with extensive epithelial destruction or ulcer formation.

Data availability. In vitro and in vivo RNA sequencing (RNA-seq) data reported in this paper have been deposited in NCBI’s GEO repository under the BioProject identifier PRJNA733127 (accession numbers SAMN19367880 to SAMN19367893).

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We declare that we have no conflict of interest with the material presented in this literature review.

REFERENCES

1. Molin G, Ternstrom A, Uprising J. 1986. Notes: \textit{Pseudomonas lundensis}, a new bacterial species isolated from meat. Int J Syst Bacteriol 36:339–342. https://doi.org/10.1099/00207713-36-2-339.

2. Arnaut-Rollier I, Zutter LD, Hoof JV. 1999. Identities of the \textit{Pseudomonas} spp. among healthy animals: concern about exolysin ExlA detection. Sci Rep 10:11667. https://doi.org/10.11667/s41598-020-68575-1.

3. Marchand S, Heylen K, Messens W, Coudijzer K, De Vos P, Dewettinck K, Herman L, De Block J, Heyndrickx M. 2009. Seasonal influence on heat-resistant proteolytic capacity of \textit{Pseudomonas lundensis} and \textit{Pseudomonas fragi}, predominant milk spoilers isolated from Belgian raw milk samples. Environ Microbiol 11:467–482. https://doi.org/10.1111/j.1462-2920.2008.01785.x.

4. de Oliveira GB, Favaron L, Luchese RH, McIntosh D. 2015. Psychrotrophic bacteria in milk: how much do we really know? Braz J Microbiol 46:313–321. https://doi.org/10.1590/1517-838246220130963.

5. Ruiz-Roldán L, Rojo-Bezares B, de Toro M, López M, Toledano P, Lozano C, Chicón G, Alvarez-Envíti L, Torres C, Sáenz Y. 2020. Antimicrobial resistance and virulence of \textit{Pseudomonas} spp. among healthy animals: concern about exolysin ExlA detection. Sci Rep 10:11667–11667. https://doi.org/10.1038/s41598-020-68575-1.
22. Vanden Bergh P, Frey J. 2014. Biofilm formation characteristics of Pseudomonas lundensis strains isolated from the lungs of humans. Genome Announc 6:e01461-17. https://doi.org/10.1128/genomeA.01461-17.

20. Hueck CJ. 1998. Type III protein secretion systems in bacterial pathogens. Microbiol Mol Biol Rev 62:379–433. https://doi.org/10.1128/MMBR.62.2.379-433.1998.

19. Balloy V, Ryffel B, Dessein R, Chignard M, Uyttenhove C, Guery B, Gosset P, Chambon P, Kipnis E. 2014. NLRC4 orchestrates type III secretion system dampens host defense by exploiting the NLRC4-coupled inammatory IL-1 signaling. FEMS Microbiol Lett 365:fny069. https://doi.org/10.1002/femsec.2669.

18. Shaver CM, Hauser AR. 2004. Relative contributions of type III secretion system gene expression interactions with promoter DNA. J Bacteriol 195:5639–5650. https://doi.org/10.1128/JB.195.18.5639-5650.2003.

17. Tryfonopoulou P, Tsikaloudis E, Nychas GJE. 2002. Characterization of Pseudomonas spp. associated with spoilage of gilt-head sea bream stored under various conditions. Appl Environ Microbiol 68:65–72. https://doi.org/10.1128/AEM.68.1.65-72.2002.

16. Finck-Barbancon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleischer SMJ, Wu C, Mende-Muel ler L, Frank DW. 1997. Exolysis expression of Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury. Mol Microbiol 25:547–557. https://doi.org/10.1111/j.1365-2958.1997.9811851.x.

15. Cohen TS, Prince AS. 2013. Activation of inammosomal signaling mediates pathologic of acute P. aeruginosa pneumonia. J Clin Invest 123:1630–1637. https://doi.org/10.1172/JCI66142.

14. Faure E, Mear J-B, Faure K, Normand S, Couturier-Maillard A, Grandjean T, Chabert J, Louwagie M, Garin J, Attree I. 2005. PscF is a major effector of the Type III secretion system of Pseudomonas aeruginosa. FEMS Microbiol Lett 28:1381–1389. https://doi.org/10.1016/j.femsle.2015.09.028.

13. Sansonetti PJ. 2001. Rupture, invasion and inammation of animals and plants. Microbiol Mol Biol Rev 62:379–433. https://doi.org/10.1128/MMBR.62.2.379-433.1998.

12. Collazo CM, Galán JE. 1997. The invasion-associated type-III protein secretion machinery. FEMS Microbiol Rev 38:802–811. https://doi.org/10.1109/1751-7915.12091.

11. Guimaraes TM, Teixeira CA, Azevedo AP, Fabrício AR, Schiavone C, Barbosa de Souza JB. 2012. Fitting pieces to the puzzle of type III secretion apparatus in the bacterial membrane and full virulence in vivo. Infect Immun 80:1789–1798. https://doi.org/10.1128/IAI.00448-15.

10. Viprey V, Del Greco A, Golinowski W, Broughton WJ, Perret X. 1998. Symbiotic implications of type III protein secretion machinery in Rhizobium. Mol Microbiol 28:1381–1389. https://doi.org/10.1111/j.1365-2958.1998.00920.x.

9. Liu Y-J, Xie J, Zhao L-J, Qian Y-F, Zhao Y, Liu X. 2015. Biofilm formation characteristics of Pseudomonas aeruginosa. FEMS Microbiol Lett 365:fny069. https://doi.org/10.1002/femsec.2669.

