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Full Length Research Paper

Identification of genes involved in long-term survival in *Pseudomonas aeruginosa* PAO1

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Pseudomonas aeruginosa **is a remarkably adaptable organism which is able to survive and persist under a broad range of environmental conditions. The traditional views of bacterial growth phases usually come from standard laboratory cultures which can be different from natural conditions. In many natural environments, bacteria could maintain a long-term surviving status. To understand bacterial long-term survival, a luxCDABE-based random promoter library of P. aeruginosa was used to screen genes persistently expressed in the long-term culture of which 45 of such genes were identified. Among them, 13 genes encode different enzymes and are involved in different metabolic processes. Eight genes encode transcriptional regulators or two-component systems. In addition, a large portion of the identified genes encode hypothetical proteins with unknown function. Six of these unknown genes (PA1216, PA2827, PA0550, PA0256, PA0057 and PA4578) were selected and gene knockout mutants were constructed. All of these mutants exhibited reduced competitiveness than the wild-type PAO1 in the long-term competition assay, suggesting that these genes may play important roles for the long-term survival in *P. aeruginosa.***

Keywords: *Pseudomonas aeruginosa*, long-term survival, random promoter library, gene knockout, competition.

INTRODUCTION

Traditionally, bacteria are mostly studied in batch growth conditions, where four growth phases, the lag phase, exponential phase, stationary phase and death phase are recognized. Bacteria experience a feast and famine cycle in these conditions. While being an essential methodology with which a great deal have been leant in bacterial physiology and genetics, the physiology and genetics in these conditions unavoidably do not always reflect those of bacteria in naturally environments.

In previous studies, the fifth phase of the bacterial life cycle, long-term stationary phase, has been identified in the long-term laboratory culture (Finkel, 2006). During the transition from exponential to stationary phase, cells undergo a variety of morphological and physiological changes that assist them in survival as preferred nutrient pools are depleted. Upon continued incubation, bacteria are able to survive for exceptionally long periods (years) without input of exogenous nutrients (Finkel et al., 2000). In many natural environments, bacteria probably exist in conditions more akin to these of long-term stationary phase culture.

Opportunistic pathogen *Pseudomonas aeruginosa,* that are capable of thriving in diverse environments ranging from water and soil to plants and animal tissues can cause acute or chronic infections in humans (Yahr and Parsek, 2006). Numerous studies of this pathogen have been routinely obtained using standard laboratory cultures. In many natural environments, the nutrients are probably limited and the bacteria do not go through a boom and burst cycle, and instead they maintain a long term surviving status where bacteria remain viable but not

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Table 1. Strains and plasmids used in this study.

