附件一

行政院國家科學委員會補助專題研究計畫 ☑成果報告 □期中進度報告

（計畫名稱）Study of swarming inhibition of *Proteus mirabilis* by p-nitrophenyl glycerol and homoserine lactones with emphasis on bacterial pathogenicity

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計畫中文摘要：關鍵詞：*Proteus mirabilis*, 致病因子, 表面移行能力, PNPG, *rsb*, hsl
研究顯示奇異變形桿菌 (*Proteus mirabilis*) 之表面移行能力和導致尿路感染有密切的關係，而且也觀察到 *P. mirabilis* 在分化為表面移行細胞的同時也伴隨著許多致病因子的表現,例如: 蛋白腌, 尿素腌, 溶血腌及鞭毛蛋白等...一種抗表面移行的化合物 "PNPG (p-nitrophenylglycerol)" 長久以來被用來分離遭 *Proteus* spp. 污染的病原菌, 但其作用機轉至今未明. 利用 miniTn5 作 mutagenesis 發現一突變株能在含有 100μg/ml PNPG 的培養基上生長, 作序列分析發現遭破壞的基因為一細菌 two-component system 成員 sensor 之相似物. 目前已成功選殖此基因, *rsb* (regulator of swarming behavior). 另一方面 細菌 quorum sensing 之訊息分子 hsl (homoserine lactone) 也被發現與表面移行能力有關, 現已知 hsl 會抑制奇異變形桿菌之表面移行能力. 若能了解 PNPG 或 hsl 抑制奇異變形桿菌表面移行能力之機轉, 將有利於設計藥物對抗此菌. 故本計畫進行如下: (I) *rsb* (PNPG 抑制奇異變形桿菌表面移行之可能媒介者) 基因功能之探討. 初步結果顯示 *rsb* 突變株之表面移行能力比野生株強,在固體培養基之表面移行模式也不同於野生株之同心圓樣式,而其haemolysin 活性高於野生株. 為了進一步了解 *rsb* 在細菌体內所扮演的角色 擬將此基因放入野生型 *P. mirabilis* 中大量表現. 分析大量表現菌株, 突變株及野生株各種表現型的差異, 包括生長, 分化, 泳動, 表面移行能力, biofilm 形成能力及各種致病因子的表現等...並進行老鼠動物實驗比較大量表現菌株, 突變株及野生株之毒力,藉由這些實驗來了解 *rsb* 如何影響表面移行能力及致病因子的表現. (II) hsl 抑制奇異變形桿菌表面移行能力之探討. (i) 找出 *P. mirabilis* hsl 訊息分子並找出轉譯合成 hsl 之基因. (ii) 確定不同 hsl 影響奇異變形桿菌表面移行之現象. (iii)探討 hsl 對 PNPG-resistant mutant 之影響. (iv) 探討 hsl 與 PNPG 之交互作用. (iv)分析受 hsl 影響之 PNPG-resistant mutant。經由揭開 PNPG 及 hsl 抑制 *P. mirabilis* 表面移行能力之謎將對表面移行有更進一步的了解也使 *P. mirabilis* 的致病機轉更為明朗而能有助於疾病之控制.

Keywords：*Proteus mirabilis*, swarming, virulence factor, PNPG, *rsb*, long chain hsl

Proteus mirabilis is an important pathogen of the urinary tract, several potential virulence factors are responsible for the pathogenicity of *P. mirabilis*. *P. mirabilis* exhibits a form of multicellular behavior known as swarming migration. It has been demonstrated that coordinate expression of virulence factors was coupled to motility and swarming differentiation. The anti-swarming agent p-nitrophenylglycerol (PNPG) has long been used to aid the isolation of small numbers of many different pathogenic bacteria from specimens contaminated with swarming strains of *Proteus* spp. Although the anti-swarming activity of PNPG on *P. mirabilis* has been known for many years, the underlying inhibitory mechanism remains unclear. Many gram-negative bacteria have been reported to use quorum-sensing (q-s) systems to coordinate activity within a population by producing extracellular signal compounds (homoserine lactone, hsl) that, when present during appropriate conditions and in sufficient quantities, trigger specific responses. Till now, no quorum-sensing system has not been identified in *P. mirabilis*. Preliminary data showed that some long chain hsls can inhibit the swarming behavior of *P. mirabilis*. By understanding the swarming inhibition mechanisms of PNPG and the long chain hsl, it will do help to the treatment of *P. mirabilis* infections. In a previous study, we found that PNPG, added to inhibit swarming differentiation, can also inhibit the ability of *P. mirabilis* to express virulence factors. To investigate the mechanism by which PNPG inhibits swarming, and to study further the intercellular signaling and genetic regulation of swarming in *P. mirabilis*, we performed mini-Tn5 transposon mutagenesis and isolated a mutant which was resistant to the inhibitory effect of PNPG. The mutant could swarm in the presence of PNPG, and have a higher haemolysin activity than the wild-type strain. The sequence was found to be the *rsb* (regulator of swarming behavior) gene, a gene which may encode a sensory protein with similarity to the histidine kinases of the bacterial two-component regulatory system. The presence of *rcsC-rcsB*, which is involved in the regulation of CPS (capsular polysaccharide) synthesis in many G (-) bacteria , in the vinicity of *rsb*, suggests the function of *rsb* may be related with CPS synthesis. In addition, it has been demonstrated that the synthesis of CPS is an important determinant of biofilm formation and bacterial biofilms are common causes of persistent infections. In order to further investigate the swarming inhibition in *P. mirabilis* by PNPG and the long chain hsl, we performed the following experiments: (1) we overexpressed the *rsb* gene in wild-type *P. mirabilis*, then differentiation, swarming, swimming, growth, and the expression of virulence factors, including biofilm formation, were characterized in this strain, comparing to those of the mutant, and the wild type *P. mirabilis*. (2) we compared the virulence among the mutant, the overexpressed and the wild type *P. mirabilis* in a mouse model. (3) we tried to identify hsl type signal molecules in *P. mirabilis*. (4) we monitored the effect of various hsls on the swarming of *P. mirabilis*. (5) we analyzed the effect of hsls on PNPG-resistant mutants. (6) we investigated the interaction of hsl and PNPG on the swarming behavior of *P. mirabilis*. (7) we characterized the PNPG-resistant clone that loses the swarming inhibitory phenomenon when certain hsls were present. These experiments will disclose the role of *rsb* and the long chain hsl in the swarming control and the virulence factor expression of *P. mirabilis*.

