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Environmental Reservoirs for *exoS*⁺ and *exoU*⁺ strains of *Pseudomonas aeruginosa*

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Summary

Pseudomonas aeruginosa uses its type III secretion system to inject the effector proteins ExoS and ExoU into eukaryotic cells, which subverts these cells to the bacterium's advantage and contributes to severe infections. We studied the environmental reservoirs of *exoS*⁺ and *exoU*⁺ strains of *P. aeruginosa* by collecting water, soil, moist substrates, and plant samples from environments in the Chicago region and neighboring states. Whole-genome sequencing was used to determine the phylogeny and type III secretion system genotypes of 120 environmental isolates. No correlation existed between geographic separation of isolates and their genetic relatedness, which confirmed previous findings of both high genetic diversity within a single site and the widespread distribution of *P. aeruginosa* clonal complexes. After excluding clonal isolates cultured from the same samples, 74 *exoS*⁺ isolates and 16 *exoU*⁺ isolates remained. Of the *exoS*⁺ isolates, 41 (55%) were from natural environmental sites and 33 (45%) were from man-made sites. Of the *exoU*⁺ isolates, only 3 (19%) were from natural environmental sites and 13 (81%) were from man-made sites ($p < 0.05$). These findings suggest that man-made water systems may be a reservoir from which patients acquire *exoU*⁺ *P. aeruginosa* strains.

Introduction

Pseudomonas aeruginosa is an important cause of severe infections in hospitalized patients and those with cystic fibrosis (Driscoll et al., 2007). This Gram-negative bacterium is primarily found as a free-living microbe in soil, water, and plants (Green et al., 1974; Hoadley, 1977; Kominos et al., 1977; Pellett et al., 1983), and the predominant selective pressures governing its evolution appear to be in the natural environment and not in human hosts (Selezska et al., 2012). The *P. aeruginosa* type III secretion system (T3SS) (Hauser, 2009) plays a critical role in the pathogenesis of infections in people (Hauser, 2009) but

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likely evolved to protect *P. aeruginosa* from predation in the environment (Ferguson et al., 2001; Matz et al., 2008). Many of the proteins encoded by this system (e.g. PscF) form a needle-like apparatus on the surface of *P. aeruginosa*. Effector proteins such as ExoS and ExoU are injected by this apparatus into the cytosol of eukaryotic cells to promote infection. Interestingly, the *exoS* and *exoU* genes are nearly always mutually exclusive; very few *P. aeruginosa* isolates contain both genes or neither gene (Feltman et al., 2001). One explanation for this exclusivity is that ExoS and ExoU provide enhanced fitness in distinct ecological niches.

Although the major reservoir of *P. aeruginosa* is the natural environment, the majority of epidemiological studies have focused on clinical rather than environmental isolates, and research into the environmental distribution of strains with specific type III secretion effector genes is limited. We cultured 120 *P. aeruginosa* isolates from largely non-healthcare sources and found that *exoU*⁺ and *exoS*⁺ isolates have different distributions in the environment. Whereas *exoS*⁺ isolates were cultured from a variety of sample types, including lakes, streams, faucets, drains, soil, and plant material, *exoU*⁺ isolates were more commonly associated with man-made water systems.

Results and Discussion

Identification and population structure of *P. aeruginosa* isolates from environmental samples

