# 行政院國家科學委員會專題研究計畫　成果報告

# 奇異變形桿菌透過 rsbA 訊息傳遞系統調控表面移行及致病因子表現之研究

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-002-361-
執行期間：92 年 08 月 01 日至 93 年 12 月 31 日
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計畫主持人：廖淑貞
共同主持人：賴信志

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中　華　民　國 94 年 3 月 28 日

# 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）The investigation of *Proteus mirabilis rsbA*-mediated two-component signal transduction pathway in the regulation of swarming motility and virulence factor expression

計畫類別：■ 個別型計畫　　□ 整合型計畫
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中　華　民　國 94 年 3 月 22 日

計畫中文摘要。

關鍵詞：*Proteus mirabilis*、表面移行、*rsbA* 訊號傳遞系統、脂肪酸

表面移行 (swarming)是細菌感應外在環境變化的一種多細胞行為, 是一個複雜的調控網路。 之前的研究顯示有許多基因參與在表面移行行為中。 包括細菌訊息傳遞系統成員(bacterial two-component system)、quorum-sensing 系統成員、細胞內蛋白質(leucine-responsive regulatory protein)、鞭毛合成相關蛋白質(FlhDC 等)以及細胞外聚合物 (polysaccharide, lipopeptide 等)等。之前我們在尿道致病菌 *Proteus mirabilis* 中找到兩個會抑制表面移行以及致病因子表現的基因 *rsbA* 及 *rsmA*。*rsbA* 乃細菌 two-component system 中之感知者(sensor)而 *rsmA* 則是廣存於許多細菌中的 global regulator。除了表面接觸之物理性刺激外 quorum-sensing 系統之訊號分子 HSL 已被證實為許多細菌進行表面移行所必需。但直至目前為止在 *P. mirabilis* 並無 HSL 類的訊號分子被發現。Gaisser 等人之前推測脂肪酸合成可能與表面移行有關。於是我們測試一系列脂肪酸對野生株 *P. mirabilis* 表面移行的影響，結果發現有些會抑制有些則會促進表面移行。其中 lauric acid 會抑制野生株 *P. mirabilis* 的表面移行但對 *rsbA* 突變株則無抑制的效果。這暗示 lauric acid 可能是透過 *rsbA* 訊號傳遞系統來抑制表面移行。先前 Aguilar 等人發現在低溫下 *Bacillus subtilis* 可以透過 two-component system 改變細胞膜脂肪酸的組成使能適應低溫環境。他們發現脂肪酸和 two-component system 的訊息傳遞有關。也許在 *P. mirabilis* 中，脂肪酸可以透過改變細胞膜的組成及 conformation 來影響 *rsbA* 訊號傳遞路徑進而影響下游基因的表現。於是進行下列實驗以釐清脂肪酸透過 *rsbA* 抑制 *P. mirabilis* 表面移行的機轉。(i)、探討不同脂肪酸對 *P. mirabilis* 表面移行的影響並在不同脂肪酸培養下萃取 *P. mirabilis* 細胞膜脂肪酸分析其組成以了解不同脂肪酸對細胞膜脂肪酸組成的影響。(ii)、由於 Hughes 等人在 1995 年發現 *cmfA* 基因 (其基因產物為 capsular polysaccharide)為 *P. mirabilis* 表面移行所必需。而之前我們找到的 *rsbA* 基因下游為 capsular polysaccharide 之調控基因相似物。故建構 *cmfA-luxCDABE* 之 operon fusion，在 lauric acid 存在及不存在下監測此 operon fusion 在 *rsbA* 突變株及野生株表現的情形。同時也測定 capsular polysaccharide 以佐證之。(iii)、探討不同脂肪酸對 *P. mirabilis rsbA* 突變株及野生株致病因子 haemolysin 及 biofilm 生成的影響。(iv)、最後進行 transposon mutagenesis 找尋其他影響表面移行的突變株。由以上之研究已釐清脂肪酸是 *P. mirabilis* 表面移行之訊號分子而且是透過改變細胞膜的組成及 conformation 來影響 *rsbA* 訊號傳遞路徑進而影響下游表面移行基因的表現。這將有助於了解 *P. mirabilis* 調控表面移行及致病因子表現的複雜網路。

