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Modulation of Secreted Virulence Factor Genes by Subinhibitory Concentrations of Antibiotics in *Pseudomonas aeruginosa*

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Recent studies have shown that subinhibitory antibiotics play important roles in regulating bacterial genes including virulence factor genes. In this study, the expression of 13 secreted virulence related gene clusters of *Pseudomonas aeruginosa*, an important opportunistic pathogen, was examined in the presence of subinhibitory concentrations of 4 antibiotics: vancomycin, tetracycline, ampicilin and azithromycin. Activation of gene expression was observed with *phzA1*, *rhlAB*, *phzA2*, *lasB*, *exoY*, and *exoS*. Subinhibitory concentrations of vancomycin resulted in more than 10-fold increase of *rhlAB* and *phzA2* transcription. Both rhamnolipid production and pyocyanin production were significantly elevated, correlating phenotypes with the increased transcription. *P. aeruginosa* swarming and swimming motility also increased. Similar results were observed with subinhibitory tetracycline, azithromycin and ampicillin. These results indicate that the antibiotics at low concentrations can up-regulate virulence factors and therefore influence bacterial pathogenesis.

***Keywords*:** *Pseudomonas aeruginosa*, virulence factors, subinhibitory concentration of antibiotics, gene regulation

Antibiotics are able to kill or inhibit the growth of bacteria and therefore have been used to treat bacterial infections in humans and animals. Most clinically used antibiotics are derived from natural compounds produced by microorganisms. It is generally perceived that naturally occurring antibiotics are weapons in intermicrobial competition, and should reduce fitness and virulence of pathogens even at subinhibitory concentrations. Many previous reports support this hypothesis (Carfartan *et al.*, 2004; Fonseca *et al.*, 2004; Wozniak and Keyser 2004; Ishikawa and Horii, 2005), but a few studies have shown that some antibiotics at subinhibitory concentrations can activate the expression of some important virulence genes in *Salmonella typhimurium* and *E. coli* (Goh *et al.*, 2002) and enhance biofilm formation (Hoffman *et al.*, 2005). There is evidence suggesting that antibiotics act as signal molecules regulating bacterial gene expression and modulating bacterial fitness (Linares *et al.*, 2006). However, the extent of this regulation especially in terms of virulence gene regulation and its mechanism are not fully understood.

Pseudomonas aeruginosa is a major opportunistic pathogen in humans, capable of causing serious infections. In patients with cystic fibrosis (CF), *P. aeruginosa* chronically colonizes the lungs contributing to loss in pulmonary function resulting in premature mortality of these patients (Stover *et al.*, 2000). The pathogenesis of *P. aeruginosa* is closely associated with the virulence factors produced by this bacterium. These include many secreted extracellular molecules, such as rhamnolipids, phenazine compounds, exoenzymes, and proteases. The production of these virulence factors are carried out by various gene products such as rhamnolipids by *rhlAB* operon (Ochsner *et al.*, 1994), phenazine compounds by two homologous operons, *phzA1B1C1D1G1* (*phzA1*), and *phzA2B2C2 D2G2* (*phzA2*) (Mavrodi *et al.*, 2001). These genes/operons are subject to complex regulation.

To examine the response of *P. aeruginosa* virulence factors to subinhibitory antibiotics and to examine possible bioactivities in addition to their inhibitory activity at high concentration, the expression of 13 secreted virulence genes of *P. aeruginosa* was examined in the presence of subinhibitory concentrations of four antibiotics. They represented four different chemical classes (tetracyclines, β-lactams, glycosides, and macrolides, respectively) and mechanisms of action. We observed transcriptional activation of six virulence factors by subinhibitory concentrations of 4 antibiotics; moreover we observed significant increase in rhamnolipid and pyocyanin production as well as *P. aeruginosa* swarm motility. The notion that antibiotics at subinhibitory concentrations can act as signaling molecules has been discussed.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Both *P. aeruginosa* PAO1 and *Escherichia*

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Table 1. Bacterial strains and plasmids used

