An important role for FlhDC in the control of *nucA* expression, cell division and flagellar synthesis in *Serratia marcescens*

Jia-Hurng Liu [(1)], Meng-Jiun Lai [(1)], Sunny Ang [(1)], Jwu-Ching Shu [(1)], Poo-Chi Su [(1)], Yu-Tze Horng [(1)], Wen-Ching Yi [(1)], Hsin-Chih Lai [(1,2) (*)], Kwen-Tay Luh [(2)], Shen-Wu Ho [(1,2)], and Simon Swift [(3)]

[(1)] *School and Graduate Institute of Medical Technology, College of Medicine, National Taiwan University, Taipei, Taiwan, R.O.C.*

[(2)] *Department of Laboratory Medicine, National Taiwan University Hospital, Taipei, Taiwan, R.O.C.*

[(3)] *Institute of Infections and Immunity, University of Nottingham, Queen's Medical* Centre, *Nottingham, NG7 2UH and School of Pharmaceutical Sciences, University Park, Nottingham, NG7 2RD, U. K.*

Key words: Serratia marcescens, *flhDC*, *nucA* expression, Cell division, Flagella synthesis.

running title: *flhDC* controls *nucA* expression and cell differentiation

flhDC Genbank accession number: AF077334

* corresponding author/to whom proofs should be sent:

tel. +886-2-23970800 extn 6931 / fax. +886-2-23711574

e-mail: hclai@ha.mc.ntu.edu.tw

includes 7 figures

SUMMARY

The gene products of the *flhDC* operon are regulatory proteins exerting global effects in enteric bacteria, especially in the areas of motility and cell division. The role of the *flhDC* operon was investigated in *Serratia marcescens*. In addition to cell division and flagellar synthesis, the regulation of expression of one of the virulence factors, nuclease (encoded by the $nucA_{Sm}$ gene) was observed. Interruption of the chromosomal *flhDC* operon in *S. marcescens* CH-1 resulted in non-flagellated cells with aberrant cell-division that were nuclease negative. Expression of $nucA_{Sm}$ and the other phenotypes were restored to the *flhDC* mutant in-trans by *flhDC* in multi-copy plasmid. Further study showed that *flhDC* in multi-copy induces the formation of differentiated (polyploid aseptate cells with peritrichous flagella over-synthesis) cells in broth culture of minimal growth medium. Expression of *flhDC* shows evidence of positive auto-regulation, and is growth-phase dependent (up-regulated in early log phase). The regulation of a P*flhDC*::*luxAB* genomic fusion was dependent upon environmental temperature (inhibited when the bacterial cell culture was shifted from 30 to 37 ℃) and osmolarity (inhibited by increasing salt concentration), but was not influenced by glucose catabolite repression as has been described for *Salmonella*.

INTRODUCTION

The products of the *flhDC* operon, FlhD and FlhC, act as global gene regulators in enteric bacteria. The expression of many genetic determinants involved in the processes of cell division, cell differentiation, swarming/swimming motility and virulence is controlled by *flhDC* (Macnab, 1992; Liu and Matsumura, 1994; Givskov *et al.*, 1995; Eberl *et al.*, 1996; Pruss and Matsumura, 1996; Pruss *et al.*, 1997; Givskov *et al.*, 1998; Dufour *et al.*, 1998). The *flhDC* operon was first noticed as an important regulator in the hierarchical system controlling the synthesis of the bacterial flagellum including *Escherichia coli* (Macnab, 1992). Subsequently, homologues have been described in *Shigella* species, *Salmonella typhimurium*, *Serratia liquefaciens* and *Proteus mirabilis* (Givskov *et al.*, 1995; Al Mamun *et al.*, 1996; Furness *et al.*, 1997; Kutsukake, 1997). In addition to the essential function of *flhDC* operon in the control of bacterial motility, it was also found to regulate important functions outside bacterial flagellar system. Pruss and Matsuyama (1996) have shown that the transcriptional activation by FlhD/FlhC is also involved in the process of cell division and affects growth rate in *E. coli*. They further showed that in *E. coli*, while flagellar synthesis is dependent on the tetrameric FlhD/FlhC complex, cell division is dependent on *cadA* under the control of FlhD only (Pruss *et al.*, 1997). Pleiotropic effects attributable to FlhD/FlhC regulation have also been observed in *Serratia liquefaciens* (Givskov *et al.*, 1995), with the induction of phospholipase synthesis and swarm-cell differentiation (flagellar over-synthesis and cell elongation) observed when *flhDC* are over-expressed in an LB rich broth culture (Eberl *et al.*, 1996).

We are interested in the roles that *flhDC* operon may play in the process of swarm-cell differentiation and virulence factor expression in *Serratia marcescens*, which is

an important opportunistic human pathogen and plays an important role in the infection (Khan *et al.*, 1997; Peeters *et al.*, 1997). In *S. marcescens* and other related bacteria, the differentiation of swarmer-cells involves a change from short motile vegetative cells with a few peritrichous flagella into multi-nucleate, aseptate swarm cells of a size up to 40~80 times the vegetative cell and and over-production of surface flagella (Harshey, 1994; Lai *et al.*, 1997b). Although Eberl *et al.*, (1996) have shown the important role that the *flhDC* operon performs in the differentiation of *S. liquefaciens* swarm-cells, their work did not rule out a role for uncharacterized factors in the media in this process. Indeed, using cell elongation, flagella over-synthesis and expression pattern of *hag* (the gene encoding the flagellin subunit) as the markers for swarmer-cell differentiation, we have demonstrated that cells with differentiation characteristics are observed in LB plate and broth cultures without the artificial over-expression of the *flhDC* operon (Lai *et al.*, 1997b). It is thus possible that cell differentiation is growth–phase dependent and occurs when *flhDC* operon expresses at its peak.