**計畫英文摘要**

Key words: *Proteus mirabilis*, swarming, *rsbA* two-component signal transduction, fatty acid

Regulation of swarming and expression of virulence factors is a complex regulatory network and involves many factors and regulatory pathways including many cellular proteins, bacterial two-component signaling system, quorum-sensing system, flagellum-biogenesis (FlhDC..) and extracellular polymer formation (capsular polysaccharide..). Previously we have identified two genes *rsbA* and *rsmA* that negatively regulate swarming and expression of virulence factors in uropathogenic *Proteus mirabilis*. *rsbA* is a gene which may encode a membrane sensor histidine

kinase of the bacterial two-component signaling system, and *rsmA* is a global regulatory gene widely distributed in many bacteria. One kind of quorum-sensing signals HSL, has been reported to regulate swarming and the expression of virulence factors in many bacteria. Till now, no such signal has been identified in *P. mirabilis*. Some evidence indicated that fatty acid biosynthesis is involved in the swarming regulation. After testing several fatty acids, we found that some fatty acids could inhibit swarming, some could enhance swarming. Among them, lauric acid can inhibit swarming of wild-type *P. mirabilis*, but fails to do so in the *rsbA* mutant. This implies that lauric acid is the possible signal and the *rsbA* two-component system may be the signal transduction pathway governing swarming in *P. mirabilis*. Previously, Aguilar et al. demonstrated that two-component signal transduction regulates the lipid composition of cell membrane and fatty acids act as specific signals regulating the signal transduction pathway. Maybe, somehow in *P. mirabilis* lauric acid could change the lipid composition and conformation of cell membrane to control swarming via the *rsbA* two-comopent system. To study the signal transduction processes from the fatty acid sensing, *rsbA* two-component signal transduction to the expression of down-stream genes, our plan was performed as follows:1.To study the effect of various fatty acids on swarming motility and the membrane lipid profiles in *P. mirabilis*. 2. We constructed the *cmfA-luxCDABE* operon fusion and monitor the effect of lauric acid on its expression in the wild-type *P. mirabilis* and the *rsbA* mutant to unravel the role of *cmfA* in this pathway. 3. To investigate if the lauric acid-mediated pathway also control the expression of virulence factors in *P. mirabilis* and monitoring the effect of lauric acid on its expression in the wild-type *P. mirabilis* and the *rsbA* mutant. 4. Finally, we performed transposon mutagenesis in *P. mirabilis* to find out other mutation that affects swarming. These experiments disclosed the processes of fatty acid-mediated *rsbA* two-component signal transduction pathway that controls swarming and virulence factor expression in *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.

*P. mirabilis* exhibits a form of multicellular behavior known as swarming migration. This is a cyclical differentiation process in which typical vegetative rods (2-4 μm) at the colony margin differentiate into long (up to 80 μm), aseptate filaments that possess up to 50-fold more flagella per unit cell surface area [1]. These swarming cells migrate rapidly and coordinately away from the original colony. Coordinate expression of virulence factors during swarm-cell differentiation and population migration of *P. mirabilis* was observed [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.

Quorum sensing, the density-dependent regulation of gene expression, is widespread among bacteria. Quorum sensing involves the synthesis and detection of extracellular signaling molecules termed autoinducers (AIs) [3]. Quorum sensing in gram-negative bacteria was first described for the marine symbiotic organism *Vibrio fischeri*. The number of acyl homoserine lactone (HSL) AI molecules in a given culture of *V. fischeri* increases as the cell density increases, and once a critical concentration of AI is reached, a signal transduction cascade that leads to the

