行政院國家科學委員會專題研究計畫 成果報告

Hfq-dependent srRNAs 在克雷白氏肺炎桿菌的生理適應與毒力潛能的調控影響

研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 98-2320-B-040-013-
執 行 期 間 ：98 年 08 月 01 日至 99 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學系

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處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 99 年 11 月 16 日

Abstract

Klebsiella pneumoniae adapts itself to various environments and is capable of causing a wild range of infections. How *K. pneumoniae* can switch physiological programs to ensure its survival in a specific ecological niche is still a mystery. Small non-coding RNAs are crucial regulators that modulate diverse cellular processes to enable prokaryotic cells to adjust physiological fitness to environmental changes. Most small regulatory RNAs (srRNAs) exert their function in the post-transcriptional level via the binding of Hfq. We aim in this project to elucidate the role of Hfq-srRNA in the physiology fitness and virulence potential of *K. pneumoniae*. An *hfq* gene-knockout mutant was generated. The Δ*hfq* strain lost its ability to disseminate into extra-intestinal organs in an oral infection mouse model. Microarray-based transcriptome analyses showed that a total of 897 genes were deregulated by the absence of Hfq that involve in numerous cellular processes revealing $\geq 1.5\log_2$ ($\cong 2.83$) fold changes in their transcripts abundance in the Δ*hfq* strain compared to that in the parental strain. In combined with the phenotypic characterization of the Δ*hfq* strain, the transcriptome result demonstrated that the expression of almost a fifth of all *K. pneumoniae* genes were controlled directly or indirectly by Hfq suggest that Hfq acts as a global regulator in *K. pneumoniae*. The manuscript regarding this part of work has been submitted.

Introduction

Since the first identification of *Klebsiella* as a pathogen of pneumonia in 1882, the remarkable ability of *K. pneumoniae* to cause a wild range of human diseases, from urinary tract infections to life-threatening systemic infections [1], has attracted increasing attention to the pathogenesis of this bacterium. Not solely confined inside the human host, *K. pneumoniae* has a great capacity for adaptation to diverse environments, including the surface water, sewage, soil, the intestinal tract of other mammals [1], and even the interior of plants [2]. How *K. pneumoniae* responds to environmental changes and thus adapts itself to a specific niche becomes an interesting question. However, our knowledge about the regulatory mechanism by which this bacterium switches among different physiological programs to ensure its survival upon different conditions remains incompletely understood.

Post-transcriptional regulations involving small non-coding RNAs (sRNAs) have received considerable attention in recent years [3]. Through rapid post-transcriptional adjustments, the regulatory sRNAs have advantages over protein regulators to promote bacterial adaptation to drastic environmental changes [4]. Studies have demonstrated that eubacterial sRNAs have crucial roles in the regulation of iron homeostasis [5], outer membrane proteins (OMPs) biogenesis [6], sugar metabolism [7], quorum sensing [8] and various stress responses [9]. The eubacterial sRNAs, in contrast to the eukaryotic siRNAs and miRNAs, show dramatic heterogeneity in size (50~250 nt) and structure, and are usually encoded by free-standing genes with a rho-independent terminator [10]. There are > 70 *Escherichia coli* sRNAs have been identified. For the *E. coli* sRNAs, two main modes of action have been established. While some sRNAs modify the activity of proteins, the majority of sRNAs act through base-pairing with partially complementary

sequences in the 5'-untranslated region of trans-encoded target mRNAs to modulate their translation and/or stability [11]. In many cases, sRNAs that act by the later mechanism do so in complex with the chaperon protein, Hfq.