S. marcescens produces a number of virulence factors including protease, chitinase and lipase, and most of them were substrate-regulated (Chen *et al.*, 1992). Unlike most catabolic enzymes, the expression of nuclease gene, *nucA*, in *S. marcescens* is reported not to be regulated by substrate catabolite repression, but by 1) a SOS-like system (Ball *et al.*, 1990) and 2) a growth-phase dependent system, which is independent of SOS induction (Chen *et al.*, 1992). Significantly, the dominant genetic regulator of $nucA_{Sm}$ expression remains to be identified. As there is evidence that expression of the virulence factor phospholipase was regulated by *flhDC* operon, we thus investigated the effect of *flhDC* operon on $nucA_{Sm}$ expression.

As part of our research programme to study the function of *flhDC* operon in *S.*

marcescens, we asked the following questions: (i) Does *flhDC*, like those reported from other bacteria, regulate cell division and flagellar synthesis in *S. marcescens*? (ii) Does *flhDC* play a dominant role in virulence factor (particularly $nucA_{Sm}$) expression? (iii) What are the factors that regulate the expression of *flhDC*? (iv) Are there any environmental factors, such as solid surface contact, or any putative signal molecules existing in the rich media that are essential to initiate the formation of differentiated swarm-cells? To answer these questions, we have cloned and sequenced the *flhDC* from *S. marcescens* and begun to correlate its relationship with cell division, morphological change, flagellar synthesis and expression of the nuclease gene $nucA_{Sm}$. Furthermore, by construction of an P*flhDC*::*luxAB* genomic fusion we have identified some factors influencing the activity of the *flhDC* promoter.

MATERIALS AND METHODS

Bacterial strains and plasmids

Table 1.

Bacterial strains	**Relevant genotype/phenotype**	**Reference/source**
S. marcescens CH-1	The wild-type cells	This Lab.
S. marcescens S-1	Phag_{Sm}::*luxAB* in the chromosome of *S. marcescens* CH-1	Lai *et al.*, (1997b).
S. marcescens F-1	*flhD*::SM, derived from *S. marcescens* CH-1	This study
S. marcescens F-3	P$flhDC_{Sm}$::*luxAB* in the chromosome of *S. marcescens* CH-1	This study
S. marcescens F-4	P$flhDC_{Sm}$::*luxAB* in the chromosome of *S. marcescens* F-1	This study
S. marcescens N-1	P$nucA_{Sm}$::*luxAB* in the chromosome of *S. marcescens* CH-1	This study
S. marcescens N-2	P$nucA_{Sm}$::*luxAB* in the chromosome of F-1	This study
S. marcescens S-2	*flhD*::SM in the chromosome of S-1	This study
Plasmids		
pCR 2.1	TA cloning vector; Amp^R; Km^R. ColE1 ori.	Invitrogen
pZero 2.1	Cloning vector; Km^R; ColE1 ori. The *ccd*B (Bernard, 1996) was used as the screening marker.	Invitrogen
pBCSK+	Cloning vector; Cm^R; ColE1 ori.	Stratagene
pBIISK+	Cloning vector; Amp^R; ColE1 ori.	Stratagene
pACYC184	Cloning vector; Tc^R; Cm^R. p15A ori.	Chang and Cohen (Chang and Cohen, 1978)
pUT miniTn5Km	A suicide vector for conjugation and transposition of the inserted DNA after modification (Lai *et al.*, 1997b).	de Lorenzo *et al.*, (1990). de Lorenzo and Timmis (1994).
pJH03	A 5kb Chromosomal *Bam*HI fragment containing the complete $flhDC_{Sm}$ operon cloned in pBIISK+.	This study
pJH04	A PCR with primers (5'-GCATGCGTGTACATCCATACACG-3' and 5'-AACAATGTGGATGGAAGGTGG-3') amplified a 1.5kb DNA fragment bearing the complete $flhDC_{Sm}$ operon including its native promoter. This fragment was T-cloned into pCR2.1 and the gene direction was with P_{lac} in the vector.	This study
pJH05	A 1.5kb *EcoRI* fragment (derived from pJH04) bearing the complete $flhDC_{Sm}$ operon with its native promoter cloned into pBCSK+. The gene direction was with P*lac* in the vector. Copy number of this plasmid is supposed to be between 15~20.	This study

Media and growth conditions

Luria-Bertani (LB) medium was used for routine growth of both *E. coli* and *S. marcescens*. *E. coli* was routinely grown at 37°C and *S. marcescens* at 30°C. Cell density was assayed by measuring absorbance at 600 nm. Minimal growth media (MGM): [5x M9 salt 200 ml, 1 M $MgSO_4$ 2 ml, 1M $CaCl_2$ 0.1 ml, 20 % glucose 20 ml, d.d. H_20 up to 1,000 ml] (Maniatis *et al.*, 1989).

DNase, phospholipase and plate gelatin assay

DNAse Test Agar medium (DIFCO) was inoculated with 100 µl of a saturated overnight bacterial culture. After culture for 6 to 8 h, levels of the nuclease production were estimated by measurement of the halo size around bacterial colonies formed after addition of 1 ml of 1N HCl. For a quantitative analysis the microplate assay described by Chen *et al.*, (1992) was performed. The phospholipase assay was performed on egg-yolk agar plate (DIFCO) (Koneman *et al.*, 1997). The plate gelatin assay was performed on 1.5 % LB plate containing 0.1 % gelatin (SIGMA) (Lai, 1994).