production of bioluminescence by cells is initiated [4]. Components of this system include LuxI, an acyl HSL synthase that directs synthesis of 3-oxo-hexanoyl-HSL (*V. fischeri* AI-1); and LuxR, a transcriptional activator necessary for responses to *V. fischeri* AI-1[5]. Homologues of the *luxI* and the *luxR* genes of have been described now for a range of gram-negative bacteria and are responsible for the density-dependent regulation of quite diverse physiological functions including sporulation, virulence, antibiotic production and biofilm-formation [3, 6, 7]. *luxS*-based signaling has recently been descried for *Escherichia. coli*, *Salmonella typhimurium*, and *Helicobacter pylori* [3, 8, 9]. In *S. typhimurium*, the expression of the *luxS* gene is controlled by environmental factors [10]. AI production and signaling activity increase at high osmolarity and low pH levels and during the mid-to-late-exponential-growth phase [10]. Since these conditions are relevant to *S. typhimurium* as an enteric pathogen, the *luxS* gene is thought to play an important role in the virulence of the organism [10].

To unravel the regulatory network that control swarming and expression of virulence factors in *P. mirabilis*, I previously studied the effect of p-nitrophenylglycerol (PNPG) [11], a swarming inhibitor, on the swarming and the expression of virulence factors. I found that PNPG not only inhibits the above activities of *P. mirabilis* but also inhibits the ability of *P. mirabilis* to invade human urothelial cells [12]. These results suggest that PNPG has the potential to be developed as an agent against *P. mirabilis* infection. To study the mechanism underlying PNPG inhibitory effect, I performed Tn*5* mutagenesis to isolate *P. mirabilis* mutants that can swarm in the presence of PNPG. Four mutants were isolated, among them three have Tn*5* inserted in the *rsbA* gene, a gene which may encode a membrane sensor histidine kinase of the bacterial two-component signaling system. These three 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. In addition, these mutants also had higher ability to express virulence factors and to invade epithelial cells than did the wild type in the absence of PNPG [13]. Together, these data suggest that RsbA may act as a repressor of swarming and virulence factor expression. In the presence of PNPG, the ability of these *rsbA*-mutated mutants to swarm, differentiate, and express virulence factors is lower than that of the wild type cell, suggesting that PNPG may target RsbA or other pathways to exert its inhibitory effect.

Several types of quorum-sensing signal molecules such as quinolones (*Pseudomonas* quinolone signal, PQS), homoserine-lactone (HSL), diketopiperazine (DKP) and AI-2 have been reported [14], among them HSL is a most common type of quorum-sensing signal molecule produced by many bacteria. While *P. mirabilis* lacks HSL signal molecules, our preliminary data showed that it possesses AI-2 like activity in the cell-free culture fluid.

Based on the knowledge given above, our plan was performed as follows:

1.To study the effect of various fatty acids on swarming motility and the membrane lipid profiles in *P. mirabilis*.

2. We constructed the *cmfA-luxCDABE* operon fusion and monitor the effect of lauric acid on its expression in the wild-type *P. mirabilis* and the *rsbA* mutant to unravel the role of *cmfA* in this pathway.

3. To investigate if the lauric acid-mediated pathway also control the expression of virulence

factors in *P. mirabilis* and monitoring the effect of lauric acid on its expression in the wild-type *P. mirabilis* and the *rsbA* mutant.

4. Finally, we performed transposon mutagenesis in *P. mirabilis* to find out other mutation that affects swarming.

**研究方法**

**Strains, plasmids and growth conditions.** Wild-type *P. mirabilis* is provided by Colin Hughes (Cambridge University). *E. coli* CC118 λ pir and *E. coli* S17-1 λ pir are provided by K.Timmis [15] and are used for maintenance and conjugation of pUT plasmids. *E. coli* INV α F' is used in TA cloning stratery (Invitrogen). Recombinant pUT plasmids are provided by K.Timmis and are used as the suicide vector for conjugation assay. All bacteria are cultured at 37℃ in LB media.

**Swarming behavior assays.** The swarming migration distance and interval migration velocity assays will be performed as described previously [13]. Briefly, an overnight bacterial culture (5 μl) will be inoculated centrally onto the surface of the dried LB swarming plates containing 2.0% (w/v) agar. The swarming migration distance and interval migration velocity will be assayed by following swarm fronts of mutant and wild type cells and recording progress at 30 min intervals. Fatty acids (lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, myristoleic acid, palmitoleic acid) will be added to the swarming plates when needed.

