行政院國家科學委員會專題研究計畫　成果報告

調控奇異變形桿菌表面移行，致病因子表現以及生物膜形成之訊號傳遞系統之研究

計畫類別：個別型計畫
計畫編號：NSC93-2314-B-002-208-
執行期間：93 年 08 月 01 日至 94 年 12 月 31 日
執行單位：國立臺灣大學醫學院醫事技術學系暨研究所

計畫主持人：廖淑貞

報告類型：精簡報告

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中　華　民　國 95 年 4 月 3 日

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）調控奇異變形桿菌表面移行， 致病因子表現以及生物膜形成之訊號傳遞系統之研究

計畫類別：■ 個別型計畫　　□ 整合型計畫

計畫編號：NSC　　93 - 2314 - B - 002 – 208

執行期間：　93 年　8 月 1 日至 94 年 12 月 31 日

計畫主持人：廖淑貞

共同主持人：

計畫參與人員：許家瑜

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫列管計畫及下列情形者外，得立即公開查詢

□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：國立臺灣大學醫學院醫學檢驗暨生物技術學系暨研究所

中　華　民　國　95　年　3月 31　日

一、中文摘要

關鍵詞

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。本實驗分析 *P. mirabilis fadD* mutant 對表面移行及致病因子表現的影響。發現 *fadD* mutant 表現 superswarming 現象，致病因子包括 haemolysin, urease, protease, flagellin 的表現也比 wild-type 強。接著進行 RT-PCR 證明 *fadD* 以及都受 *rsbA* 正調控，但 *fadD* 以及 *rsbA* 之間無調控關係。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 *rsbA* 訊號傳遞路徑進而影響下游 *fadD* 以及 *rsbA* 的表現來調控表面移行相關基因的表現。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。

二、英文摘要

keywords: *fadD*, swarming-related phenotypes

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. *fadD* mutant 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 *fadD*. Together, these data suggest that FadD may act as a repressor of swarming and virulence factor expression. Together, these data reveal a novel mechanism through which bacteria may negatively regulate swarming differentiation and virulence factor expression by FadD. We also demonstrates that *fadD* and *rsmA* were positively regulated by RsbA, a membrane sensor histidine kinase.

三、報告內容:

前言、文獻探討: *Proteus mirabilis* is an important pathogen of the urinary tract, especially in patients with indwelling urinary catheters (1). It is believed that the ability of *P. mirabilis* to colonise the urinary tract is associated with its swarming motility. Moreover, the ability of *P. mirabilis* to express virulence factors, including urease, protease, haemolysin and flagellin, and to invade human uroepithelial cells, is coupled to swarming differentiation (2-4). 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 coordinated 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. The coordination 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 signal molecules to coordinate activity within a population (5,6). These bacteria communicate among themselves by producing extracellular signal compounds (such as HSL, AI-2..) that, when present during appropriate conditions and in sufficient concentrations, trigger specific responses. Swarmer cell differentiation and swarming behavior are the results of complex sensory transduction and global control mechanisms. Characterisation of swarming-defective *Proteus* transposon mutants has indicated that many proteins are involved in the regulation of differentiation and subsequent swarming migration. These include FlhA, a protein involved in flagella assembly and swarm-cell differentiation (7); $FlhD_2C_2$, a transcription activator that regulates the expression of the flagellar regulon (8); and RsbA, a putative bacterial two-component sensor kinase involved in the regulation of swarming (9). Another gene that has been observed to affect swarming behavior is *cmfA* (colony migration factor), which encodes capsular polysaccharide (CPS) (10). The presence of *rcsC-rcsB*, which is involved in the regulation of CPS synthesis in many G (-) bacteria, in the vinicity of *rsbA* , suggests the function of *rsbA* may be related with CPS synthesis. Among them, FlhDC, FlhA and the CmfA proteins probably function as part of a broader regulatory network that may include bacterial two-component systems and the chemotaxis phosphorelay (8).

RsmA is a homologue of CsrA (for carbon storage regulator) (11,12), a critical component of the *Escherichia coli* Csr system, a global regulatory system which represses a variety of stationary-phase genes (11). CsrA inhibits glycogen biosynthesis and catabolism, gluconeogenesis, and biofilm formation in *E. coli* (11,13). Searches in the GenBank databases have shown that homologues of *csrA* can be found in many

gram-negative bacteria and some gram-positive bacteria (14). RsmA, a homologue of CsrA, represses stationary-phase genes in *Pseudomonas fluorescens* (15) and negatively controls several genes involved in motility, secondary metabolism, pathogenesis and quorum-sensing in *Erwinia carotovora* subsp. *carotovora* (12, 16). Previously, we also found that *P. mirabilis* RsmA could inhibit swarming and virulence factor expression (17).