Enzymes and chemicals

Restriction and modification enzymes were purchased from Boehringer Mannheim. *Taq* polymerase and PCR related products were from Perkin Elmer or Takara Biomedicals. Other chemicals were purchased from SIGMA.

DNA manipulation and analysis

DNA was sequenced by an ABI 373A DNA Sequencer with the *Taq* DyeDeoxy Terminator Cycle sequencing kit (Perkin-Elmer). DNA/amino acid sequence analysis

was achieved by GCG (version 9.1). Unless mentioned specifically, standard protocols were used for Southern hybridization, cloning, the isolation of plasmid and chromosomal DNA, transformation, PCR, restriction digests, elution and ligation of DNA (Maniatis *et al.*, 1989). Hybridization and washes were carried out at 68°C. DNA detection was achieved by DIG system (Roche). *S. marcescens* CH-1 chromosomal DNA library was constructed by lambda DashII system according to the protocol supplied by the manufacturer (Stratagene).

Inactivation of *flhD*

A DNA fragment containing the complete *flhD* structural gene and a partial *flhC* were amplified in a PCR reaction from *S. marcescens* CH-1 chromosomal DNA template using the amplimers (5'-CATCCATACACGTTGGTTTACGCT-3'/5'TGCTCGTCCAGCAGTTGAGGAATA-3'). The fragment was then cloned into pCR2.1 vector. A *Sma*I digested streptomycin-resistant gene (SM) was inserted into the *Sal*I site of the amplified DNA fragment by blunt-ended ligation. Through transfer by modified pUT suicide vector (Lai *et al.*, 1997b) and homologous recombination (de Lorenzo and Timmis, 1994), the wild-type *flhD* was replaced by the recombinant DNA fragment to create a new strain designated F-1. Similar procedures were performed on strain S-1 (main genetic feature, i.e. Phag_{Sm}::*luxAB*, see Table 1) (Lai *et al.*, 1997b) to form S-2.

Construction of isogenic strains N-1 and N-2

The procedures were similar to those described in Lai *et al.*, (1997b) for the creation of a chromosomal Phag_{Sm}::*luxAB* fusion. Primers (5'-CTGTTGGACGCCGTTTTTATTT-3' and 5'-ATTCATATCCTCAATAAGTTAA-3')

were designed from the promoter region of nuclease gene (Chen *et al.*, 1992), and a 170 bp promoter region of the nuclease structure gene $nucA_{Sm}$ was PCR amplified, T-cloned, sequenced, and then ligated in the same direction into the upstream region of bacterial *lux*AB reporter genes as a *NotI/SfiI* fragment. The P$nucA_{Sm}$::*lux*AB fragment was randomly transferred into the chromosome of wild-type *S. marcescens* through conjugation and transposition.

Detection of luciferase activity

A cell suspension, 100 µl, was added to Con's tubes (Genetek, Taiwan) containing 900 µl 0.85 % normal saline before measurement using the Autolumat LB 953 luminometer (EG & G). N-decyl aldehyde 100 µl [1 % (v/v) in ethanol] was used as the enzyme substrate. The result was expressed in relative light units (R. L. U.).

Cell differentiation assays

Fixed amounts of bacteria were harvested hourly after a 1/100 dilution of an overnight culture into fresh MGM broth. Mean cell length was estimated by phase-contrast microscopy BH_2 (Olympus) of at least 50 cells fixed in 1 % formaldehyde/0.85 % normal saline. For microscopic examination, bacteria were fixed on slides by mild flaming. Flagella silver stain followed the procedures of Koneman *et al.* (1997).

RESULTS

Identification of *S. marcescens flhDC* operon

A pair of primers *flhDF2* (5'-CATCCATACACGTTGGTTTACGCT-3') and *flhCR2* (5'-TGCTCGTCCAGCAGTTGAGGAATA-3') designed from the conserved nucleotide acid sequence region of *flhD*/*flhC* from *E. coli* (GenBank accession number AB001340) and *S. liquefaciens* (Givskov *et al.*, 1995) were used for PCR with *S. marcescens* chromosomal DNA as the template. An amplification product of 1093 bp was obtained and sequenced after cloning. This DNA fragment contained a high nucleotide sequence identity (95 %) with *flhD*C of *S. liquefaciens*. This DNA fragment was used to probe a λ-DASHII (Stratagene) phage library of partial *EcoRI* fragments from the wild-type *S. marcescens* CH-1 chromosome. Five phage clones hybridized and restriction mapping by Southern blot hybridization using the 1093 bp DNA as the probe was performed. A 5Kb *Bam*HI fragment high-lighted was subcloned from phage clone JH-1.

A 1.7 kb DNA fragment containing the complete *flhD and flhC* genetic determinants was sequenced. The physical map, DNA and the translated amino acid sequences were shown in Fig. 1. As predicted, no putative promoter sequence was observed in the intergenic region of *flhD* and *flhC*, suggesting that similar to those of *E. coli* and *S. liquefaciens*, *flhD* and *flhC* also form an operon (*flhDC*$_{Sm}$ and hereafter). The sequence of *flhD* and *flhC* are predicted to encode a protein of 116 amino acids (a.a.) (nucleotide sequence 656~1003), and 194 a.a. (nucleotide sequence 1006~1587), respectively. There is no open reading frame found on the complementary strand or immediately upstream or down stream of the coding sequence.