A total of 700 environmental samples of soil, water, plants and moist substrates were collected from sites in the Chicago region and neighboring states (Supporting Information Fig. S1). Samples were enriched for *P. aeruginosa* (Supporting Information Fig. S2), and the resulting bacteria were sequenced, which identified 97 *P. aeruginosa* isolates. This collection was supplemented with 23 isolates previously collected from environmental sites in central Minnesota (Supporting Information Fig. S1), yielding a total of 120 *P. aeruginosa* isolates from environmental sites (Supporting Information Table S1). We next generated a maximum likelihood phylogenetic tree based upon the core genomes of the 120 isolates (Fig. 1). As previously reported (Wiehlmann et al., 2007; Freschi et al., 2015; Thrane et al., 2015; Klockgether and Tummeler, 2017), nearly all the isolates fell into one large clade and one smaller clade. Five isolates were closely related to the reference strain PA14, which is a member of a widespread clonal complex of *P. aeruginosa* isolates (Wiehlmann et al., 2007). Interestingly, none of the isolates were highly similar to the reference strain C-NN2, which is a member of a clonal complex of clone C-like strains commonly cultured in Europe from aquatic environments and individuals with cystic fibrosis (Romling et al., 1994; Romling et al., 2005). Closer inspection of the tree indicated that nearly identical isolates and distantly related isolates were often found at sites within close proximity of each other. Also, some nearly identical isolates were found at sites quite distant from each other. For example, isolates from Wisconsin (env091), Ohio (env189), and Iowa (env126) had core genomes that were nearly identical (Fig. 1, Supporting Information Table S1). These results agree with prior studies demonstrating that local populations of *P. aeruginosa* are nearly as diverse as global populations (Kiewitz and Tummeler, 2000; Pirnay et al., 2005; Kidd et al., 2012; Selezska et al., 2012) and that clonal groups of *P. aeruginosa* can be found across broad

geographic distances (Romling et al., 1994; Pirnay et al., 2002; Dinesh et al., 2003; Romling et al., 2005). Taken together, these findings suggested the absence of a correlation between *P. aeruginosa* genotype and geographical location. Indeed, the pairwise genetic distance between isolates was not appreciably correlated with the geographic distance between the collection sites (Supporting Information Fig. S3). These results confirm that *P. aeruginosa* has a nonclonal epidemic population structure consisting of widely distributed epidemic clones dispersed among a background of non-clonal strains (Romling et al., 1994; Johnson et al., 2007; Wiehlmann et al., 2007; Pirnay et al., 2009; Maatallah et al., 2011).

We next compared the genotypes of our isolates to those previously reported by Wiehlmann and colleagues from a large global collection of 2921 *P. aeruginosa* isolates, of which 278 were environmental isolates (Wiehlmann et al., 2015). Each isolate had been genotyped using a set of 16-genetic markers (Wiehlmann et al., 2007). We searched the genomes of our isolates for these markers to similarly genotype them. We found that our 120 isolates comprised 53 genotypes, of which 31 were also in the Wiehlmann collection (Supplemental Information Table S2). Of the four most common genotypes in the Wiehlmann collection, two were found in our collection, indicating that some isolates from the Midwestern United States had a global distribution. A total of 51 (43%) of the 120 environmental isolates in our collection had genotypes that were not present in the Wiehlmann collection (Supplemental Information Table S3). These results indicate that some *P. aeruginosa* clones are not geographically isolated but rather widely dispersed, while most environmental isolates represent distinct clones.

The ecological niche of *P. aeruginosa*

We next examined how frequently *P. aeruginosa* was cultured from the different types of environmental samples. Data on sample collection were not available for the 23 central Minnesota isolates, so they were excluded from this analysis. The yield of *P. aeruginosa* was highest in soil and moist substrates, somewhat lower in water samples, and lowest in plant samples (Table 1). Our results are in agreement with previous reports that *P. aeruginosa* is found ubiquitously throughout moist environments (Hoadley, 1977; Pellett et al., 1983; Pirnay et al., 2005).

Type III secretion genotypes and environmental sites

Next we examined the type III secretion genotypes of the 120 environmental isolates. Each isolate contained the *pscF* gene, which is located in a large chromosomal cluster of genes that encode the structural and regulatory components of the type III secretion system. All contained the *exoT* gene, 115 (96%) contained the *exoY* gene, 100 (83%) contained the *exoS* gene, and 19 (16%) contained the *exoU* gene (Supporting Information Table S1). These values are within the ranges observed by others of 100%, 95%, 61–80%, and 18–33% for *exoT*, *exoY*, *exoS*, and *exoU*, respectively, in *P. aeruginosa* isolates from the natural environment (Feltman et al., 2001; Pirnay et al., 2009; Selezska et al., 2012). Interestingly, these values differ somewhat from those reported for isolates from acute infections, in which the *exoT* gene was found in 92–100% of isolates, the *exoY* gene in 89%, the *exoS* gene in 56–75%, and the *exoU* gene in 21–44% (Fleiszig et al., 1997; Feltman et al., 2001; Lomholt et al., 2001; Berthelot et al., 2003; Wareham and Curtis, 2007; Garey et al., 2008; Agnello