研究計畫之背景及目的：*Proteus mirabilis* is an important pathogen of the urinary tract, especially in patients with indwelling urinary catheters [1]. Several potential virulence factors may be responsible for the pathogenicity of *P. mirabilis*. *P. mirabilis* exhibits a form of multicellular behavior known as swarming migration. The ability of *P. mirabilis* to differentiate into swarming cells capable of rapid surface migration plays an important role in renal infections which involve colonization of the lower urinary tract followed by ascending migration of bacteria [2]. The presence of a lag period prior to swarming behavior and the coordination of the swarming colony suggests that some form of cell-cell interaction and communication occurs to control these processes. Coordinate expression of virulence factors during swarm-cell differentiation and population migration of *P. mirabilis* was observed [3]. The anti-swarming agent p-nitrophenylglycerol (PNPG) has long been used to aid the isolation of small numbers of many different pathogenic bacteria from specimens contaminated with swarming strains of *Proteus* spp. [4]. Although the anti-swarming activity of PNPG on *P. mirabilis* has been known for many years, the underlying inhibitory mechanism remains unclear. To investigate the mechanism by which PNPG inhibits swarming, and to study further the intercellular signaling and genetic regulation of swarming in *P. mirabilis*, we performed mini-Tn5 transposon mutagenesis and isolated a mutant which was resistant to the inhibitory effect of PNPG. The mutant could swarm in the presence of PNPG, albeit with a reduced ability, and exhibited a super-swarming phenotype in the absence of PNPG (Fig 1) and have a higher haemolysin activity than the wild-type strain (Fig 3). The sequence was found to be the *rsb* (regulator of swarming behavior) gene (a homologue of bacterial two-component sensor kinase). Populations of many bacteria exhibit abilities that extend beyond the individual cells. To coordinate activity within a population, bacteria communicate among themselves by producing extracellular signal compounds that, when present during appropriate conditions and in sufficient quantities, trigger specific responses. Many gram-negative bacteria have been reported to use quorum-sensing (q-s) systems to coordinate activity within a population [5,6]. Although a quorum-sensing system has not been identified in *P. mirabilis*, some evidence points to this mechanism for regulating swarming and expression of virulence factors in this bacterium [7]. In this respect, it is worth noting that *P. mirabilis* can produce extracellular diketopiperazines (DKPs) which may act as signal molecules that regulate quorum sensing [7]. In addition, preliminary data indicate that the long chain hsl (C12) can inhibit the swarming of *P. mirabilis*. In gram-positive bacteria, the molecular mode involved in the quorum-sensing modules is mediated by peptide pheromones and two-component regulatory systems in which the extracelluar signal is sensed by the two-component sensor [8]. If *rsb* can sense the long chain hsl in *P. mirabilis*, it will be judged by the mutant selection experiment.

By understanding the swarming inhibition mechanisms of PNPG and the long chain hsl, it will do help to the treatment of *P. mirabilis* infections. Based on the knowledge given above, we performed the following experiments: (1) we overexpressed the *rsb* gene in wild-type *P. mirabilis*, then differentiation, swarming, swimming, growth, and the expression of virulence factors, including biofilm formation, were characterized in this strain, comparing to those of the mutant, and the wild type *P. mirabilis*. (2) we compared the virulence among the mutant, the

overexpressed and the wild type *P. mirabilis* in a mouse model. (3) we tried to identify hsl type signal molecules in *P. mirabilis*. (4) we monitored the effect of various hsls on the swarming of *P. mirabilis*. (5) we analyzed the effect of hsls on PNPG-resistant mutants. (6) we investigated the interaction of hsl and PNPG on the swarming behavior of *P. mirabilis*. (7) we characterized the PNPG-resistant clone that loses the swarming inhibitory phenomenon when certain hsls were present.

研究方法

Part I: Characterization of *rsb* gene:

1. Overexpress the *rsb* gene in wild-type *P. mirabilis*, then characterizethe phenotypic traits in this strain, comparing to those of the mutant, and the wild type *P. mirabilis*.
2. Compare the virulence among the mutant, the over-expressed and the wild type *P. mirabilis* in a mouse model.

Part II: Investigation of the swarming inhibition in *P. mirabilis* by hsls

1. we tried to identify hsl type signal molecules in *P. mirabilis*..
2. we monitored the effect of various hsls on the swarming of *P. mirabilis*.
3. we analyzed the effect of hsls on PNPG-resistant mutants.
4. we investigated the interaction of hsl and PNPG on the swarming behavior of *P. mirabilis*.
5. we characterized the PNPG-resistant clone that loses the swarming inhibitory phenomenon when certain hsls were present.

Transposon mutagenesis. Transposon mutagenesis. *P. mirabilis* super-swarming mutants were constructed by mini-Tn5 Cm (chloramphenicol) mutagenesis as described previously [9].

Cloning and sequencing of the mutated gene. The mutant genomic DNA were partially digested with *Alu*I, and fragments larger than 4 kb were cloned into *Eco*RV-digested pZErO-2.1. Following transformation of *E. coli* TOP10, chloramphenicol-resistant Tn5 Cm-containing clones were selected. The nucleotide sequences of the cloned DNA fragments were determined using a 373A DNA sequencer.

Swarming behavior assays. The swarming migration distance and interval migration velocity assays were performed as described previously [10]. Briefly, an overnight bacterial culture (5 μl) was inoculated centrally onto the surface of the dried LB swarming plates containing 2.0% (w/v) agar, in the presence and absence of PNPG or various chain hsl.