**Membrane fatty acid analysis.** Exponentially growing cells will be harvested by centrifugation at 6000xg for 20 min, washed with 20 mM HEPES (pH 8), and then broken with a sonicator for 3 min. Unbroken cells will be removed by centrifugation at 6000xg for 20 min. Membranes will be pelleted by centrifugation at 100000xg for 1 h and washed with the same buffer. Membrane fatty acid analysis will be performed by gas-liquid chromatography with the Microbial Identification System (MIDI, Del.). The system includes a model 5890A gas chromatograph with a capillary column, a FID, an autosampler, an integrator, and a microcomputer. The manufacturer's protocol will be followed for all stages of the saponification, methylation, extraction, and chromatography procedures.

**Construction of *cmfA*, operon fusions with *luxCDABE*.** The fusion was constructed as described by Hakkila et al. [16]. The promoter activity of *cmfA* was monitored during growth and the swarming cycle using the Autolumat LB953 luminometer ( EG&G, Berthold, Germany) in the presence or absence of lauric acid.

**Assay of haemolysin activities.** The method used was as described by Liaw et al. [13].

**Capsular polysaccharide assay.** The method used was as described by Liaw et al. [17].

**Transposon mutagenesis of *P. mirabilis*.** The method used was as described by Liaw et al. [13]. Bacteria that were resistant to the antibiotics and could swarm on the plates were selected for further studies.

**結果**

Till now no HSL quorum-sensing system has not been identified in *P. mirabilis*. Besides cell density and glutamine, fatty acids have been implied as the environmental signals for swarming of *P. mirabilis*. We tested the effect of a series of fatty acids on swarming of wild-type *P. mirabilis*, and found that lauric acid (L)、myristic acid (M)、palmitic acid (P) and stearic acid (S) could inhibit swarming, and oleic acid (O) could enhance swarming (Fig. 1). Among them, L, M

and P exert their inhibitory effect on swarming and haemolysin activity (Fig. 2, 3) through RsbA. Addition of fatty acids did change the membrane fatty acid composition, and extent of swarming inhibition is directly proportional to the ratio of total percentage of saturated straight- chained fatty acids/total percentage of unsaturated and branched-chained fatty acids (Table 1). This implies that *P. mirabilis* can sense the environmental cues (such as the existence of fatty acids) to change the membrane fatty acid composition and the fluidity, then to activate the two-component sensor (RsbA) to trigger the expression of downstream genes. In 1995, Hughes et al. demonstrated that *cmfA*, a gene encoding CPS, is essential for swarming in *P. mirabilis* [18]. We found unexpectedly that the *rsbA* mutant produces less extracellular polysaccharide (EPS) than the wild-type does (Fig. 4). Maybe, except CmfA, there are other capsular polysaccharides involving in swarming and CmfA only provides basal level. Biofilms are surface-attached microbial communities with characteristic architecture and phenotypic properties distinct from their planktonic counterparts [19]. The biofilm cells have increased resistance to antibiotics and oxidative stress [19]. The ability to produce biofilm is reduced in our *rsbA* mutant (Fig. 5). We found that RsbA may act as a positive regulator of biofilm formation and EPS production. Myristic acid was found to slightly stimulate biofilm formation and EPS production and this stimulation was mediated through an RsbA-dependent pathway. Together, these data suggest that fatty acids may act as environmental cues to regulate swarming and virulence in *P. mirabilis* and that RsbA may play an important role in this process. Finally, we found out other *P. mirabilis* Tn*5* mutants that showed abnormal swarming behavior (Table 2). This study contributes to the understanding of complex swarming regulatory network in *P. mirabilis* .