8. Liu Y-J, Xie J, Zhao L-J, Qian Y-F, Zhao Y, Liu X. 2015. biofilm formation characteristics of Pseudomonas aeruginosa. FEMS Microbiol Lett 365:fny069. https://doi.org/10.1002/femsec.2669.

7. Redondo Nieto M, Barret M, Morri ssey JP, Germaine K, Francesco MG, Barahona E, Navazo A, Maria SC, Moynihan JA, Giddens SR, Coppoolse ER, Muriel C, Stie kema WJ, Rainey PB, Dowling D, O’Gara F, M artin R, Rivilla R. 2012. Genome sequence of the biocontrol strain Pseudomonas aeruginosa Fl3.1. J Bacteriol 194:1273–1274. https://doi.org/10.1128/jb.00661-11.

6. Beaton A, Loo J, Cunningham-Oakes E, MacFadyen A, Mullins AJ, Bestawry WE, Botelho J, Chevalier S, Coleman D, Dalzell C, Dolan SK, Facenda A, Ghequire M, Higgins S, Kutscher A, Murray J, Redway M, Sali th T, da Silva AC, Smith SM, Smith NM, Thomson R, Woodcock S, Welch M, Cornelis P, Lavigne R, van Noort V, Tucker NP. 2018. Community-led comparative genomic and phenotypic analysis of the aquaculture pathogen Pseudomonas baetica a390T sequenced by Ion semiconductor and N ano-pore technologies. FEMS Microbiol Lett 365:fny069. https://doi.org/10.1093/femsle/fny069.

5. Sawa T, Hamaoka S, Kinumma A, Naito Y, Akiyama K, Kato S, Shibata K, Kato M, Shin H, Cornelis GR. 2007. Type III secretion translocation pores of Salmonella typhimurium: Making sense of prokaryote type-3 secretion machin ies. Toxins (Basel) 8:307. https://doi.org/10.3390/toxins8010307.

4. Pastor A, Chabert J, Louwagie M, Garin J, Attree I. 2005. PscF is a major component of the Pseudomonas aeruginosa type III secretion needle. FEMS Microbiol Lett 253:95–101. https://doi.org/10.1016/j.femsec.2005.09.028.

3. Shaver CM, Hauser AR. 2004. Relative contributions of Pseudomonas aeruginosa ExoU and its predicted homologs. Toxins (Basel) 8:307. https://doi.org/10.3390/toxins8110307.

2. Galle M, Carpenter I, Beyer R. 2012. Structure and function of the type III secretion system of Pseudomonas aeruginosa. Curr Protein Pept Sci 13:831–842. https://doi.org/10.2174/1389203128471210.

1. Ravi et al. 2019. Pseudo monas aeruginosa uses type III secretion system pore technologies. FEMS Microbiol Lett 365:fny069. https://doi.org/10.1093/femsle/fny069.
T/Y independent pathogenic role during acute lung infection. PLoS One 7:e41547. https://doi.org/10.1371/journal.pone.0041547.

45. Yahr TL, Wolfgang MC. 2006. Transcriptional regulation of the Pseudomonas aeruginosa type III secretion system. Mol Microbiol 62:631–640. https://doi.org/10.1111/j.1365-2958.2006.05412.x.

46. Nydam SD, Shah DH, Call DR. 2014. Transcriptome analysis of Vibrio para-haemolyticus in type III secretion system 1 inducing conditions. Front Cell Infect Microbiol 20:1. https://doi.org/10.3389/fcimb.2014.00001.

47. Y Ting AS, Ting SY, Tan HC. 2009. Hydrocarbon-degradation by isolate Pseudomonas lundensis UTAR FPE2. Malaysian J Microbiol 5:104–108.

48. Rask C, Adlerberth I, Berggren A, Ahrén IL, Wold AE. 2013. Differential effect on cell-mediated immunity in human volunteers after intake of different lactobacilli. Clin Exp Immunol 172:321–332. https://doi.org/10.1111/cei.12055.

49. Lopez-Medina E, Neubauer MM, Pier GB, Koh AY. 2011. RNA isolation of Pseudomonas aeruginosa colonizing the murine gastrointestinal tract. J Vis Exp 3293. https://doi.org/10.3791/3293.

50. Vernikos GS, Parkhill J. 2006. Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the Salmonella pathogenicity islands. Bioinformatics 22:2196–2203. https://doi.org/10.1093/bioinformatics/btl369.

51. Bertelli C, Laird MR, Williams KP, Lau BY, Hoad G, Winsor GL, Brinkman FSL, Simon Fraser University Research Computing Group. 2017. Island-Viewer 4: expanded prediction of genomic islands for larger-scale datasets. Nucleic Acids Res 45:W30–W35. https://doi.org/10.1093/nar/gkx343.

52. Xie Z, Tang H. 2017. ISEScan: automated identification of insertion sequence elements in prokaryotic genomes. Bioinformatics 33:3340–3347. https://doi.org/10.1093/bioinformatics/btx433.

53. Guy L, Roat Kultima J, Andersson SGE. 2010. genoPlotR: comparative gene and genome visualization in R. Bioinformatics 26:2334–2335. https://doi.org/10.1093/bioinformatics/btq413.

54. McDermott AJ, Falkowski NR, McDonald RA, Pandit CR, Young VB, Huffnagle GB. 2016. Interleukin-23 (IL-23), independent of IL-17 and IL-22, drives neutrophil recruitment and innate inflammation during Clostridium difficile colitis in mice. Immunology 147:114–124. https://doi.org/10.1111/imm.12545.