By using GCG (version 9.1) and BLASTP (version 2.0.5), a search of the data base

with the protein sequences indicated similarity to a variety of FlhD/FlhC proteins. The highest similarity to known FlhD/FlhC was, as expected, with the *S. liquefaciens* FlhD/FlhC (Givskov *et al.*, 1995). There were 99/95 % identity and 99/96 % similarity for FlhD/FlhC at the protein level between *S. marcescens* and *S. liquefaciens*. Identity with the *E. coli* FlhD/FlhC was 70/83 % (76/84 % similarity) at the protein level.

Inactivation of the chromosomal *flhD* gene by homologous recombination

To unravel the possible physiological functions that $flhDC_{Sm}$ may play, a *flhD* knock-out mutant strain was constructed. A streptomycin-resistant antibiotic marker was inserted into the chromosomal *flhD* as described in the materials and methods. A mutant strain named *S. marcescens* F-1 was selected. To confirm that insertional mutagenesis into *flhD* had occurred in strain *S. marcescens* F-1, PCR was performed using (5'- CATCCATACACGTTGGTTTACGCT-3' and 5'-TGCTCGTCCAGCAGTTGAGGAATA-3') as primers and F-1 chromosomal DNA as the template. Compared with the strain CH-1 as the control, a 3.1 kb amplified DNA fragment was observed in *S. marcescens* F-1 (Fig. 2a), indicating the F-1 mutant genotype. Further experiments by Southern blot hybridization confirmed a double cross-over event had taken place with the replacement of *flhD* by *flhD*::SM (Fig. 2b).

Cellular motility, growth and elongation were defective in $flhDC_{Sm}$ mutant

S. marcescens F-1 was used for characterization of $flhDC_{Sm}$ function in cell division and flagellar synthesis. It was first inoculated into 0.4 % and onto 0.8 % LB agar to assess the swimming and swarming behaviors. As predicted, no phenomena of

swarming and swimming were observed (Fig. 3a). Flagella silver stain further showed no evidence of flagella (data not shown). However, the red pigment prodigiosin and the transparent zone supposed to be the surfactant serrawettin (Matsuyama *et al.*, 1995; Lindum *et al.*, 1998) were not affected in *S. marcescens* F-1, suggesting that they were not regulated by *flhDC*$_{Sm}$. The growth rate, cell elongation, and expression profile of *hag* (the flagellin structural gene of *S. marcescens*. Harshey *et al.*, (1989) throughout growth in LB broth culture were compared for *S. marcescens* CH-1 and F-1 cells. Fig. 3b showed that the growth rate expressed as O. D. (A_{600}) of F-1 was higher than that of CH-1 cells, and similar phenomenon was also observed in *E. coli flhD* mutant (Pruss and Matsumura, 1996). Measurement of cell elongation showed that significant difference in cell length was observed, especially during the period of early exponential phase, when the length of F-1 cells was about two thirds of the length of CH-1 cells (Fig. 3b). The *hag* promoter activity in *S. marcescens* S-2 was significantly decreased compared with that of *S. marcescens* S-1, (1/1000 activity was left). These data showed that, as expected, in *S. marcescens* swarmer-cell differentiation is regulated by *flhDC*$_{Sm}$ operon.

NucA activity was abolished in *flhDC*$_{Sm}$ mutant

The role of *flhDC*$_{Sm}$ in the control of secreted virulence factors, such as the expression of *nucA*$_{Sm}$ was compared in *S. marcescens* CH-1 and N-2. A qualitative DNase plate assay (Koneman *et al.*, 1997) was first performed and showed a difference between the nuclease activity of these two strains was observed (about 1/2 activity was left). As expected, following similar procedures described from *S. liquefaciens* by Givskov *et al.*, (1995), the phospholipase-negative *S. marcescens* CH-1 cells were also observed in the *flhD* mutant (Fig.4a). Plate gelatin degradation assay

(Lai, 1994) to see the effect of *flhDC*$_{Sm}$ operon on the secreted protease activity did not show significant difference in the transparent zones around bacterial colonies.

A microplate assay was performed to quantify the nuclease activity. The result showed that the relative nuclease activity detected in the spent supernatant of *S. marcescens* CH-1 was higher than that of F-1 for about 4~8 times. These results demonstrate that *flhDC*$_{Sm}$ has an important role in the regulation of nuclease synthesis.

To determine whether regulation of *nucA*$_{Sm}$ by *flhDC*$_{Sm}$ occurs at the transcriptional level, a P*nucA*$_{Sm}$::*luxAB* reporter system was constructed in *S. marcescens* CH-1. This approach offers high sensitivity of reporting of the *nucA*$_{Sm}$ promoter activity, not obtained with a *lacZ* reporter system, where mitomycin has to be added for detection of *nucA*$_{Sm}$ promoter activity (Chen *et al.*, 1992). Also, by using this assay, it is easier and faster than measuring the nuclease activity directly. The bacterial strains (N-1 and N-2) containing the promoter region of *nucA*$_{Sm}$ in front of *luxAB* in the chromosome were constructed. Intensity of light emission was measured following the growth process in LB broth culture. A significant difference in bioluminescence emission was observed between N-1 and N-2 cells and only 1/10 of N-1 P*nucA*$_{Sm}$ activity was left in the *S. marcescens* N-2 mutant (Fig. 4b), suggesting that expression of *nucA*$_{Sm}$ was regulated by FlhD/FlhC$_{Sm}$ directly or indirectly at the promoter level. For complementation, *flhDC*$_{Sm}$ constructed in pJH05 was transformed into *S. marcescens* N-2 cells. Chloramphenicol resistant transformants were assayed for *nucA*$_{Sm}$ expression and cell differentiation. As expected, cell division, flagellar synthesis and swimming/swarming ability were restored to slightly better than the level of wild-type cells. The *nucA*$_{Sm}$ expression pattern was also restored to normal in the *flhDC*$_{Sm}$ mutant *S. marcescens* N-2 (Fig. 4b), showing that *flhDC*$_{Sm}$ operon was the genetic determinant controlling *nucA*$_{Sm}$ expression.

