Characterisation of p-nitrophenylglycerol-resistant *Proteus mirabilis* super-swarming mutants

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*p*-Nitrophenylglycerol (PNPG) inhibits the co-ordinately regulated activities of swarming behaviour and virulence factor expression in *Proteus mirabilis*. The inhibitory action of PNPG was investigated by the isolation of *Tn*5 insertion mutants that could swarm, albeit with much reduced ability, in the presence of PNPG. The mutants exhibited a super-swarming phenotype in the absence of PNPG; i.e., they migrated further in a given time than did the wild-type cells. Cloning and sequence analysis of the mutants indicated that *Tn*5 was inserted into the *rsbA* gene, which may encode a membrane sensor histidine kinase of the bacterial two-component signalling system. In the absence of PNPG, the mutants exhibited several swarming-related phenotypes that were different from those of the wild type; they initiated swarming earlier and had a less conspicuous consolidation phase, they differentiated earlier and maintained a differentiated state for longer, they started to express virulence factors earlier and maintained high expression levels of these factors for longer, and they had higher cell invasion ability than the wild type. These mutant phenotypes could be complemented by a plasmid-borne copy of *rsbA*. Together, these data suggest that RsbA may act as a repressor of swarming and virulence factor expression. In the presence of PNPG, these *rsbA*-mutated mutants could still swarm, differentiate and express virulence factors, whereas the wild type could not, suggesting that PNPG may target RsbA or RsbA-regulated pathways to exert its inhibitory effect. Together, these data reveal a novel mechanism through which bacteria may negatively regulate swarming differentiation and virulence factor expression and identify a potential target of PNPG action.

Introduction

*Proteus mirabilis*, a motile gram-negative bacterium, is a principal cause of urinary tract infections in catheterised patients and those with urinary tract abnormalities. It often infects the upper urinary tract where it can lead to acute pyelonephritis, bladder or renal stones and bacteraemia [1–4]. It is believed that the ability of *P. mirabilis* to colonise the surfaces of catheters and the urinary tract is aided by a characteristic first described over a century ago and currently referred to as swarming differentiation and migration [5].

Swarming migration in *P. mirabilis* involves the co-ordinate differentiation of short, motile, vegetative cells with a peritrichous flagella into multinucleate, aseptate swarm cells of up to 40 times the vegetative cell length and with >50-fold greater surface density of flagella. Swarm cells migrate rapidly away from the colony as multicellular rafts until they pause (consolidation) and undergo some de-differentiation. Regular cycles of migration and consolidation generate a colony on the agar surface with a characteristic pattern of concentric rings [6–8]. After inoculation on to an agar plate, *P. mirabilis* undergoes the first cycle of swarming migration and consolidation in which there is induction of swarm cell differentiation, a lag period before the onset of swarming migration, active motile swarming migration, and finally consolidation, a phase in which migration stops and cell morphology returns to that of a short, undifferentiated vegetative cell. How these processes are regulated co-ordinately is largely unknown.

Swarming in *P. mirabilis* is the result of the co-
ordinated, multicellular effort of groups of differentiated, swarming cells and is regulated by population density [7, 9]. Moreover, the ability of *P. mirabilis* to express virulence factors, including urease, protease, haemolysin and flagellin, and to invade human urothelial cells, is coupled to swarming differentiation [10–12]. The co-ordination of swarmer cells to form concentric rings and to express virulence factors suggests that some form of cell–cell interaction and communication occurs to control these processes. Many gram-negative bacteria use quorum-sensing systems to co-ordinate activity within a population [13–16]. These bacteria communicate among themselves by producing extracellular signal compounds that, when present during appropriate conditions and in sufficient concentrations, trigger specific responses. Quorum sensing acts in response to population density and has been found to regulate the swarming behaviour and the expression of virulence genes in some bacteria [17, 18]. Although a quorum-sensing system has not been identified in *P. mirabilis*, some evidence points to this mechanism regulating swarming and expression of virulence factors in this bacterium [12, 19]. In this respect, it is worth noting that *P. mirabilis* can produce extracellular dkketopiperazines (DKPs) which may act as signal molecules that regulate quorum sensing [19].

*p*-Nitrophenylglycerol (PNPG) has long been used to inhibit the swarming behaviour of *Proteus* spp. to facilitate the identification of other bacteria in specimens contaminated with swarming strains [20, 21]. PNPG can also inhibit the ability of *P. mirabilis* to express virulence factors and to invade human urothelial cells [12]. This study investigated the mechanism by which PNPG inhibited swarming and swarming-related phenotypes.