and Wong-Beringer, 2012; Pena et al., 2014). Together, these data suggest two points. First, nearly all environmental isolates contain the *exoT* and *exoY* genes, perhaps because these genes play an important role in the survival of *P. aeruginosa* in diverse natural environments. Second, the *exoU* gene is more commonly present in *P. aeruginosa* isolates causing acute infections than isolates found in the environment.

As previously noted, the larger clade of the phylogenetic tree was comprised of the *exoS*⁺ isolates and the smaller clade of the *exoU*⁺ isolates (Fig. 1) (Ozer et al.; Wiehlmann et al., 2007; Selezska et al., 2012; Freschi et al., 2015; Klockgether and Tummler, 2017). Such cladogenesis may have resulted from ecological divergence, in which *exoU*⁺ and *exoS*⁺ strains thrived in distinct niches (Cohan and Koeppl, 2008). Selezska and colleagues noted similar findings with regard to a largely *exoU*⁺ “extended clonal complex” that they identified and named eccB. These authors state that eccB “is quite a diverse but clonal group, with an apparently elevated sexual isolation to other lineages” (Selezska et al., 2012). Only one of our 120 isolates lacked both *exoS* and *exoU* (env068); it was distantly related to the *exoS*⁺ and *exoU*⁺ clades (Fig. 1). Closer examination of env068 indicated that it is distinct from PA7-like strains (which also lack *exoU* and *exoS*) in that it contains several type III secretion genes (e.g. *exoT*, *exsA*, *pscF*, and *pscJ*) but lacks the type V secretion gene *exlA* (Roy et al., 2010; Huber et al., 2016).

To validate our findings, we compared the type III secretion genotypes of our isolates to those of other environmental *P. aeruginosa* isolates with publically available sequences. We included all *P. aeruginosa* strains with whole-genome sequences deposited in the NCBI FTP site (ftp.ncbi.nlm.nih.gov accessed on January 7, 2018) with a source label of “water,” “plant,” “soil,” or “environment” (excluding isolates from hospital or industrial settings). We identified 56 isolates, of which 37 (66%) were *exoS*⁺, 16 (29%) were *exoU*⁺, 3 (5%) were *exoS*⁻/*exoU*⁻, and 0 were *exoS*⁺/*exoU*⁺. We expect that these results differ somewhat from ours because the proportion of each environmental sample type also differed from ours. Nonetheless, these results confirm that the majority of environmental isolates are *exoS*⁺.

Environmental niches of *exoS*⁺ and *exoU*⁺ isolates

We next examined the types of sites from which the *exoS*⁺ and *exoU*⁺ isolates were cultured. We categorized sites as either “natural” or “man-made,” based upon whether they were naturally occurring (e.g. lakes, streams, soil, plants) or fabricated (e.g. sinks, drains, toilets, fountains, hoses, pipes) (Supporting Information Table S1). To prevent bias from repeat sampling of the same bacterial clone at the same site, we included only one clonal isolate per sample type from each collection site in these analyses. This left a total of 90 *P. aeruginosa* isolates, 74 of which contained the *exoS* gene and 16 of which contained the *exoU* gene. While *exoS*⁺ isolates were frequently cultured from soil and plant matter, no *exoU*⁺ isolates were grown from these sources. Of the *exoS*⁺ isolates, 41 (55%) were from natural environments and 33 (45%) were from man-made environments (Table 2). Of the 16 *exoU*⁺ isolates, only 3 (19%) were from natural environments and 13 (81%) were from man-made environments. Thus relative to *exoS*⁺ isolates, *exoU*⁺ isolates of *P. aeruginosa* were more frequently found in man-made environments ($p < 0.01$). Our results are consistent with those of Vincent and colleagues (Vincent et al., 2017), who noted that all 16 *P. aeruginosa*

