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# R-Type Pyocin is Required for Competitive Growth Advantage Between *Pseudomonas aeruginosa* Strains

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**Abstract** R-type pyocin is a bacteriophage tail-shaped bacteriocin produced by *Pseudomonas aeruginosa*, but its physiological roles are relatively unknown. Here we describe a role of R-type pyocin in the competitive growth advantages between *P. aeruginosa* strains. Partial purification and gene disruption revealed that the major killing activity from the culture supernatant of PA14 is attributed to R-type pyocin, neither F-type nor S-type pyocins. These findings may provide insight into the forces governing *P. aeruginosa* population dynamics to promote and maintain its biodiversity.

**Key words:** R-Type pyocin, *Pseudomonas aeruginosa*, competition, population

Understanding population dynamics in a mixed microbial community leads to elucidating the mechanisms dictating the strain diversification and the evolution of a microorganism, which involves genetic diversity and phylogenetic divergence of relevant properties of a microorganism, such as virulence factors or drug resistance [22]. Microbial population dynamics are best characterized in *Escherichia coli*, in which bacteriocin production and its resistance/sensitivity play a crucial role [9, 11]. Bacteriocins are bacteriocidal proteinous substances, which are produced by many bacteria and have a killing activity against the strains of the same or closely related species [20]. At least sixteen classes of bactericins have been listed so far and named on the basis of the species that produce them [23], which include colicins of *E. coli*, cloacins of *Enterobacter cloacae*, pestisins of *Yersinia pestis*, pyocins of *Pseudomonas aeruginosa* (formerly, *P. pyocynia*), monocins of *Listeria monocytogenes*, cerecins of *Bacillus cereus*, staphylococcins of *Staphylococcus* species, and pediocin-like bacteriocins produced by lactic acid bacteria [10, 13, 14, 24].

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Pyocins are produced by more than 90% of *P. aeruginosa* which is an organism found ubiquitously in nature and a human pathogen infecting a host when local or general defense mechanisms are impaired, such as cystic fibrosis patients and hospitalized burned and/or ventilated patients [1]. Each strain of *P. aeruginosa* may synthesize several pyocins of three distinct families, designated S, F, and R pyocins [16], whose genetic determinants are usually found in variable regions of the genome [5, 28]. As well, they differ by their morphology and mode of killing. The S-type (soluble) pyocins are like colicins in their structure and mode of action; they have an effector and an immunity component, with the effector component possessing DNase and lipase activity [3, 25]. S pyocins are proteinase-sensitive and cannot be sedimented nor observed by electron microscopy, due to their small size. The F-type (flexuous) pyocins are curved, non-contractile rods with distal filaments. They vary in their host ranges but are structurally, morphologically, and antigenically similar [12]. The R-type (rod) pyocins resemble bacteriophage tails of T-even phages, being composed of a contractile sheath, a core and tail fibers [8]. The receptors for R pyocins are the lipopolysaccharides or lipooligosaccharides found in the outer membrane of Gram-negative bacteria [4]. The mode of killing is most likely the disruption of the membrane potential by forming pores in the target cell membranes [26]. The nucleotide sequences and the genetic organizations in R and F pyocin gene clusters are similar to those of coliphages P2 and λ, respectively [18]. The production of pyocins in *P. aeruginosa* involves two transcriptional regulators, PrtN and PrtR and is inducible by treatments that cause DNA damage in a *recA*-dependent manner, which may cleave PrtR, a homologue of phage λ cI repressor normally repressing the pyocin gene expression [15, 18].