**Materials and methods**

**Bacterial strains and plasmids**

The bacterial strains and plasmids are listed in Table 1. Bacteria were grown as described previously [12].

**Transposon mutagenesis**

*P. mirabilis* super-swarming mutants were constructed by mini-Tn5 Cm (chloramphenicol) mutagenesis as described previously [22]. Donor *Escherichia coli* (S17-1) cells harbouring pUTmini-Tn5 Cm [22] and recipient cells (wild-type *P. mirabilis* P19) were grown separately overnight at 37°C in LB broth with appropriate antibiotics (tetracycline 12.5 μg/mL for *P. mirabilis*, ampicillin 50 μg/mL for *E. coli*). Then 50 μl of both donor and recipient cell cultures were mixed in 5 ml of 10 mM MgSO4 and filtered through a 0.45-μm pore membrane (Millipore). The filter was then placed on the surface of an LB agar plate and incubated for 8 h at 37°C. The filter was removed from the agar surface and the bacteria were suspended by vortex mixing in 5 ml of 10 mM MgSO4. Samples of 100 μl were spread on LB agar plates containing PNPG 100 μg/mL, Cm 30 μg/mL and tetracycline 12.5 μg/mL and the plates were incubated overnight at 37°C. Bacteria that were resistant to the antibiotics and could swarm on the plates were selected for further studies.

**Cloning and sequencing of the mutated gene**

Chromosomal DNA was extracted from the mutants, partially digested with *Alu*I and fragments >4 kb were cloned into EcoRV-digested pZERO-2.1 (Invitrogen, USA). Following transformation of *E. coli* TOP10, Cm-resistant Tn5 Cm-containing clones were selected. The nucleotide sequences of the cloned DNA fragments were determined step by step with a 373A DNA sequencer (Applied Biosystems, USA). The nucleotide sequence of the mutated locus was obtained by analysing the sequences of the overlapping clones.

**Southern blot analysis**

Genomic DNA of the wild type and the Tn5-inserted mutants was digested with *Ssp*I. Southern blot analysis with the probe specific to the mini-Tn5 Cm-resistant gene was performed with a DIG DNA labelling and detection kit according to the manufacturer’s recommendations (Boehringer Mannheim, Germany).

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristic</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. mirabilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P19</td>
<td>Wild-type, RfΔ TeΔ</td>
<td>24</td>
</tr>
<tr>
<td>P1100</td>
<td>Mini-Tn5-Cm insertion at nt 1100 of <em>robA</em> gene in P19, RfΔ CmΔ TeΔ</td>
<td>This study</td>
</tr>
<tr>
<td>P301</td>
<td>Mini-Tn5-Cm insertion at nt 1301 of <em>robA</em> gene in P19, RfΔ CmΔ TeΔ</td>
<td>This study</td>
</tr>
<tr>
<td>P2399</td>
<td>Mini-Tn5-Cm insertion at nt 2399 of <em>robA</em> gene in P19, RfΔ CmΔ TeΔ</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>Donor cell for conjugation; CmΔ TeΔ</td>
<td>22</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR2.1</td>
<td>PCR TA cloning vector, ApΔ KmΔ</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCR2.1 TA cloning vector containing PCR ampiclon with intact <em>robA</em> (nt 228–3086) orientated behind <em>lac</em> promoter</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pUT mini-Tn5 Cm</td>
<td>Delivery plasmid for mini-Tn5 Cm, ApΔ CmΔ</td>
<td>22</td>
</tr>
</tbody>
</table>

ApΔ, ampicillin resistance; KmΔ, kanamycin resistance; RfΔ, rifampin resistance; TeΔ, tetracycline resistance; CmΔ, chloramphenicol resistance; TeΔ, tetracycline sensitive.
Construction of wild-type RshA-expressing plasmid

The full-length rshA sequence (nucleotides 228–3086 in Fig. 4) was obtained by PCR amplification of genomic DNA from wild-type P. mirabilis P19 as template with primers (sense: 5′-TGATGGTTGTG TAGCCAAT 3′ and anti-sense: 5′-AAGATTAC GAATTCGGAAT 3′). The amplified DNA fragment was cloned into plasmid pCR2.1 with the TA Cloning Kit (Invitrogen) to generate pSI228.

