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Profiling Pyocins and Competitive Growth Advantages of Various *Pseudomonas aeruginosa* Strains

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Abstract *Pseudomonas aeruginosa* produces a variety of bacteriocidal substances including pyocins that are active against the same species, but their physiological roles are relatively unknown. Here, we profiled the bacteriocidal activities in the culture supernatants of various *P. aeruginosa* isolates and describe the competitive growth advantages of strains PAO1 and PA14 over some strains including PAK, which are sensitive to their bacteriocidal activities. These findings suggest that the factors governing the production of pyocins and the resistance to them play important roles in controlling *P. aeruginosa* populations in its local environments.

Key words: Bacteriocins, pyocins, *Pseudomonas aeruginosa*, killing, competition

Bacteriocins are narrow spectrum antibiotics, generally effective against the closely related bacterial species. These compounds are produced by many γ-proteobacteria and include the colicins of *Escherichia coli* and the pyocins of *Pseudomonas aeruginosa* [19, 23]. Much attention has been paid to pyocins as promising therapeutic agents to control the infections caused by *P. aeruginosa*, an opportunistic human pathogen that is one of the pathogens most frequently isolated from nosocomial infections, primarily causing chronic lung infections in cystic fibrosis patients and posing fatal problems for immunocompromised AIDS and cancer patients [3, 20].

Pyocins are produced by more than 90% of *P. aeruginosa* strains, and each strain may synthesize pyocins of three types (R, F, and S; 19). Both R- and F-type pyocins are phage tail-like particles, but have different shapes [8]; R pyocins are rod-shaped with a contractile sheath, whereas F pyocins appear as flexuous, noncontractile rods. The soluble (S-type) pyocins share structural and functional features with a subclass of the nuclease colicins of *E. coli*, and their gene clusters also show similar gene organizations, with autoimmunity genes tightly linked to the cognate bacteriocin genes [19]. The *P. aeruginosa* reference strain PAO1 is known to secrete all three types of pyocin (R2, F2, and S2 subtypes) and the gene clusters encoding R2 and F2 pyocins are located adjacently within the third variable segment between *trpE* (PA0609) and *trpG* (PA0649) on the chromosome [24, 27]. The nucleotide sequences and the genetic organizations in R2 and F2 pyocin gene clusters are similar to those of coliphages P2 and λ, respectively [17]. The production of pyocins in *P. aeruginosa* 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 [14, 18]. These genetic, regulatory, and functional similarities between pyocins and bacteriophages suggest their evolutionary relatedness. However, why and how the pyocin gene clusters have been so diversified and their physiological roles are relatively unknown.

To gain an insight into the roles of the bacteriocidal activities of pyocins, we investigated the intraspecific competitions between *P. aeruginosa* strains including PAO1, PAK, and PA14, because the bacteriocidal activities of pyocins have not extensively been characterized under normal growth conditions of mixed populations of *P. aeruginosa* strains. We demonstrate here that PAO1 and PA14 outcompete PAK, resulting in the complete loss of PAK in mixed planktonic cultures. The competitive growth advantage displayed by PAO1 and PA14 is attributed to secreted bacteriocidal components, most likely pyocins, whose production is observed during the earlier growth phase in the planktonic culture. Furthermore, we performed a comprehensive profiling of pyocin production and pyocin

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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	25
PAK	Wild-type laboratory strain	S. Lory
57RP	Environmental isolate	2
PA2	Human clinical isolate; Rif^R	13
PKL7Z1	PAK harboring a miniCTX-derived *katA::lacZ* fusion; Tc^R, $LacZ^+$	This study
PMM1–28[b]	*P. aeruginosa* clinical isolates from various Asian countries	Lab collection

a: Rif^R, rifampin-resistant; Tc^R, tetracycline-resistant; $LacZ^+$, β-galactosidase-producing.
b: Twenty-eight clinical isolates of *P. aeruginosa* from the Asian Bacterial Bank (ABB) of Asian-Pacific Research Foundation for Infectious Diseases (ARFID) and renamed as PMM strains in our laboratory.