A universally conserved adaptation response observed among bacteria and most poikilothermic organisms is the adjustment of membrane lipid composition at low temperatures (18). As the growth temperature is lowered, the proportion of unsaturated fatty acids in the membrane lipids increases. This regulatory mechanism system called thermal control of fatty acid synthesis, is thought to be designed to ameliorate the effects of temperature changes on the physical state of the membrane phospholipids (18). Previous studies suggest that membranes can sense environmental changes and, as a consequence of changes in their phase state and microdomain organization, transmit signals that activate transcription (19). This signaling mechanism was proposed to control the expression of cold-induced desaturases from cyanobacteria (19). For signal transduction across the cell membrane, bacteria extensively use two-component systems, which have an input-sensing domain (histidine kinase) and an output effector domain (response regulator). Because two-component sensor kinases are generally integral membrane proteins that respond to environmental signals, it seems likely that temperature regulation of membrane lipid in bacilli could be controlled by members of the family of two-component regulatory proteins. In *Escherichia coli*, *fadD* encodes a long-chain acyl coenzyme A synthetase, a partially membrane-associated protein required for the import of long-chain fatty acids and the activation of long-chain fatty acids with coenzyme A before either the first step of β–oxidation or their incorporation into phospholipids (20). Previous studies have related FadD activity to the regulation of gene expression in some bacteria. For example, in *E. coli*, long-chain fatty acyl coenzyme A, the product of FadD, modulates the activity of transcriptional regulator FadR (21), leading to the expression of either fatty acid biosynthesis (*fab*) or fatty acid degradation (*fad*) genes. Interestingly, in some cases, FadD-mediated regulation of gene expression has been associated with the establishment of pathogenic association. For instance, in *Salmonella enterica*, the loss of *fadD* represses the expression of a gene, *hilA* whose product is involved in activating the expression of invasion genes (20). Another example can be found in the plant pathogenic bacterium *Xanthomonas campestris*, in which a FadD homolog has also been involved in the regulation of gene expression. A mutation in the gene leads to reduced virulence (20). Soto et al. reported that, in *Sinorhizobium meliloti*, in response to environmental changes, fatty

acid derivatives dependent on FadD activity may act as intracellular signals that control swarming and symbiosis through gene expression (20).

Biofilms are surface-attached microbial communities with characteristic architecture and phenotypic and biochemical properties distinct from their free-living, planktonic counterparts. Biofilm bacteria were more resistant to antibacterial treatments, e.g. antibiotics, detergents and reactive oxygen species and hence, bacterial biofilms are common causes of persistent infections (22). It has been demonstrated that the synthesis of capsular polysaccharides (CPS) is an important determinant of biofilm formation.

Resveratrol is a naturally occurring phytoalexin produced by grapevines, in response to injury 23. Invasion of grapevines by fungi induces the production of resveratrol to ward off the damaging microbes (23). Resveratrol has been shown to have antiviral and antioxidant activities (23). 2,3-butanediol is a volatile bacterial metabolite produced from anaerobic fermentation (24). Ryu et al. demonstrated that plant growth-promoting rhizobacteria release 2,3-butanediol to stimulate plant growth (24). This establishes a function for volatile bacterial metabolites as signaling molecules to mediate microbe-host interaction (24). We have found that resveratrol inhibit and 2,3-butanediol enhance the swarming of *P. mirabilis*. 2,3-butanediol is a common volatile bacterial metabolite. Both resveratrol and 2,3-butanediol are naturally occurring products. This indicates many signaling molecules exist in the environment to modulate *P. mirabilis* swarming.

研究目的:

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*. Previously, 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 could inhibit swarming, and oleic acid could enhance swarming. Among them, L, M and P exert their inhibitory effect on swarming and haemolysin activity 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. 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. To understand the role of *rsmA* in the pathway, we will construct *rsmA-luxCDABE* operon fusion and monitor the effect of fatty acids on its expression in the wild-type *P. mirabilis* and the *rsbA* mutant. *fadD* gene is a partially membrane-associated protein

required for the fatty acid metabolism. Maybe, fatty acids will affect the activity of FadD and the membrane conformation, thus to modulate the *rsbA*-mediated signaling pathway. We will clone the gene and characterize its functions and investigate its role in swarming, expression of virulence factors and biofilm formation. The ability to produce biofilm is reduced in our *rsbA* mutant. We are going to analyse the characteristics of biofilms formed by mutant and wild-type strains and perform uroepithelial cell invasion assay to assess their virulence.

研究方法：

Swarming behavior assays. The method used was as described by Liaw et al.(9)

Assay of haemolysin activities. The method used was as described by Liaw et al. (9)

Gene-knockout by homologous recombination. Full length *fadD* gene was amplified by PCR and mutagenised by omega cassette (25), constructed in pUT and transferred to wild-type *P. mirabilis* through conjugation.