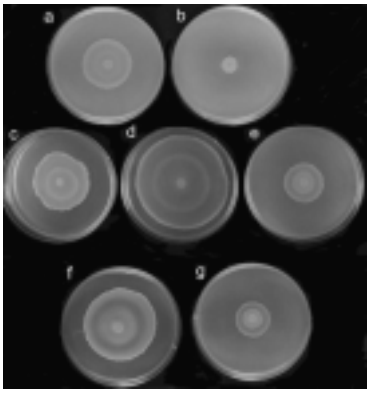



**Fig. 1** Effect of fatty acids on the swarming of wild-type *P. mirabilis*. Cells were grown overnight in LB broth, washed once in 1x PBS and a 5 μl-spot of inoculum was added to the center of the 2% LB agar plate with or without 0.01% (w/v) respective fatty acid. The plates were incubated at 37℃ and observed after 8 h. a, stearic acid; b, myristoleic acid; c, palmitic acid; d, oleic acid; e, lauric acid; f, no fatty acid control; g, myristic acid.

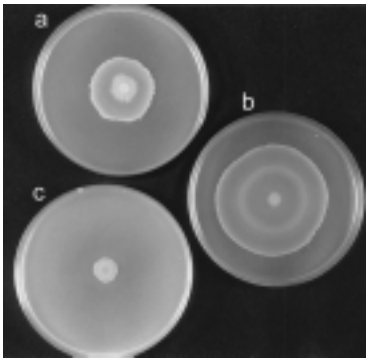
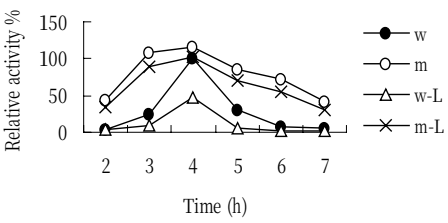


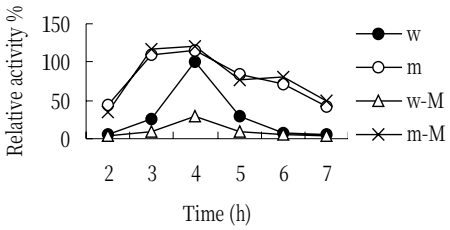

**Fig. 2** Effect of lauric acid on the swarming of *P. mirabilis rsbA* mutant and the wild-type. Cells were grown overnight in LB broth, washed once in 1x PBS and a 5 μl-spot of inoculum was added to the center of the 2% LB agar plate with or without 0.01% (w/v) lauric acid. The plates were incubated at 37℃ and observed after 7 h. a, the wild-type with no fatty acid added; b, the mutant with lauric acid; c. the wild-type with lauric acid. Lauric acid has no effect on swarming of the mutant.

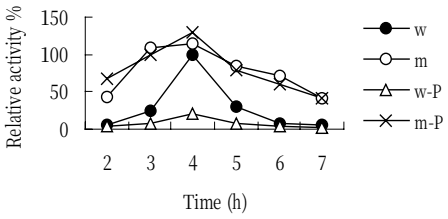
Table 1. Percentage of cellular fatty acids of *Proteus mirabilis rsbA* mutant and wild-type cells.