Cell differentiation assay, assay of flagellin and haemolysin, urease, and protease activities. All were determined as described [11]

Biofilm formation assay. Biofilm formation was assayed by the ability of cells to adhere to the wells of 96-well microtitre dishes made of PVC (Falcon 3911; Becton Dickinson). LB broth was inoculated with cells from a 1:100 dilution from an overnight LB culture. After inoculation, plates were incubated at 30 ℃ for 10 h, then 25 μl of a 1% solution of crystal violet (CV) was be added to each well (this dye stains the cells but not the PVC), the plates were incubated at room temperature for approximately 15 min, rinsed thoroughly and repeatedly with water. CV stained biofilm was solubilized in 95% ethanol then transferred to a new microtitre well and the absorbance was determined with a plate reader at 600 nm.

CBA mouse model of ascending UTI. A mouse model of ascending UTI described by Johnson

et al. [12] was used. Mice, tested for the absence of bacteriuria, were anaesthetized and inoculated with the mutant, the over-expressed or the wild type *P. mirabilis* through a sterile catheter inserted into the bladder through the urethra. After 1 week, the mice were killed by administration of an overdose of methoxyflurane. Urine will be collected and the bladder and both kidneys will be removed. Each sample was quantitatively cultured, and viable counts were determined as c.f.u. (ml urine)$^{-1}$ or c.f.u. (g tissue)$^{-1}$.

偵測 *P. mirabilis* AHL 類訊號分子. 首先以 *E. coli* JM109(pSB401) 及 *Chromobacterium violaceum* CV026 偵測系統測定 *P. mirabilis* 是否具 AHLs 訊號分子 [13]。我測試 *P. mirabilis* P19 及 160 株臨床上引起尿路感染的 *P. mirabilis* 菌株，觀察其產生 AHLs 分子的情形。 另外我也將 *P. mirabilis* P19 培養過夜之上清液 (10 ml)， 以等體積 ethyl acetate 萃取二次， 蒸乾後以 150 µl methanol 溶之， 取 5~100 µl， 加入 1.2 ml 之 *E. coli* JM109(pSB401) 培養液(OD_{600nm} = 0.9)， 於 37℃ 培養 30 分鐘, 然後測其發光情形。

***P. mirabilis luxI* 相似基因之選殖.** *P. mirabilis* 染色體 DNA 以不同酵素切割後， 與 pBluescriptII KS 接合，送入 *E. coli* JM109(pSB401)偵測菌株內，以 X 光壓片法找尋會發光的菌落，並用 luminometer 法確定其發光。 然後抽取質體，進行序列分析。此外也從已發表的 *luxI* 相似基因 *swrI* 及 *carI* 〔Eberl *et al.*, 1996b〕的保守序列， 設計一對 PCR 引子，IF：5'AGACTGGCTCGTTAATTGTGA3' 及 IR：5'CCACCGAGA- TATGCCAACCGG，用以放大 *P. mirabilis* 之 *luxI* 相似基因。 將 PCR 片段定序，確定其為 *luxI* 相似基因後，利用此片段當 probe 進行 Southern-blot 實驗。將 *P. mirabilis* 染色體 DNA 以不同酵素切割後進行 Southern-blot, 以選取可能含 *luxI* 相似基因的 DNA 片段， 並將此片段與載體接合，送入 *E. coli* Top10 中進行基因選殖。

各種 AHLs 訊號分子對 *P. mirabilis* 表面移行的影響. 本實驗所用 AHLs 訊號分子由 Paul Williams 在英國的實驗室所合成，AHL 相關資料列於 Table 1。 首先將各個訊號分子以 acetonitrile 調成 10 mM 濃度，加到表面移行培養基之最終濃度為 100 µM，將 *P. mirabilis* P19 接種到含 AHL 或不含 AHL 的培養基中，然後觀察其表面移行。 若對表面移行能力有影響，這些 AHL 訊號分子，將被加到 seeding plates，測定其對細菌溶血素、蛋白酶、尿素酶活性及細菌長度的影響。

結果

Characterization of the phenotypic traits in the wild type (P19), the mutant (P1100), and the RsbA overexpression strain (Pov)

The migration velocity and cycle frequencies

In Fig. 2, after 2.5h, swarming migration was observed in P1100, but the start of swarming in P19 was visible only 30min later. Migration initiation of Pov is delayed further 30min. The mutant moved out farther than the wild type and Pov over a given time and the consolidation zone was less conspicuous. The migration velocity of P19 and P1100 was comparable. Translocation speed was observed to increase after initiation of migration to peak at about 1.4 cm/h and 1.2 cm/h in the first and the second swarming cycle respectively. The most conspicuous difference between P19 and P1100 is the difference in time spent during the initial lag phase of swarming migration. P1100 spent about 30 min less in this phase. The migration pattern of Pov is similar to that of P19, but at a reduced rate.

Alteration of expression of flagellin, haemolysin, protease, and urease, and cell length (Fig. 3).

Alterations of the expression of these virulence factors and cell elongation in P19 and Pov followed an identical pattern, being highest at time 4h for flagellin, haemolysin, urease, and cell length or 5h for protease, then decreasing toward 2h and 7h. The expression of these virulence factors in Pov was slightly lower than that in P19 strain. The P1100 exhibited highest expression of flagellin, haemolysin, urease, at time 3h & 4h, and at time 4h & 5h for protease. The change of cell length for P1100 followed the tendency as that of virulence factors. In addition, the mutant exhibited longer cell morphology during the differentiation cycle than P19 or Pov, and the time to reach maximal expression was 1h earlier than P19 or Pov. The duration of high expression was also longer in the mutant. In conclusion, the level of expression of all virulence factors parallels with the cell length alteration and the mutant cell differentiate earlier and the differentiated form is present for a longer time than P19 or Pov.

