The effect of *pmpR* on the type III secretion system in *Pseudomonas aeruginosa*

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The type III secretion system (T3SS) plays important roles in *Pseudomonas aeruginosa* pathogenicity. Previously, we reported that the uncharacterized protein PmpR could regulate *pqsR*, an important regulator in the quorum-sensing system, by directly binding to its promoter region. As the T3SS is controlled by the quorum-sensing system, here, we investigated the relationship between PmpR and the T3SS. Our data showed that expression of the T3SS genes *exoS*, *exoY*, *exoT*, and *exsD* was dramatically increased in a *pmpR*-deletion mutant compared with that in the wild-type *P. aeruginosa* strain PAO1. Data from DNA mobility assays indicated that PmpR affects the T3SS indirectly. It is unlikely that PmpR controls the T3SS via the *Pseudomonas* quinolone signal (PQS) because the PQS negatively regulates the T3SS, while *pmpR* negatively regulates the PQS. The effect of PmpR on the T3SS seems to be independent of the PQS; further investigation is required to uncover the underlying regulatory pathways.

***P. aeruginosa*, *pmpR*, PQS, type III secretion system**

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Pseudomonas aeruginosa is an opportunist pathogen that causes serious infectious diseases in humans. Its infection in the lungs of cystic fibrosis patients is the main cause of morbidity and mortality in these patients. Complex factors are involved in *P. aeruginosa* pathogenicity, and among them, quorum-sensing (QS) and the Type III secretion system (T3SS) play important roles.

P. aeruginosa possesses at least 2 well-defined acyl-homoserine lactone (HSL)-based quorum-sensing systems, *las* and *rhl*, which control numerous virulence factor genes including *lasA*, *lasB*, and *rhlA* [1,2]. Each system comprises a transcriptional activator and an autoinducer synthase. The *las* system consists of the transcriptional activator LasR, and the QS signal synthase LasI that directs the biosynthesis of N-3-oxo-dodecanoyl-HSL (3OC₁₂-HSL). The *rhl* system consists of the transcriptional activator RhlR, and the synthase Rhl that is responsible for the biosynthesis of N-butanoyl-HSL (C₄-HSL). 3O-C₁₂-HSL and C₄-HSL acti-

vate the transcription of LasR and RhlR respectively [3]. Activation of RhlR by C₄-HSL regulates the production of rhamnolipid, hydrogen cyanide, pyocyanin, and proteins of the T3SS pathway. Recent microarray analyses have revealed that the *las* and *rhl* systems also regulate the expression of many additional *P. aeruginosa* genes [4,5].

Besides 3O-C₁₂-HSL and C₄-HSL, *P. aeruginosa* produces additional intercellular signaling molecules termed the AHQ signals [6]. The major AHQ signals include 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal, PQS) and 2-heptyl-4-quinolone [6–8]. The PQS is synthesized by enzymes encoded by the *pqsABCDE* and *phnAB* operons as well as *pqsH* [8]. The transcription of these two operons is regulated by the PQS transcriptional regulator PqsR, also known as MvfR [8]. Mutations in *pqsR* interfere with PQS synthesis and affect the production of pyocyanin and other QS-controlled virulence factors [8,9].

The T3SS is a highly specialized protein secretion apparatus that facilitates the translocation of effector proteins from the bacterial cytoplasm directly into host cells. The

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T3SS plays important roles in *P. aeruginosa* pathogenesis especially during acute infections. In *P. aeruginosa*, a single set of genes encodes the structural components of the T3SS. This pathogen has 4 known effectors, ExoS, ExoT, ExoU, and ExoY [10]. These T3SS effectors are major virulence factors of *P. aeruginosa*. All T3SS genes, both structural genes and effectors, such as *exoS*, are under the direct transcriptional control of ExsA [11]. Moreover, ExsA interacts with and is influenced by ExsC, ExsD, and ExsE. These protein interactions, which are affected by Ca^{2+} concentration and T3SS secretory activity, eventually determine the availability of free ExsA and hence T3SS activity [12,13]. Intriguingly, the T3SS is also affected by the QS system in *P. aeruginosa*. It has been reported that the T3SS is negatively regulated by the *rhl* system [14].

In our previous study, we found that PmpR could directly bind to the *pqsR* promoter region and regulate the PQS. Considering that the PQS is connected to the *rhl* system, in this study we investigated the relationships between PmpR and the T3SS. Our data indicate that the PQS system negatively controls the T3SS and that PmpR indirectly affects the T3SS, possibly via a pathway independent of the PQS.