resistance using a total of 33 *P. aeruginosa* strains and found that pyocins of PA14 were required for the killing and the subsequent competitive growth advantage over some other clinical isolates as well. Based on these results, we propose that the production of and the resistance to pyocins may function in cell death within the mixed population of *P. aeruginosa*, similar to the *E. coli* colicins important in controlling population dynamics, probably in a way to enhance the adaptive fitness in their localized abiotic and biotic environments.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

E. coli, *P. aeruginosa* strains, and their derivatives were grown at 37°C using Luria-Bertani (LB) broth or on 2% Bacto-agar (Difco, Detroit, MI, U.S.A.) LB plates as described elsewhere. Stationary phase cultures were made to inoculate fresh LB broth with the initial inoculum size of 1.6×10^7 CFU/ml, which were grown and used for experiments. Likewise, the mixed cultures were made using mixtures of cells that had been grown separately, with the initial inoculum size of 1.6×10^7 CFU/ml for each strain. Antibiotics such as rifampin (Rif, 100 μg/ml), tetracycline (Tc, 100 μg/ml or 20 μg/ml), ampicillin (Ap, 50 μg/ml), and carbenicillin (Cb, 200 μg/ml) were supplemented as required. Table 1 lists the *P. aeruginosa* strains used in this study.

PCR Detection Assay of Each Strain from the Mixed Populations

Based on the hypervariable regions described by Wolfgang *et al.* [27] and Pf1-related genome diversity that have been shown to discriminate the three strains, we selected four sets of primers as summarized in Table 2. The 601 bp fragment was amplified by katM-N1 and katM-UC primers from the *katM* (PA2185) gene residing in the 12th variable segment of the PAO1 genome [12, 27]. The 759 bp fragment was amplified only from PAK by the *att1* primers (0714D and 0730U), since some genetic segments are integrated at the *att1* sites of PAO1 and PA14 genomes. Likewise, the *att2* primers (1190D and 1192U) amplified the 1,676 bp amplicons present only in PAO1 and PAK. The PA14-specific *orf71* amplicon targeting a Pf1 prophage region (552 bp) of the PA14 genome was amplified by N-out and orf71U primers. Appropriate amount of cells were taken from the mixed cultures at the given time point, diluted in water, and used as the template for the PCR detections using approximately 1.6×10^4 cells of the major population per reaction.

Two pairs of primers (1190D and 1192U and N-out and orf71U) were used for the multiplex PCR to specifically

Table 2. PCR detection primers used in this study.

Amplicon (size)	Sequences of relevant primer pairs[a]		Amplifiable in[b]
katM	katM-N1	5'-CCATGAATTCGAACACATAGCC-3'	PAO1
(601 bp)	katM-UC	5'-CTGGATCCGCTTGTCATGTACG-3'	PMM1, 2, 3, 7, 20, and 26
att1	0714D	5'-CATGAGGTCTATCTGGAACC-3'	PAK
(759 bp)	0730U	5'-TCGACGTCGAGCACAAGGCC-3'	PMM3
att2	1190D	5'-CATCGCCAGGTAGGTGAGGACCG-3'	PAO1 and PAK
(1,676 bp)	1192U	5'-CTGGGAATAGGCCTCGATGTCC-3'	PMM1, 2, 3, 7, 16, 20, and 26
orf71	N-out	5'-CAAATGTGCGGTATCCAGGGATC-3'	PA14
(552 bp)	orf71U	5'-TTCTTCGAATTCCATGGCCTTTGC-3'	

a: Underline denotes mutations for restriction enzyme sites.
b: These results are shown in Figs. 1 and 4A.

detect each strain from the PA14-PAK coculture. Both primer pairs could amplify the 1,676 bp *att2* amplicon of PAK and the 552 bp *orf71* amplicon of PA14, under the following conditions; denaturation at 94°C for 3 min followed by 28 cycles of the amplification (denaturation at 94°C for 30 sec, annealing at 42°C for 30 sec, and extension at 72°C for 1 min) and final extension at 72°C for 10 min.