Hfq, a RNA-binding protein, assembles into homohexameric rings which are structurally similar to those formed by the Sm and Sm-like proteins in eukaryotic and archaeal cells. Except for stabilizing sRNAs and enhancing the formation of sRNA-mRNA pairs to modulate gene expression post-transcriptionally, Hfq can also binds directly to mRNAs to influence messenger stability, polyadenylation, and ribosome binding. Hfq has a broad and diverse impact on bacterial physiology and virulence beyond its original role as a host factor required for replication of Qβ RNA bacteriophage [12]. Defects including reduced growth, impaired resistance to various stresses, and altered virulence are detected in *E. coli* cells lacking *hfq* [13]. Virulence of several pathogenic bacteria, including *Brucella abortus* [14], *Vibrio cholera* [15], *Listeria monocytogenes* [16], *Legionella pneumophila* [17], *Pseudomonas aeruginosa* [18], *Yersinia* [19, 20], *Salmonella* Typhimurium [21], and uropathogenic *E. coli* [22], were significantly attenuated by hfq mutations. While Hfq homologues are encoded in approximately half of the sequenced bacterial genomes, they are absent in several phyla (eg. *Cyanobacteria*, green-sulphur bacteria and *Chlamydia*), in some proteobacteria (eg. *Bruchnera spp.* and *Campylobacter spp.*), and in a number of Gram-positive bacteria (eg. *Streptococcus spp.* and *Mycoplasma spp.*) [23].

Although the presence of homologues to *E. coli hfq* and a number of sRNA genes in *K. pneumoniae* genome suggests the existence of conserved mechanisms, the roles of Hfq-sRNA mediated regulation in *K. pneumoniae* still remains undiscovered. Considering the significance of post-transcriptional regulation on numerous pathways for a variety of bacteria, we aimed in this study to understand

how Hfq might contribute to the physiology and virulence of *K. pneumoniae*.

Results and Discussion

Deletion of *hfq* attenuates *K. pneumoniae* virulence.

The *hfq* gene is located in clockwise orientation at bps 446148-446456 in the genome of *K. pneumoniae* strain NTHU-K2044 [24]. As in *E. coli*, it is located in the *miaA-hfq-hflX* cluster of genes with three promoter regions as indicated in Fig. 1A. The *K. pneumoniae* Hfq protein has a 76.7% identity with other Hfq proteins which were reported in other *Enterobacteriaceae* bacteria. The *Enterobacteriaceae* Hfq proteins share the conserved Sm1 and Sm2 motifs with all amino acid derivatives locating in the C-terminal region (Fig. 1B). The sequence of *hfq* region of *K. pneumoniae* CG43 used in this study is identical to that of strain NTHU-K2044. Based on the sequence data, a nonpolar *hfq* deletion mutant, named H0201, and two trans-complemented strains, H0201-C1 and H0201-C2, which carried the *hfq* gene under the control of pBAD promoter or its native promoters, respectively, were constructed as described in Materials and Methods.

To examine the role of *hfq* in the *in vivo* fitness of *K. pneumoniae* during infections, competitive assays were performed in 8-week-old male BALB/c mice by orally inoculating them with the bacterial mixture containing equal amount of the Δ*hfq* strain, H0201, and its parental strain, CG43S. No significant difference was observed in the ability of H0201 to colonize the small intestine at 24 hpi (hour post-inoculation) (Fig. 2A). However, the bacterial load of H0201 was undetected in the liver, but all the infected mice had a burden of the CG43S strain approaching 10^2 to 10^4 CFU in the liver at the same time point (Fig. 2A). The significant decline in the bacterial load of H0201 in the liver indicated that the loss of *hfq* attenuated the ability of *K. pneumoniae* to disseminate to extra-intestinal organs. To further

investigate if *hfq* is also required for a systemic infection of *K. pneumoniae*, groups of mice were infected via the intraperitoneal route with H0201 or CG43S and survival was monitored for two weeks. This allowed the infection to bypass the step where *K. pneumoniae* colonies the small intestine. When 10^4 CFU of bacteria were inoculated, 80% of mice infected with H0201 survived in the experimental course, but all of the mice infected with CG43S died by day 2. Similarly, when the inoculums decreased to 2×10^3 CFU, 60% of CG43S-infected mice succumbed to the infection. The requirement of *hfq* in a systemic *K. pneumoniae* infection was also supported by the *in vivo* competition assays. At 6 h after intraperitoneal inoculation, the average CI values of H0201 in the livers and spleens were 0.11 and 0.01, respectively (Fig. 2C). The significant decline in the *in vivo* CI values was not due to an inability to compete under nutrient-limiting conditions, as the growth of H0201 approximated to that of CG43S with the *in vitro* CI values >0.82 in M9 medium.