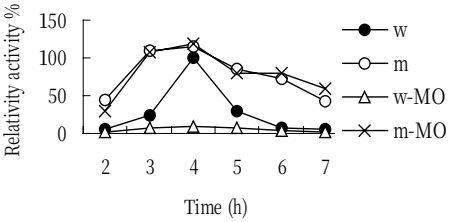
| | | **Percentage of total fatty acids** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Ratio*** | 12:0 | 14:0 | 15:0 | 16:0 | 18:0 | 15:0 anteiso | 17:0 cyclo | 18:1 w7c | 18:1 w9c |
| **Wt** | 2.29 | 0.83 | 10.8 | | 35.93 | 0.69 | 0.58 | 0.5 | 19.99 | |
| **-P** | 2.48 | 1.5 | 11.35 | 0.59 | 39.47 | 0.67 | 0.22 | 0.35 | 21.03 | |
| **-S** | 2.5 | 1.31 | 10.96 | 0.58 | 35 | 4.23 | 0.89 | 1.83 | 17.9 | |
| **-O** | 1 | 0.68 | 10.54 | 0.26 | 27.27 | 0.64 | 0.38 | | 10.51 | 28.38 |
| **-L** | 2.5 | 14.18 | 8.44 | 0.36 | 26.84 | 0.83 | 0.56 | 1.00 | 17.9 | |
| **-M** | 2.6 | 2.71 | 22.11 | | 28.46 | 0.98 | 0.72 | 0.58 | 18.8 | |
| **Mt** | 2 | 0.47 | 10.06 | 0.13 | 32.28 | 0.48 | 0.26 | 0.23 | 21.2 | |
| **-P** | 1.73 | 0.38 | 10.3 | 0.1 | 28.91 | 0.54 | 0.12 | 0.15 | 22.98 | |
| **-S** | 2.6 | 1.55 | 11.36 | 0.32 | 37.24 | 4.26 | 0.33 | 0.4 | 20.32 | |
| **-O** | 1.1 | 0.74 | 10.84 | 0.31 | 28.69 | 0.62 | 0.35 | | 10.18 | 26.33 |
| **-L** | 1.54 | 9.4 | 8.04 | 0.16 | 24.44 | 0.59 | 0.22 | 0.36 | 27.01 | |
| **-M** | 2 | 0.66 | 17.68 | 0.16 | 26.47 | 0.85 | 0.34 | 0.28 | 22.23 | |

**Ratio*** calculated as the total percentage of 12:0, 14:0, 15:0, 16:0, and 18:0 divided by total percentage of 15:0 anteiso, 17:0 cyclo, 18:1 w7c and 18:1 w9c. Wt, wild type; Mt, mutant; S, stearic acid; P, palmitic acid; O, oleic acid; L, lauric acid; M, myristic acid. P, S, L, and M inhibit warming of the wild-type, while O stimulates swarming of the wild-type. P, L and M have no effect on swarming of the mutant. O and S have same effect of swarming on both wild-type and the mutant.



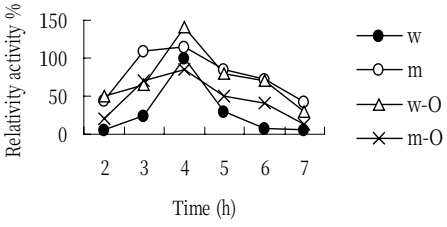



**Fig. 3** Effect of lauric acid (L), myristic acid (M), palmitic acid (P), myristoleic acid (MO) and oleic acid (O) on the haemolysin activity of *P. mirabilis rsbA* mutant (m) and the wild-type (w). 200 µl stationary-phase LB culture was spread onto 9- cm diameter LB agar plates and incubated at 37°C for 2 h, after which cells from the entire surface will be harvested by washing into 3ml LB broth at 1h-intervals till 7 h after incubation. These cells were then subjected to the haemolysin assays. For activity measurements, the value obtained with wild-type *P. mirabilis* at 4-h post-seeding was set at 100% and all other values were expressed relative to this value. The data represent the average of three independent experiments. L, P, M and MO decrease the haemolysin activity of wild-type cells but have no effect on the mutant cells.

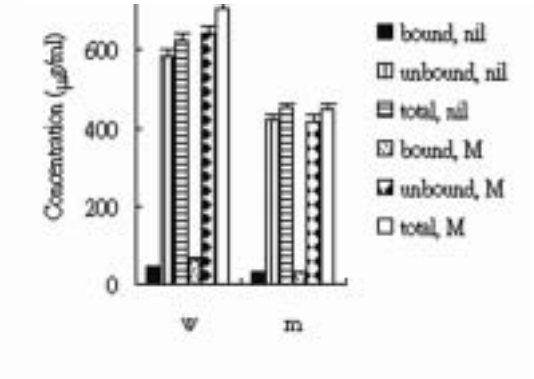



**Fig. 4** Production of EPS is reduced in the *P. mirabilis rsbA*-defective mutant. Bound-form, unbound-form and total EPS produced by the wild-type strain and *rsbA*-defective mutant of *P. mirabilis* in the absence or presence of myristic acid (0.01%, w/v) were determined as described in Materials and Methods. The data represent the average of three independent experiments with standard deviation. w, wild-type; m, mutant; nil, no