Viable Count Assay of PA14 and PAK Strains from the Coculture

Viable cell counting was more sensitive than PCR detection, since it ensured to monitor the presence of a single culturable cell. The CFU of PA14 during the coculture was determined on a plate containing Rif, since PA14 is Rif-resistant [21]. For PAK viable counting, we generated a tetracycline-resistant PAK strain (PKL7Z1), in which a miniCTX-*lacZ* [5] derivative containing the *katA* promoter region [12] had been integrated on the genome by the phage CTX *att*-directed homologous recombination. The miniCTX integration of PKL7Z1 was verified by Tc-resistance and β-galactosidase production. The CFU of PKL7Z1 cells during the cocultures with PA14 were assayed on a Tc-containing plate, or its proportion was easily visualized on a plate containing 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Pyocin Experiments

Spotting of pyocin samples on the lawns of *P. aeruginosa* cells was performed as described previously [25], with some modifications. The pyocin samples were obtained by chloroform ($CHCl_3$) extraction of the culture supernatant from the overnight cultures in LB broth, to remove contaminating cells and other insoluble impurities. Residual $CHCl_3$ of the aqueous phase was evaporated prior to use, to minimize its harmful effect. Droplets (3 μl) of the pyocin samples were spotted onto an LB medium top layer containing 0.7% agar and 50 μl of *P. aeruginosa* cells from the late logarithmic cultures (OD_{600}=1.0). The plates were incubated at 37°C for 18 h.

Serial dilutions (10^6 to 10^2 CFU per droplet) of the late logarithmic cultures were spotted onto an LB medium containing $CHCl_3$-extracted pyocin samples (500 μl). The plates were incubated at 37°C for 18 h.

Results and Discussion

P. aeruginosa Strains PAO1 and PA14 Outcompete PAK Strain in the Mixed Planktonic Cultures

To test for the possibility of the intraspecific competitions in the mixed populations of *P. aeruginosa* strains under normal culture conditions, we selected for liquid planktonic mixed cultures three representative "wild-type" strains, PAO1, PA14, and PAK, that display different virulence and survival characteristics [2, 4, 21, 22]. Among them, PA14 may be the most virulent with a broader host spectrum that includes mammals, insects, nematodes, slime molds, fungi, and plants. PAO1 and PA14 produced a green-colored phenazine, pyocyanin, and its related compounds, some of which have a pivotal role in *P. aeruginosa* infection [10]. In contrast, PAK was not prone to produce pyocyanin under our laboratory culture conditions (data not shown). The secretion of 4-hydroxy-2-alkylquinolines (HAQs) such as *Pseudomonas* quinolone signal (PQS) was nearly undetectable in the LB culture supernatant of PAK, unlike in that of PAO1 or PA14 [13]. These results suggest that the three strains may possess different virulence and/or survival characteristics in their natural or host environments, since the production and secretion of phenazines and HAQs, in addition to the hostspectra, are important in the virulence pathways of *P. aeruginosa*.

The subpopulation of each strain in mixed cultures was monitored by two methods; PCR detection and viable cell count assay. Strain-specific PCR markers were devised, as shown in Table 2 and Fig. 1A. Amplifications of *katM*, *orf71*, and *att1* amplicons are specific to PAO1, PA14, and PAK, respectively [12], whereas the *att2* amplicon is amplified from PAO1 and PAK, but not from PA14 as previously reported, indicating that the combinations of the PCR markers enabled us to specifically monitor the presence of each strain in the mixed populations.