We hypothesize that the wide spread and regulatory natures of "pyocinogeny" may be associated with population dynamics of *P. aeruginosa* as in *E. coli*, resulting in

**Table 1.** *P. aeruginosa* strains used in this study.

| Strain | Strain name and relevant characteristics[a] | Reference or source |
|---|---|---|
| PAO1 | Wild-type laboratory strain | Lab collection |
| PA14 | Wild-type laboratory strain; $Rif^R$ | Lab collection |
| PAK | Wild-type laboratory strain | Lab collection |
| PA2 | Wild-type laboratory strain | Lab collection |
| 57RP | Wild-type laboratory strain | Lab collection |
| U1089 | PA14 Tn*phoA* insertion mutant in PA0620 (R-pyocin); $Rif^R$, $Km^R$ | MGH-PGA[b] |
| U483 | PA14 Tn*phoA* insertion mutant in PA0622 (R-pyocin); $Rif^R$, $Km^R$ | MGH-PGA |
| U1189 | PA14 Tn*phoA* insertion mutant in PA0636 (F-pyocin); $Rif^R$, $Km^R$ | MGH-PGA |
| U527 | PA14 Tn*phoA* insertion mutant in PA0639 (F-pyocin); $Rif^R$, $Km^R$ | MGH-PGA |
| U120 | PA14 Tn*phoA* insertion mutant in PA0641 (F-pyocin); $Rif^R$, $Km^R$ | MGH-PGA |
| U1272 | PA14 Tn*phoA* insertion mutant in PA0646 (F-pyocin); $Rif^R$, $Km^R$ | MGH-PGA |
| PRH801 | PA14 in-frame deletion mutant of PA0622 (R-pyocin); $Rif^R$ | This study |
| PKL7Z1 | PAK harboring a miniCTX-derived *katA::lacZ* fusion; $Tc^R$, $LacZ^+$ | 6 |
| PMM1-PMM28 | *P. aeruginosa* clinical isolates from various Asian countries | 6 |

[a]$Rif^R$, rifampin-resistant; $Km^R$, kanamycin-resistant; $Tc^R$, tetracycline-resistant; $LacZ^+$, β-galactosidase producing.
[b]PA14 transposon insertion mutant library supported by Massachusetts General Hospital-ParaBioSys/National Heart, Lung, and Blood Institute Program for Genomic applications (http://pga.mgh.harvard.edu/cgi-bin/pa14/mutants/retrieve.cgi).

diversity in the virulence and survival potentials of different strains of this bacterium. As an initial attempt to test this hypothesis, we investigated the competitive growth advantages/disadvantages between *P. aeruginosa* strains such as PAO1, PAK, and PA14. In our previous study, we showed that PAO1 and PA14 outcompete PAK, resulting in the complete loss of PAK in mixed cultures [6]. The competitive growth advantage displayed by PAO1 and PA14 is attributed to a secreted bacteriocidal component, whose production is observed early during the growth phase in the planktonic culture. And the killing activity is associated with the competitive growth advantage of PA14 over several sensitive strains, which is most likely pyocins as suggested previously.

In the present study, we decided to identify and characterize the killing substance from PA14 and, at first, tried to partially purify it from the culture supernatant of PA14. The *P. aeruginosa* strains used in this study are listed in Table 1. As shown in Fig. 1A, the killing activity is sedimentable by ultracentrifugation (pellet 2), characteristic of the phages or phage-related particles such as R- and F-type pyocins rather than the small soluble proteins such as S-type pyocins. The killing activity could not likely replicate either, since conventional plaque assays had failed to produce halos at any dilution of the killing sample (data not shown). As well, the killing activity was not significantly diffusible, because the concentration of the killing activity affected the halo transparency, but not the halo size (data not shown). Therefore, we tentatively concluded that the PAK killing activity from the PA14 culture supernatant is most likely the sedimentable pyocins such as R- or F-type pyocins. To validate the tentative conclusion, the supernatants of the 6 Tn*phoA* mutants whose insertion sites were within the R pyocin cluster [PA0620 (U1089) and PA0622 (U483)] or the F pyocin cluster [PA0636 (U1189), PA0639 (U527), PA0641 (U120), and PA0646 (U1272)] were