Cell differentiation was observed in multi-copy *flhDC*$_{Sm}$ in MGM broth culture

The pJH05 was transformed into *S. marcescens* CH-1. Following the growth in MGM broth culture at 30°C, cellular morphology was observed closely. The result in Fig. 5 showed that elongated cells of cell length up to 40~80 vegetative cells without septum formation were observed at early-to-mid log phase, suggesting that the *flhDC*$_{Sm}$ operon has a dominant role in the cell differentiation process. We have previously hypothesized that there may be putative signal factors existing in the rich broth media, which in combination with *flhDC* $_{Sm}$ operon, stimulate the formation of differentiated cells. The over-expression of *flhDC*$_{Sm}$ suggests that either a signal is not required for formation of differentiated cells or over-expression of *flhDC*$_{Sm}$ overcomes the need for a signal.

Expression of *flhDC*$_{Sm}$ is self-regulated

To monitor the expression pattern of *flhDC*$_{Sm}$ throughout growth, *S. marcescens* F-3 (P*flhDC*$_{Sm}$::*luxAB*) was constructed. Following the growth curve at 30°C in LB broth culture, bioluminescence measurements (indicating *flhDC*$_{Sm}$ transcription) were taken (Fig. 6). Expression from P*flhDC*$_{Sm}$ is growth-phase dependent, with the peak activity occurring in the early log phase. To determine whether FlhD/FlhC auto-regulates, an *flhD* knock-out mutation was constructed in *S. marcescens* F-3 and designated *S. marcescens* F-4. The patterns of light emission were measured in *S. marcescens* F-3 and F-4 (Fig. 6) and show that a 30 % ~50 % decrease in P*flhDC*$_{Sm}$ occurs in the *flhD* mutant. Using a different reporter system, a similar result was found in *Salmonella typhimurium* (Kutsukake, 1997). The results show that *flhDC*$_{Sm}$ autoregulation does not dominate the regulation of P*flhDC*$_{Sm}$ and that some other factors should exist for

the regulation.

Expression of *flhDC*$_{Sm}$ is influenced by temperature, osmolarity, but not by glucose catabolite repression

Our data showed that the remaining 50 % of *flhDC*$_{Sm}$ activation was not achieved by autoregulation. As the expression of nuclease gene *nucA*$_{Sm}$, the synthesis of flagella and the swarming phenotype are regulated by environmental temperature and osmolarity [Li *et al.*, 1993, Lai *et al.*, unpublished data], we investigated the role of these important environmental parameters in the regulation of *flhDC*$_{Sm}$. The P*flhDC*$_{Sm}$::*luxAB* and P*hag*$_{Sm}$::*luxAB* DNA fusions were used as the reporter systems and the patterns of bioluminescence emission of *S. marcescens* F-3 and S-1 were monitored closely throughout growth in MGM broth culture. Results in Fig. 7a showed that the activity of P*flhDC*$_{Sm}$ in F-3 at 37℃ was decreased for 25 % compared with that at 30℃, and expression of P*hag*$_{Sm}$ in S-1 was decreased for about 50 %.

Although it has been reported that expression of *flhDC*$_{Sm}$ is regulated by the EnvZ/OmpR signal transduction system in *E. coli* (Shin and Park, 1995), the *ompR* mutation did not affect the motility phenotype or *flhD* expression in *S. typhimurium* (Kutsakake 1997). EnvZ and OmpR are the sensor and response regulator proteins of a two-component system that controls the porin regulon of *Escherichia coli* in response to osmolarity (Hsing and Silhavy, 1997). To investigate the role of osmolarity in the control of P*flhDC*$_{Sm}$, *S. marcescens* F-3 was used. Increase of NaCl concentration (8.5, 100, 250, and 500 mM) in MGM broth cultures inhibits the cell growth and down-regulates the expression of P*flhDC*$_{Sm}$ (Fig 7b).

It has also been reported that a small increase in the glucose concentration of the medium up to 27.8 mM (0.5 %) completely inhibited the synthesis of flagella in *E.*

coli (Adler and Templeton, 1967), a phenomenon known as the glucose catabolite repression (Emmer *et al.*, 1970). However, a similar phenomenon was not observed in our experiments including *E. coli* strains (Lai *et al.*, 1997a). For confirmation, *S. marcescens* F-3 was inoculated into MGM broth cultures containing different concentrations of glucose (0.4 %, 0.8 %, 1.2 %, and 2.0 %), and the light emission pattern was recorded. The result in Fig. 7c showed that instead of inhibition, increase of glucose concentration generally up-regulates the expression of P*flhDC*$_{Sm}$. This result confirms our previous observation.