Strain or plasmid	Phenotype	Source
E. coli		
DH5α	*F⁻ φ80lacZ ΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk⁻, mk⁺)phoA supE44 thi-1 gyrA96 relA1 tonA*	Invitrogen
P. aeruginosa		
PAO1	Wild type	
ΔPA1216	PA1216 knockout mutant of PAO1; Gm^r	This study
ΔPA2827	PA2827 knockout mutant of PAO1; Gm^r	This study
ΔPA0550	PA0550 knockout mutant of PAO1; Gm^r	This study
ΔPA0256	PA0256 knockout mutant of PAO1; Gm^r	This study
ΔPA0057	PA0057 knockout mutant of PAO1; Gm^r	This study
ΔPA4578	PA4578 knockout mutant of PAO1; Gm^r	This study
Plasmids		
pEX18Ap	*oriT*⁺ *sacB*⁺ gene replacement vector with multiple-cloning site from pUC18; Ap^r	(Hoang et al., 1998)
pZ1918-*lacZ*Gm	Source plasmid of Gm^r cassette; Gm^r	(Schweizer, 1993)
pRK2013	Broad-host-range helper vector; Tra^+, Kn^r	(Ditta et al., 1980)
pMS402	Expression reporter plasmid carrying the promoterless *luxCDABE* gene; Kn^r, Tmp^r	(Duan et al., 2003)
pEX-1216	PA1216 knockout plasmid, pEX18Ap with upstream region, Gm^r-*lacZ* fragment from pZ1918-*lacZ*Gm and downstream of PA1216; Ap^r, Gm^r	This study
pEX-2827	PA2827 knockout plasmid, pEX18Ap with upstream region, Gm^r-*lacZ* fragment from pZ1918-*lacZ*Gm and downstream of PA2827; Ap^r, Gm^r	This study
pEX-0550	PA0550 knockout plasmid, pEX18Ap with upstream region, Gm^r-*lacZ* fragment from pZ1918-*lacZ*Gm and downstream of PA0550; Ap^r, Gm^r	This study
pEX-0256	PA0256 knockout plasmid, pEX18Ap with upstream region, Gm^r-*lacZ* fragment from pZ1918-*lacZ*Gm and downstream of PA0256; Ap^r, Gm^r	This study
pEX-0057	PA0057 knockout plasmid, pEX18Ap with upstream region, Gm^r-*lacZ* fragment from pZ1918-*lacZ*Gm and downstream of PA0057; Ap^r, Gm^r	This study
pEX-4578	PA4578 knockout plasmid, pEX18Ap with upstream region, Gm^r-*lacZ* fragment from pZ1918-*lacZ*Gm and downstream of PA4578; Ap^r, Gm^r	This study

actively dividing.

In order to understand the long-term survival, a lux-based random promoter library of *P. aeruginosa* constructed by Duan et al. (2003) was used to screen persistently expressed genes in the long-term culture according to luminescence intensity. Forty five (45) genes were identified from more than 3000 clones of the promoter library. A large portion of the identified genes encode hypothetical proteins with unknown function. Long-term competition assays showed that six of such unknown genes (PA1216, PA2827, PA0550, PA0256, PA0057 and PA4578) tested may play important roles in long-term survival in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are described in Table 1. *P. aeruginosa* and *Escherichia coli* were routinely grown on

Table 2. Primers used in this study.

Primer	Sequence (5'→3')	Restriction site
pZE.05	CCAGCTGGCAATTCCGA	
pZE.06	AATCATCACTTTCGGGAA	
PA1216-1S	TAAGAATTCGTCGGCGCTGCCGAGATG	*Eco*RI
PA1216-1A	ATTGGATCCGTGTCGCCGGCGGAAACC	*Bam*HI
PA1216-2S	AATGGATCCGCTGAAGCGGATCTCCGG	*Bam*HI
PA1216-2A	TAAAAGCTTCGCTACACGCCGATGCAC	*Hin*dIII
PA2827-S	AGTGAATTCGTTGCCGAGGTAGATGTC	*Eco*RI
PA2827-A	GCTAAGCTTGAAGTTCCACTTGATGCC	*Hin*dIII
PA0550-S	GCTGGATCCAGTGAAAGGCGCCAATTATG	*Bam*HI
PA0550-A	TATAAGCTTGCCGGAGATCCTGGTGCT	*Hin*dIII
PA0256-1S	GTCGAATTCAATCGGCAATGACAGTGC	*Eco*RI
PA0256-1A	ATAGGATCCCTCTACAAGCCGGCCTTC	*Bam*HI
PA0256-2S	ATAGGATCCGCTTGTTCCGCGCATAGG	*Bam*HI
PA0256-2A	ATTAAGCTTCAAGACCTGCCTCACCGC	*Hin*dIII
PA0057-S	TACAAGCTTGACATGTCCAGACGCTCG	*Hin*dIII
PA0057-A	CTAGAATTCGCAGAGCGGATCGTAGAG	*Eco*RI
PA4578-S	AGTAAGCTTTTCGATAGCCTCGCCACAGC	*Hin*dIII
PA4578-A	TATGAATTCAGCGCCTTTAGCGTGGCC	*Eco*RI

Luria-Bertani (LB) agar or in LB broth at 37°C unless otherwise specified. Antibiotics were used at the following concentrations: for *P. aeruginosa,* gentamicin (Gm) at 50 µg/ml in LB or 150 µg/ml in Pseudomonas isolation agar (PIA), and trimethoprim (Tmp) at 300 µg/ml in LB; for *E. coli,* kanamycin (Kn) at 50 µg/ml, ampicillin (Ap) at 100 µg/ml, and Gm at 15 µg/ml in LB.