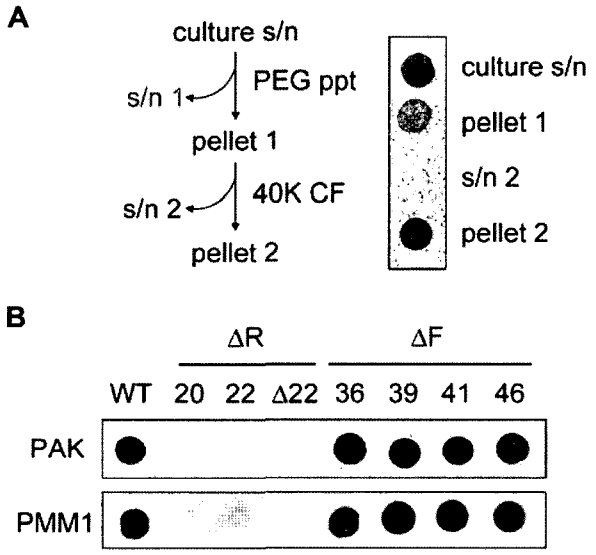


**Fig. 1.** Characterization of the killing substance from the PA14 culture supernatant.
**A.** The culture supernatant (s/n) from the late stationary phase culture of PA14 was partially purified by successive selective precipitations. Polyethylene glycol precipitation (PEG ppt) resulted in the separation of the supernatant (s/n 1) and the pellet, which was dissolved in 10 mM $MgSO_4$ (pellet 1) and subjected to ultracentrifugation (40K CF). The pellet was dissolved in 10 mM $MgSO_4$ (pellet 2) and spotted onto the PAK lawns together with s/n 2, pellet 1, and s/n 1. The visible halos were made after 18 h at 30°C. **B.** PAK killing was performed using the culture supernatants from R pyocin mutants (U1089, U483, and PRH801) and F pyocin mutants (U1189, U527, U120, and U1272) with PA14 (WT) as the control. The visible halos were made after incubation at 30°C for 18 h. Mutant abbreviations (on the top) indicate the mutations in PA0620 (20), PA0622 (22), PA0636 (36), PA0639 (39), PA0641 (41), and PA0646 (46) for Tn*phoA* mutants and an in-frame deletion of PA0622 (Δ22).