DISCUSSION

It is becoming increasingly apparent that *flhDC*$_{Sm}$ has an important global role in the regulation of physiology in Gram-negative bacteria. Here we report that in *S. marcescens flhDC* (*flhDC*$_{Sm}$) activates the expression of nuclease gene *nucA*$_{Sm}$ in addition to previously described activation of expression of phospholipase gene, control of cellular motility, and cell division. This result shows that FlhD/FlhC is a multiple-functional transcriptional activator involved in the process of cell differentiation, swarming and virulence factor expression.

Many extracellular proteins made by *S. marcescens* are partly co-ordinated in the regulation of their expression. The nuclease, chitinase, and phospholipase are all found at increased levels when bacterial growth slows down. However, the signals specifying growth-phase regulation have not yet been determined for these proteins. In this work we extend our studies to nuclease regulation. Our results show that in addition to growth–phase and SOS regulation, *nucA*$_{Sm}$ is activated by FlhDC$_{Sm}$ at the promoter level. To add further complexity, expression of *nucA*$_{Sm}$ is co-ordinately regulated with cell division and flagellar synthesis. As it is reported that regulation of *nucA*$_{Sm}$ expression is growth-phase dependent, and occurs at the transcriptional level in *S. marcescens* (Chen *et al.*, 1992), it is quite possible that this effect actually takes place via the expression of *flhDC*$_{Sm}$ operon. Furthermore, as the expression of both *flhDC*$_{Sm}$ and *nucA*$_{Sm}$ are influenced by osmolarity and temperature in *S. marcescens* (Lai *et al.*, unpublished data), such an effect might also work through *flhDC*$_{Sm}$.

It is not clear whether FlhD$_{Sm}$ alone or FlhD/FlhC$_{Sm}$ controls the expression of *nucA*$_{Sm}$. What is known is that the flagellar synthesis is controlled by FlhD/FlhC in *E. coli*, and that cell division is regulated by FlhD alone (Pruss *et al.*, 1997). We are currently investigating whether both FlhD/FlhC are essential for *nucA*$_{Sm}$ expression.

Another question we hope to address is whether there is direct interaction between FlhD/FlhC and the *nucA*$_{Sm}$ promoter. DNA footprinting will be performed for such purpose.

It is interesting to note that differentiated swarmer-cells were observed in *S. marcescens* containing multi-copy of *flhDC*$_{Sm}$, in the absence of other environmental factors such as solid agar surface contact and signal molecules. This observation suggests that initiation of cell differentiation in *S. marcescens* might be growth–phase dependent. In fact, in LB rich broth media, differentiated cells could also be observed in early log phase, when *flhDC*$_{Sm}$, and then the *hag* were over-expressed (Lai *et al.*, 1997b). More experiments have to be performed to confirm the supposition.

Our data showed that expression of *flhDC*$_{Sm}$ was growth-phase dependent, autoregulated, and influenced by temperature and osmolarity, but no glucose catabolite repression was observed in *S. marcescens*. These data suggested that a feedback regulation was observed from *flhDC*$_{Sm}$, and that the environmental factors affecting *flhDC*$_{Sm}$ expression might indirectly affects the phenotypes regulated by *flhDC*$_{Sm}$. The data also confirms our previous observation that the glucose effect observed on the inhibition of cell swimming is not observed in enterobacteria (Lai et al., 1997a). Although from the nucleotide sequence data, a consensus CRP binding site was found in the upstream region of *flhDC*$_{Sm}$, our result showed that no significant effect was observed from glucose catabolite repression, suggesting that such effect does not play a dominant role in the regulation of *flhDC*$_{Sm}$. Similar effect was also observed from *S. typhimurium* (Kutsukake, 1997). Also, it was found that a σ^{28} consensus sequence was found between the nucleotide sequence 182~208, suggesting that the P*flhDC*$_{Sm}$ might be regulated by FliA (the σ^{28} factor) of *S. marcescens*. Indeed, some genetic evidence was also observed in *S. typhimurium* (Kutsukake,

1997). To confirm this, an experiment was performed to see whether an interaction between FliA and P$flhDC_{Sm}$ exists in *S. marcescens*. *S. marcescens* does not swarm at 37°C and our result showed that the promoter activity of $flhDC_{Sm}$ was decreased for about 25 % when the environmental temperature was shifted from 30 to 37 °C. This may partly explain the mechanism of swarming inhibition at higher temperature in *S. marcescens*.

Acknowledgements

This work was supported by the National Science Council grants NSC88-2314-B002-287, and NSC88-2314-B002-330, Taiwan, R. O. C. We thank Wei-Ming Chen and Ya-Lan Cheng for technical and facility assistance.

References

Adler, J. & Templeton, B. (1967), The effect of environmental conditions on the motility of *Escherichia coli*. *J. Gen. Microbiol.*, 46, 175-184.

Al Mamun, A.A., Tominaga, A. & Enomoto, M. (1996), Detection and characterization of the flagellar master operon in the four *Shigella* subgroups. *J. Bacteriol.*, 178, 3722-3726.

Ball, T.K., Wasmuth, C.R., Braunagel, S.C. & Benedik, M.J. (1990), Expression of *Serratia marcescens* extracellular proteins requires *recA*. *J. Bacteriol.*, 172, 342-349.

Bernard, P. (1996), Positive selection of recombinant DNA by CcdB. *Biotechniques*, 21, 320-323.

Chang, A.C.Y. & Cohen S.N. (1978), Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.*, 134, 1141-1156.

Chen, Y.C., Shipley, G.L., Ball, T.K. & Benedik, M.J. (1992), Regulatory mutants and transcriptional control of the *Serratia marcescens* extracellular nuclease gene. *Mol. Microbiol.*, 6, 643-651.