To determine whether *P. aeruginosa* strains grew together (i.e. coexisted) or competed with (i.e. outcompeted) one another, we prepared an 18 h mixed culture of the three strains, since the growth advantages, if they exist, may be augmented by saturating the populations. Using viable cell count assay, we determined that the concentrations of the separate and the mixed cultures of the three strains at saturation were ~1.6×10^9 CFU/ml under our experimental conditions (data not shown). Approximately 1.6×10^4 cells

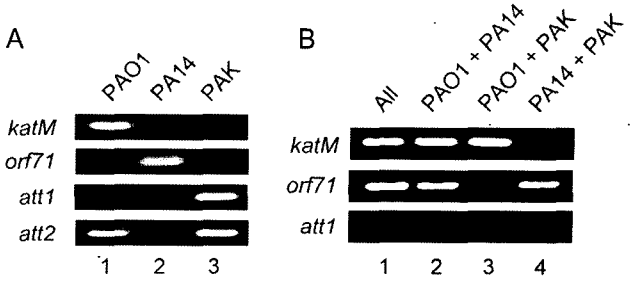

Fig. 1. Growth competitions between *P. aeruginosa* strains.
(A) PCR markers for PAO1 (lane 1), PA14 (lane 2), and PAK (lane 3) as summarized in Table 2. Cells were separately inoculated to LB and incubated at 37°C for 18 h, and 1.6×10^4 cells were subjected to the PCR reactions to detect the designated amplicons (left). (B) The PCR reactions to target the designated amplicons (left) were performed using the samples (1.6×10^4 cells) from the cocultures as indicated (top).

Table 3. Growth parameters of the wild-type *P. aeruginosa* strains.[a]

Strain	Lag time (min)	Doubling time (min)[c]
PAO1	21.8±2.1[b]	31.1±2.9
PA14	21.3±2.0	32.4±3.3
PAK	22.4±2.2	30.2±2.1

a: Cell growth was monitored by OD_{600} measurement.
b: Error ranges represent standard deviations.
c: Doubling times were estimated using the linear regression method (r^2>0.996).

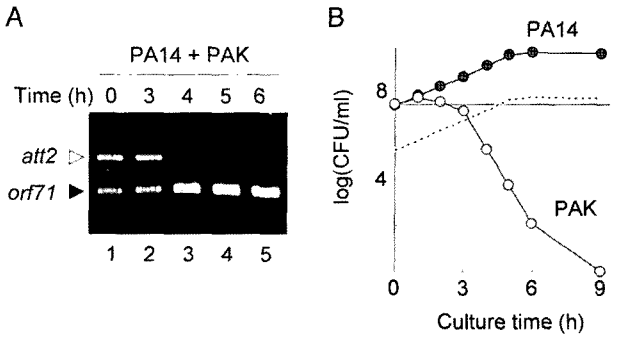

Fig. 2. Time-course of growth competition between *P. aeruginosa* strains, PA14 and PAK.
(A) Multiplex PCR to detect PA14 and PAK strains from the coculture. The multiplex PCR reactions to target PA14 (*orf71*) and PAK (*att2*) were performed using the samples at the designated time points from the cocultures that had been prepared as shown in Fig. 1B. (B) Growth curves were constructed based on the viable cell count assay results of PA14 (●) and a PAK-derivative, PKL7Z1 (○; designated as PAK) from the coculture. The dotted line denotes the 1/100 of the outgrowing PA14 population, indicating the PCR detection limit, and the solid line denotes the growth cessation without viability loss (~7.2 for 1.6×10^7 CFU/ml). The doubling time of PA14 in the coculture was estimated as 32.3 min (r^2=0.996).

taken from the 18 h mixed culture were used for PCR detection, which revealed that PAK was outcompeted by PAO1 and PA14 in the mixed culture containing all the three strains (Fig. 1B, lane 1).

Population analyses from the three cocultures of the two strains further supported the finding that PAO1 and PA14 outcompeted PAK in mixed cultures and showed that PAO1 and PA14 could coexist (Fig. 1B, lane 2). Viable counting of PA14, which is resistant to Rif [19], revealed that the numbers of PAO1 and PA14 cells remaining in the coculture did not differ significantly (data not shown), which was further verified by PCR amplifications of *katM* and *orf71* amplicons from the 96 individual colonies from an LB plate that allowed growth of both PAO1 and PA14 (46 colonies of PA14 and 50 colonies of PAO1; data not shown).

