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Effects of PNPG on cell growth cycle, motility machinery and quorum sensing in *Serratia marcescens*

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p-Nitrophenylglycerol (PNPG) effectively inhibits swarming of the enterobacterium *Proteus mirabilis*. The underlying mechanism of inhibition is unclear. We have now found that both PNPG also inhibits motility and swarming in another enterobacterium, *Serratia marcescens*. While the peak promoter activities of the flagellar master operon ($flhDC_{Sm}$), the flagellin structural gene (hag_{Sm}) and the nuclease gene ($nucA_{Sm}$) in *S. marcescens* increased with increasing PNPG concentration, the expression of these genes was delayed in accordance with the reduced growth rate. As the quorum-sensing system is involved in the regulation of swarming in *S. marcescens*, we also examined the effect of PNPG on the production of quorum-sensing signal molecules and found that their expression was delayed with a reduced level. PNPG, therefore, had a pleiotropic effect on all aspects of *S. marcescens* physiology relating to swarming. The underlying molecular mechanism remains to be elucidated.

Key words: Bacterial genes, bacterial proteins, gene expression regulation, movement, *Serratia marcescens*

Serratia marcescens is a member of the family of Enterobacteriaceae and is an opportunistic human pathogen [1]. It is a dimorphic bacterium capable of undergoing dramatic morphological and physiological changes following the swarming cycles on 0.8% Luria-Bertani (LB) swarming plates [2,3]. The swarmer cell differentiation (from short, vegetative cells with few peritrichous flagella to elongated, multi-nucleoid swarmer cells covered with over-produced flagella) is required for producing the flagella-assisted population surface migration known as swarming [2,4-6].

A variety of factors have been shown to influence the ability of this organism to migrate over surfaces. The bacterial flagellar motility machinery, including about 40 genetic determinants under the control of the *flhDC* master regulatory operon [7-10], is essential. Another important factor reported to regulate the swarming motility of a related bacterium *Serratia liquefaciens* is a cell-density dependent regulatory system named the *swrR/I* quorum-sensing system [11, 12]. The predominant *N*-acylhomoserine lactone (AHL) signal synthesized at a high cell density by the *swrI* gene product is *N*-butanoyl homoserine lactone (BHL) in *S. liquefaciens* [12]. Accumulation of BHL above a certain threshold concentration has been shown to be important for the initiation of swarming [12]. In *S. marcescens* SS-1, the genetic determinants encoding the quorum-sensing signal system (named *spnI* and *spnR*) have been cloned, sequenced and partially characterized, and the structure of AHL signal synthesized has also been analyzed [13]. The relationship between *S. marcescens* SS-1 *spnI*/*R* quorum-sensing system and swarming has been determined [13].

The antiswarming agent p-nitrophenylglycerol (PNPG) has been found invaluable for the recognition and isolation of pathogenic bacteria from specimens contaminated with swarming strains of *Proteus* spp. [14]. In addition, PNPG has little effect on the results of a variety of identification tests performed directly on colonies from media containing PNPG [14-16]. It was further shown that PNPG could also inhibit swimming of the eukaryotic cells such as spermatozoa [17,18]. Despite these observations, there has been no further study on how PNPG would affect the bacterial cells.

To gain insight into the mechanism of PNPG inhibition of swarming, we investigated its effects on the expression of flagellar gene hierarchy and quorum-sensing systems in *S. marcescens*. In addition, we investigated the effects of PNPG on $nucA_{Sm}$, the gene coding for *S. marcescens* nuclease [19], which is co-regulated with sliding [13]. We then examined the relationship between PNPG and the production of

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quorum-sensing signals [12] from *S. marcescens*. From these data, a possible underlying mechanism of the inhibitory effect of PNPG on *S. marcescens* swarming is proposed.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* was cultured at 37°C and *S. marcescens* at 30°C in LB broth media [26]. The agar concentration in the LB plates used in the swarming and swimming assays was 0.8% and 0.4%, respectively.

Swimming and swarming motility assay

Bacteria were inoculated onto LB swimming/swarming plates at 30°C (for *S. marcescens* strains) or at 37°C (for the other bacteria). After overnight incubation, the distances of the bacterial swimming/swarming were measured and recorded. The promoters of genes to be analyzed were ligated in front of the luciferase genes LuxAB using a reporter system for monitoring gene expression activity.