Promoter screening and clustering

A lux-based random promoter library of *P. aeruginosa* ATCC27853 constructed by Duan et al. (2003) was used to screen persistent expression genes. The luminescence of each promoter clone was measured as counts per second (cps) of light production in a Wallac Victor 2 model 1450 multilabel counter (Perkin-Elmer Life Sciences) during long-term culture in multiwell plates. The promoter clustering was performed according to the similarity in their expression profiles using the CLUSTER program and visualized using TREEVIEW.

Sequence analysis of the persistent expression promoters

A subset of persistently expressed promoters was polymerase chain reaction (PCR) amplified using primers pZE.05 and pZE.06 (Table 2), which flank the BamHI site of pMS402. The PCR products were sequenced, and DNA sequences obtained were compared with the *P. aeruginosa* chromosome sequences (http://www.pseudomonas.com/) to verify related genes.

Construction of gene knockout mutants

For construction of gene knockout mutants, the previously described sacB-based strategy was used (Hoang et al., 1998). The DNA regions of the target genes were PCR amplified using primers listed in Table 2. Restriction sites were incorporated into the primers to facilitate cloning. The PCR products obtained were digested with restriction enzymes; and then cloned into pEX18Ap. The DNA fragment containing the Gmr-lacZ from pZ1918-lacZGm (Schweizer, 1993) was inserted into the target genes. Gene knockout mutants were obtained using the triparental mating procedure in which the strain carrying the helper plasmid pRK2013 (Ditta et al., 1980) was used together with the donor and recipient. The resultant mutants were verified by PCR.

Competition assay

A competition assay was used to compare the long-term survivability of the wild type and the mutants. Overnight cultures of *P. aeruginosa* strains were adjusted to an OD_{600} of 0.5, then 1 ml adjusted cultures of different mutant strains were co-inoculated into 50 ml fresh LB medium with the wild-type PAO1 at a ratio of 1:1 respectively. These co-cultures were incubated at 37°C with shaking (200 rpm) for 20 days. Total cell counts and mutant numeration were performed on LB agar or antibiotic selective agar after 1 day, 3, 14, and 20 days incubation.