We speculated that the loss of PAK cells from the cocultures with PAO1 and PA14 might be due to the slower growth rate of PAK. To address this possibility, we determined the doubling and lag times of the separate cultures of each strain under the same conditions as in the cocultures. The growth parameters of the three strains did not differ significantly (Table 3), suggesting that the competition and coexistence phenomena observed were not the consequence of a difference in growth rates, but rather some other strain-specific competitive growth advantage.

Competitive Growth Advantage of PA14 Accompanies the Viability Loss of PAK

To quantitatively determine the competitive growth advantages between the outcompeting strains (PAO1 and PA14) and the outcompeted strain (PAK) in the mixed population, we tried time-resolved detections of PAK during the coculture with PA14. As described in Materials and Methods, the multiplex PCR to monitor the presence of PAK and PA14 during the coculture were optimized. Furthermore, quantitative analyses of our multiplex PCR procedures revealed that the PCR condition resulted in about 10^8 fold amplification of the target molecules (corresponding to the effective 27 cycle PCR amplifications with other elements not limiting) in our experimental conditions (data not shown), and the detection limit was calculated as ~10^2 targets (for 1 kb DNA), because more than 10^{10} molecules (i.e., ~10 ng for 1 kb) were detectable in the conventional ethidium bromide-dependent visualization of DNA. The samples containing 1.6×10^4 cells taken from the coculture were tested for the presence of PAK at distinct time points over the course of an 18 h coculture with PA14, where the actual proportion of PAK might decrease as its growth was arrested, even without the loss of viability. The presence of PAK was detected at 3 h, but not at 4 h post-inoculation, indicating that the proportion of PAK cells at that time point was below the detection limit (about 1/100 of the total cells) between 3 and 4 h (Fig. 2A). However, the determined growth parameters of PAK in a separate culture (Table 3) enabled us to predict that it would take about 4.15 h for the number of PAK cells to fall below the detectable limit in the sample containing 1.6×10^4 cells, as long as PA14 continued to grow as in its separate culture and the outcompetition had simply accompanied the growth cessation of PAK (the intersection between the dotted and solid lines in Fig. 2B). Therefore, our inability to detect PAK after 4 h of the coculture with PA14 was due to the loss of viability (or culturability), but not simply due to growth arrest of PAK cells.

To further investigate the viability loss of PAK cells after 4 h of coculture with PA14, the PAK subpopulation was monitored by viable count assay using an isogenic PAK strain (PKL7Z1) in place of PAK (Fig. 2B). PKL7Z1 contains a miniCTX-*lacZ* derivative containing a catalase (*katA*) promoter on its genome that confers Tc-resistance (Tc^R) and β-galactosidase production ($LacZ^+$) phenotypes [12]. Both the doubling and lag times of PKL7Z1 were

almost identical to those of PAK, and the chromosome-derived LacZ$^+$ and Tc^R phenotypes were highly stable in our separate or mixed culture conditions without Tc selection, as verified by PCR detection of miniCTX (data not shown). Since PKL7Z1 is LacZ$^+$ and Tc^R, whereas PA14 is Rif^R, both subpopulations could be independently determined, even when the major population was PA14. The PAK subpopulation decreased after 1 h post-inoculation, substantiating the notion that cell death, not growth cessation, may be involved. We postulate, therefore, that PA14 may be the source of bacteriocidal activity responsible for the loss of viability of PAK cells in the PA14-PAK mixed culture.

The Killing Factors are Secreted in PAO1 and PA14 Cultures

The competitive growth phenomenon between the *P. aeruginosa* strains observed in this study was similar to that observed in *E. coli* strains, where colicin production, resistance, and sensitivity may be important [23]. We suspected that pyocins, the *P. aeruginosa* bacteriocins, might be associated with this phenomenon.