Transcriptional profile globally affected by Hfq.

Given Hfq contributes to the regulation of numerous cellular pathways in *E. coli*, to gain insight into the range of genes with expression that is regulated by Hfq in *K. pneumoniae*, DNA microarrays were performed to compare the transcriptome of H0201 with CG43S. Probes were made corresponding to RNA transcribed during log phase growth in LB medium at 37^oC. Of the 5116 genes in the *K. pneumoniae* genome, the expression of 897 genes (approximately 17.5% of all *K. pneumoniae* genes) showed a $>1.5\log_2$ fold change (2.83-fold) in transcript abundance in H0201 when compared to CG43S. Among the 897 Hfq-dependent genes, down-regulated genes (n=610) were more than twice as common as up-regulated genes (n=287), suggesting that Hfq may function more frequently as an activator than as a repressor. Based on the genome annotation of *K. pneumoniae* NTHU-K2044 (NC012731;[24]), these Hfq-dependent genes belong to more than 19 functional categories (Fig. 3).

The expression of several categories of genes was notably affected by Hfq. Approximately 34.3% of genes belonging to the class of signal transduction mechanism were Hfq-dependent [13.3% (14/105) for up-regulated; 21% (22/105) for down-regulated). Besides, Hfq-dependency also accounts for 35.7%, 30.6%, 26.36%, and 18% of genes belonging to the classes of energy production and conversion and transport and metabolism of lipid, carbohydrate and amino acid, respectively.

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Figures

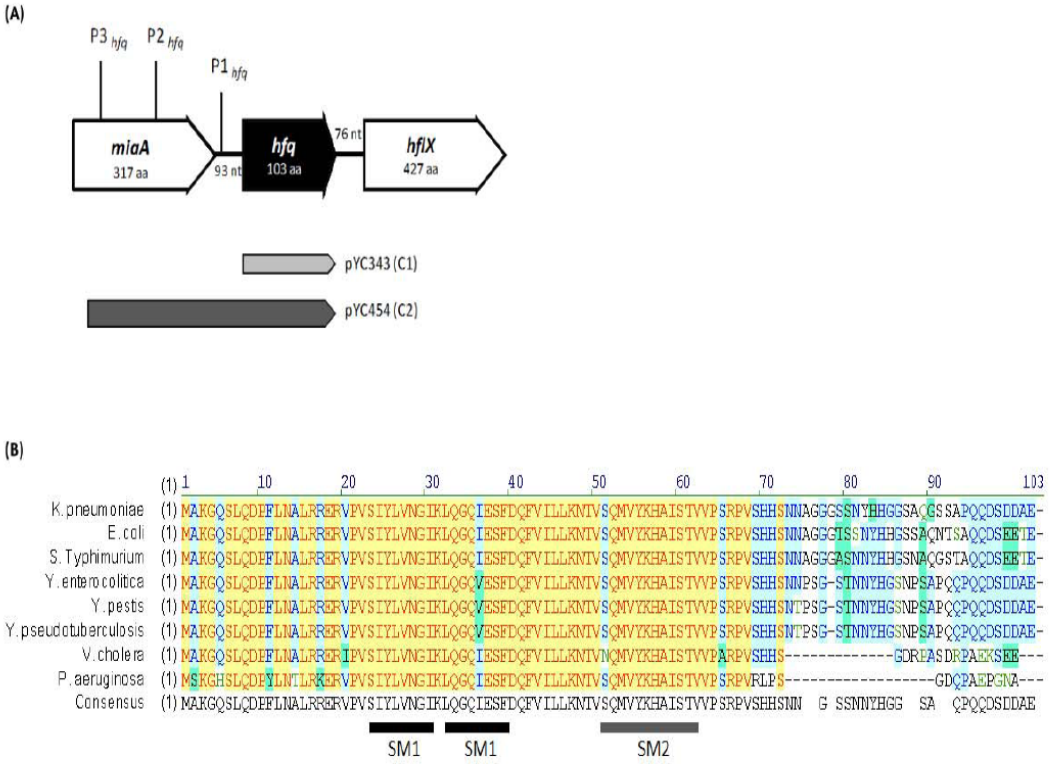

Figure 1. (A) Genomic localization of *hfq* in *K. pneumoniae* CG43. The region cloned on complementation plasmid, pYC343, is indicated as grey arrow. The black arrow indicates the region containing the coding region with three hfq promoters cloned on pYC454. (B) Alignment of *hfq* sequences of the pathogens with their Hfq functionally identified. The highly conserved SM1 and SM2 motifs are indicated as black and grey lines, respectively.