P1100 Biofilm 形成的能力：結果顯示 P1100 biofilm 之形成比 P19 及 Pov 少，而 Pov 與 P19 形成 biofilm 的能力相當 (Fig. 4)。老鼠實驗看不出 P19, P1100, Pov 致病力之差別。

偵測 *P. mirabilis* AHLs 的存在：以 *P. mirabilis* P19 及 160 株臨床菌株為實驗對象，不管是細菌培養上清液或經萃取濃縮的細菌培養上清液，皆無法使 *E. coli* JM109(pSB401)發光情形明顯增加，顯示這些 *P. mirabilis* 菌株的培養上清液中可能無 AHL 類的訊號分子存在。將以上菌株與 *C. violaceum* CV026 做 co-streaking，也未見藍色色素產生，再次顯示這些菌株可能無法分泌 AHL 類的訊號分子。最後我將細菌打破，取其 lysate〔13〕，分別以 *E. coli* JM109(pSB401) 或 *C. violaceum* CV026 測試菌株偵測 AHL 類的訊號分子，結果也測不到 AHLs 的存在。

***luxI* 相似基因之選殖**：雖然以 *E. coli* JM109(pSB401) 及 *C. violaceum* CV026 測試菌株皆無法證明 *P. mirabilis* 可以產生 AHL 類訊號分子，但這並不表示 *P. mirabilis* 確實無法產生 AHL，有相當多的原因可能讓此二偵測系統無法偵測出 *P. mirabilis* 的 AHL，例如此二系統的敏感度可能不夠。因此嘗試直接選殖 *luxI* 相似基因。當 *P. mirabilis* P19 之基因庫以 pBluescriptII KS 送入 *E. coli* JM109(pSB401) 偵測菌株後，以 X 光壓片法尋找發光菌落，很多發光菌落被找到，但以 luminometer 測定其發光情形時，發現其和只含載體的控制組菌落無明顯差異，因此可能是假陽性的放光。我也將此基因庫送入 *E. coli* Top10 宿主細胞，挑選抗 ampicillin 的菌落，然後將這些菌株和 *C. violaceum* CV026 做 co-streaking，藉以篩選可以使 *C. violaceum* 變藍色的菌株。我共篩選了約 6000 個抗 ampicillin 的菌株，但無一菌株會讓 *C. violaceum* CV026 變藍。接下來嘗試利用由 *luxI* 相似基因所設計的引子，以 *P. mirabilis* P19 染色體 DNA 為模板放大 *luxI* 相似基因，我得到 4 個可能含 *luxI* 相似基因的 DNA 片段，其大小分別為 1 kb、0.9 kb、0.6 kb 及 0.3 kb，但經序列分析比對後，證實它們不是 *luxI* 相似基因。

各種 AHLs 訊號分子對 *P. mirabilis* 表面移行的影響：由於未能成功找到 *P. mirabilis* 之 AHL 訊號分子及其合成的基因，我於是利用 Paul Williams 實驗室所合成的 AHL 類訊號分子 (Table 1)，觀察這些合成的 AHL 對 *P. mirabilis* 的影響。首先觀察這些合成的 AHL 分子對 *P. mirabilis* 表面移行的影響。由 Fig. 5 可知在所用的 AHL 訊號分子中 OC4、OC8、OC12 及 OC14 會促進表面移行，而 OC10 及 C12 則扮演抑制的角色。其餘如 C4、C6、OC6、C8 及 C10 則對表面移行無影響。由於表面移行與致病因子的表現有關，所以接下來測試 OC14 及 C12 對細胞分化、溶血素、蛋白胨及尿素胨等活性的影響。由 Fig. 6 可見 OC14 對細胞長度、溶血素等致病因子的表現有些微的促進作用，而 C12 則可明顯地抑制細胞變長及溶血素等致病因子的表現 (Fig. 7)。可見 OC14 及 C12 在調控表面移行的同時，也調控了致病因子的表現。

合成的 AHL 分子對 PNPG-resistant *P. mirabilis* 突變株表面移行的影響：為了了解 PNPG-resistant

P. mirabilis 突變株基因缺陷所在是否和 quorum-sensing 訊息傳遞有關， 於是測試 OC4、OC8、OC12、OC14、OC10 及 C12 (最終濃度為 100 μM，已知這些 AHL 分子會影響 *P. mirabilis* 野生株的表面移行) 對 18 株 PNPG-resistant 突變株表面移行的影響。 由 Table 2 可知 AHL 對各個突變株的影響，可以是促進，抑制或沒有影響，未來將挑選表面移行不能被 OC4、OC8、OC12 及 OC14 促進，或不能被 OC10 及 C12 抑制的抗 PNPG 突變株，找出其基因缺陷所在，以了解這些 AHL 訊號分子分別透過什麼基因來調控表面移行。

AHL 與 PNPG 對表面移行的影響：

由於 PNPG 會抑制 *P. mirabilis* 之表面移行，而 OC14 可以促進表面移行， 於是想了解 OC14 是否可以頡抗 PNPG 的抑制作用？ 故在分別只有 PNPG 及 PNPG／AHL 同時存在下比較 *P. mirabilis* P19 表面移行情形。由 Table 3 可知，當 OC14 濃度愈高時 （120 μM 對 70 μM)， 其頡抗 PNPG 抑制作用的能力愈強，所以細菌表面移行起始的時間也提早了。

第四節 討論

許多致病菌藉由與細胞密度有關的 Q-S 系統來調控致病因子的表現，Q-S 系統所調控的表現型包括表面移行，細胞外酵素的分泌、接合作用、抗生素的產生等等。細菌藉由 Q-S 系統來協調群體行為，有利於感染的進行，科學家們正致力於開發抑制 Q-S 系統的藥物以阻斷細菌感染的發生。此外 *P. aeruginosa* Q-S 系統的訊號分子 OC12 已被發現具有調節宿主免疫力的活性。 可見 Q-S 訊號分子在致病菌-宿主之間交互作用扮演重要角色。

由於目前尚無 *P. mirabilis* Q-S 系統被發現， 因此著手找尋 *P. mirabilis* 之訊號分子，雖然本實驗無法找到 *P. mirabilis* 產生之訊號分子，也無法成功選殖出 *luxI* 相似基因，但發現合成的 AHLs 可促進或抑制 *P. mirabilis* 表面移行的現象。 其中 OC14、C12 促進或抑制表面移行的情形和它們調控致病因子表現的情形有一致的現象。 無法找到 *P. mirabilis* AHL 類訊號分子的可能原因如下： 一、 *P. mirabilis* 上清或菌體所含的 AHL 訊號分子太少，以 *E. coli* JM109(pSB401) 或 *C. violaceum* 偵測菌株皆無法偵測到。 二、 也許 *P. mirabilis* 所產生的訊號分子非 AHL 類， 但一樣具有促進或抑制表面移行及相關 phenotype 的功能。 現已發表的 Q-S 訊號分子除了 AHL 類外，尚有 PQS、DKPs、A factor、PAME、環形胜肽類，及 *luxS* 所合成的訊號分子。也許 *P. mirabilis* 的訊號分子是其中一種， 也可能是尚未被發表的訊號分子。往後要致力於萃取訊號分子方法的改良，例如加大培養體積， 改用 dichloromethane 萃取，或改用 LasR/I (pSB1075) 或 RhlR/I (pSB406) 偵測系統 以偵測不同類型的 AHL 分子, 同時尋找其他非 AHL 類訊號分子。