1 Materials and methods

1.1 Bacterial strains and media

The bacterial strains and plasmids used in this study are

listed in Table 1. *P. aeruginosa* wild-type strain and derivatives were routinely grown at 37°C on LB agar plates or in LB broth with orbital shaking at 200 r/min. Antibiotics were purchased from Sigma (St. Louis, USA) and used at the following concentrations where appropriate: for *E. coli* in all media, kanamycin at 50 $\mu\text{g mL}^{-1}$, tetracycline at 15 $\mu\text{g mL}^{-1}$, and ampicillin at 100 $\mu\text{g mL}^{-1}$; for *P. aeruginosa*, gentamicin at 75 $\mu\text{g mL}^{-1}$ in LB or 150 $\mu\text{g mL}^{-1}$ in *Pseudomonas* Isolation Agar (PIA), tetracycline at 75 $\mu\text{g mL}^{-1}$ in LB or 200 $\mu\text{g mL}^{-1}$ in PIA, carbenicillin at 500 $\mu\text{g mL}^{-1}$ in PIA, trimethoprim at 300 $\mu\text{g mL}^{-1}$ in all media.

1.2 Construction of the promoter-reporter fusion plasmids

The plasmid pMS402 carrying a promoter-less *luxCDABE* reporter gene cluster was used to construct promoter-*luxCDABE* reporter fusions of *exsD*, as reported previously [15,20]. The promoter regions of *exsD* were PCR-amplified using high-fidelity *Pfx* DNA polymerase and primers synthesized according to the *P. aeruginosa* PAO1 strain genome data [21]. The promoter regions were cloned into the *Xho* I-*Bam*HI site upstream of the *lux* genes in pMS402. Then, the resultant plasmid was digested with *Pac* I, and the larger fragment was isolated by gel extraction. Next, the purified DNA was ligated with the CTX6.1 plasmid, which was also digested with *Pac* I. The construct was integrated into the PAO1 chromosome by bi-parental mating [17].

Table 1 Strains and plasmids used in this study^{a)}

Strains or plasmids	Relevant feature	Source or reference
<i>P. aeruginosa</i>		
PAO1	<i>P. aeruginosa</i> wild type	This laboratory
$\Delta pmpR$	<i>pmpR</i> replacement mutant of PAO1, Gm ^r	[15]
$\Delta pqsR$	<i>pqsR</i> replacement mutant of PAO1, Gm ^r	This study
<i>E. coli</i>		
DH5 α	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>)80 <i>dlacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara leu</i>)7697 <i>galU</i> <i>galK</i> λ - <i>rpsL</i> <i>nupG</i>	Invitrogen
SM10	Mobilizing strain, RP4 integrated in the chromosome; Kn ^r	[16]
Plasmids		
pEX18Ap	<i>oriT</i> ⁺ <i>sacB</i> ⁺ gene replacement vector with multiple-cloning site from pUC18, Ap ^r	[17]
pZ1918Gm	Source plasmid of Gm ^r cassette	[18]
pRK2013	Broad-host-range helper vector; Tra ⁺ , Kn ^r	[19]
CTX6.1	Integration plasmid; Tet ^r	This laboratory
HTb- <i>pmpR</i>	Protein expression construct, <i>pmpR</i> cloned in HTb vector	[15]
pMS402	Expression reporter plasmid carrying the promoterless <i>luxCDABE</i> ; Kn ^r , Tmp ^r	[20]
pKD- <i>exsD</i>	pMS402 containing <i>exsD</i> promoter region; Kn ^r	This study
pKD- <i>exoS</i>	pMS402 containing <i>exoS</i> promoter region; Kn ^r	[20]
pKD- <i>exoY</i>	pMS402 containing <i>exoY</i> promoter region; Kn ^r	[20]
pKD- <i>exoT</i>	pMS402 containing <i>exoT</i> promoter region; Kn ^r	[20]
CTX- <i>exsD</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exsD</i> ; Kn ^r , Tmp ^r , Tc ^r	This study
CTX- <i>exoS</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> ; Kn ^r , Tmp ^r , Tc ^r	This study
CTX- <i>exoY</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoY</i> ; Kn ^r , Tmp ^r , Tc ^r	This study
CTX- <i>exoT</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoT</i> ; Kn ^r , Tmp ^r , Tc ^r	This study

a) Kan, kanamycin; Tc, tetracycline; Ap, ampicillin; Gm, gentamicin; Tc, tetracycline; Cb, carbenicillin; Tmp, trimethoprim.

Chromosome-integrated reporters for *exoS*, *exoY*, and *exoT* were constructed in the same way by using previously reported fusion constructs (Table 1). DNA manipulation, PCR, and transformation were performed following standard procedures. Cloned promoter sequences were confirmed by DNA sequencing.