Dufour, A., Furness, R.B. & Hughes, C. (1998), Novel genes that upregulates the *Proteus mirabilis flhDC* master operon controlling flagellar biogenesis and swarming. *Mol. Microbiol.*, 29, 741-751.

Eberl, L., Christiansen, G., Molin, S., Givskov, M., Guynn, L.J., Dai, W. & Benedik, M.J. (1996), Differentiation of *Serratia liquefaciens* into swarm cells is controlled by the expression of the *flhD* master operon. *J. Bacteriol.*, 178, 554-559.

Emmer, M., deCrombrugghe, B., Pastan, I. & Perlman, R. (1970), Cyclic AMP

receptor protein of *E. coli*: Its role in the synthesis of inducible enzymes. *Proc. Natl. Acad. Sci.*, 66, 480-487.

Furness, R.B, Fraser, G.M., Hay, N.A. & Hughes, C. (1997), Negative feedback from a *Proteus* class II flagellum export defect to the *flhDC* master operon controlling cell division and flagellum assembly. *J. Bacteriol.*, 179, 5585-5588.

Givskov, M., Eberl, L., Christiansen, G., Benedik, M.J. & Molin, S. (1995), Induction of phospholipase- and flagellar synthesis in *Serratia liquefaciens* is controlled by expression of the flagellar master operon *flh*D. *Mol. Microbiol.*, 15, 445-454.

Givskov, M., Ostling, J., Eberl, L., Lindum, P.W., Christensen, A.B., Christiansen, G.,

Molin, S. & Kjelleberg, S. (1998), Two separate regulatory systems participate in control of swarming motility of *Serratia liquefaciens* MG1. *J Bacteriol.*, 180, 742-745.

Harshey, R.M. (1994), Bees are not the only ones: swarming in Gram-negative bacteria. *Mol. Microbiol.*, 13, 389-394.

Harshey, R.M., Estepa, G. & Yanagi, H. (1989), Cloning and nucleotide sequence of a flagellin-coding gene (*hag*) from *Serratia marcescens* 274. *Gene*, 79, 1-8.

Hsing, W. & Silhavy, T.J. (1997), Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli. J. Bacteriol.*, 179, 3729-3735.

Khan, E.A., Wafelman, L.S., Garcia-Prats, J.A. & Taber, L.H. (1997), *Serratia marcescens* pneumonia, empyema and pneumatocele in a preterm neonate. *Pediatr. Infect. Dis. J.*, 16, 1003-1005.

Koneman, E. W., Allen, S. D., Janda, W. M., Schreckenberger, P. C. & Winn, W.C.

(1997), Color Atlas and Textbook of Diagnostic Microbiology. Fifth Edition. J. B. Lippincott Company. Philadelphia, U. S. A.

Kutsukake, K. (1997), Autogenous and global control of the flagellar master operon, *flhD*, in *Salmonella typhimurium*. *Mol. Gen. Genet.*, 254, 440-448.

Lai, H.C. (1994), Molecular studies on the swarming migration of *Proteus mirabilis*. Ph.D thesis, Wolfson College, Cambridge University. England, U.K.

Lai, H.C., Shu, J.C., Ang, S., Lai, M.J., Fruta, B., Lin, S., Luh K.T. & Ho, S.W. (1997a), Effect of Glucose Concentration on Swimming Motility in Enterobacteria. *Biochem. Biophy. Res.Commu.*, 231, 692-695.

Lai, H.C., Lai, M.J., Lin-Chao, S., Luh, K.T. & Ho, S.W. (1997b), Population cell differentiation of *Serratia marcescens* on agar surface and in broth culture. *Chin. J. Microbiol. Immunol.*, 30, 242-254.

Li, C., Louise, C.J., Sh, W. & Adler, J. (1993), Adverse conditions which cause lack of flagella in *Escherichia coli*. *J. Bacteriol.* 175, 2229-2235.

Lindum, P.W., Anthoni, U., Christophersen, C., Eberl, L., Molin, S. & Givskov, M. (1998), N-acyl-l-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. *J. Bacteriol.*, 180, 6384-6388.

Liu, X.Y. & Matsumura, P. (1994), The FlhD/FlhC complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. *J. Bacteriol.*, 176, 7345-7351.

de Lorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K.N. (1990), Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *J. Bacteriol.*, 172, 6568-6572.

de Lorenzo, V. & Timmis, K.N. (1994), Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Bacterial pathogenesis part A. Identification and regulation of virulence factors. *Methods. Enzymol.*, 235, 386-405.

Macnab, R.M. (1992), Genetics and biogenesis of bacterial flagella. *Annu.Rev. Genet.*, 26, 131-158.

Maniatis, T., Sambrook, J. & Fritsch, E.F. (1989), Molecular cloning. A laboratory manual, second edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N. Y.

Matsuyama, T., Bhasin, A., & Harshey, R.M. (1995), Mutational analysis of flagellum-independent surface spreading of *Serratia marcescens* 274 on a low-agar **medium**. *J. Bacteriol.*, 177, 987-991.

Peeters, A., Vandercam, B., Sindic, C.J., Hantson, P.& Mahieu, P. (1997) Community-acquired *Serratia marcescens* meningitis. *J. Infect.Dis.*, 35, 303-304.

Pruss, B.M. & Matsumura, P. (1996), A regulator of the flagellar regulon of *Escherichia coli*, *flhD*, also affects cell division. *J. Bacteriol.*, 178, 668-674.