本實驗所用的十一種合成 AHLs 中，C4、C6、OC6、C8 及 C10 等五種對 *P. mirabilis* 之表面移行沒有影響，而 OC4、OC8、OC12 及 OC14 則有促進的作用， 其中 OC12 及 OC14 為長鏈分子（10 個碳以上)，而 OC4 及 OC8 為短鏈訊號分子。而具抑制作用的 OC10 及 C12 則皆為長鏈分子，這些訊號分子之所以能促進或抑制表面移行是否是因 *P. mirabilis* 存在這些分子的接受器或運輸系統呢？ 不同訊號分子是否可共用接受器或運輸系統？ AHLs 一般被認為可以經由擴散作用自由進出細胞，但 Pearson 等人發現 OC12 的運輸須靠 MexAB-OprM 幫浦來完成，MexAB-OprM 在 *P. aeruginosa* 負責色素等次級代謝物以及多種藥物的主動輸出，他們並推測 AHL 的 *N*-acyl 長度及取代基種類，會決定其是否經由主動運輸的方式進出細胞。 此外長鏈 AHL 已被發現可以抑制短鏈 AHL 的作用，也許長鏈分子 C12、OC10 的抑制作用是由於抑制了某一種短鏈 AHL 的訊息傳遞， 但這不能解釋 OC12、OC14 等長鏈分子依然可以促進表面移行。 未來可從 *P. mirabilis* 抗 PNPG 突變株中, 挑選表面移行不能被 OC4、OC8、OC12、OC14 促進以及不能被 OC10、C12 抑制的菌株，找出遭破壞的基因，才能了解各個訊號分子調控表面移行的可能機轉。本實驗中發現 OC14 可以拮抗 PNPG 的抑制作用，而且其抑制濃度與 OC14 的劑量成正相關。 表示 OC14 或其

類似物可能會和 PNPG 互相競爭訊息傳遞媒介分子的結合位，來達到拮抗表面移行的目的。此媒介物可以是細菌 two-component 系統的感受者或是細胞內蛋白質。未來只有研究 OC14 促進表面移行的機轉，才能釐清這個問題。

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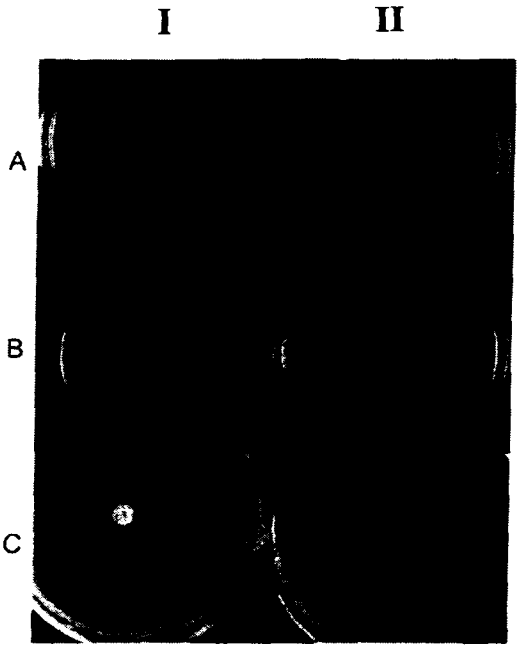


Fig. 1 Swarming of wild type *P. mirabilis* (I) and the super-swarming *P. mirabilis* mutant (II) on LB swarming plates. (A) Swarming in the presence of PNPG. Five micro-liters of the wild type (P19) or the super-swarming mutant (P1100) cells were inoculated centrally onto LB swarming plates containing 80 μg/ml PNPG. The plates were incubated at 37℃ and observed after 24-h incubation. (B) The 24-h swarming patterns of the wild type and the super-swarming mutant in the absence of PNPG. Five micro-liters of the wild type (P19) or the super-swarming mutant (P1100) cells were inoculated centrally onto the LB swarming plates without PNPG. Photographs were taken after incubation at 37℃ for 24 h. (C) The 5-h swarming patterns of the wild type and the super-swarming mutant in the absence of PNPG. Experiments were performed as in (B) except that photographs were taken after 5-h incubation.