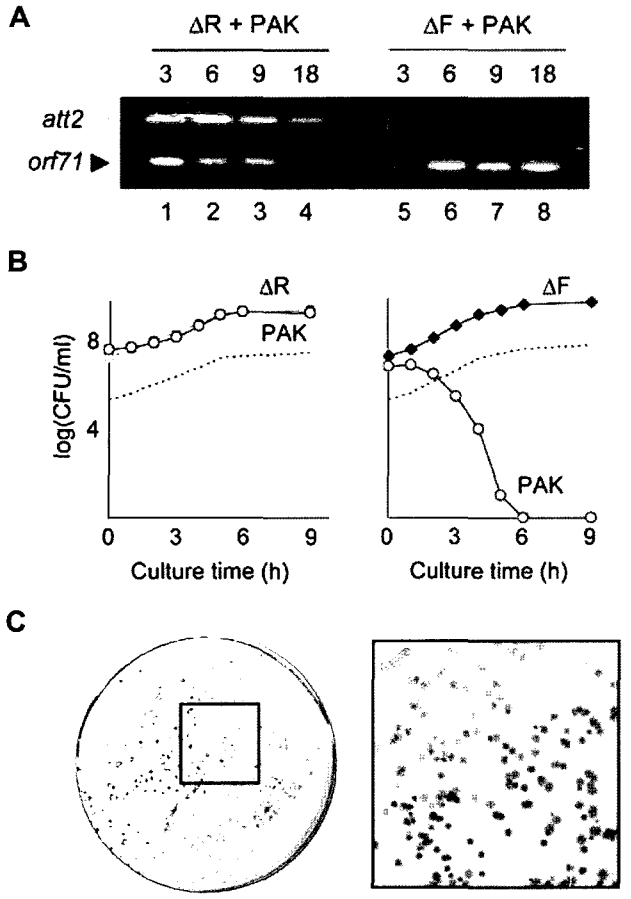


**Fig. 2.** Growth competitions between PAK and PA14 R-type pyocin mutant.

**A.** Multiplex PCR to detect PAK and PA14 derivatives from the coculture. The multiplex PCR were performed as described elsewhere [6], from the PAK cocultures with an R pyocin mutant (PRH801 for PA0622) or with an F pyocin mutant (U527 for PA0639). **B.** Growth curves were constructed based on the viable count assay results of an R pyocin mutant (PRH801, ■; designated as ΔR), an F pyocin mutant (U527, ◆; designated as ΔF) and a PAK derivative, PKL7Z1 (○; designated as PAK) from the coculture of PKL7Z1 with either PRH801 or U527. The dotted lines denote the 1/100 from the population of the R or F pyocin mutants, indicating the PCR detection limit. The doubling times of PRH801 and U527 in the cocultures were estimated as 35.4 min ($r^2$=0.995) and 34.1 min ($r^2$=0.993), respectively. The doubling time of PKL7Z1 in the coculture with PRH801 was calculated as 32.3 min ($r^2$=0.984). **C.** Coexistence of PRH801 and PAK from the coculture. About $8.0\times10^2$ cells were taken from the 18 h coculture of PRH801 ($LacZ^-$) and PKL7Z1 ($LacZ^+$) and plated on an LB agar medium containing X-gal (left). The numbers of PRH801 (white, 361 colonies), and PKL7Z1 (blue, 372 colonies) are almost identical. The squared region is magnified for comparison of the proportions of both strains (right). PAK derivatives are *att2*-positive and *orf71*-negative, whereas PA14 derivatives are *att2*-negative and *orf71*-positive [6].

subjected to the killing assay as described elsewhere [6], including an in-frame deletion mutant of PA0622 (PRH801) to exclude the possible polar effect (Fig. 2B). The PA14 Tn*phoA* transposon insertion mutants have been obtained from the PA14 Transposon Insertion Mutant Database (http://pga.mgh.harvard.edu/cgi-bin/pa14/mutants/retrieve.cgi). To create a PA0622 in-frame deletion mutant, we amplified 1,870 bp DNA fragment encompassing PA0622 (PRF17) using two primers (0622N1, 5'-AGCAATGAA-TTCCGACGTGAAGG-3' and 0622C1, 5'-CGTACTTGA-ATTCGGCTTTCTCG-3', where the underlined denote mutations for EcoRI digestion) based on the PA14 genome sequences [17]. The resulting construct was digested with NcoI to create a 432 bp (37.3% of the full-length) in-frame deletion of the PRF17 coding region, which was subsequently cloned into pEX18T followed by sucrose selection of double crossover replacement [7]. As a result, the mutations in the R pyocin cluster, but not those in the F pyocin cluster resulted in the failure to generate the PAK killing activity. We also found that the major killing activity from the PA14 culture supernatants toward various clinical isolates including PMM1 disappeared completely by R pyocin mutations, but not by F pyocin mutations (Fig. 1B and Table 2).

These biochemical and genetic evidences indicate that R-type pyocin rather than F- or S-type pyocins in the PA14 culture supernatant may directly inhibit the growth of PAK. An R pyocin (R1) has been shown not to affect the respiration nor induce cell lysis, but instead cause a decrease in the intracellular ATP level by depolarizing the cytoplasmic membrane of the target cells [26]. The R pyocin-mediated dissipation of the proton motive force leads to the inhibition of active transport and ATP synthesis. This killing mechanism of R-type pyocin may explain the viability loss of the PAK strain that was initiated when it begins to actively grow and thus may become metabolically active, as observed previously [6].