Measurement of LuxAB luciferase activity

100 μL of bacterial suspension was added to Con's tubes containing 900 mL of 0.85% normal saline and the bioluminescence emitted from each tube was measured with Autolumat LB953 luminometer (EG&G, Berthold, Germany). N-dedanol (N-dodecyl aldehyde, Sigma Chemical Co. St. Louis, MO, USA) 100 μL [1% (v/v) in ethanol] was used as the substrate and all procedures followed the protocol supplied by the manufacturer. The results were expressed as specific relative light unit (RLU) activity [RLU/OD(A_{600})].

Measurement of AHL levels

The spent culture supernatants (100 μL) of a bacterial culture with known OD(A_{600}) were added into a suspension of *E. coli* JM109/pSB401 (1 mL) [25]. The mixture was incubated at 30°C for 15 min and then the light emission was measured by Autolumat LB953 luminometer using the program 'replicates'. N-dedanol [1% (v/v) in ethanol] (100 mL) was used as the substrate. The experiments were repeated at least 3 times.

Results

PNPG inhibits swarming and swimming of *S. marcescens*

To see whether PNPG had any effect on the swarming of *S. marcescens* strains CH-1 and BG-1, a series of PNPG concentrations ranging from 0 to 200 μg/mL were added to LB swarming plates containing 0.8% agar. For comparison of swarming behavior, *Proteus mirabilis*

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Origin/reference
Strains		
Proteus mirabilis P19	Wild-type	Hughes C
Serratia marcescens CH-1	Wild-type; swarms well on 0.8% LB plates	Hughes C
Serratia marcescens BG-1	A clinically isolated strain; swarms well on 0.8% LB plates	NTUH
Serratia marcescens SS-1	Environmentally isolated strain; swarms poorly on 0.8% LB plates but spreads well	[20,21]
S. marcescens N-1	P$nucA_{Sm}$::*luxAB* (promoter transcriptional fusion) in the chromosome of *S. marcescens* CH-1. The $nucA_{Sm}$ encodes for a nuclease virulence factor in *S. marcescens* CH-1	[18]
S. marcescens S-1	Phag_{Sm}::*luxAB* (promoter transcriptional fusion) in the chromosome of *S. marcescens* CH-1. The *hag* is the flagellin-coding gene of *S. marcescens* CH-1	[22]
S. marcescens F-3	P$flhDC_{Sm}$::*luxAB* in the chromosome of *S. marcescens* CH-1. The $flhDC_{Sm}$ is the flagellar class I regulatory operon cloned from *S. marcescens* CH-1	NTUH
Chromobacterium violaceum CV026	Double mini-Tn5 mutant derived from *C. violaceum* 017; non-AHL producer	[23]
E. coli JM109	PSB401	[24,25]
Plasmid		
pSB401	Tc^r with ori pACYC184; containing *luxRCDABE*	[24,25]

Abbreviations: LB = Luria-Bertani; AHL = *N*-acylhomoserine lactone; NTUH = National Taiwan University Hospital

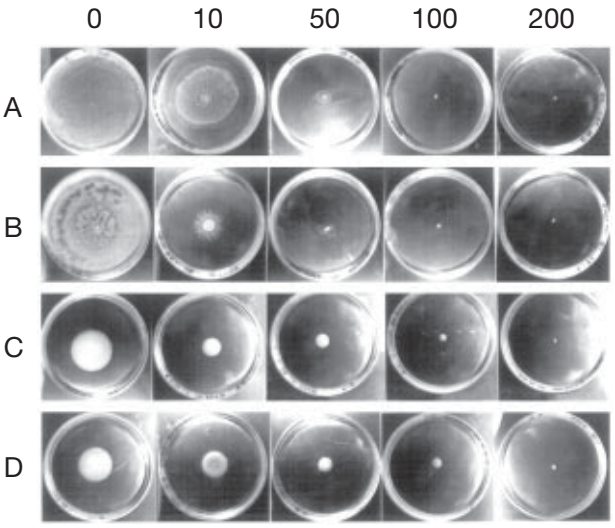

Fig. 1. Effect of PNPG on the swarming and swimming behaviors of *P. mirabilis* p19 and *S. marcescens* CH-1. The overnight bacterial culture (5 μL) was inoculated into LB swarming (0.8% agar for *S. marcescens*; 1.5% agar for *P. mirabilis*) and swimming plates (0.4% agar for both bacteria) followed by incubation at 30°C (*S. marcescens*) and 37°C (*P. mirabilis*) overnight. **(A)** Swarming of *P. mirabilis* P19. **(B)** Swarming of *S. marcescens* CH-1. **(C)** Swimming of *P. mirabilis* P19. **(D)** Swimming of *S. marcescens* CH-1.