To this end, we tested whether the culture supernatant from a separate PA14 or PAO1 culture could kill PAK cells (Fig. 3A). As described in Materials and Methods, the culture supernatants from stationary phase culture of either PAO1 or PA14 were subjected to chloroform ($CHCl_3$)-extraction and then spotted on the lawns of the PAK cells. After incubation at 37°C, visible zones of lysis (plaques) appeared, indicating that PAO1 and PA14 culture-derived factors are bacteriocidal to PAK. As might be expected, all strains were resistant to the supernatants derived from the same culture, and PAO1 and PA14 were resistant to the culture supernatants from PAK.

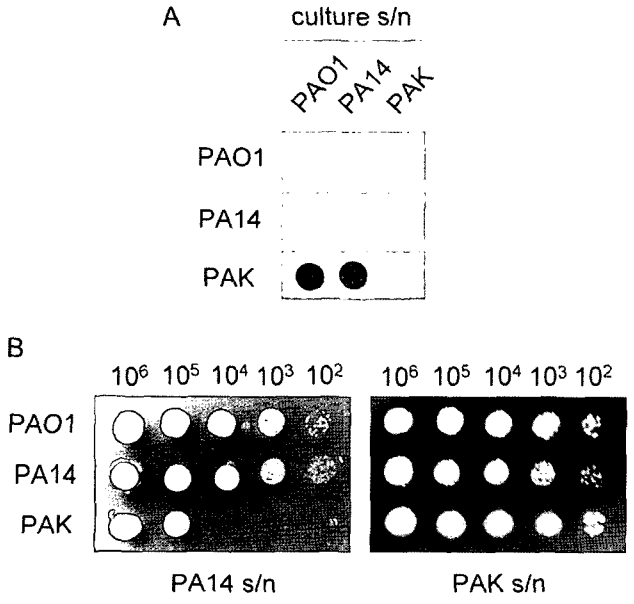

Fig. 3. Culture supernatant sensitivity of *P. aeruginosa* strains.
(A) The culture supernatant (s/n) from the late stationary phase cultures of the indicated strains were spotted onto an LB agar medium containing lawns of the strains indicated on the left. (B) Cells were grown to the late logarithmic growth phase, and their 10-fold serial dilutions in LB broth were spotted onto an LB agar medium containing either PA14-derived culture supernatant (left) or PAK-derived culture supernatant (right). The numbers (from 10^6 to 10^2) indicate the CFU of the cell spots.

The semi-quantitative assays of the PA14 and PAO1-derived killing revealed that the activity was present during the earlier logarithmic growth phase (before 2 h) and was maximal (~2 fold increase) after the late logarithmic growth phase (~3 h) (data not shown). This timing may be correlated with the growth inhibition of PAK in the mixed culture that apparently occurred after 1 h post-inoculation, when PA14 cells in the mixed cultures seemed to be in the logarithmic growth phase, as shown in Fig. 2B. However, considering the growth parameters, it is more likely that PAK growth inhibition might require the cell division or other normal cellular activity that might be sufficiently initiated after 1 h post-inoculation.

For the side-by-side comparison of susceptibilities of the four strains, we applied cell spots of serial dilutions on the plates containing $CHCl_3$-extracted culture supernatant of either PA14 or PAK (Fig. 3B). The growth of PAK cells was inhibited on the plates containing PA14-derived culture supernatant, whereas that of PAO1 and PA14 cells was not affected at all: All strains grew normally in the presence of PAK culture supernatant. These results suggest that the secreted killing activities of PAO1 and PA14 in planktonic cultures might confer the competitive growth advantage over the PAK by killing it, when it becomes metabolically active.