Swarming behaviour assays

The swarming migration distance and interval migration velocity assays were performed as described previously [23, 24]. Briefly, an overnight bacterial culture (5 μl) was inoculated centrally on to the surface of dry LB swarming plates containing agar 2% w/v and PNPG (0 or 80 μg/ml), which were incubated at 37°C. The swarming migration distance and interval migration velocity were assayed by following swarm fronts of mutant and wild-type cells and recording progress at 30-min intervals.

Measurement of cell length, flagellin level and haemolysin, urease, protease and cell invasion activities

When vegetative cells of a wild-type P. mirabilis stationary-phase liquid culture are spread evenly on to LB agar plates and incubated at 37°C, they differentiate and de-differentiate in a way similar to that in the regular swarm cycle [25]. Therefore, differentiation was initiated by spreading 200 μl of stationary-phase LB cultures on to LB agar plates with PNPG 80 μg/ml or without PNPG, and incubating them at 37°C for various time periods. After incubation, cells from the entire surface were harvested by washing into 5 ml of LB. These cells were then subjected to the assays described below. Cell length was estimated by phase-contrast microscopy (Olympus BH2, Japan). Cell membrane-associated haemolysin activity was assayed as described previously [26]. Protease activity was determined by the method of Gibson and Macfarlane [27]. The urease activity of whole-cell suspensions was determined by the phenol red colorimetric assay [28]. Flagellin determination was performed as described previously [25].

After incubation for 2, 4 or 7 h, the bacterial cells were harvested and subjected to a cell invasion assay according to the protocol of Liaw et al. [12] with some modifications. Briefly, human urothelial NTU1B1 cells were grown in monolayer cultures in RPMI 1640 medium (Gibco, USA) supplemented with fetal bovine serum 10% v/v at 37°C in a humidified CO2 (5% v/v in air) incubator. After growing to 90% confluence, cells were washed twice with Hank’s Balanced Salts Solution (HBSS) and then infected at 37°C with 1 ml of a bacterial suspension containing c. 5 x 10^3 bacteria in incubation solution (HBSS + minimal medium [29] + 0.2% Trit-HCl, pH 7.5; 80:10:10 v:v:v) for 1 or 2 h (a longer incubation time resulted in urothelial cell lysis). Urothelial cells were then washed twice with HBSS and incubated at 37°C in 1 ml of RPMI 1640 medium containing streptomycin sulphate 250 µg/ml for another 1.5 h. Cells were washed twice again with HBSS and then lysed by incubation with 1 ml of lysis solution (Tween 20 1%, v/v and trypsin 0.025% w/v in 0.01 M sodium phosphate buffer, pH 8.0) at 37°C for 30 min. Cell lysates were diluted serially in saline and viable bacteria were counted by plating on MacConkey agar. All experiments were performed in triplicate, and the results were expressed as the percentage of viable bacteria that survived the streptomycin treatment versus total inoculum.

Results

Isolation and characterisation of P. mirabilis mutants that swarm in the presence of PNPG

To investigate the mechanism by which PNPG inhibits swarming and the genetic regulation of swarmer cell differentiation in P. mirabilis, the minitransposon mini-Tn5 Cm [22] was used to construct a transposon mutant library. Selection was then made for mutants that swarmed in the presence of PNPG 100 µg/ml. From c. 3000 transconjugants, three mutants (P1100, P1301 and P2399) were isolated that were resistant to the swarming-inhibitory effect of PNPG. Because all three mutants exhibited similar swarming and swarming-related phenotypes in the presence and absence of PNPG (unpublished observations), only the data on mutant P1100 are reported. Mutant P1100 could swarm in the presence of PNPG 80 µg/ml, although more slowly than in the absence of PNPG (compare Fig. 1A and B). In contrast, the wild-type P. mirabilis, P19, could not swarm in the same concentration of PNPG (Fig. 1A). In the absence of PNPG, mutant P1100 showed a more rapid translocation across the agar surface and created a swarm colony that extended further than the wild type over a given time (Fig. 1B). The swarming pattern of mutant P1100 was also different from that of the wild-type strain P19. As shown in Fig. 1B, whereas the wild-type strain formed a swarm colony with clear consolidation zones, the mutant formed a more homogenous swarm colony with less conspicuous consolidation zones.