P19 was also tested in the assay. After overnight culture at 30°C, the swarming of *P. mirabilis* P19 (Fig. 1A) and *S. marcescens* CH-1 (Fig. 1B) was significantly inhibited by increasing PNPG concentration and completely inhibited at the PNPG concentration of 100 μg/mL. Similar results were also observed in *S. marcescens* BG-1 strain (data not shown). The spreading behavior [20,21] of *S. marcescens* SS-1 was completely inhibited at the PNPG concentration of 50 μg/mL (data not shown). These results showed that the swarming behaviors of not only *P. mirabilis* P19, but also *S. marcescens* CH-1 and BG-1 were inhibited by PNPG, suggesting a common inhibitory effect of PNPG on swarming.

As bacterial flagellar motility is essential for the swarming behavior, it is possible that PNPG inhibits the motility, and thus the swimming phenomenon. To confirm this, the bacteria tested above were subjected to swimming assay in the media with serial concentrations of PNPG (0, 10, 50, 100, and 200 μg/mL). Interestingly, for *P. mirabilis* P19 (Fig. 1C) and *S. marcescens* CH-1 (Fig. 1D), the swimming diameter was decreased as the PNPG concentration was increased, and the swimming behavior was completely inhibited at a PNPG concentration of 100 μg/mL. This suggested that not only swarming, but also swimming behaviors of *P. mirabilis* and *S. marcescens* were significantly inhibited by PNPG.

PNPG stimulates the flagellar motility machinery and $nucA_{Sm}$ expression while inhibiting cellular growth

As both swarming and swimming behaviors of *S. marcescens* CH-1 and BG-1 were inhibited by PNPG, it was possible that the underlying mechanism was due to the inhibition of flagellar activity. To test this, expression patterns of the master flagellar regulatory operon $flhDC_{Sm}$ and the flagellin structural gene hag_{Sm} (both were cloned from *S. marcescens* CH-1) were monitored following the growth process in LB broth containing different concentrations of PNPG (0, 10, 50, 100, and 200 μg/mL) at 30°C. *S. marcescens* F-3 and *S. marcescens* S-1 were used and the intensity of bioluminescence emission was measured hourly following the growth curve. The temporal expression patterns of both $flhDC_{Sm}$ (Fig. 2A) and hag_{Sm} (Fig. 2B) were significantly delayed compared with the control, which contained no PNPG. However, it was interesting to note that as PNPG concentration was increased, the peak promoter activities detected were not inhibited, but rather up-regulated. It was also observed that the bacterial growth rate (and thus the temporal cell cycle) was significantly inhibited in proportion to the PNPG concentration gradients (Fig. 2A and 2B). However, the final bacterial growth density [OD(A_{600})] after overnight culture was similar among these cultures [about OD (A_{600}) 3.0; data not shown], suggesting that bacterial cells were not killed by PNPG. These results showed that while PNPG inhibited the swimming of *S. marcescens*, it did not inhibit the flagellar activities ($flhDC_{Sm}$ and hag_{Sm}); rather, it delayed the expression pattern of these 2 genetic determinants, the bacterial growth rate and cell cycle temporally.

As expression of $nucA_{Sm}$, the genetic determinant encoding for the virulence factor nuclease, was found to be dominantly regulated by $flhDC_{Sm}$ in *S. marcescens* CH-1 [26], we then asked whether a similar effect of PNPG on the expression pattern of $flhDC_{Sm}$ and hag_{Sm} would also be observed in $nucA_{Sm}$. To test this, *S. marcescens* N-1 (P$nucA_{Sm}$ in the chromosome) was used and the pattern of light emission (the promoter activity of $nucA_{Sm}$) was detected. Similar to $flhDC_{Sm}$ and hag_{Sm}, the $nucA_{Sm}$ promoter activity was not inhibited (Fig. 2C), but as the PNPG concentration was increased, the temporal expression pattern of $nucA_{Sm}$ together with the temporal cell cycle pattern were delayed.