1.3 Construction of a *pqsR*-deletion mutant

For gene replacement, the previously described *sacB*-based strategy was employed [17]. A suicide plasmid of pKD*pqsR* for gene replacement was constructed by inserting the *Bam*H I-digested *Gm^r-lacZ* cassette derived from pZ1918-*lacZ* [18] in between 2 PCR fragments of *pqsR* cloned into pEX18Ap. The upstream fragment of *pqsR* was amplified using the primers L1 and H1 (Table 2). The downstream region of *pqsR* was generated using primers L2 and H2. The pKD*pqsR* plasmid was transferred to PAO1 using a tri-parental mating procedure employing the helper vector pRK2013 [19]. Briefly, overnight cultures (25 mL of each) of *P. aeruginosa* PAO1 and an *E. coli* strain containing plasmid pKD*pqsR* or pRK2013 were centrifuged and the pellets resuspended in phosphate-buffered saline, then mixed in equal proportions and spotted onto an LB agar plate. Following incubation overnight at 37°C, bacteria were removed from the agar surface and resuspended in 1 mL of phosphate-buffered saline. Appropriate dilutions were plated onto PIA containing gentamicin at 150 µg mL⁻¹. Subsequently, strains that had undergone a second crossover event were selected by plating on LB supplemented with 5% sucrose and gentamicin. The *pqsR* gene replacement mutant, designated Δ *pqsR*, was verified by assessing gentamicin resistance and carbenicillin sensitivity and by PCR analysis.

1.4 Gene expression assays

Using the *lux*-based reporters, gene expression in liquid cultures was measured as counts per second of light production in a Synergy 2 (Biotek, Winooski, Vermont, USA), as previously described by our group [15,20]. Overnight cultures of the reporter strains were diluted to an A_{600} of 0.2

and cultivated for an additional 2 h before use. The cultures were inoculated into parallel wells of a black 96-well plate with a transparent bottom. A 5-µL volume of the fresh cultures was inoculated into the wells containing a total volume of 95 µL medium plus other components, and the A_{600} value in the wells was adjusted to approximately 0.07. A 60-µL volume of filter-sterilized mineral oil was added to prevent evaporation during the assay. Promoter activities were measured every 30 min for 24 h. Bacterial growth was monitored at the same time by measuring the A_{600} in a Synergy². Expression on solid medium was assessed by plating the reporter strains onto soft-top LB agar and imaging using a LAS300 Imaging System (Fuji Corp., Tokyo, Japan).

1.5 Protein expression and purification

PmpR protein was expressed as a His-tag fusion using the pProEX-HT protein expression system and purified using the high-affinity nickel-nitrilotriacetic acid resin column (Qiagen, Hilden, Germany) as described previously [15].

1.6 Electrophoretic Mobility Shift Assay (EMSA)

The promoter regions of *pqsR*, *exoT*, and *exsD* were PCR-amplified according to standard protocols. The template was *P. aeruginosa* genomic DNA. The PCR product and the PmpR protein were incubated for 20 min at room temperature in binding buffer (40 mmol L⁻¹ Tris-HCl [pH 7.5], 0.1 mmol L⁻¹ EDTA, 70 mmol L⁻¹ KCl, 0.1 mmol L⁻¹ dithiothreitol, 4 mmol L⁻¹ MgCl₂) and subjected to electrophoresis on a 6% native polyacrylamide gel. The gel was stained with SYBR green (Invitrogen, Carlsbad, California, USA) and photographed using a LAS-3000 imaging system.

2 Results

2.1 The effect of *pmpR* on the T3SS

It has been shown that the quorum-sensing *rhl* system negatively regulates the T3SS in *P. aeruginosa* [14]. In our previous study, we reported that PmpR regulates the PQS by directly binding to the *pqsR* promoter region. Considering

Table 2 Primers used in this study

Primers		Sequence (5'→3')	Restriction site (underlined in sequence)
<i>pqsR</i> upstream	L1	GGCGAATTCATCGAGTCCTCGACGCTT	<i>Eco</i> R I
	H1	ATACCCGGGTCGATGGTGATGGCGATA	<i>Sma</i> I
<i>pqsR</i> downstream	L2	ATACCCGGGCCGCTTCAACCAAGTGCG	<i>Sma</i> I
	H2	GGCAAGCTTCTACTCTGGTGCGGCGCG	<i>Hind</i> III
<i>exsD</i> promoter	S1	CGTCTCGAGCGACATGAGCATCGT	<i>Xho</i> I
	S2	ATAGGATCCAGCCACCAACGATACCC	<i>Bam</i> HI
<i>exoT</i> promoter	S3	CACTCGAGATCCAGGGAAAGCGTCATGC	<i>Xho</i> I
	S4	GAGGATCCAGTTGTTGCGCCTCCCGG	<i>Bam</i> HI I
<i>lacZ</i>	<i>lacZ</i> f	AGATCGCACTCCAGCCAG	None