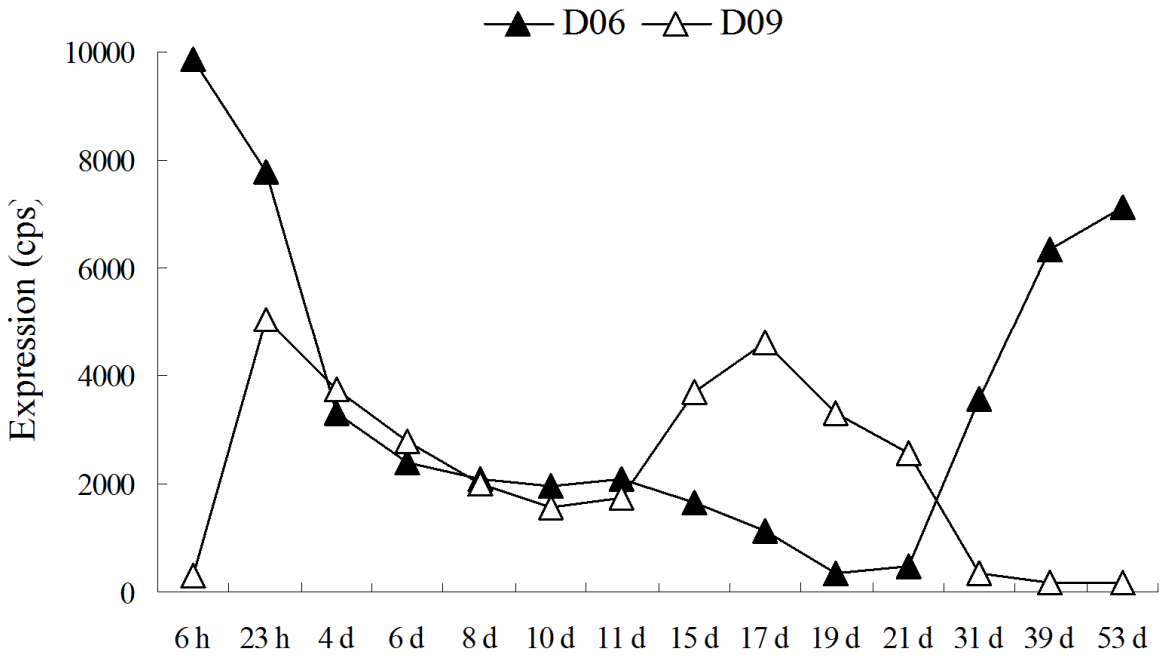

Figure 1. The expression profiles of clones D06 and D09 during the long-term culture. The Y-axis indicates cps value; the X-axis represents time course (h, hour; d, day). This experiment was repeated at least three times, and curves shown are representatives of similar results.

RESULTS

Screening of *P. aeruginosa* genes persistently expressed in long-term culture

In order to investigate the bacterial state in long-term survival, a *lux*-based random promoter library of *P. aeruginosa* was used to screen persistently expressed genes in the long-term culture. The random promoter library was constructed by a low-copy-number plasmid pMS402 carrying a promoterless *luxCDABE* reporter gene cluster. The activity of individual promoter was represented by the amount of light generated by the clone containing the construct. By measuring luminescence in a multilabel plate counter, the *P. aeruginosa* library can be screened under different conditions to identify differentially regulated genes.

Using this method, we screened more than 3000 *P. aeruginosa* clones for persistently expressed promoters. During the long-term culture in multiwell plates, most of the genes were only expressed in the first few days. A number of genes were found to express further for more than 20 days. Actually, the other expression peak appeared in the seventeenth day (Figure 1, D09). Certain genes even had enhanced expression after 20 days incubation (Figure 1, D06). We hypothesized that these persistently expressed genes may have important function for bacterial long-term survival. The clones which had persistent expression profiles were picked out for further study.

Characterization of the persistent expression genes in *P. aeruginosa*

The persistent expression promoters were sequenced and compared with the annotated P. aeruginosa PAO1 genome to identify relevant genes. Table 3 lists the 45 operons that are persistently expressed during the long-term incubation. These genes can be classified into five groups by function.

Among the genes that were persistently expressed in long-term culture, many genes were involved in metabolic process (Table 3, the first group). Two genes, argF and metX, were involved in amino acid biosynthesis and metabolism (Itoh et al., 1988; Bourhy et al., 1997). codA, which encodes cytosine deaminase (Danielsen et al., 1992), participates in nucleotide metabolic process. Ten other genes which encode different enzymes were involved in different metabolic processes.

Eight of the identified genes encode transcriptional regulators or two-component systems (Table 3, the second group). mucC encodes a positive regulator for alginate biosynthesis (Boucher et al., 1997). parR and cbrA are two-component system genes which are involved in regulating antibiotic resistance and utilization

Table 3. List of genes that persistently expressed in long-term culture.