Strain or plasmid	Description or sequence	Source or reference
Strains		
P. aeruginosa PAO1	Wild type	Holloway (1955)
PDO100	*rhlI* mutant of PAO1	Pearson *et al.* (1997)
PAO-214	*lasI* mutant of PAO1	Pearson *et al.* (1997)
PAO-JP2	*lasI-rhlI* double mutant of PAO1	Pearson *et al.* (1997)
A. tumefaciens A136 (pCF218) (pMV26)	HSL detection strain	Chambers *et al.* (2005)
E. coli DH10B		Invitrogen
Plasmids		
pMS402	Expression reporter plasmid carrying the promoterless luxCDABE	Duan *et al.* (2003)
pKD-*rhlAB*	*rhlAB* (Rhamnolipid synthesis, rhamnosyltransferase chain AB) promoter region cloned upstream of the *luxCDABE* in pMS402	Duan *et al.* (2003)
pKD-*phzA1*	pMS402 containing the promoter region of the *phzA1* operon (phenazine synthesis)	Duan *et al.* (2003)
pKD-*phzA2*	pMS402 containing the promoter region of *phzA2* operon (second phenazine synthesis operon)	Duan *et al.* (2003)
pKD-*exoT*	pMS402 containing *exoT* (exoenzyme T) promoter region	Duan *et al.* (2003)
pKD-*exoY*	pMS402 containing *exoY* (exoenzyme Y, adenylate cyclase) promoter region	Duan *et al.* (2003)
pKD-*exoS*	pMS402 containing *exoS* (exoenzyme S, ADP-ribosyltransferase) promoter region	Duan *et al.* (2003)
pKD-*lasA*	pMS402 containing *lasA* (staphylolytic protease) promoter region	Duan *et al.* (2003)
pMS404	pMS402 containing *lasB* (elastase) promoter region	Duan *et al.* (2003)
pKD-*toxA*	pMS402 containing *toxA* (toxin A) promoter region	Duan *et al.* (2003)
pKD-*algD*	pMS402 containing *algD* (alginate synthesis, GDP-mannose 6-dehydrogenase) promoter region	Duan *et al.* (2003)
pKD-*plcH*	pMS402 containing *plcH* (hemolytic phopholipase C, hemolysin precursor) promoter region	Duan *et al.* (2003)
pKD-*aprA*	pMS402 containing *aprA* (alkaline metalloproteinase precursor) promoter region	Duan *et al.* (2003)
pKD-*rnr*	pMS402 containing *rnr* (exoribonuclease RNase R) promoter region	Duan *et al.* (2003)

coli DH10B strains were grown and maintained in Luria-Bertani (LB) broth or on LB agar. Cultures were grown at 37°C. Trimethoprim and kanamycin were added at 300 μg/ml and 50 μg/ml respectively where appropriate. All antibiotics were purchased from Amresco (USA).

Construction of reporter fusions

The plasmid pMS402 was used to construct promoter-*luxCDABE* reporter fusions of the secreted virulence genes in *P. aeruginosa* as reported previously (Duan *et al.*, 2003). The promoterless *luxCDABE* operon allows the level of gene expression to be monitored as light production. The promoter regions of the 13 secreted virulence genes were PCR amplified using primers synthesized according to the genome data of *P. aeruginosa* (Stover *et al.*, 2000). The secreted virulence factors investigated are listed in Table 1.

Monitoring gene expression

Serial dilutions were made to determine the MIC of the antibiotics by measuring growth in clear 96-well plates (Costar, USA). Effect of subinhibitory concentrations of the antibiotics on 13 secreted virulence genes/operons was measured in black clear-bottom 96-well plate (Costar 3614). Overnight cultures were diluted to OD_{600}=0.2 and cultivated for two additional hours before used as inoculants. 10 μl of fresh culture was inoculated to the wells of the assay plates. Forty microliter filter-sterilized mineral oil was added to prevent evaporation during the assay. Both luminescence and absorbance (595 nm) were measured every 30 min for 24 h in a Victor^2 Multilabel Counter (PerkinElmer, USA). Expression assays with and without the presence of sub-inhibitory antibiotics were carried out on the same plate.

Rhamnolipid measurement

Rhamnolipids in the *P. aeruginosa* cultures were quantified according to the method described previously (Pearson *et al.*, 1997). PTSB medium (5% Tryptone, 0.25% Tryptone Soya broth, pH 7.0) (Ohman, 1980) and nitrogen-limited Guerra-Santos (GS) medium (Guerra-Santos, 1986) with 0.1 M glucose were used in the assay. The measurements were repeated six times, and the combined data from the experiments are reported.