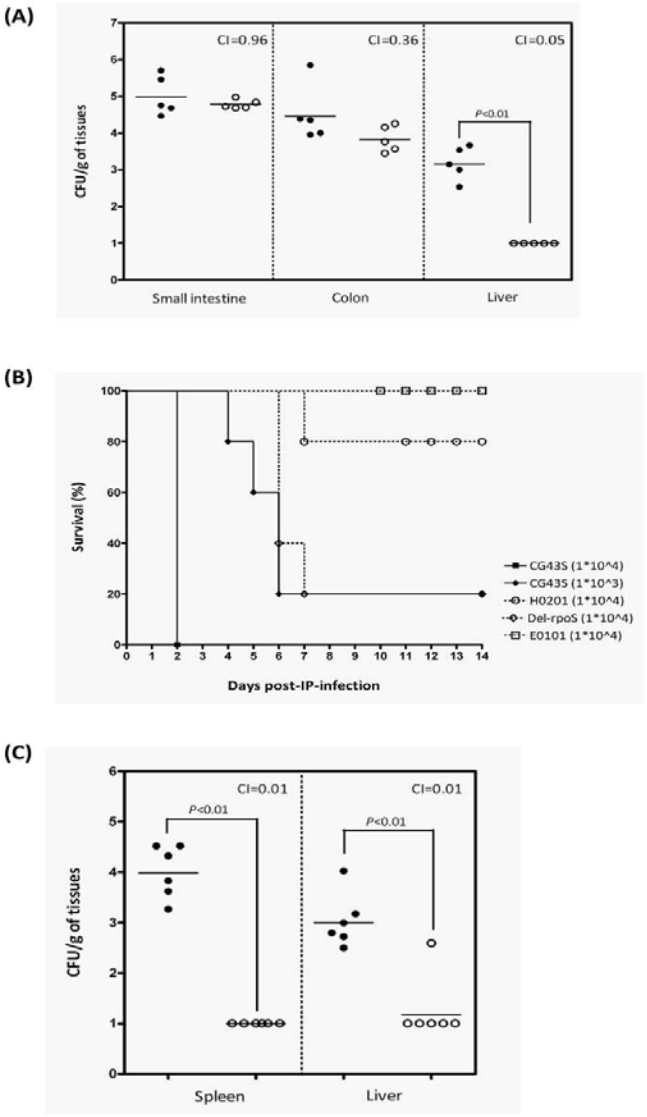

Figure 2. (A) Groups of five mice were inoculated orally with bacterial suspension containing equal amount of *K. pneumoniae* CG43S (5×10^8 CFU) and H0201 (5×10^8 CFU). Bacterial loads of CG43S (filled circles) and H0201 (open circles) in small intestine, colon, and liver were determined at 48 h post-inoculation. Horizontal bars indicate geometric means. The limit of detection was approximately 10 CFU. Samples which yielded no colonies were plotted having the value as 10 CFU g^{-1} tissues. Competitive index is defined as $H0201_{output}/CG43S_{output} \div H0201_{input}/CG43S_{input}$. The indicated *P* values were determined using the Student's *t*-test. (B) Survival of *K. pneumoniae*-infected mice. Groups of five mice were inoculated by intraperitoneal injection with 1×10^3 CFU of CG43S (filled diamonds), or with 1×10^4 CFU of CG43S (filled squares), H0201 (open circles), Del-rpoS (open diamonds), or E0101 (open squares), and monitored for 14 days. (C) Groups of six mice were inoculated intraperitoneally with bacterial suspension containing equal amount of *K.*

pneumoniae CG43S (1×10^3 CFU) and H0201 (1×10^3 CFU). Bacterial loads of CG43S (filled circles) and H0201 (open circles) in spleen and liver were determined at 24 h post-inoculation.