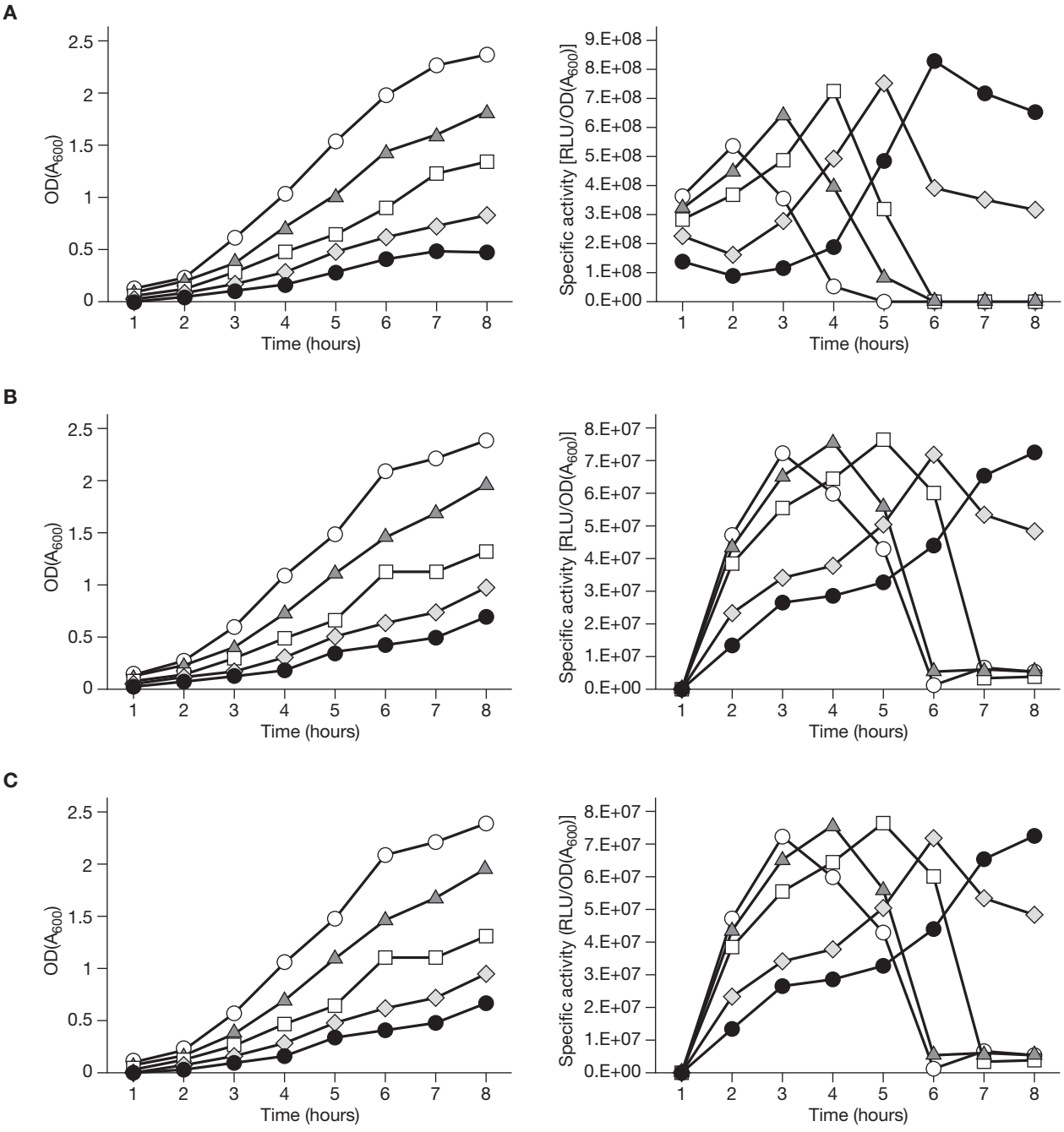

Fig. 2. Effect of PNPG on the growth and promoter activities of *Serratia marcescens* strains following the growth process in LB broth at 30°C. Bacteria were cultured overnight in LB broth followed by 1/100 dilution for further growth and detection of promoter activity by using *luxAB* as the reporter genes. **(A)** *S. marcescens* F-3 (P*flhDC*$_{Sm}$::*luxAB* in the chromosome). **(B)** *S. marcescens* S-1 (P*hag*$_{Sm}$::*luxAB* in the chromosome). **(C)** *S. marcescens* N-1 (P*nucA*$_{Sm}$::*luxAB* in the chromosome). (○), without PNPG; (△), PNPG 10 μg/mL; (□), PNPG 50 μg/mL; (◇), PNPG 100 μg/mL; (●), PNPG 200 μg/mL. The bacterial growth was expressed as OD(A_{600}). Data presented are the means of 3 determinations (SEM<5%).