Measurement of pyocyanin production

Pyocyanin was extracted from culture supernatants and measured using the previously described method (Kurachi,

Table 2. Regulation of secreted virulence factors by subinhibitory antibiotics

Gene	Fold-change of expression[a]			
	Vancomycin	Tetracycline	Azithromycin	Ampicillin
rhlAB	11.4	5.7	2.5	7.7
phzA2	11.3	10.6	6.7	23.6
phzA1	2.5	15.6	7.0	1
lasB	2.9	2.7	2.2	3.2
exoY	2.7	2.2	2.2	4.1
exoS	1.4	2.7	2.6	1.7
aprA	1.4	1.7	1	1
plcH	1	1	1	1
lasA	1	1	1	1
rnr	1.1	1.5	1.5	1
algD	1	1	1	1
exoT	1.1	1.2	1.3	1
toxA	1	1	1	1

[a] ratio between maximum expression levels (cps) during the whole growth course in LB medium and those in LB supplemented with subinhibitory antibiotics. The subinhibitory concentrations of these antibiotics used were vancomycin at 1/4 MIC; tetracycline at 1/32 MIC; azithromycin at 1/4 MIC; ampicillin at 1/128 MIC.

1958; Essar *et al.*, 1990). Briefly, 3 ml of chloroform was added to 5 ml culture supernatant. After extraction the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 N HCl. After centrifugation, the top layer (0.2 N HCl) was removed and its absorption measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per mililiter of culture supernatant, were determined by multiplying the optical density at 520 nm (OD_{520}) by 17.072 (Kurachi, 1958).

Swarm and swim motility assay

Swarm medium used is based on M8 minimal medium, supplemented with 0.2% glucose, 2 mM $MgSO_4$, and solidified with agar (0.5%). Glutamate was added at a final concentration of 0.5% (Kohler *et al.*, 2000). Swim agar contents were identical to swarm agar contents except that it was solidified with 0.3% agar. 2.5 μl of bacterial cultures grown overnight in LB was spotted on swarm and swim agar plates. The plates were incubated for 24 h at 37°C and then incubated an additional 24 h at room temperature for a total of 48 h. All swarm or swim agar plates were poured from the same batch of preparation to minimize variation in agar concentrations. Photographs were taken using the LAS-3000 Imaging System (Fuji Corp., Japan).

N-acyl-homoserine lactone measurement

In order to measure HSL in bacterial cultures, we used the methods based on an *A. tumefaciens* system (Chambers *et al.*, 2005) and the *rhlA-luxCDABE* reporter (Duan and Surette, 2007) as reported previously except that *P. aeruginosa* strain PDO100 (Pearson *et al.*, 1997) harboring *rhlA-luxCDABE* was used in place of JP2 strain. Briefly, an overnight culture of the reporter strains was diluted 1:300 in LB medium and 90 μl was added to the wells of a 96-well microtitre plate. 10 μl of the samples or medium control was added to the wells and the luminescence and OD values were measured every half hour for a total of 24 h in a Vctor^2 1420 Multilabel Plate Reader and relative levels of HSL were calculated from the maximal cps values minus the medium control.

Results

Regulation of secreted virulence genes by subinhibitory concentrations of antibiotics

To investigate the effect of subinhibitory concentrations of antibiotics on the expression of the secreted virulence factors, the promoter regions of 13 known secreted virulence factor genes or operons were amplified and cloned upstream of the promoterless *luxCDABE* reporter on plasmid pMS402 (Table 1). The MICs of 4 antibiotics, tetracycline, ampicillin, vancomycin, and azithromycin were determined using 2-fold serial dilutions in LB medium on 96-well plates. They belong to four different chemical classes (tetracylclines, β-lactams, glycosides, and macrolides, respectively) with two mechanisms of action, i.e. cell wall synthesis inhibitors and protein synthesis inhibitors. The MIC for vancomycin, tetracycline, azithromycin, and ampicillin were 125 μg/ml, 10 μg/ml, 6.25 μg/ml, and 250 μg/ml, respectively.