The Killing Factors are Most Likely Pyocins

We tested for the stability of the killing activities upon heating, phenol extraction, or protease treatments and showed that the killing activities were due to proteinous substance, as completely abrogated by the above treatments (data not shown). Thus, the killing factors are most likely pyocins or bacteriophages. We ruled out the bacteriophages, however, since conventional plaque assays failed to produce discrete phage plaques at any dilution of the culture supernatants from PAO1 and PA14. Instead, the killing activities showed a diffuse thining of cell growth, becoming a less marked zone of lysis with increasing dilution (data not shown). Therefore, we tentatively concluded that the killing activity of the PAO1 and PA14 culture supernatant was most likely due to pyocins, in agreement with a previous suggestion [1].

Profiling Killing Activities from and toward Various *P. aeruginosa* Strains

We then examined whether the pyocin-mediated competitive growth advantage could generally be observed in the

Table 4. Profiling pyocin production and resistance.[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
PAO1	–	–	–	–	+	–	–	–	+	+	+	–	–	++	–	+	–	+	–	–	–	+	–	++	++	–	++	+	–	–	–	++	–
PA14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+[b]	–	–	–	–	–	–
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	–	–	–	–	–	–	–	–	++	++	++	–	++	++	++	++	–	++	–	+	–	++	++	++	++	+	++	++	–	–	–	++	–

a: ++, Highly killing; +, slightly killing or unclear; –, no killing.
b: Several phage plaques are formed.

broader range of *P. aeruginosa* strains. Additional 30 *P. aeruginosa* isolates from clinical and environmental reservoirs were included to obtain a comprehensive profiling of pyocin production and resistance, in order to shed some insights into the relationships between the production of and the resistance to pyocins and the competitive growth advantage in the mixed populations of *P. aeruginosa*. As shown in Table 4, culture supernatants from the 33 *P. aeruginosa* strains were subjected to the killing assay on the cell lawns of the same strains. Out of the 33 strains, 10 strains were sensitive to the supernatants of PA14 and PAO1. More importantly, the killing spectra of both PAO1 and PA14 supernatants were identical in our profiling experiments, whereas the resistance spectra to the given killing activities were different (Table 4); Unlike PAO1, PA14 was highly resistant to most killing activities, except for some weak, discrete plaques by the culture supernatant from PMM22, (Table 4; data not shown). Most notably, because the PAK culture supernatant was bacteriocidal toward at least 10 strains, the competitive growth disadvantage of PAK during the coculture with PAO1 and/or PA14 might not be due to the inability to produce pyocins, but rather to the resistance of PAO1 and PA14 to the pyocins that PAK produces, as well as the susceptibility of PAK to the pyocins that PAO1 or PA14 produced. It was noted that one strain (PMM1) was completely unkilling toward the other strains tested. These results suggest that both pyocin production and its killing spectra are important in directing the competitive growth advantage in the mixed populations.

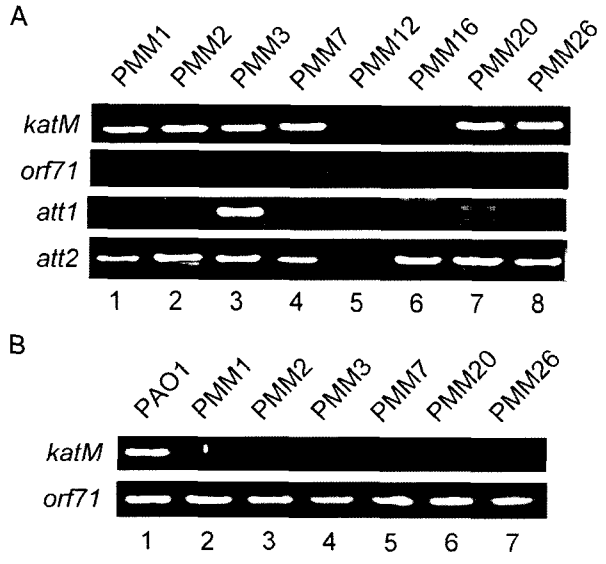

Fig. 4. Growth competitions between PA14 and PMM strains. (A) PCR detection of *katM*, *orf71*, *att1*, and *att2* amplicons from the clinical isolates that were sensitive to the PA14 culture supernatant (Table 4). Culture and sample preparations were performed as in Fig. 1A. (B) The PCR reactions to target the *katM* (for PMM strains and PAO1) and *orf71* (for PA14) amplicons were performed using the samples from the cocultures of PA14 and strains as designated (top).