PNPG inhibits the levels of quorum-sensing signal molecules

Given the important role of quorum-sensing AHL signal (s) in the regulation of swarming behaviors of *S. liquefaciens* [12] and *S. marcescens* [13], and that PNPG not only inhibits the swarming, but also bears a structure similar to that of AHL signals, it was important to determine whether PNPG affected the production of AHL signals from *S. marcescens*. *S. marcescens* SS-1 and BG-1 which produced *Chromobacterium violaceum*

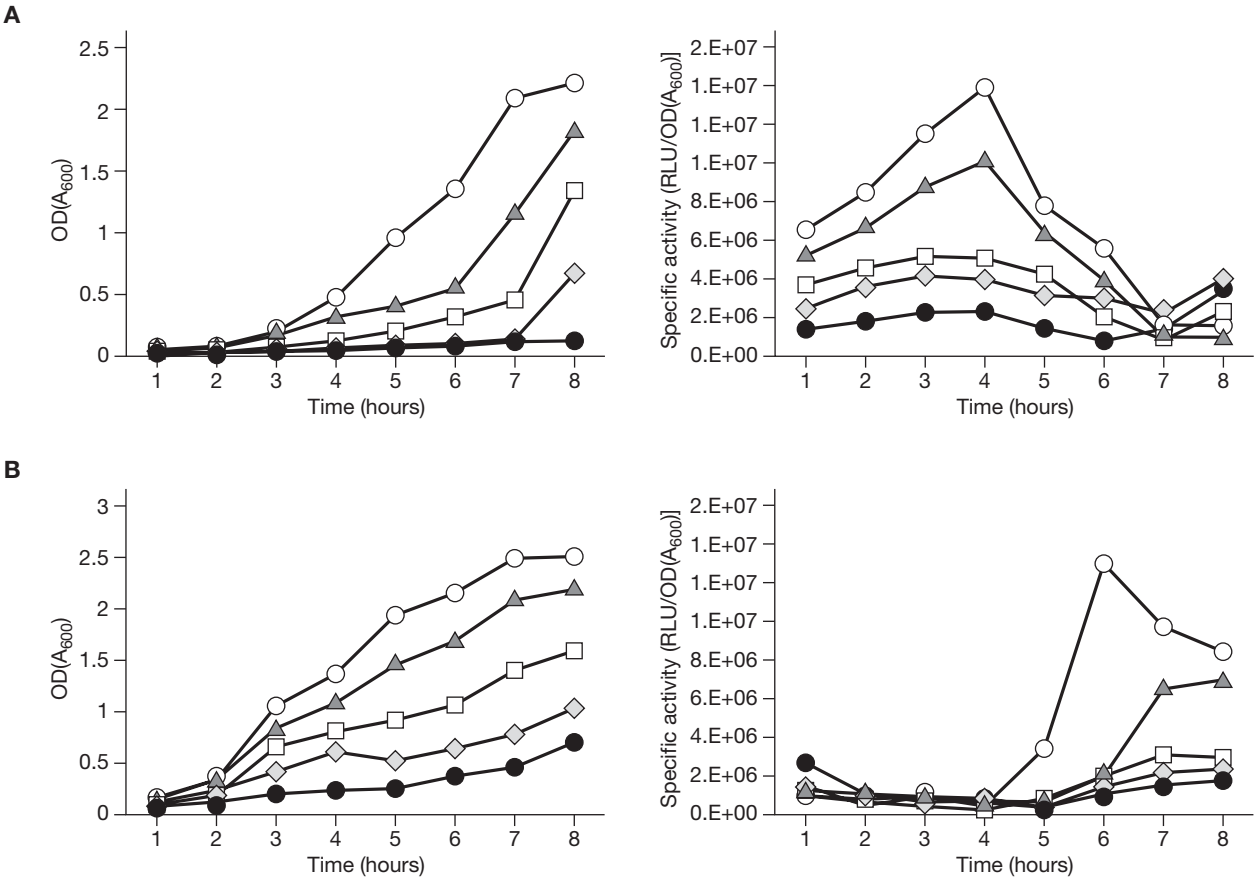

Fig. 3. Effect of PNPG on the production of AHL quorum-sensing signal molecules of *S. marcescens*. After overnight culture, bacteria were diluted 1/100 before being grown in LB broth containing different PNPG concentrations at 30°C. The growth [OD(A_{600})] of each sample was determined and the AHL signal activities in the spent supernatants were measured by *E. coli* JM109/pSB401. **(A)** AHL signal activities of *S. marcescens* SS-1. **(B)** AHL signal activities of *S. marcescens* BG-1. (○), without PNPG; (▲), PNPG 10 mg/mL; (□), PNPG 50 mg/mL; (◇), PNPG 100 mg/mL; (●), PNPG 200 mg/mL. Data presented are the means of 3 independent experiments (SEM<6%).