Next, we conjectured that the R-type pyocin of PA14 was decisive for the competitive growth advantage over PAK or other sensitive strains, if the killing activity is attributed to the R-type pyocin. PAK (or PKL7Z1) cells were cocultured with an R pyocin deletion mutant (PRH801) and the remaining population of PAK was monitored by PCR detection and viable count assays as described elsewhere [6] (Fig. 2). We found that PRH801 could not outcompete PAK and was not outcompeted by PAK either (Fig. 2A). The doubling times for PRH801 and PAK were 35.4 and 32.3 min, respectively, indicating the independent and comparable growth of both strains in the coculture (Fig. 2B). Similar results were found with a Tn*phoA* insertion mutant for PA0620 (U1089) or PA0622 (U483) instead of PRH801 (data not shown). The number of PKL7Z1 cells was comparable to that of PRH801 even at the 18 h post-inoculation of the coculture by viable counting in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), as shown in Fig. 2C. As observed with the wild type PA14, no viable PAK cells were recovered from the 6 h coculture with an F pyocin mutant (U1189 for PA0636), which is no less killing than the wild type (Fig. 2B).

To generalize the relationship between the competitive growth advantage and the ability to produce R pyocins

**Table 2.** Killing spectra of R and F pyocin mutants[a].

| Strains / s/n | PAO1 | PA14 | PAK | 57RP | PA2 | PMM1 | PMM2 | PMM3 | PMM4 | PMM5 | PMM6 | PMM7 | PMM8 | PMM9 | PMM10 | PMM11 | PMM12 | PMM13 | PMM14 | PMM15 | PMM16 | PMM17 | PMM18 | PMM19 | PMM20 | PMM21 | PMM22 | PMM23 | PMM24 | PMM25 | PMM26 | PMM27 | PMM28 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PA14 | – | – | ++ | ++ | – | ++ | ++ | ++ | – | – | – | ++ | – | – | – | – | ++ | – | – | – | ++ | + | – | – | ++ | – | – | – | – | – | ++ | – | – |
| PKL7Z1 | – | – | – | – | – | ++ | ++ | – | + | – | – | + | + | ++ | ++ | ++ | – | + | – | – | – | + | ++ | ++ | ++ | – | + | ++ | – | – | ++ | + | – |
| PRH801 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| U527 | – | – | ++ | ++ | – | ++ | ++ | ++ | – | – | – | ++ | – | – | – | – | ++ | – | – | – | ++ | + | – | – | ++ | – | – | – | – | – | ++ | – | – |

[a]++, highly killing; +, slightly killing or unclear; –, no killing.

of PA14, other susceptible strains including PMM1 were cocultured with the R pyocin mutant of PA14 and the survival was monitored by PCR. Fig. 3 shows that the competitive growth advantage over PMM1 was lost for the R pyocin mutant, whereas the F pyocin mutant was able to outcompete PMM1. The finding that susceptible strains such as PAK and PMM1 and an R-type pyocin mutant of PA14 coexisted without killing each other suggests that the competitive growth advantage of PA14 over those strains requires the capability to produce R-type pyocins which had been acquired probably at no metabolic cost.

The R and F pyocin clusters are located in a hypervariable region and the genomic diversity involving this region is relatively profound among various *P. aeruginosa* isolates [28]. To provide more information about the R pyocin gene cluster in the outcompeted strain, PAK, we performed PCR-based detection of the individual genes in the R pyocin regions and revealed that only PA0621 (PRF16) is missing in PAK, but is present in PAO1 and PA14 (data not shown). PRF16 is homologous to Orf21 of phage CTX and supposed to have a role in tail fiber assembly as the gpG of coliphage P2 [19], but its genuine role in the production and function of R pyocin remains to be experimentally verified. Wolfgang *et al.* [28] showed that this gene was most likely absent in the most (16 out of 18) *P. aeruginosa* isolates including PAK. It is still uncertain whether the outcompeting strains (PA14 and PAO1) are resistant to the