Before conducting the coculture experiments to investigate the competitive growth advantage of PAO1 and PA14 over the sensitive strains other than PAK, PCR detections targeting the *katM*, *att1*, *att2*, and *orf71* amplicons were performed (Fig. 4A). All 8 strains showed no *orf71* amplicon. Out of the 8 sensitive strains, 6 *katM*-positive strains were subjected to the mixed cultures with PA14, and PCR detection assay using 18 h coculture revealed that PA14 had outcompeted all 6 strains (Fig. 4B). These results suggest that pyocins are generally the primary bacteriocidal compounds responsible for the competitive growth advantage in the mixed population of *P. aeruginosa* cells.

The involvement of pyocins in competitive advantage of PAO1 and PA14 and the identical killing spectra of both strains, shown in Table 4, implies that both killing components are identical. Furthermore, the nucleotide sequence comparison of the R and F pyocin gene clusters (between *trpE*/PA0609 and *trpG*/PA0649) from both PAO1 and PA14 genomes revealed that a deletion (~3.6 kb) encompassing PA0641 and PA0646 was found on the PA14 genome, which belongs exclusively to the F pyocin gene cluster, whereas the R pyocin gene clusters of both strains were almost identical [16, 26]. Therefore, the actual killing factors in the culture supernatants of PAO1 and PA14 were most likely R-type pyocins, the verification of which is currently underway.

This study describes a physiological role of pyocins in competitive growth advantage of *P. aeruginosa* strains, due to its bacteriocidal activity upon susceptible *P. aeruginosa* strains during the mixed planktonic cultures, and suggests that a comprehensive profiling of pyocin production and resistance of the various strains may reveal the evolutionary relatedness of the *P. aeruginosa* strains from various environmental and clinical reservoirs.

Many bacterial species have a strong proclivity toward forming complex populations in their natural habitats as well as in their host environments. Even though a population originates from a single cell, not all individual cells therein are genetically identical, probably because of population dynamics associated with the localized multicellular interactions and unequal microenvironments within the spatial architecture of the population [6]. The spontaneous generation of this heterogeneity may be accelerated by the fluctuating environmental conditions and, consequently, population dynamics may be under the combined control of such external and some intrinsic selective pressures in order to promote the survival capability of the given species at the expense of the altruistic cell death within the populations at the local level. We propose that pyocins may be one of the intrinsic selective forces involved in such cell death within *P. aeruginosa* populations, whereas the ability to produce pyocins and/or the ability or inability to resist particular subtypes of pyocins have fortuitously been acquired. Lessons from the colicin-mediated *E. coli*

strain competitions that constitute a rock-paper-scissors mode of population dynamics [8, 9] suggest that some *P. aeruginosa* strains might be present to complete the similar mode of experimental population dynamics involving at least three parties, to understand the dynamic maintenance of all the strains in the natural habitats. Besides, *P. aeruginosa* strains need to be analyzed also for the pyocin-mediated growth advantages in biofilms and in animal infections, to address the involvement of pyocins in strain competitions and population dynamics at the local and global levels of the natural habitats and host tissues.

Most importantly, because the PAO1 and PA14 strains are generally considered to be more virulent than the PAK strain, the acquisition of capability of pyocin-mediated outcompetition might have provided an opportunity for a fraction of cells in a population to promote their survival and possibly virulence potentials. A detailed understanding of how population control involves pyocins may provide a new perspective on the evolution and diversity of *P. aeruginosa*, and population behavior may enhance the survival and/or virulence potentials of this bacterium.

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