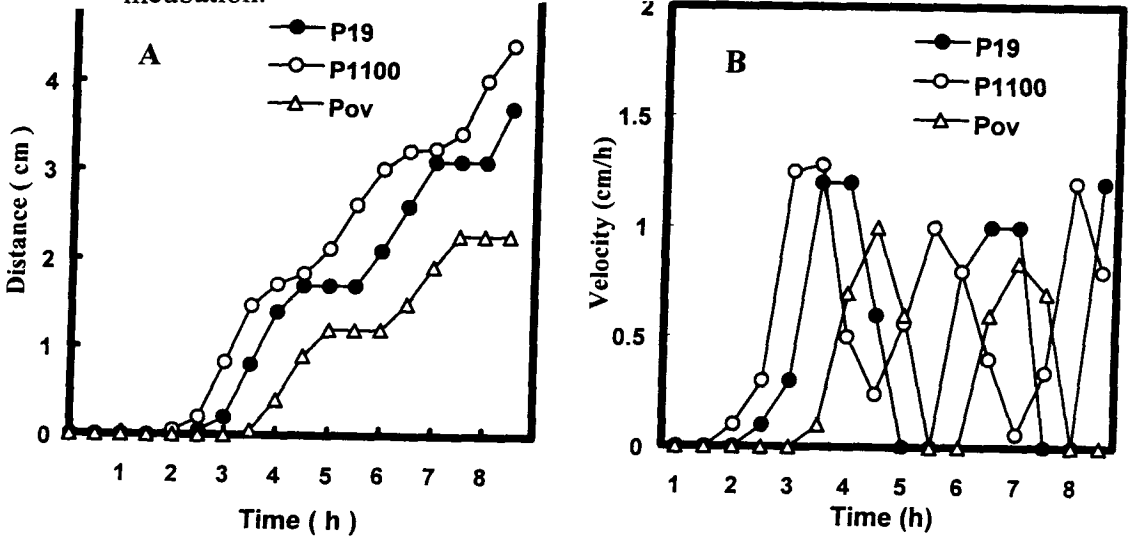


Fig. 2 (A) The swarming migration distance of the wild type (P19), the super-swarming mutant (P1100) and the strain overexpressing RsbA (Pov) in the absence of PNPG. The experiments were performed in triplicate and the mean migration distance was plotted against incubation time. (B) The swarming migration velocity of P19, P1100 and Pov in the absence of PNPG.

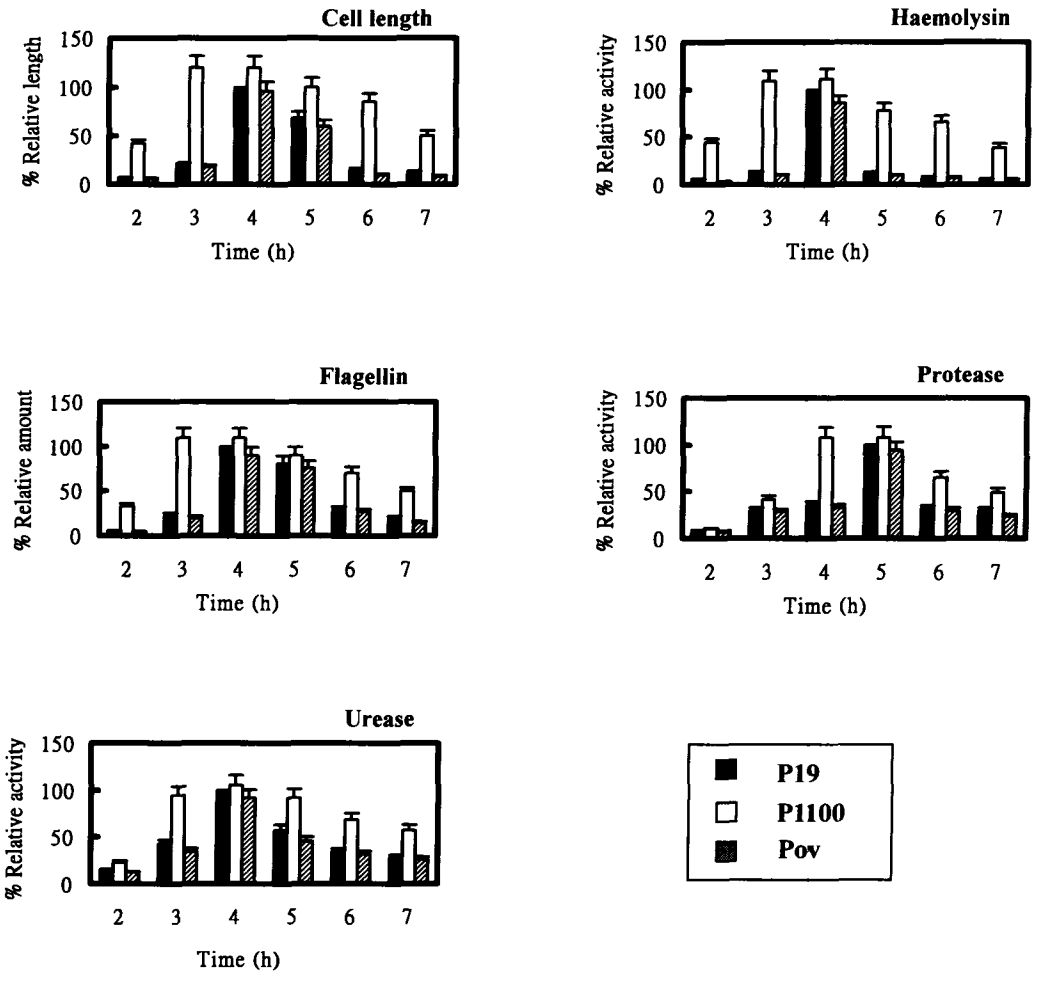


Fig. 3 Cell differentiation and expression of virulence factors following plating the wild type (P19), the super-swarming mutant (P1100) and the strain overexpressing RsbA (Pov) on the LB agar plates without PNPG. The increase in cell length was taken as a sign of cell (swarming) differentiation. For protease activity measurements, the value obtained with the wild type cells at 5-h post-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 post-seeding were set at 100%. The data represent the average of three independent experiments with standard deviation.

Fig. 4 Biofilm formation of (A) the wild type (P19), (B) the super-swarming mutant (P1100) and (C) the strain overexpressing RsbA (Pov). Overnight bacterial cultures were inoculated into LB broth and were placed in polystyrene petri dishes. After 10 h incubation at 30℃, the petri dishes were rinsed with distilled water. The remaining attached bacteria were then fixed and stained with crystal-violet.

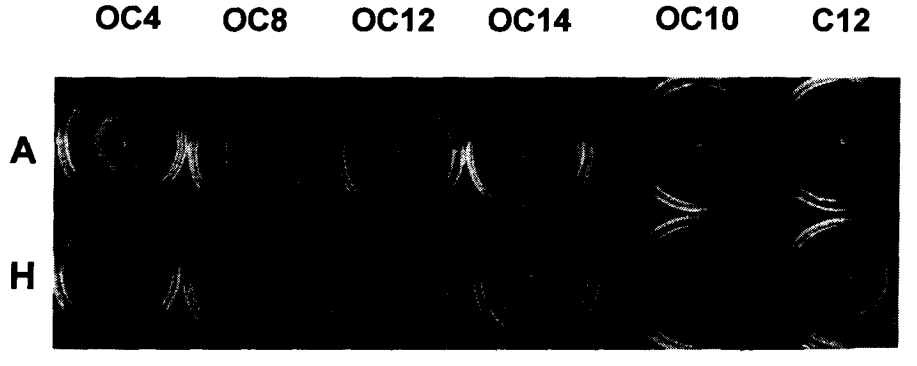


Fig. 5 The swarming behavior of wild-type *P. mirabilis* in the presence (H) and the absence (A) of OC4, OC8, OC12, OC14, OC10 and C12. Five micro-liters of bacterial cells were inoculated centrally onto LB swarming plates. The plates were incubated at 37℃ and observed after 8-h incubation.