isolates from dental water lines fell into a phylogenetic clade corresponding to our *exoU*⁺ group. These findings suggest that *exoS* provides a fitness advantage in many types of ecological niches, whereas fitness attributable to *exoU* may be limited predominantly to man-made water systems. One explanation is that ExoS targets and confers protection against a broad variety of predatory eukaryotic organisms, whereas ExoU is active against a more narrow range of organisms that inhabit man-made water systems. Free-living amoebae are known to colonize and form biofilms in domestic plumbing systems (Shoff et al., 2008; Thomas et al., 2010), and *P. aeruginosa* bacteria can survive and multiply within these amoebae (Michel et al., 1995) or even kill them to prevent predation (Matz et al., 2008). Furthermore, the effector complement of *P. aeruginosa* strains influences their ability to kill *Acanthamoeba castellanii* amoebae (Matz et al., 2008). At first glance, the presence of both *exoS*⁺ and *exoU*⁺ isolates in man-made water systems argues against the ecological isolation of these two types of *P. aeruginosa* strains. Indeed, *exoS*⁺ and *exoU*⁺ isolates were cultured from the same drinking fountain (env113a and env113b) and from a shower drain and sink drain within the same bathroom (env189 and env187). It is possible, however, that within these water systems the *exoS*⁺ and *exoU*⁺ strains inhabit distinct micro-niches, as has been described for *Vibrio cyclitrophicus*, which is associated with different zoo- and phytoplankton or organic particle types in the same samples of seawater (Shapiro et al., 2012).

Our results are consistent with several published reports that have found *exoS*⁺ isolates in soil (Ferguson et al., 2001) and a paucity of *exoU*⁺ *eccB* isolates in a Belgian river (Pirnay et al., 2005; Selezska et al., 2012). However, Selezska and colleagues found *eccB* to be common in a river system in Northern Germany (Selezska et al., 2012) and Wiehlmann and colleagues reported that 18% of soil, plant, and freshwater isolates were *exoU*⁺, compared to 7% in our study (Wiehlmann et al., 2015). These findings may reflect differing levels of pollution or contamination of natural water systems with human-manipulated water such as sewage. In support of the latter hypothesis, two of the three *exoU*⁺ isolates we identified in natural water systems came from the Chicago River, which is highly affected by industrial and residential development and has even been engineered to reverse its direction of flow in several segments. These river isolates had nearly identical core genomes to isolates cultured from sink and shower drains and a drinking fountain (Fig. 1 and Supporting Information Table S1). Interestingly, *exoU*⁺ *eccB* strains had a survival advantage relative to *exoS*⁺ *eccS42* strains when incubated in autoclaved highly polluted river water (Selezska et al., 2012), suggesting that high levels of pollution may favor *exoU*⁺ strains. Additional studies over wider geographical areas will be necessary to more definitively define the factors that are associated with the presence of *exoU*⁺ strains.

These findings shed light on potential reservoirs from which patients acquire their *P. aeruginosa* infections. *exoU*⁺ strains are over-represented in hospital-associated infections relative to the natural environment (21–44% vs. 16%, respectively), suggesting that many infected patients acquire their strains from man-made water systems. Indeed, hospital-acquired infections have been linked to the presence of *P. aeruginosa* in hospital water supplies (Trautmann et al., 2005; Trautmann et al., 2006; Kizny Gordon et al., 2017; Lefebvre et al., 2017), and isolates collected from the sinks and washtubs of intensive care units were more likely to be *exoU*⁺ (Bradbury et al., 2010). We would predict that