The promoter activity of secreted virulence factor genes in the presence and absence of subinhibitory antibiotics was measured as light production in a black clear-bottom 96-well plate in the Victor^2 Multilabel Counter while the bacterial growth was monitored at OD_{600}. The results indicate that some of these genes were clearly regulated by subinhibitory concentrations of the antibiotics. The fold-changes of these virulence factors in the presence of the antibiotics are listed in Table 2. The expression of five of the 13 secreted virulence genes (rhamnolipid synthesis genes *rhlAB*, elastase gene *lasB*, one of phenazine synthesis operons *phzA2*) was up-regulated by all 4 antibiotics tested. Exoenzyme genes *exoY* and *exoS* were activated about 2-fold by these anti-

biotics. *exoY* was activated more markedly by ampicillin (~5 fold). *phzA1* was activated by subinhibitory vancomycin, tetracycline, and azithromycin, but not ampicillin. The expression of *aprA, rnr*, and *exoT* was slightly affected by

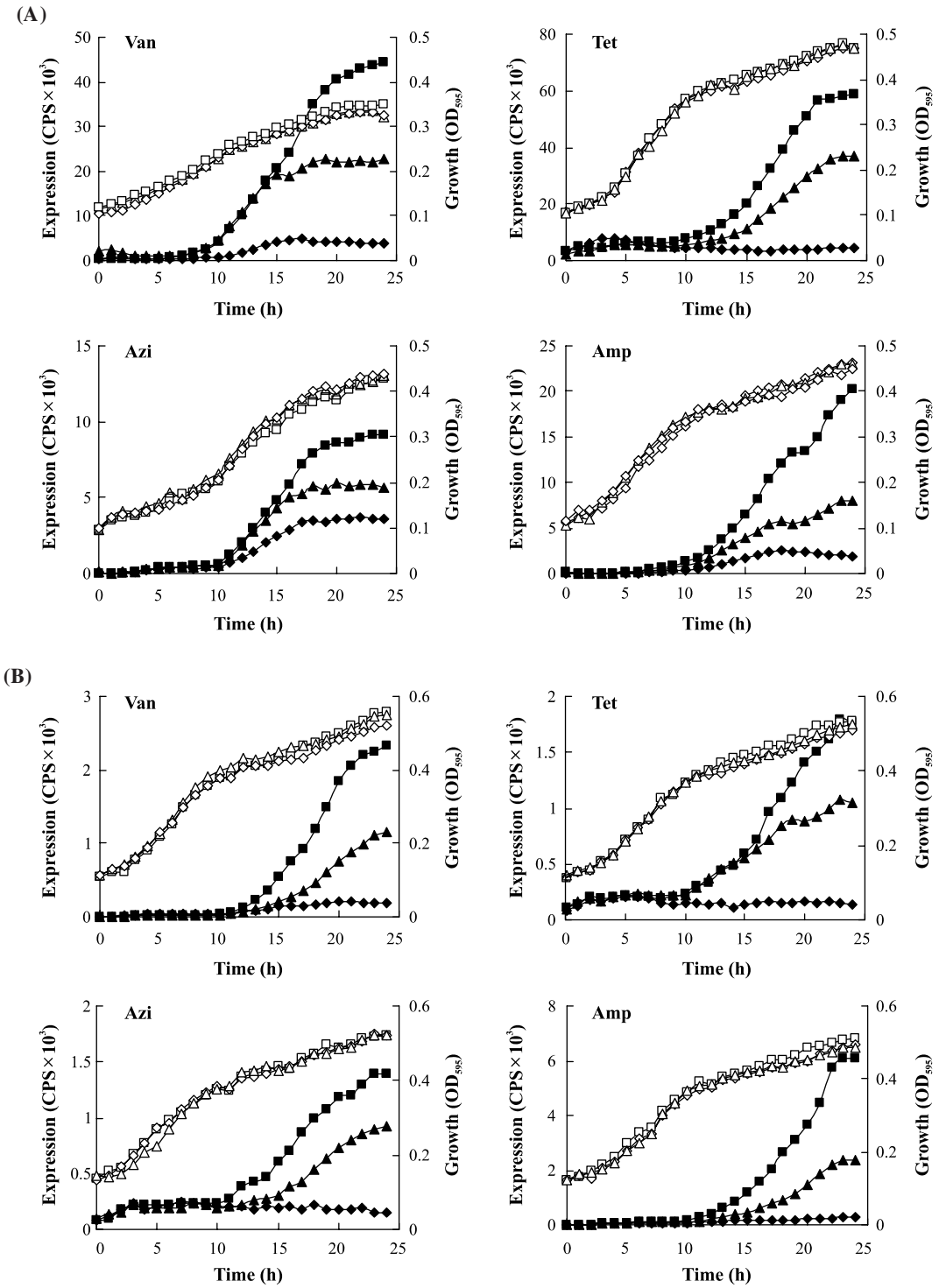

Fig. 1. The expression profiles and growth of PAO1 with reporter pKD-*rhlAB* (A) and *phzA2* (B) in the presence or absence of subinhibitory antibiotics. Van, vancomycin; Tet, tetracycline; Azi, azithromycin; Amp, ampicillin. The subinhibitory concentrations were at 1/8 MIC and 1/4 MIC for vancomycin and azithromycin, 1/32 MIC and 1/16 MIC for tetracycline, and 1/256 MIC and 1/128 MIC for ampicillin. (◊) growth without antibiotics (Δ) growth with the lower concentrations of antibiotics (□) growth with the higher concentrations of antibiotics (♦) luminescence without antibiotics, (▲) luminescence with the lower concentrations of antibiotics (■) luminescence with the higher concentrations of antibiotics.