that the *pmpR* gene negatively controls *pqsR* and *rhlR* expression [16], we speculated that PmpR also controls the T3SS. To test this possibility, we firstly constructed promoter-*lux* fusions of the T3SS genes *exsD*, *exoS*, *exoY* and *exoT*. These constructs were integrated into wild-type PAO1 and a *pmpR*-deletion mutant, respectively, and their expression was compared. As shown in Figure 1, the expression of *exoS*, *exoY*, *exoT* and *exsD* was dramatically increased in the *pmpR* mutant compared with that in the wild type, while the bacterial growth remained the same (data not shown). The expression of these genes was increased approximately 5-fold in the *pmpR* mutant during the stationary phase of growth. This suggests that *pmpR* negatively regulates expression of the T3SS.

2.2 PmpR affects the T3SS indirectly

To test whether the regulation of PmpR on the T3SS is direct, we performed EMSAs using purified PmpR and DNA fragments containing the promoter regions of the T3SS genes. As shown in Figure 2, purified PmpR protein was able to bind to the *pqsR* promoter region, but could not bind to the promoter regions of *exoT* or *exsD* (Figure 2(b)). Similarly, PmpR was not able to bind to either the *exoS* or *exoY* promoter region (data not shown). These results indicate that, unlike its effect on *pqsR* expression, PmpR affects ex-

pression of the T3SS indirectly.

2.3 Regulation of the T3SS by the PQS

It has been shown that the *rhl* system negatively regulates the T3SS [14]. In addition, the *rhl* system negatively regulates the PQS, but the PQS positively regulates the *rhl* system. To examine the relationships between QS and T3SS, we tested whether the PQS affects the T3SS. The promoter-*lux* fusion constructs of *exoS*, *exoY*, *exoT*, and *exsD* were integrated into the chromosomes of wild-type PAO1 and the *pqsR* mutant, respectively, and the expression of these genes was compared. As shown in Figure 3, the expression of *exoS* and *exoT* was strongly increased in the *pqsR* mutant, but the expression of *exoY* and *exsD* remained the same, indicating that the effect of PqsR on the T3SS is complex and gene-dependent.

3 Discussion

Prompted by our previous observations, we investigated the effect of PmpR on the T3SS. We constructed *pqsR*- and *pmpR*-deletion mutants, and integrated into them promoter-reporter fusions of the T3SS genes. We showed that the expression of the T3SS genes *exoS*, *exoY*, *exoT* and *exsD*

Figure 1 Expression of the T3SS genes (*exoS*, *exoY*, *exoT*, and *exsD*) in the wild-type PAO1 and the *pmpR*-deletion mutant. The assays were performed three times and the data are expressed as mean $\pm$ SD. * $P < 0.005$, significant difference between the wild type and the mutant (log-rank test).

Figure 2 Electrophoretic mobility shift assays (EMSAs). (a) Purification of the PmpR protein. 1, protein marker; 2, cell sample before induction; 3, cell lysate after induction; 4, flow-through solutions from His-column; 5, cell lysate without induction; 6, cell sample after induction; 7–9, purified PmpR protein. (b) EMSA testing of the binding of PmpR to the promoter regions of the T3SS genes. PCR products containing the *pqsR*, *exoT*, and *exsD* promoter regions (*PpqsR*, *PexoT*, and *PexsD*, respectively) were added to the reaction mixtures at approximately 50, 30, and 30 ng respectively. The PmpR protein was added to the reaction mixtures in lanes B–D at 320, 640, and 960 ng, respectively. No protein was added to the mixture in lane A. DNA containing the *pqsR* promoter region was bound specifically by PmpR (*PpqsR*-PmpR). No PmpR binding to the *exoT* or *exsD* promoter regions was observed.

Figure 3 Expression of the T3SS genes (*exoS*, *exoY*, *exoT*, and *exsD*) in wild type and *pqsR* mutant. These assays were performed three times and the data are expressed as mean $\pm$ SD. * $P < 0.005$, significant difference for the expression of *exoS* and *exoT* between the wild type and the mutant (log-rank test).

increased dramatically in the *pmpR* mutant, indicating that PmpR negatively regulates the T3SS. Previously, we demonstrated that PmpR regulates the PQS by directly binding to the *pqsR* promoter region. Using EMSAs, we showed that PmpR influences the T3SS indirectly.