The swarming behaviour of the wild type (P19) and the mutant (P1100) were analysed by recording the position of the swarm front through two cycles of migration and consolidation in the absence of PNPG at 30-min intervals. Plots of the migration distances and the interval migration velocities of these bacteria against time are shown in Fig. 2. The wild type exhibited a typical swarming pattern of alternating active migration and consolidation (migration velocity be-
came zero) phases, whereas the mutant exhibited a less conspicuous consolidation phase (migration velocity did not become zero) (Fig. 2a, b). Another difference between the wild type and the mutant was the duration of the initial lag phase. Before the onset of swarming migration this was 2 h for wild-type cells, but about 30 min less for the mutant cells. Because of this and an incomplete consolidation phase (migration velocity did not become zero), the mutant translocated further in a given time than did the wild-type cells (Fig. 2a). The swarming abilities of the wild-type (P19) and the mutant (P1100) strains in the presence of PNPG were also measured. As shown in Fig. 3, the mutant could migrate in the presence of PNPG, but at a much reduced speed, whereas the wild-type strain could not migrate at all. In the presence of PNPG, the mutant P1100 had migrated c. 2 cm after incubation for 24 h, whereas in the absence of PNPG, it migrated >8.5 cm in the same time (unpublished observations). Because the mutants exhibited faster swarming than the wild-type strain, they were referred to as 'super-swarming mutants'.

Fig. 1. Swarming of wild-type *P. mirabilis* and the super-swarming *P. mirabilis* mutant on LB swarming plates. (A) Swarming in the presence of PNPG of the wild-type P19 (L) or the super-swarming mutant P1100 (R) after incubation for 24 h. (B) The 5-h swarming patterns of the wild-type strain (L) and the super-swarming mutant (R) in the absence of PNPG.

Fig. 2. (a) The swarming migration distance of the wild-type strain (P19; O) and the super-swarming mutant (P1100; ●) in the absence of PNPG. The experiments were performed in triplicate and the mean migration distance was plotted against incubation time. (b) The swarming migration velocities of strain P19 (O) and the super-swarming mutant (P1100; ●) in the absence of PNPG. The interval migration velocities were calculated from the data in (a).
PNPG-RESISTANT P. MIRABILIS SUPER-SWARMING MUTANTS

Fig. 3. The swarming migration distance of the wild-type (P19; –○–) and the super-swarming mutant (P1100; –●–) in the presence of PNPG 80 µg/ml. The experiments were performed in triplicate and the mean migration distance was plotted against incubation time.

Cloning and analysis of the mutated gene

The nucleotide sequence was obtained from each of the cloned DNA fragments flanking the transposon in the three super-swarming mutants (P1100, P1301 and P2399) and the open reading frames were identified. As shown in Fig. 4, the locus was found to contain three open reading frames and to have 99% nucleotide sequence identity to the rsbA-rscB-rscC locus [30]. rsbA may encode a membrane sensory protein with similarity to the histidine kinases of the bacterial two-component signalling system [31]. rscC and rcsB may encode proteins highly homologous to E. coli RscC and RcsB, which are members of a two-component regulatory circuit controlling capsular synthesis [32], in which RscC is a histidine kinase and RcsB is its cognate response regulator. DNA sequence analysis of the cloned Tn5-containing fragments indicated that Tn5 was inserted into the rsbA gene in all three super-swarming mutants (Fig. 4). To investigate whether these mutants contained other Tn5 insertion sites in their chromosomes, Southern blot analyses were performed. As shown in Fig. 5, all three super-swarming mutants contained a single fragment of c. 4.2 kb that hybridised to the probe. The sizes of the hybridised fragments corresponded to the sizes predicted from the SphI restriction map: 4.27 kb for mutant P1100 and mutant P1301 and 4.12 kb for mutant P2399 (Fig. 4). This result indicated that the mutants contained a single Tn5 insertion site within the rsbA gene. As they exhibited more aggressive swarming than the wild-type strain both in the presence and absence of PNPG, the data suggest that RsbA may be involved in the regulation of swarming and PNPG may target this protein or its regulated pathways to inhibit swarming.

Cell differentiation and expression of virulence factors in the super-swarming mutants

Swarming differentiation is regulated co-ordinately with the expression of virulence factors and both are

Fig. 4. Location of the Tn5 insertion in the P. mirabilis chromosome. Vertical arrows indicate the Tn5 insertion sites of the three super-swarming mutants (P1100, P1301 and P2399) isolated in this study. Vertical thin lines indicate the SphI restriction sites within the rsbA gene.