some of the antibiotics (<2-fold), while the expression of *lasA*, *algD*, *toxA*, and *plcH* was not influenced by any of the antibiotics tested.

Regulation of *rhlAB* and *phzA2* by subinhibitory concentrations of the antibiotics

Among the genes most affected by the subinhibitory concentrations of antibiotics are *rhlAB*, *phzA1*, and *phzA2*. They were selected for further investigation. *rhlAB* involves in the biofilm formation (Davey *et al.*, 2003) and the motility of *P. aeruginosa* (Caiazza *et al.*, 2005). *phzA*1 and *phzA*2 are the two homologous operons involved in phenazine compound synthesis. As shown in Fig. 1, the expression of *rhlAB* was up-regulated 11.4-fold, 5.7-fold, 2.5-fold, and 7.7-fold in the presence of sub-MIC vancomycin, tetracycline, azithromycin, and ampicillin, respectively. In the mean time, the bacterial growth was not affected (Fig. 1A). The expression of *phzA2* operons were up-regulated in the similar way, but to different extents by different antibiotics (Fig. 1B).

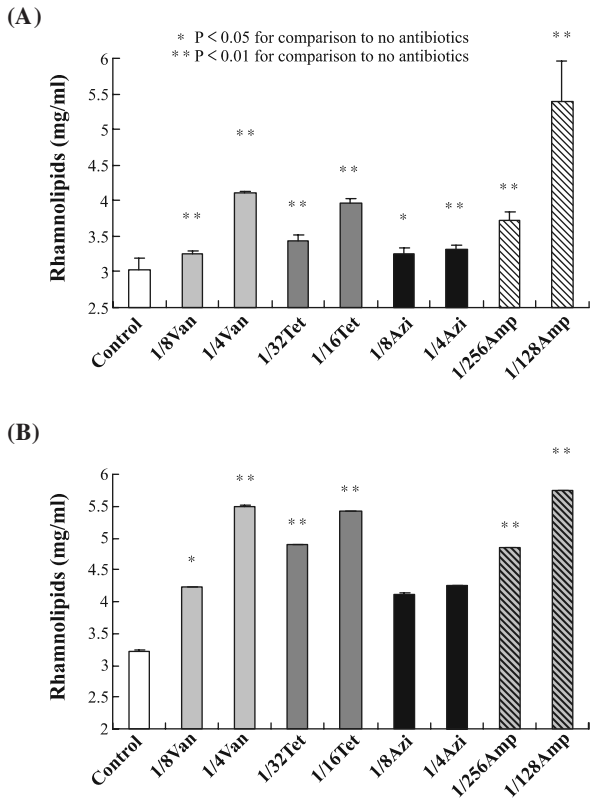

Fig. 2. Rhamnolipid (A) and pyocyanin (B) production in response to subinhibitory concentrations of antibiotics. The values represent the averages of six independent experiments. Van, vancomycin; Tet, tetracycline; Azi, azithromycin; Amp, ampicillin.