It is unlikely that PmpR affects the T3SS via the PQS pathway. Although the expression of T3SS genes such as *exoS* and *exoT* was strongly increased in the *pqsR* mutant,

the expression of *exoY* and *exsD*, however, did not change, indicating that the regulation by PqsR of the T3SS is not the same as that of PmpR, which affected all these genes similarly. In addition, because PmpR negatively regulates both the PQS and the T3SS, and the PQS negatively affects the T3SS, it is not possible for PmpR to affect the T3SS via the PQS system. We speculate that there are other pathways through which PmpR exerts its effect on the T3SS. One

Figure 4 Model of the proposed effect of PmpR on the T3SS. PmpR regulates the T3SS (dashed line) via two possible pathways that are independent of the PQS: 1, PmpR directly regulates the PQS, and the PQS regulates the T3SS through unknown genes (solid black lines); 2, PmpR affects the T3SS via other unknown genes (gray lines). An arrowhead at the end of the line represents positive regulation and a bar at the end of the line represents negative regulation.

possibility is that PmpR first regulates the PQS, and the PQS then regulates the T3SS through some unknown genes (Figure 4). Another possibility is that PmpR influences the T3SS via an unknown pathway that is independent of the PQS. To investigate the regulation pathways, we have constructed a transposon mutagenesis library of *P. aeruginosa*, and are analyzing mutations that alter the effect of PmpR on the expression of T3SS genes. Several interesting genes have been identified. Among them, the expression level of *exoS* in a *pmpR/PA2583* double mutant was the same as in the wild type, and PA2583 directly binds to the *exoS* promoter region, as shown by EMSA (unpublished data). Therefore, PA2583 is likely to regulate the T3SS positively and represents a candidate protein through which PmpR affects the T3SS. Clearly, the regulatory pathways by which PmpR affects the T3SS are complex. The results reported in this study provide a cue for further investigations.

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- Pearson J P, Passador L, Iglewski B H, et al. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA*, 1995, 92: 1490–1494
- Quinones B, Dulla G, Lindow S E. Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Mol Plant Microbe Interact*, 2005, 18: 682–693
- Latifi A, Foglino M, Tanaka K, et al. A hierarchical quorum-sensing

- cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol*, 1996, 21: 1137–1146
- Schuster M, Lostroh C P, Ogi T, et al. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: A transcriptome analysis. *J Bacteriol*, 2003, 185: 2066–2079
- Wagner V E, Bushnell D, Passador L, et al. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J Bacteriol*, 2003, 185: 2080–2095
- Pesci E C, Milbank J B J, Pearson J P, et al. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA*, 1999, 96: 11229–11234
- Diggle S P, Cornelis P, Williams P, et al. 4-quinolone signalling in *Pseudomonas aeruginosa*: Old molecules, new perspectives. *Int J Med Microbiol*, 2006, 296: 83–91
- Déziel E, Lépine F, Milot S, et al. Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci USA*, 2004, 101: 1339–1344
- Gallagher LA, McKnight S L, Kuznetsova M S, et al. Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol*, 2002, 184: 6472–6480
- Frank D W. The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Mol Microbiol*, 1997, 26: 621–629
- Dasgupta N, Lykken G L, Wolfgang M C, et al. A novel anti-anti-activator mechanism regulates expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol*, 2004, 53: 297–308
- Kim J, Ahn K, Min S, et al. Factors triggering type III secretion in *Pseudomonas aeruginosa*. *Microbiology*, 2005, 151: 3575–3587
- McCaw M L, Lykken G L, Singh P K, et al. ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. *Mol Microbiol*, 2002, 46: 1123–1133
- Blevess S, Soscia C, Nogueira-Orlandi P, et al. Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. *J Bacteriol*, 2005, 187: 3898–3902
- Liang H, Li L, Dong Z, et al. The YebC family protein PA0964 negatively regulates the *Pseudomonas aeruginosa* quinolone signal system and pyocyanin production. *J Bacteriol*, 2008, 190: 6217–6227
- Simon R, Priefer U, Pühler A. A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in gram negative bacteria. *Bio/Technology*, 1983, 1: 784–791
- Hoang T T, Karkhoff-Schweizer R R, Kutchma A J, et al. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: Application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene*, 1998, 212: 77–86
- Schweizer H P. Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. *Gene*, 1993, 134: 89–91
- Ditta G, Stanfield S, Corbin D, et al. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA*, 1980, 77: 7347–7351
- Duan K, Dammel C, Stein J, et al. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol Microbiol*, 2003, 50: 1477–1491
- Stover C K, Pham X Q, Erwin A L, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 2000, 406: 959–964

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