Fig. 5. Southern blot analysis of the super-swarming mutants. Genomic DNA of the wild-type (P19) and super-swarming mutants (P1100, P1301 and P2399) was digested with SphI and hybridised with a probe specific for the mini-Tn5 Cm-resistant gene.
related to the swarming behaviour of *P. mirabilis* [10–12, 24]. Knowing that swarming behaviour was altered in the super-swarming mutants, tests were made to determine whether swarming differentiation and expression of virulence factors, such as haemolysin, protease, urease and flagellin, were also altered in these bacteria. The wild-type strain (P19) and the super-swarming mutant (P1100) were spread on to LB swarming plates without PNPG, and cell length and expression of virulence factors were determined 2 h after seeding and hourly thereafter. As shown in Fig. 6, in the absence of PNPG, the super-swarming mutant P1100 formed longer cells than the wild-type strain during the 7-h incubation period and became the longest about 1 h earlier than wild-type cells. Moreover, the P1100 mutant retained its elongated cell shape for longer than the wild-type strain. These data indicate that the super-swarming mutant differentiated earlier and maintained a differentiated state for longer than the wild-type strain. In parallel with the above characteristics, the P1100 mutant also expressed higher levels of haemolysin, protease, urease and flagellin than the wild-type strain and they reached maximal expression levels c. 1 h earlier than in the wild-type strain (Fig. 6). Moreover, the P1100 mutant retained high expression levels of these factors for longer than the wild-type strain (Fig. 6). Furthermore, swarming differentiation and virulence factor expression was co-ordinately regulated both in the wild-type strain and the P1100 mutant (Fig. 6).

The abilities of the wild-type strain (P19) and super-swarming mutant (P1100) to differentiate and to express virulence factors in the presence of PNPG were examined. As shown in Fig. 7, swarming differentiation was completely blocked and the expression of virulence factors was inhibited to the basal level by PNPG in the wild-type strain. In contrast, the super-swarming P1100 mutant could differentiate to a long cell morphology in the presence of PNPG, but at a

![Fig. 6. Cell differentiation and expression of virulence factors after plating of the wild-type (P19; ■) and the super-swarming mutant (P1100; □) on the LB agar plates without PNPG. a, Haemolysin; b, protease; c, urease; d, cell length – the increase in cell length was taken as a sign of cell (swarming) differentiation; e, flagellin. For protease activity measurements, the value obtained with the wild-type cells at 5 h after seeding was set at 100% and all other values were expressed relative to this value. For all other measurements, the values obtained with the wild-type cells at 4 h after seeding were set at 100%. The data represent the average of three independent experiments with standard deviation.](image-url)
Fig. 7. Cell differentiation and expression of virulence factors after plating the wild type (P19; ■) and the super-swarming mutant (P1100; □) on the LB agar plates with PNPG (80 μg/ml). a, Haemolysin; b, protease; c, urease; d, cell length; e, flagellin. For each measurement, the values obtained in the presence of PNPG were compared to that obtained with the wild-type at 5 h (for protease measurement) or 4 h (for all other measurements) after seeding in the absence of PNPG (the latter value was set at 100%). The data represent the average of three independent experiments with standard deviation.

slower rate and to a shorter cell length than in the absence of PNPG (compare Figs. 6 and 7). The P1100 mutant could also express virulence factors in the presence of PNPG, but at much lower levels than in its absence (compare Figs. 6 and 7).

**Cell invasion ability in the super-swarming mutants**

Swarming differentiation and expression of virulence factors are correlated with the ability of *P. mirabilis* to invade cells [10, 11, 24]. Therefore, the cell invasion ability of the super-swarming mutants was investigated. As shown in Table 2, the super-swarming mutant P1100 had a greater cell invasion ability than the wild-type strain at all stages of cell differentiation, and especially at the late stage (7 h) during which cells of the wild-type strain had undergone de-differentiation. The cell invasion abilities of the wild-type strain were at their highest at 4 h, at which time the cells had differentiated into long cells, and at their lowest at 7 h by which time the cells had reverted to short cells.