Rhamnolipid and pyocyanin production affected by subinhibitory antibiotics

To verify that the effect of subinhibitory antibiotics on virulence gene expression at the transcriptional level actually results in altered phenotypes, the production of rhamnolipids and pyocyanin was analyzed. Rhamnolipids are the final products catalyzed by RhlAB, and pyocyanin synthesis is determined by the *phzA* operons. Rhamnolipid and pyocyanin production were measured in the presence of different subinhibitory concentrations of the 4 antibiotics. The results indicate that rhamnolipid production was increased by 35%, 31%, 9%, and 78% in the presence of subinhibitory vancomycin, tetracycline, azithromycin, and ampicillin, respectively (Fig. 2A). This is consistent with the transcriptional effect. Under the same conditions, pyocyanin production increased 72%, 64%, 30%, and 77% compared to that in the absence of antibiotics (Fig. 2B). These results indicate that the transcriptional increases caused by subinhibitory antibiotics did lead to phenotypic changes.

Enhanced swarm and swim motilities in the presence of subinhibitory antibiotics

Motility is closely associated with bacterial pathogenesis under specific conditions. Rhamnolipids are amphipathic molecules (Caiazza *et al.*, 2005) that promote surface translocation on semisolid surfaces (Kohler *et al.*, 2000). Since we observed elevated rhamnolipid production in the presence of subinhibitory antibiotics, *P. aeruginosa* motility was examined. As shown in Fig. 3, both swarm and swim motilities were significantly increased in the presence of subinhibitory vancomycin, indicative of a correlation between rhamnolipid production and *P. aeruginosa* motility.

Discussion

Antibiotics at high concentrations kill or inhibit the growth of bacterial pathogens, and may act as signal molecules regulating bacterial gene expression and modulating bacterial fitness at subinhibitory concentrations (Linares *et al.*, 2006). In human hosts receiving antibiotic therapy, pathogens will

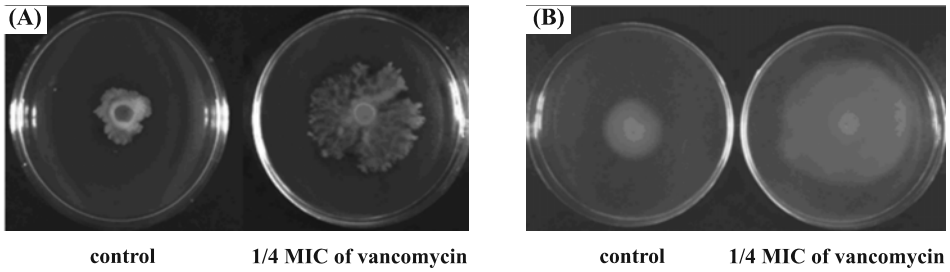

Fig. 3. Increased swarming (A) and swimming (B) motility of PAO1 in the presence of subinhibitory vancomycin.

encounter variable concentrations of antibiotics (Mukhopadhyay *et al.*, 1994). It is therefore important to understand how antibiotics at different concentrations may affect the pathogenesis of the bacteria. We tested the effect of subinhibitory concentrations of 4 different antibiotics on 13 secreted virulence factors in *P. aeruginosa*. Six of the 13 secreted virulence factor genes/operons, i.e. *rhlAB*, *phzA1, phzA2*, *lasB, exoS,* and *exoY*, were activated at least 2-fold by at least one of the antibiotics. Most dramatic activation of gene expression was observed with the *rhlAB*, *phzA1*, and *phzA2* operons which were activated more than 10-fold by at least one antibiotic. *rhlAB* encodes rhamnosyltransferase whose final catalyzed product is rhamnolipids (Ochsner *et al.*, 1994), and *phzA1* and *phzA2* operons are involved in the synthesis of phenazine compounds including pyocyanin (Mavrodi *et al.*, 2001). All these final products are important virulence factors and involved in biofilm architecture formation, motility, and bacteria-host interactions (Davey *et al.*, 2003; Lau *et al.*, 2004; Caiazza *et al.*, 2005). Measuring rhamnolipid production and phenazine compound levels in the presence of subinhibitory antibiotics revealed significant increased production, demonstrating activation of gene expression at the transcriptional level resulted in phenotypic changes. The observed activation of virulence factors by subinhibitory antibiotics is in agreement with some of the recent research (Bagge *et al.*, 2004; Hoffman *et al.*, 2005; Linares *et al.*, 2006; Yun *et al.*, 2006), and suggest that subinhibitory concentrations antibiotics may increase bacterial pathogenicity in certain conditions, and thus complicate the effect of antibiotic chemotherapy.