Group	Gene	Description	Class
Metabolism	PA3537 (*argF*)	Ornithine carbamoyltransferase, anabolic	Class 1
	PA0390 (*metX*)	Homoserine *O*-acetyltransferase	Class 2
	PA0437 (*codA*)	Cytosine deaminase	Class 2
	PA1986 (*pqqB*)	Pyrroloquinoline quinone biosynthesis protein B	Class 2
	PA3029 (*moaB2*)	Molybdopterin biosynthetic protein B2	Class 2
	PA3976 (*thiE*)	Thiamin-phosphate pyrophosphorylase	Class 2
	PA5046	Malic enzyme	Class 2
	PA0224	Probable aldolase	Class 3
	PA1266	Probable oxidoreductase	Class 3
	PA1856	Probable cytochrome oxidase subunit	Class 3
	PA2355	Probable FMNH2-dependent monooxygenase	Class 3
	PA3723	Probable FMN oxidoreductase	Class 3
	PA5349	Probable rubredoxin reductase	Class 3
Transcriptional regulators and two-component systems	PA0765 (*mucC*)	Positive regulator for alginate biosynthesis mucc	Class 1
	PA1799 (*parR*)	Two-component response regulator, parr	Class 1
	PA4725 (*cbrA*)	Two-component sensor cbra	Class 1
	PA0225	Probable transcriptional regulator	Class 3
	PA2121	Probable transcriptional regulator	Class 3
	PA2798	Probable two-component response regulator	Class 3
	PA4381	Probable two-component response regulator	Class 3
	PA4886	Probable two-component sensor	Class 3
Transporter	PA1342	Probable binding protein component of ABC transporter	Class 3
	PA2114	Probable major facilitator superfamily (MFS) transporter	Class 3
Other functional protein	PA0423 (*pasP*)	Pasp	Class 1
	PA4228 (*pchD*)	Pyochelin biosynthesis protein pchd	Class 1
	PA4527 (*pilC*)	Still frameshift type 4 fimbrial biogenesis protein pilc	Class 1
	PA2652	Probable chemotaxis transducer	Class 3
Unknown protein	PA0550	Conserved hypothetical protein	Class 4
	PA1533	Conserved hypothetical protein	Class 4
	PA2827	Conserved hypothetical protein	Class 4
	PA3880	Conserved hypothetical protein	Class 4

Table 3. Contd.

PA4879	Conserved hypothetical protein	Class 4
PA0046	Hypothetical protein	Class 4
PA0057	Hypothetical protein	Class 4
PA0256	Hypothetical protein	Class 4
PA0553	Hypothetical protein	Class 4
PA1216	Hypothetical protein	Class 4
PA1414	Hypothetical protein	Class 4
PA1571	Hypothetical protein	Class 4
PA2171	Hypothetical protein	Class 4
PA2747	Hypothetical protein	Class 4
PA3022	Hypothetical protein	Class 4
PA3855	Hypothetical protein	Class 4
PA4364	Hypothetical protein	Class 4
PA4578	Hypothetical protein	Class 4

of carbon and nitrogen sources, respectively (Nishijyo et al., 2001; Fernandez et al., 2010). All these regulatory genes may participate in regulating the expression of genes required for long-term survival in P. aeruginosa.

Two genes in the third group encode probable transporter proteins. Other known genes are collected as the fourth group, including pilC which encodes the still frameshift type 4 fimbrial biogenesis protein PilC (Nunn et al., 1990), pchD encodes the pyochelin biosynthesis protein PchD (Serino et al., 1997), and pasP encodes a protease PasP (Marquart et al., 2005).

In addition to the above genes, 18 of the identified genes encode hypothetical proteins with unknown function. This number represents 40% of the 45 characterized promoters. These data imply that some of the unknown genes may only be functional under long-term survival conditions.

Loss of viability of mutants in long-term co-culture with wild-type strain

To investigate the function of these persistently expressed genes in bacterial long-term survival, six of these unknown genes (PA1216, PA2827, PA0550, PA0256, PA0057 and PA4578) were selected for further investigation. Gene knockout mutants were generated by inserting a Gm^r-*lacZ* cassette into these genes. A long-term competition assay was used to test the competitiveness of these mutants. Different mutant strains were co-inoculated with the wild-type PAO1 at a ratio of 1:1. These co-cultures were incubated at 37°C for 20 days. The survival rates of parental and mutant strains were calculated after one, three, 14, and 20 days incubation.