**Table 2. Invasion of uroepithelial cells by *P. mirabilis* strains**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Characteristics</th>
<th>2 h (SD)</th>
<th>4 h (SD)</th>
<th>7 h (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P19</td>
<td>Wild-type <em>P. mirabilis</em></td>
<td>0.14 (0.01)</td>
<td>0.20 (0.013)</td>
<td>0.10 (0.01)</td>
</tr>
<tr>
<td>P1100</td>
<td>Tn5 insertion <em>rshL4</em> mutant</td>
<td>0.17 (0.013)</td>
<td>0.29 (0.020)</td>
<td>0.18 (0.01)</td>
</tr>
</tbody>
</table>

Values represent the mean (SD) of three experiments.
Complementation of the super-swarming mutant by rshA

Because the Tn5 insertion sites in the super-swarming mutants were scattered over rshA (Fig. 4), the super-swarming phenotypes of these mutants probably arose from the defectiveness of the rshA gene. If this inference is correct, then provision of RshA to the super-swarming mutants should restore wild-type swarming behaviour. To test this, plasmid pSI228, which encoded a full-length RshA protein, was transformed into the super-swarming mutant (P1100) to generate an RshA-complemented strain (Pc). As shown in Fig. 8a and b, in the absence of PNPG, the P1100 mutant exhibited a super-swarming phenotype as described above, whereas the Pc strain exhibited a similar swarming behaviour to the wild type (P19). The Pc strain also exhibited a pattern of expression of haemolysin similar to the wild type, in marked contrast to the super-swarming mutant P1100, which exhibited a super-expression pattern (Fig. 8c). Similar changes were found when the other two super-swarming mutants were transformed with plasmid pSI228 DNA. Taken together, these data indicate that expression of RshA in the super-swarming mutants led to the restoration of the wild-type swarming-related phenotype and suggest that the super-swarming phenotype arose from the defectiveness of the rshA gene, and not from other mutations in the chromosome.

Discussion

Swarmer cell differentiation and swarming behaviour are the results of complex sensory transduction and global control mechanisms. Three temporally discrete stages are evident in the multicellular swarming migration of *P. mirabilis*: (i) the initiating event of swarmer cell formation, presumably involving signal recognition and triggering of morphological differentiation of vegetative cells; (ii) the co-ordinated migration of the differentiated swarmer cell population; and (iii) the consolidation of swarmer cells under the influence of signals for swarmer cell de-differentiation and termination of swarming migration. Many *P. mirabilis* mutants that are defective in different aspects of swarming differentiation and multicellular migration have been isolated by Tn5 or TnphoA transposon mutagenesis [6, 23, 25, 33–35]. In this study, three Tn5 insertion mutants that swarmed, albeit with much reduced ability, in the presence of PNPG (an effective swarming suppression agent), were isolated (Fig. 1A). In the absence of PNPG the mutants exhibited a super-swarming phenotype; i.e., they migrated further in a given time than did the wild-type cells (Fig. 1B), initiated swarming earlier than the wild-type strain, and had a less conspicuous consolidation phase (migration did not completely stop during the consolidation phase, Fig. 2). The mutants also differentiated earlier and maintained a differentiated state longer on LB swarm-

Fig. 8. Complementation of the super-swarming mutant by rshA. Plasmid pSI228, which encodes the full-length RshA protein, was transformed into the super-swarming mutant (P1100) to generate an RshA-complemented strain (Pc). The swarming migration distance (a), the interval migration velocity (b) and the haemolysin activity (c) of the wild-type (P19; ■, △, □) and the RshA-complemented strain (Pc; ▽, ●, ○) at various times after inoculation in the absence of PNPG were measured as described in Fig. 2 and Fig. 6. Cloning and sequence

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analysis of the Tn5-inserted DNA indicated that, in all three mutants, Tn5 was inserted into the rshA gene (Fig. 4), a gene which may encode a membrane sensor histidine kinase of the bacterial two-component signal-
ing system [30]. Because these Tn5-inserted rshA mutants were altered in both swarming differentiation and expression of virulence factors, RsbA may act as a membrane sensor that regulates a hierarchical network that controls swarming differentiation and expression of virulence factors. PNPG inhibited swarming differentiation and virulence factor expression in the wild-type strain, but could not do so in the rshA-defective super-
swarming mutants (Fig. 7), suggesting that PNPG may target RsbA or RsbA-regulated pathways to exert its inhibitory effects. Together, these studies identify a  
protein (RsbA) that may be involved in regulation  
of swarming differentiation and virulence factor expres-
sion and illustrate a possible mechanism underlying PNPG swarming-inhibitory effects.