It is noted that the observed effect of subinhibitory azithromycin on virulence factors differ from some previous reports that subinhibitory concentrations antibiotics could reduce virulence factor expression and thereby reduce pathogenicity of the (Tateda *et al.*, 2001; Carfartan *et al.*, 2004; Nalca *et al.*, 2006). Similar discrepancy in the effect of azithromycin on genes regulated by the quorum sensing systems has been reported (Nalca *et al.*, 2006). The differences of these results might be accounted for by the difference of the experimental conditions especially the concentrations of the antibiotics used. Profound differences may be observed when bacteria are exposed to different concentrations of antibiotics. At high concentrations (i.e. above or close to MIC), bacteria are usually killed or bacterial growth inhibited and many genes are differentially regulated as part of general stress responses (Yun *et al.*, 2006; Yim *et al.*, 2007). However, when sub-MIC concentrations of antibiotics are present, a broad range of genes are regulated which are not necessarily related to stress. This may reflect the ability of the bacteria to recognize antibiotics as environmental cues and respond by altering expression of specific genes (Davies, 2006; Yim *et al.*, 2007; Surette and Davies, 2008).

Several of the genes regulated by subinhibitory antibiotics such as *lasB*, *rhlAB*, *phzA1*, and *phzA2* are also under the control of quorum sensing which is a global regulatory system controlling bacterial group behavior and is central to *P. aeruginosa* pathogenecity. It has been reported that subinhibitory concentrations of ceftazidime and tobramycin reduce the quorum sensing signals of *P. aeruginosa* (Garske *et al.*, 2004). Macrolides such as azithromycin have also been reported to reduce quorum sensing activity and virulence factor expression (Tateda *et al.*, 2004; Nalca *et al.*, 2006). In contrast, our results indicate that the subinhibitory antibiotics tested significantly activated the expression of these quorum-sensing controlled virulence factors. The measurement of acyl-homoserine lactone signals showed that the amount of the quorum-sensing signals didn't vary significantly in the presence of these 4 antibiotics at subinhibitory concentrations while *rhlR* expression was activated to various extents by these subinhibitory antibiotics (data not shown). Some of the observed activation of the virulence factors by subinhibitory antibiotics may be accomplished through their interactions with the quorum-sensing systems. It is clear that further studies are required to understand the connections of the complex regulation networks in *P. aeruginosa.*

Most of the antibiotics used clinically are natural products produced by microorganisms. In the natural soil environments of the producing organisms, antibiotics are thought be present at lower concentrations and may play a more frequent role as signaling molecules (Davies, 2006; Yim *et al.*, 2007). *P. aeruginosa* also lives in soil environment and probably have evolved specific responses to antibiotics at these lower concentrations (Yim *et al.*, 2007). Many of the virulence factors produced by *P. aeruginosa* may have roles in microbial community interactions (Duan *et al.*, 2003). Phenazine compounds such as pyocyanin produced by *P. aeruginosa* are antibiotics in their own right that can function as competitive agents and/or signaling molecules in microbial communities. The regulation of virulence factors such as rhamnolipids and phenazine compounds by subinhibitory concentrations of antibiotics suggests that the antibiotics secreted by other microorganisms in microbial communities could serve as a signal to alert *P. aeruginosa* of the existence or aggression of other bacteria, and the increased rhamnolipids and pyocyanin production and subsequent motility in response would help *P. aeruginosa* to compete with the other microbes and/or better adapt to these environments.

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