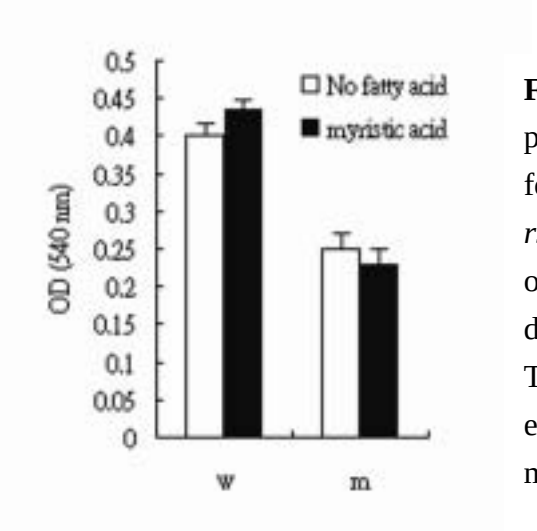



**Fig. 5** The *rsbA*-defective mutant of *P. mirabilis* was partially defective in biofilm formation. Biofilm forming abilities of the wild-type strain and *rsbA*-defective mutant of *P. mirabilis* in the absence or presence of myristic acid (0.01%, w/v) were determined as described in Materials and Methods. The data represent the average of four independent experiments with standard deviation. w, wild-type; m, mutant.