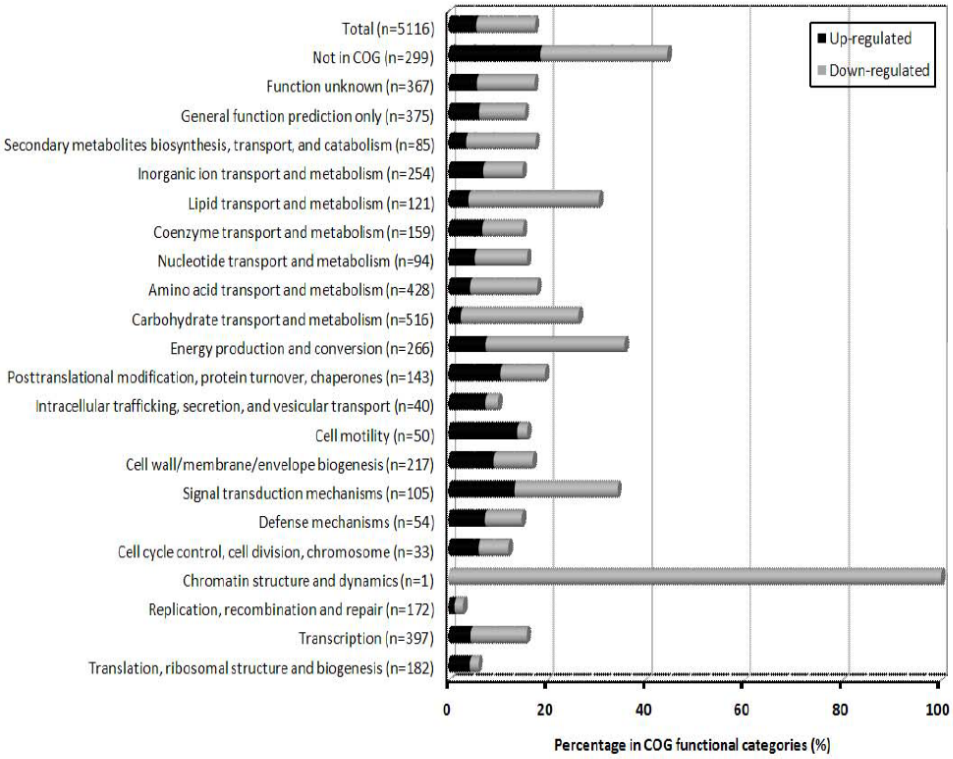

Figure 3. Functional classification of Hfq-dependent genes according to the genome of *K. pneumoniae* NTHU-K2044 (NC012731;[Wu, 2009 #208]). The values represent the percentage of genes affected by Hfq in H0201 versus CG43S within the respective class. Black bars: up-regulated genes; grey bars: down-regulated genes.

國科會補助計畫衍生研發成果推廣資料表

日期:2010/11/16

國科會補助計畫	計畫名稱: Hfq-dependent srRNAs在克雷白氏肺炎桿菌的生理適應與毒力潛能的調控影響
	計畫主持人: 賴怡琪
	計畫編號: 98-2320-B-040-013- 學門領域: 微生物及免疫學

無研發成果推廣資料

98 年度專題研究計畫研究成果彙整表

計畫主持人：賴怡琪	計畫編號：98-2320-B-040-013-
計畫名稱：Hfq-dependent srRNAs 在克雷白氏肺炎桿菌的生理適應與毒力潛能的調控影響	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	2	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 ■未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

Through the determination of the molecular basis of Hfq-dependent modulation in the physiology of K. pneumoniae, several interesting molecular mechanisms which may be unique in this bacterium were identified. In particular, the identification of Hfq-dependent genes provide us insights into how K. pneumoniae adapt itself to various environments by rapidly fine-tuning several cellular processes with RNA molecules. Certain regulatory RNA molecules may serve as an ideal chemical scaffold for discovery of novel antimicrobial drugs.