Although the super-swarming mutants contained a  
single Tn5 insertion in the rshA gene (Fig. 5), because the rcsB gene is located downstream of rshA, and is transcribed in the same direction (Fig. 4), it was possible that Tn5 insertion in rshA may affect rcsB expression via polar effects. However, complementation experiments in which expression of rshA alone in the super-swarming mutants restored wild-type swarming  
behaviour and wild-type pattern of virulence factor expression (Fig. 8) indicated that the super-swarming phenotype was caused primarily by alteration of rshA function.

The super-swarming mutants differentiated and expressed flagellin earlier than the wild-type strain on LB swarming plates (Fig. 6), causing them to start swarming 30 min earlier than the wild type (Fig. 2). Furthermore, the super-swarming mutants retained a differentiated state, with high expression levels of flagellin, for longer than the wild type (Fig. 6), causing them to have a less conspicuous and shorter conserva-
tion phase. By spending less time in the initial lag period and in the consolidation phase, the super-
swarming mutants thus translocate further in a given time than wild-type cells. The maximal expression  
level of flagellin was similar in the super-swarming  
muts and the wild type cells (Fig. 6), and this was consistent with the finding that the maximal migration velocities of these strains were similar (Fig. 2). This suggests that the super-swarming mutants have normal flagellar function and that the rshA mutation does not affect flagellar rotation, and is supported by the observation that the super-swarming mutants swim normally in semi-solid agar (unpublished observations).

Because Tn5 insertion sites in the super-swarming mutants were scattered over the rshA gene (Fig. 4), it is unlikely that the super-swarming phenotypes were caused by truncated RsbA proteins, but more probable that they resulted from knockout mutations in the rshA gene. If this inference is correct, rshA should encode a repressor that inhibits swarming differentiation and  
virulence factor expression. Alternatively, rshA may encode a positive regulator of swarming and the super-
swarming phenotypes may be caused by truncated versions of RsbA that more actively activate swarming-
related pathways. However, overexpression of RsbA in the super-swarming mutants led to the restoration of a wild-type swarming phenotype (Fig. 8). Moreover, when RshA was overexpressed in wild-type P. mirabilis P19, the RsbA-overexpressed cells were found to have a lower migration velocity and initiated swarming about 1 h later than the wild-type cells (unpublished observations).

Together, these data argue against the proposition that RsbA is a positive regulator of swarming and that the super-swarming phenotype is caused by truncated RsbA. Rather, they suggest that RsbA is a repressor of  
swarming and that the super-swarming phenotype is a consequence of a knockout mutation in rshA.

Quorum sensing has been reported to regulate the expression of virulence factors in many pathogenic bacteria [14, 18] and swarming behaviour in Serratia liquefaciens [17, 18]. In S. liquefaciens, N-butanolyl-L-

homoserine lactone and N-hexanoyl-L-homoserine lac-
tone act as diffusible signal molecules that positively  
regulate swarming [17], whereas DKPs, such as cyclo-
(L-Pro-L-Tyr) and cyclo(Ala-L-Val), can inhibit  
swarming [19]. Although a quorum-sensing system has not been identified in P. mirabilis, there is some evidence that its swarming and expression of virulence factors is regulated through quorum sensing [12, 19]. In this  
respect, it is worth noting that P. mirabilis can produce extracellular DKPs, including cyclo(L-Pro-L-

Tyr) and cyclo(Ala-L-Val) [19], that inhibit swarming  
[19]. It is possible that RsbA, a membrane sensor, may bind DKPs or other swarming-inhibitory signal mole-
cules and transduce an inhibitory signal to a hierarchi-
cal regulatory network that controls swarming dif-
ferentiation and expression of virulence factors.  
Although not proven, this model is attractive because it can explain why P. mirabilis cells undergo de-
differentiation (consolidation) after a wave of active  
migration. PNPG may act as a homologue of swarm-
ing-inhibitory signal molecules, such as DKPs, and  
inhibit swarming and virulence factor expression  
through binding to RsbA. The swarming velocity of the rshA-mutated super-swarming mutants was much lower in the presence than in the absence of PNPG (compare Figs 2 and 3), indicating that PNPG can still inhibit swarming to some extent in these mutants. This suggests that PNPG may act on pathways other than  
RsbA to inhibit swarming. Experiments aiming to  
identify other PNPG targets are in progress.

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