In the first three days, there were no significant difference in survival rates between the wild-type and the mutants. However, after 14 days incubation, distinct difference in survival rates was observed for the PA2827 mutant and PAO1 (Figure 2A). PA0550 mutant also exhibited obviously reduced competitiveness than the wild-type strain (Figure 2B). Moreover, the rest of the mutants displayed decreased competitiveness in different degrees (data not shown). The results suggest that these persistently expressed genes may indeed play a role for the long-term survival in *P. aeruginosa*.

DISCUSSION

P. aeruginosa strains are found in various environmental habitats as well as in animal and human hosts. The high potential for adaptation to new environmental conditions is mostly due to the large genomes of P. aeruginosa strains (Stover et al., 2000). The physiology and genetics of P. aeruginosa strains are usually leant through conventional laboratory culture. Some of the genes which do not express in normal conditions could always be ignored.

In an attempt to investigate this problem, we set up a long-term laboratory culture condition without continuously input of exogenous nutrients. A luxCDABE-based random promoter library of *P. aeruginosa* was used to

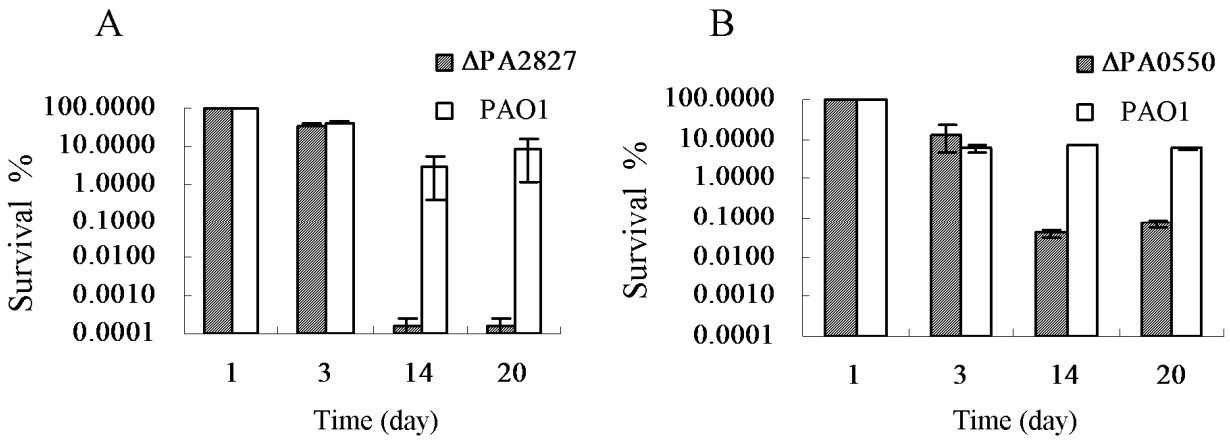

Figure 2. The survival rates of mutants and wild-type PAO1 during long-term culture. The Y-axis indicates survival rates; the X-axis represents time course. Error bars indicate one standard deviation.

screen persistent expression genes in the long-term culture. Using this method, 45 genes which can continuously express during long-term incubation were identified from the promoter library. Lots of genes encoding metabolic enzymes, transcriptional regulators and two component regulatory systems were found in this process. Successful adaptation of living organisms to different niches depends on their ability to regulate gene expression as specified by the needs of the environment (Stock et al., 2000). Persistently expressing genes involved in metabolism and transcriptional regulation may enable the bacteria to adapt to this infertile environment. In addition to these well-characterized genes, a large part of the identified genes encode hypothetical proteins. These unknown genes may have essential functions for bacterial long-term survival.

In order to investigate the functions of these persistent expression genes in long term survival, six of the unknown genes were selected for gene knockout mutagenesis. A long-term competition assay was used to test the competitiveness of these mutants. The survival rates of parental and mutant strains were compared during the long-term co-culture. As expected, all of these six mutants showed significant decrease in competitiveness. This result indicates that these persistent expression genes may play important roles in long-term survival in *P. aeruginosa*.

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