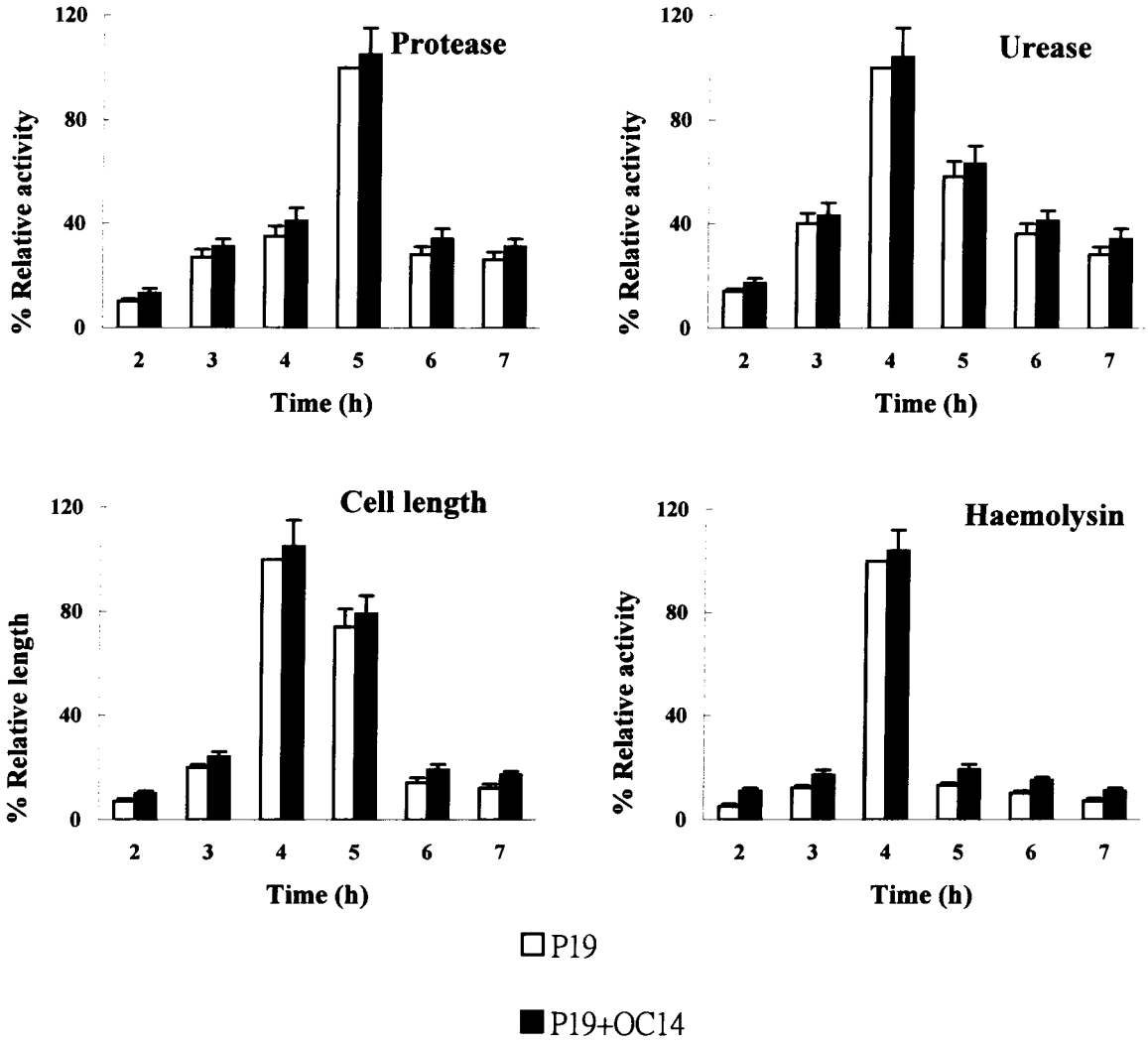


Fig. 6 Effect of OC14 on the cell length and expression of virulence factors in *P. mirabilis* P19 following plating the P19 on the LB agar plates. The increase in cell length was taken as a sign of cell (swarming) differentiation. For protease activity measurements, the value obtained in the absence of OC14 at 5-h post-seeding was set at 100% and all other values were expressed relative to this value. For all other measurements, the values obtained in the absence of OC14 at 4-h post-seeding were set at 100%. The data represent the average of three independent experiments with standard deviation.