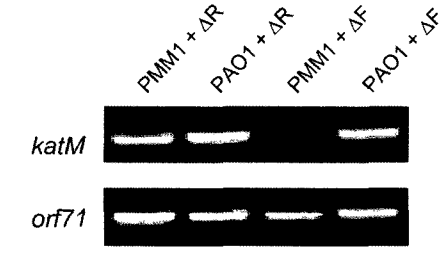


**Fig. 3.** Growth competitions between *P. aeruginosa* strains.
PCR detection of the designated amplicons (left) were performed using the samples from the cocultures as indicated (top); PA14 derivatives (PRH801 designated as ΔR and U527 designated as ΔF) and the competing strains (PAO1 and PMM1). Cells of each strain ($4.8\times10^6$ CFU) were subjected to the mixed culture. Both PMM1 and PAO1 are *katM*-positive and *orf71*-negative, whereas PA14 is *katM*-negative and *orf71*-positive [6].

R pyocins from the outcompeted strain (PAK) or whether PAK lacks the biogenesis of R pyocin, which might be attributed to the absence of PRF16. Although PAK is sensitive to PA14 R pyocins, it still displays the killing activity toward several clinical and environmental strains (17 out of 33 strains tested) (Table 2), the apparent function of PA0621 and the R-pyocin variations remains to be revealed. It is noticeable that PA14 is killing toward 11 strains out of the tested 33 strains, where 11 strains are sensitive to the PAK-derived killing activity, but resistant to the killing activity from PAO1 and PA14 [6]. The rock-scissor-paper mode of killing/resistance may be established, when we could have profiled killing activities from more *P. aeruginosa* strains. We are currently trying to introduce the PRF16 of PA14 into the genome of PAK or to isolate PA14 mutants that die when grown together with PAK, to further elucidate the importance of R-type pyocin and the resistance to it in *P. aeruginosa* population dynamics.

The spontaneous generation of the heterogeneity within microbial community may be accelerated by the fluctuating environmental conditions and population dynamics may be under the combined control of such external and some intrinsic selective pressures, presumably at the expense of the altruistic cell death within the populations. Based on the previous and present studies, we propose that R-type pyocin may be one of the selective forces involved in cell death within *P. aeruginosa* populations, while the ability to produce R-type pyocins and/or the ability or inability to resist the particular subtypes of R pyocins have been fortuitously transferred via horizontal gene transfer.

Recently, an R pyocin gene (PA0620) was shown to be required for the survival of PAO1 in a rat model of chronic respiratory infection [21], indicating a role for R-type pyocin in survival of the *P. aeruginosa* cells within the population at least in that host environment. Because the receptors to R pyocins are associated with the lipopolysaccharide (LPS) core region, the resistance and sensitivity to R pyocins are also likely related with the LPS structures [29], which affect the antigenicity and toxicity and consequently the virulence of *P. aeruginosa*.

Insights from the colicin-mediated *E. coli* strain competitions that constitute a rock-scissors-paper mode of

population dynamics [9, 11] and from the different pyocin profiles [6] suggest some *P. aeruginosa* strains might be present for the similar mode of population dynamics involving at least three parties. Nevertheless, it will be promising to address the mechanism that involves R pyocins for the strain competition in our laboratory mixed planktonic culture conditions. The killing unit of an R pyocin is 1 to 2, meaning only 1 or 2 particles are required for killing [16], which is much lower than those of F and S pyocins (280 and 300, respectively), suggesting that it may be much more effective to involve R-type pyocin for strain competitions. However, it is not completely ruled out that F- or S-type pyocins and other factors including phages might be implicated in strain competitions, possibly in other conditions and/or in other strains, since the F pyocin gene cluster is located adjacent to the R pyocin cluster, exhibiting a profound degree of genetic diversity [28] and S-type pyocins are evolutionarily related with colicins of *E. coli* [2, 20]. Cell death within *P. aeruginosa* biofilm is known to involve the induction of a Pf1-related filamentous prophage [27]. *P. aeruginosa* strains that have been isolated from diverse environmental and clinical reservoirs need to be analyzed for the R pyocin-mediated growth advantages in biofilm culture as well as in host tissues, to validate and generalize the involvement of R pyocins in strains competitions and population dynamics, which are more relevant situations to the real world.

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