Assay of outer membrane proteins. The method used was as described by Fukuoda et al.(26)

RT-PCR. Bacteria will be allowed to attach to uroepithelial cells at 37℃ for 30 min and then total RNA was extracted from the bacteria. cDNA was synthesized using the first strand cDNA synthesis kit and the resulting RNA/cDNA hybrid was used as a template in PCR reactions using the primer sets designed from *rsbA*, *rsmA*, or *fadD*. The resulting products were analyzed on an agarose gel.

Biofilm formation assay. The method used was as described by Liaw et al.(27)

Biofilm susceptibility to antibiotics and oxidative stress. The method used was as described by Liaw et al.(27)

Cell invasion assay. The method used was as described by Liaw et al. (9)

Quantitative measurement of extracellular polysaccharide (EPS) Production. The method used was as described by Liaw et al.(27)

結果： 1. The swarming behaviour of the wild type (P19) and the *fadD* mutant were analysed. by recording at 30-min intervals the position of the swarm front through two cycles of migration and consolidation. The wild type exhibited a typical swarming pattern of alternating active migration and consolidation phases, whereas the mutant exhibited a less conspicuous consolidation phase. Another difference between the wild type and the mutant was the duration of the initial lag phase. This was 2 h for wild-type cells in the initial lag phase prior to the onset of swarming migration, whereas the mutant cells spent about 1 h less in this phase. Because of this and an incomplete consolidation phase, the mutant translocated further in a given time than did the wild-type cells. The swarming abilities of the wild type (P19) and the mutant were also measured. Because the mutants exhibited faster swarming than the wild type, they were referred to as “super-swarming mutants”. 2. Swarming

differentiation is regulated co-ordinately with the expression of virulence factors, and both are related to the swarming behaviour of *P. mirabilis*. Knowing that swarming behavior 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 (P19) and the super-swarming mutant were spread on to LB swarming plates, and cell length and expression of virulence factors 2 h after seeding and hourly thereafter were determined. The super-swarming *fadD* mutant formed longer cells than the wild type during the 7 h incubation period and became the longest about 1 h earlier than wild type cells. Moreover, the mutant retained its long cell shape for longer than the wild type. These data indicate that the super-swarming mutant differentiated earlier and maintained a differentiated state longer than the wild type. In parallel with the above characteristics, the mutant also expressed higher levels of haemolysin, protease, urease and flagellin, than the wild type and they reached maximal expression levels about 1 h earlier than the wild type. Moreover, the mutant retained high expression levels of these factors for longer than the wild type. Furthermore, swarming differentiation and virulence factor expression was coordinately regulated both in the wild type and the *fadD* mutant. 3. Swarming differentiation and expression of virulence factors are correlated with the ability of *P. mirabilis* to invade cells. Therefore, the cell invasion ability of the super-swarming mutants was investigated. The *fadD* mutant had a higher cell invasion ability than the wild type at all stages of cell differentiation, and especially at the late stage (7 h) during which cells of the wild type had undergone de-differentiation. The cell invasion abilities of the wild type 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. 4. Complementation of the super-swarming mutant by *fadD*. The super-swarming phenotypes of these mutants probably arose from the defectiveness of the *fadD* gene. If this inference is correct, then provision of *fadD* to the super-swarming mutants should restore wild type swarming behaviour. To test this, plasmid pSJfadD, which encoded a full-length FadD protein, was transformed into the super-swarming mutant to generate a FadD-complemented strain (Pc). The f*adD* mutant exhibited a super-swarming phenotype as described above, whereas the Pc strain exhibited a similar swarming behaviour to the wild type. The Pc strain also exhibited a similar pattern of expression of haemolysin to the wild type, in marked contrast to the super-swarming mutant, which exhibited a super-expression pattern. These data together indicate that expression of FadD 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 *fadD* gene, and not

from other mutations in the chromosome. 5. To disclose the relationship of *rsmA*, *fadD* and *rsbA* in the swarming regulatory network, we performed RT-PCR assays. We found that both *rsmA* and *fadD* are positively regulated by *rsbA* and the expression of *rsmA* and *fadD* is independent. 6. We also found that 2,3-butanediol enhance swarming and virulence expression of *P. mirabilis*.

討論: For the first time, *fadD* gene was cloned in *P. mirabilis* by us and this is the first finding about the role of *fadD* in the regulation of swarming and virulence expression in *P. mirabilis*. We also demonstrate that *rsbA* can positively regulate the expression of *rsmA* and *fadD* in *P. mirabilis* either directly or indirectly. Further experiments to analyze the Tn*5* mutagenesis mutants that exhibit aberrant swarming behavior will unravel the detailed of the complex network.

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五、計畫成果自評:

1. For the first time, *fadD* gene was cloned in *P. mirabilis* by us

2.This is the first finding about the role of *fadD* in the regulation of swarming and virulence expression in *P. mirabilis*.

3.We also demonstrate that *rsbA* can positively regulate the expression of *rsmA* and *fadD* in *P. mirabilis* either directly or indirectly.