community-acquired outbreaks of *P. aeruginosa* dermatitis-folliculitis, which are associated with man-made water systems such as hot tubs and swimming pools (Fiorillo et al., 2001; Wu et al., 2011a; Michl et al., 2012), would be caused largely by *exoU*⁺ strains. Although to our knowledge such strains have not been analyzed for T3SS genotypes, they are commonly serogroup O-11 (Ayi, 2015), which tend to be *exoU*⁺ (Berthelot et al., 2003; Zhu et al., 2006). Perhaps the most intriguing disease manifestation in this regard is keratitis, in which *exoU*⁺ strains are associated with contact lens use and *exoS*⁺ strains are more common in patients who do not wear contact lenses (Lomholt et al., 2001; Winstanley et al., 2005; Zhu et al., 2006; Choy et al., 2008; Shankar et al., 2012; Yamaguchi et al., 2014). It is conceivable that keratitis in contact-lens wearers originates from *P. aeruginosa* strains in tap water that contaminates contact lenses through the washing of the lenses and cases (Stapleton et al., 2007), whereas keratitis in the absence of contact lenses is caused by *P. aeruginosa* acquired from the natural environment. Additional studies are necessary to more clearly define the roles of environmental reservoirs in dictating the prevalence of *exoU*⁺ vs. *exoS*⁺ strains in different types of infections.

Our study has several limitations. First, it examined only a localized region within the Midwestern United States, and it is not clear whether our results can be generalized to other areas. Second, our isolation protocols may not have detected the full diversity of *P. aeruginosa* strains present. Third, our sample size was limited to 120 *P. aeruginosa* isolates. Finally, we largely excluded hospital and other medical sites from the study. This was done intentionally to ensure that the results represented the distribution of *P. aeruginosa* outside of the healthcare setting. In future studies, we plan to collect *P. aeruginosa* isolates from healthcare settings within the same region and to compare these isolates to those of the current study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Originality-Significance Statement

ExoU and ExoS are important virulence factors of *Pseudomonas aeruginosa*, and isolates of this bacterium tend to contain either the *exoU* or the *exoS* gene. Our results confirm previous findings that *exoU*⁺ and *exoS*⁺ strains of *P. aeruginosa* comprise distinct phylogenetic clades. To our knowledge, this is the first study to associate *exoU*⁺ strains with man-made water systems. This finding has implications regarding how patients acquire the *P. aeruginosa* strains that subsequently cause severe infections.

Figure 1.

The phylogeny of environmental *P. aeruginosa* isolates. A maximum likelihood tree was generated from core genome alignments following removal of sequences under the influence of recombination. Isolates in red are *exoU*⁺, those in blue are *exoS*⁺, and the isolate in green is *exoS*⁻/*exoU*⁻. Reference strains PA14 (*exoU*⁺) and C-NN2 (clone c, *exoS*⁺) and three previously sequenced environmental isolates, M18 (Wu et al., 2011b), F9676 (Shi et al., 2015), and USDA-ARS-USMARC-41639 (Winsor et al., 2016), are shown in black.

Table 1Yield of *P. aeruginosa* from different sample types.

	earth/soil	water	moist substrates	plant
total # samples *	113	164	293	113
# samples from which <i>P. aeruginosa</i> grew (%)	18 (15.9)	16 (9.8)	47 (16.0)	8 (7.1)

* samples from central Minnesota (site 44) are excluded

Table 2

Number of *exoS*⁺ and *exoU*⁺ *P. aeruginosa* isolates from man-made and natural environments, using only one clonal isolate per collection site and sample type.

	<i>exoS</i> ⁺	<i>exoU</i> ⁺	Total
Natural environment	41 (55%)	3 (19%)	44
water	14	2	
moist substrates	4	1	
earth/soil	16		
plant matter	7		
Man-made environment	33 (45%)	13 (81%)	46
water	4	1	
moist substrates	28	12	
earth/soil	1		
plant matter			
Total	74	16	90

% values reflect percentage of all *exoS*⁺ isolates or of all *exoU*⁺ isolates