Pruss, B.M., Markovic, D. & Matsumura, P. (1997), The *Escherichia coli* flagellar transcriptional activator *flhD* regulates cell division through induction of the acid response gene *cadA*. *J. Bacteriol.*, 179, 3818-3821.

Shin, S. & Park, C. (1995), Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. *J. Bacteriol.*, 177, 4696-4702.

Figure Legends

Fig. 1. The physical map and sequence characterization of the *flhDC*$_{Sm}$ genetic locus.

(a) The physical map of *S. marcescens flhDC*$_{Sm}$ genetic locus. PI, *PstI*; EI, *EcoRI*; SI, *SalI*; XI, *XhoI*; SpI, *SphI*.

(b) DNA and predicted protein sequences of the *flhDC*$_{Sm}$ locus. A potential Shine-Dalgarno (S.D.) sequence was indicated. A sequence TAAAAT, similar to the *E. coli* consensus –10 box, is found between the nucleotide sequence 413~418. The sequence, TTGCGC, similar to the *E. coli* consensus –35 box, is found between positions 387~392 (The shaded area). A σ^{28} consensus sequence was found between 182~208, and a putative consensus CRP binding site was found between 311~331, with the conserved sequence being boxed.

Fig. 2. Confirmation of inactivation of *flhD*$_{Sm}$ by streptomycin (SM) through homologous recombination.

(a) A pair of primers (5'CATCCATACACGTTGGTTTACGCT-3' and 5'-TGCTCGTCCAGCAGTTGAGGAATA-3') were used in the PCR for confirmation of insertion mutagenesis. *S. marcescens* CH-1 chromosomal DNA (lane 1), pJH04 (lane 2), pCR2.1*flhDC*$_{Sm}$::Sm (lane 3), *S. marcescens* F-1 chromosomal DNA (lane 4), and *S. marcescens* S-2 chromosomal DNA (lane 5) were used as the template. The arrows indicate the DNA size markers.

(b) Southern blot hybridization using the PCR amplified 1.1 Kb DNA fragment (the same as a) as the probe. Chromosomal DNA of *S. marcescens* CH-1 (lane 1, 4, 7), *S. marcescens* F-1 (lane 2, 5, 8) and *S. marcescens* S-2 (3, 6, 9) were digested by *Sal*I, *EcoR*I/*Hind*III, and *Pst*I separately as indicated. Arrows indicate the DNA size markers.

Fig. 3. The *flhD*$_{Sm}$ mutant phenotypes.

(a) The swimming of F-1 (A) and CH-1 (B), and swarming of F-1(C) and CH-1 (D) in LB plates at 30℃. A and B, 0.4 % agar; C and D, 0.8 % agar.

(b) The growth curve [CH-1 (O); F-1 ( )] and mean cell length units [CH-1 (filled column); F-1 (empty column)] of *S. marcescens* CH-1 and F-1 following the growth process in LB broth culture at 30℃. Results are the means of at least 3 independent experiments (SEM < 5 %).

Fig. 4. Effect of *flhDC*$_{Sm}$ on the phospholipase activity and *nucA*$_{Sm}$ expression.

(a) The phospholipase–negative cells (upper arrow) were observed in *flhD* mutant (*S. marcescens* F-1) after O/N culture on egg-yolk agar plate (Koneman *et al.*, 1997). The lower arrow indicates the wild-type control cells.

(b) Expression of *nucA*$_{Sm}$ was activated by *flhDC*$_{Sm}$. Following the growth process in LB broth culture at 30℃, the light emission of *S. marcescens* N-1/ pBCSK+ ( ), N-2/ pBCSK+ (O), and N-2/pJH05 (ì) were measured and the specific activity (spe. act.) was expressed as (R.L.U./O.D.). Results are the means of three independent experiments (SEM < 6 %).

Fig. 5. Cells with differentiated characteristics were observed in *S. marcescens* CH-1/pJH05 in MGM broth culture.

S. marcescens CH-1/pJH05 was inoculated in MGM broth culture at 30℃. Following the growth process, cells were harvested for observation of cellular morphology. The cells shown in the left (A) were taken from early log phase (O.D. 0.2). The cells shown in the right (B) was taken from *S. marcescens* CH-1/ pBCSK+ control cells (O.D. 0.2).

Fig. 6. P*flhDC*$_{Sm}$ was self-regulated.

Following the growth process in LB broth culture at 30℃, the light emissions of *S. marcescens* F-3 ( ) and F-4 (O) were measured and expressed as specific activity (spe. act., R.L.U./O. D.). Results are the means of three independent experiments (SEM < 6 %).

Fig. 7. Effect of temperature, osmolarity and glucose concentration on the expression of P*flhDC*$_{Sm}$.

(a) *S. marcescens* F-3 (P*flhDC*$_{Sm}$::*luxAB*) was cultured in LB broth at either 30 (O) or 37℃ ( ). The light emission was measured following the growth process. For comparison, *S. marcescens* S-1 (P*hag*$_{Sm}$::*luxAB*) was also assayed at 30 (ì) or 37℃ ( ).

(b) *S. marcescens* F-3 was cultured at increasing NaCl concentration [8.5 (O), 100 ( ), 250 (ì) and 500 ( ) mM] in LB broth, followed by measuring the light emission. (c) *S. marcescens* F-3 was

cultured at increasing glucose concentration [0.4 % (O), 0.8 % (Ì), 1.2 % ( ) and 2.0 % ( )] in MGM broth culture. Results are the means of three independent experiments (SEM < 5 %). spe. act., specific activity (R. L. U./O. D.).