CV026 [23] and *E. coli* JM109/pSB401 [24,25] detectable AHL signals were used for study. The *lux*-based detection system using *E. coli* JM109 containing an AHL biosensor plasmid such as pSB401 was used to detect the amount of AHL signaling molecules produced. Before the assay, it was already observed that the normal patterns of AHL synthesis in *S. marcescens* SS-1 and BG-1 were basically different from each other [26]. For *S. marcescens* SS-1, the peak total activity occurred at the mid-log growth phase and that the level of the activity (about 1×10^7 RLU) was about 100 times higher than that of *S. marcescens* BG-1. For *S. marcescens* BG-1, the peak total activity occurred at the late stationary phase and showed a significantly lower AHL signal activity (about 1×10^5 RLU).

S. marcescens SS-1 and BG-1 were grown in LB broth containing different concentrations of PNPG (0, 10, 50, 100, and 200 mg/mL), and the spent culture supernatants were taken hourly for detection of AHL level following the growth curve. Although the patterns of AHL levels from both *S. marcescens* SS-1 (Fig. 3A) and *S. marcescens* BG-1 (Fig. 3B) were different, the total levels of AHL signals throughout the growth curve from both strains were consistently lower in proportion to the increase of PNPG concentrations in the media. The onset of AHL accumulation was also delayed in accordance with the decrease in growth rate, and thus the increase in PNPG concentration.

Discussion

The regulation of bacterial multicellular behavior such as swarming is a complex process. In *S. marcescens*, several of the genes involved in flagellar motility and quorum-sensing are found to play a regulatory role in this process [13]. In this study, we have shown that

PNPG, has a negative regulatory effect on the swarming and swimming behaviors of *S. marcescens* strains.

PNPG was previously shown to not only inhibit the swarming behavior of *P. mirabilis* but also inhibit the swimming behavior of spermatozoa, suggesting potential for use as a contraceptive [17]. However, the underlying inhibitory mechanism was unknown. In searching for any extrinsic or intrinsic factors that may influence the swarming of *S. marcescens*, we found that PNPG also inhibited the swarming/swimming of *S. marcescens*. In addition, PNPG delayed the cell cycle by inhibiting the cellular growth rate. An interesting phenomenon was observed in that PNPG stimulated the promoter activities of $flhDC_{Sm}$, hag_{Sm} and $nucA_{Sm}$, suggesting that PNPG in fact affected cellular growth, and thus rendered cell populations unable to swarm or swim.

Finally, we looked at the effect of PNPG on the production of the quorum-sensing AHL signal. In both *S. marcescens* SS-1 and BG-1 strains, synthesis of AHL levels was substantially lowered in the presence of PNPG. Although AHL signals were reported to be important for the initiation of swarming in *S. liquefaciens* [12], their role in the swarming of *S. marcescens* remains to be determined, as different AHL concentrations show either stimulatory or inhibitory effects on the swarming of *S. marcescens* [26]. The significance of the finding that PNPG inhibits AHL signals is still not clear. It is possible that such an effect is just a result of inhibition of cellular growth, or that PNPG competes with the native AHL signals. The genetic determinants responsible for synthesis of the quorum-sensing system (named *spnI*/*spnR*) have been cloned and more experiments are being performed in order to unravel the relationship between AHL signals and *S. marcescens* swarming.

One question that arises from this work is why and how PNPG inhibits bacterial cell growth and delays the cell cycle. It is known that PNPG has a common inhibitory effect on *P. mirabilis* p19 and *S. marcescens* CH-1 and BG-1. *E. coli* JM109/pSB401 detectable AHL signals were not produced from *P. mirabilis* P19 and *S. marcescens* CH-1, but were produced from *S. marcescens* SS-1 and BG-1, the inhibitory effect of PNPG should not act through regulation of the quorum-sensing system. Transposon mutagenesis study to select for mutants that are insensitive to high concentrations of PNPG will be required to aid the identification of genetic determinants involved in the regulation of swarming and control of the cellular growth rate and cell cycle. Irrespective of its mechanism of action, it is clear from this study that PNPG affects the multiple and diverse physiological traits in both bacteria and eukaryotic cells [17,18].

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