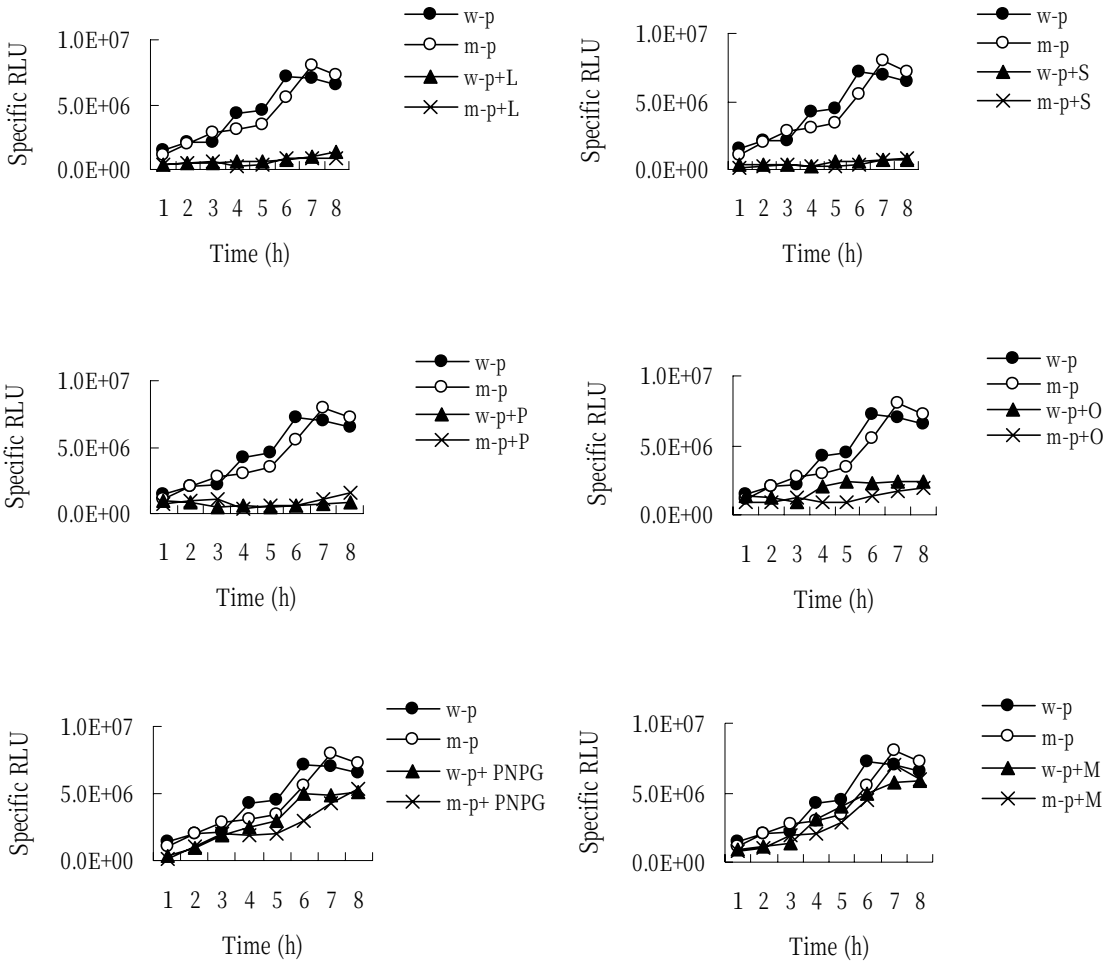



Fig.6 Effect of various fatty acids and PNPG on the expression of *cmfA-luxCDABE* operon fusion. w-p, wild-type with the fusion; m-p, mutant with the fusion; L, lauric acid; M, myristic acid, P, palmitic acid, O, oleic acid; S, stearic acid; PNPG, p-nitrophenylglycerol.

**Table 2.** Phenotypes of *P. mirabilis* Tn*5* swarming mutants.

| | Very fast(VF)* | Fast (F)* | Comparable(C)* | Slow (S)* |
|---|---|---|---|---|
| No. of mutants | 20 | 30 | 8 | 6 |
| % Total | 31 | 47 | 13 | 9 |
| Diameter (cm) of swarming colony (PNPG－)[a] | 4.8-6.1 | 3.2-4.2 | 2.6-2.7 | 2.0-2.2 |
| Diameter (cm) of swarming colony (PNPG＋)[b] | 2.2-2.6 | 1.6-2.0 | 1.2-1.3 | 1.2-1.3 |
| Consolidation | - or ± | ± | WT# | WT |

*Classified by the migration distance compared to that of wild-type *P. mirabilis* after 8 h incubation at 37℃ on LB agar plate without PNPG.

[a] Migration distance after 8 h incubation at 37℃ on LB agar plate without PNPG.

[b] Migration distance after 18 h incubation at 37℃ on LB agar plate with PNPG (80 μg/ml).

# Wild-type migration pattern.

## 討論

After sensing external signals, *Proteus mirabilis* undergoes a multicellular behavior called swarming which is coordinately regulated with the expression of virulence factors. Here we report that exogenously added fatty acids could act as signals to regulate swarming in *P. mirabilis*. Specifically, while oleic acid enhanced swarming, some saturated fatty acids, such as lauric acid, myristic acid, palmitic acid, and stearic acid inhibited swarming. We also found that expression of haemolysin, which has been shown to be coordinately regulated with swarming, was also inhibited by the above saturated fatty acids. Previously we identified a gene, *rsbA*, which may encode a membrane sensor kinase and act as a repressor of swarming and virulence factor expression in *P. mirabilis*. We found that while myristic acid, lauric acid, palmitic acid exerted their inhibitory effect on swarming and haemolysin expression through an RsbA-dependent pathway, the inhibition by stearic acid was mediated through an RsbA-independent pathway. Biofilm formation and extracellular polysaccharide (EPS) production play an important role in *P. mirabilis* infection. We found that RsbA may act as a positive regulator of biofilm formation and EPS production. Myristic acid was found to slightly stimulate biofilm formation and EPS production and this stimulation was mediated through an RsbA-dependent pathway. Together, these data suggest that fatty acids may act as environmental cues to regulate swarming and virulence in *P. mirabilis* and that RsbA may play an important role in this process. This is a new finding about swarming of *P. mirabilis*.

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