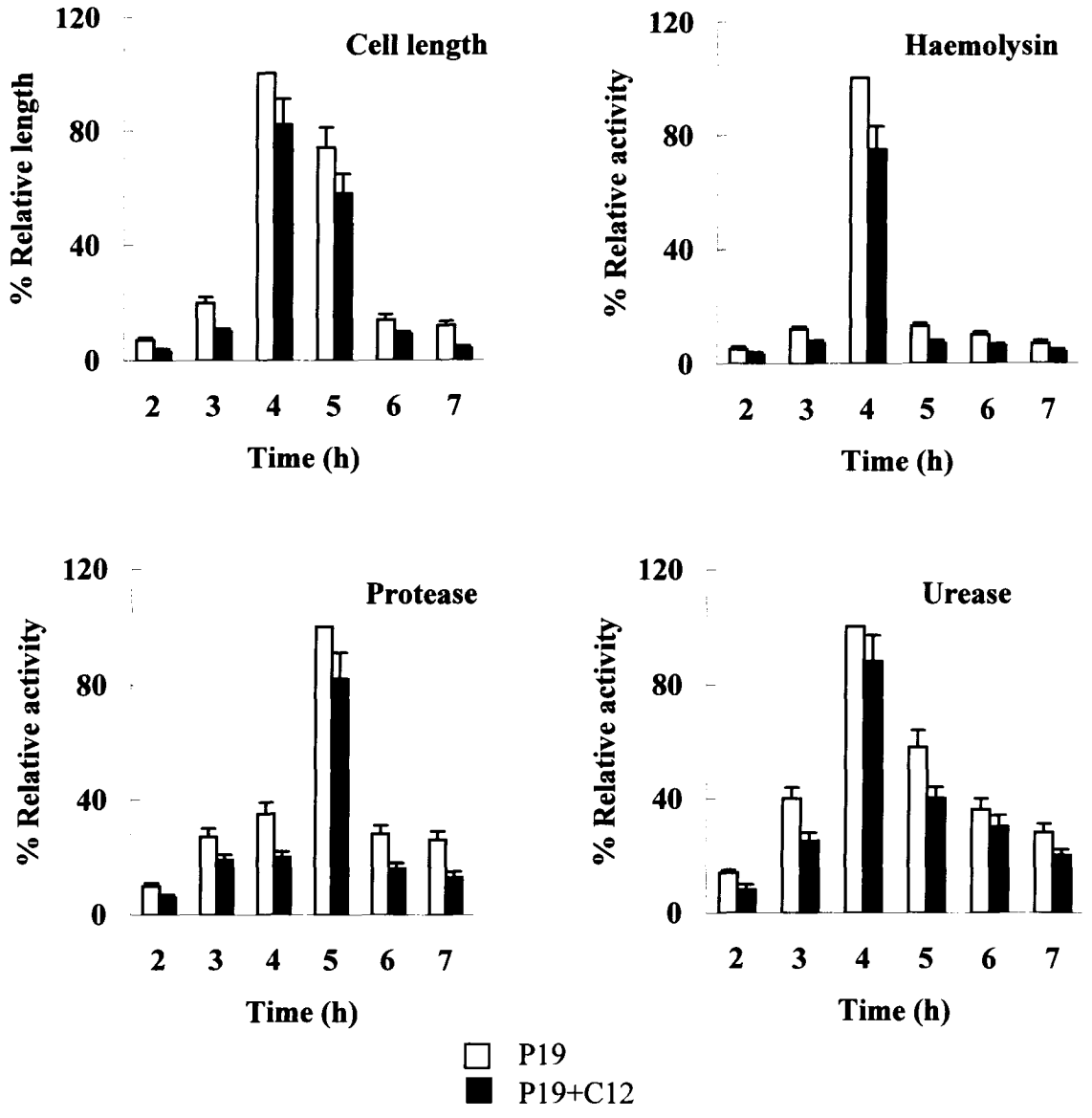
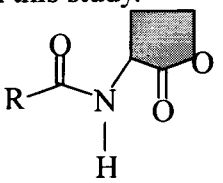


Fig. 7 Effect of C12 on the cell length and expression of virulence factors of *P. mirabilis* P19 following plating the P19 on the LB agar plates. The increase in cell length was taken as a sign of cell (swarming) differentiation. For protease activity measurements, the value obtained in the absence of C12 at 5-h post-seeding was set at 100% and all other values were expressed relative to this value. For all other measurements, the values obtained in the absence of C12 at 4-h post-seeding were set at 100%. The data represent the average of three independent experiments with standard deviation.

Table 1. The synthetic AHLs used in this study.



R	Chemical name	Abbreviation
CH3CH2CH2	N-butanoyl-L-homoserine lactone	BHL, C4
CH3COCH2	3-oxo-butanoyl- L-homoserine lactone	OBHL, OC4
CH3(CH2)4	N-hexanoyl-L-homoserine lactone	HHL, C6
CH3(CH2)2 COCH2	3-oxo-hexanoyl- L-homoserine lactone	OHHL, OC6
CH3(CH2)6	N-octanoyl-L-homoserine lactone	OHL, C8
CH3(CH2)4 COCH2	3-oxo-octanoyl- L-homoserine lactone	OOHL, OC8
CH3(CH2)8	N-decanoyl-L-homoserinelactone	DHL, C10
CH3(CH2)6 COCH2	3-oxo-decanoyl- L-homoserine lactone	ODHL, OC10
CH3(CH2)10	N-dodecanoyl-L-homoserine lactone	dDHL, C12
CH3(CH2)8 COCH2	3-oxo-dodecanoyl-L-homoserine lactone	OdDHL, OC12
CH3(CH2)12 COCH2	3-oxo-tetradecanoyl-L-homoserine lactone	OtDHL, OC14

Table 3 The interaction of OtDHL and PNPG.

OtDHL (μM)	PNPG (μM or μg/ml)	Swarming initiation time
0	328 or 70	>72 h
70	328 or 70	72 h
120	328 or 70	48 h
0	234 or 50	>24 h
70	234 or 50	24 h
120	234 or 50	14h

Table 2 Effect of various AHL signal molecules on the swarming motility of the 18 PNPG-resistant *P. mirabilis* mutants

* AHL / Mutant	OC4	OC8	OC12	OC14	OC10	C12
F11	N**	N	N	N	N	N
F12	N	N	N	N	N	N
F13	N	N	N	N	N	N
F14	N	N	–	N	N	N
F15	+**	N	–	N	–	N
F16	+	–	+	+	N	N
F17	N	N	+	+	N	N
F18	N	+	N	+	N	N
F19	N	–	N	N	N	N
F20	N	N	N	N	N	–
F21	N	N	–	–	N	N
F22	+	N	N	N	N	N
F23	N	–	N	N	+	+
F24	–**	–	–	+	N	N
F25	+	–	N	+	N	N
F26	N	N	–	N	N	–
F27	N	N	–	N	N	–
F28	N	N	–	N	N	–

* OC4, OC8, OC12 and OC14 have stimulatory effect on the swarming of the wild-type *P. mirabilis*; OC10 and C12 have inhibitory effect on the swarming of the wild-type *P. mirabilis*.

** N, no effect; –, inhibitory effect; +, stimulatory effect.