科技部補助專題研究計畫成果報告
期末報告

探討 Sigma E-dependent small RNA RybB 與 MicA 在克雷白氏肺炎桿菌的生理與致病上的後轉錄調控機制

計 畫 類 別 ：個別型計畫
計 畫 編 號 ：NSC 101-2320-B-040-013-
執 行 期 間 ：101 年 08 月 01 日至 102 年 12 月 31 日
執 行 單 位 ：中山醫學大學醫學系

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中 華 民 國 103 年 04 月 09 日

中 文 摘 要 ： 克雷白氏肺炎桿菌 (Klebsiella pneumoniae) 可適應環境變化並具有引起多樣化的臨床感染的能力，究竟 K. pneumoniae 如何轉換調節生理機制以允許其在特定環境中生存仍然是個謎團。 近來研究證實 Small non-coding RNAs 在原核細胞適應多樣的環境變化中扮演著重要調控角色。兩個 K. pneumoniae 的 sRNAs，RybB 和 MicA 在 Sigma E (钖 E)-overproduced strain 中大量增加其表現量。 相對於 Sigma S (钖 S) 缺失的菌株，钖 E 的缺失除造成 K. pneumoniae 的毒性喪失外亦會減損其在不同極端環境下的抗壓性。 雖然钖 E 是轉錄活化因子，全基因組 DNA 微陣列分析顯示在 K. pneumoniae 有 45%的钖 E-dependent 的基因受到負向調控。由於 RybB 是可以後轉錄抑制基因表現的 RNA 調節子，負向調控钖 E-regulon 的基因可能是源自於這種钖 E-driven sRNAs 的後轉錄抑制作用。 全基因組 DNA 微陣列分析顯示有 31 個 K. pneumoniae 基因在 RybB 的短時間脈衝表現下，mRNA 的量相較於對照組顯著減少四倍以上，而這些基因與受到大腸桿菌 RybB 調控的基因不盡相同。 為探討 钖 E-dependent RybB 在後轉錄層次上如何調節 K. pneumoniae 基因表現，在這個一年期的計畫中，我們利用同源重組的方法建構 rybB 基因剔除菌株，並將這個菌株命名為岛 rybB。 相較於野生型菌株，在小鼠模式中，岛 rybB 對小鼠的毒性顯著下降；在體外培養的條件中發現岛 rybB 在高鹽環境中的生長也受到影響。為進一步了解 RybB 如何藉由調控其目標基因影響 K. pneumoniae 的致病能力，我們由 31 個受到 RybB 負調控的基因當中挑選四個基因，包括 ompC, galU, hdeB, and KP1_0760，運用遺傳學的策略，建構 GFP-translation fusions 以瞭解 RybB 與目標基因的交互作用機制。 藉由比較在 arabinose 存在與否的條件下，GFP 蛋白表現量上的差異，我們觀察到在 arabinose 誘導大量產生 RybB 時，這四個基因的 GFP-translational fusions 在八小時的時間點，表現的 GFP 蛋白量分別有 90%，60%，50% 和 40%的顯著減少。由於 galU, hdeB, and KP1_0760 是 K. pneumoniae-specific RybB targets，究竟 RybB 對這些基因的負調控如何影響該菌的生長與致病性值得後續的研究加以闡明。

中文關鍵詞： 克雷白氏肺炎桿菌，Small RNAs, RybB

英 文 摘 要 ： Klebsiella pneumoniae adapts itself to various environments and is capable of causing a wild range of infections. How K. pneumoniae switches its physiological programs to ensure survival in a specific niche is still a mystery. Recently, it has

become clear that small non-coding RNAs are crucial regulators modulating diverse cellular processes to enable prokaryotic cells to adjust physiological fitness to environmental changes. RybB and MicA, two of K. pneumoniae sRNAs, were strongly activated by the overproduction of sigma E (σE). Deletion of σE-encoding gene rpoE dramatically attenuated K. pneumoniae virulence and abolished its tolerance to diverse stressful conditions. Although σE is a transcriptional activator, 45% of σE-dependent genes were negatively regulated in K. pneumoniae. Given RybB and MicA are RNA regulators repressing gene expression at the post-transcriptional level; it is possible that the negative regulation of genes belonging to the σE-regulon is mediated through the action of σE-driven sRNAs. Upon pulse expression of RybB, DNA microarray analysis revealed that mRNA abundances for 31 genes were significantly decreased with more than 4-fold changes as compared to that of the vector control. The majority of these genes were different from that targeted by E. coli RybB. Because RybB behave like a global regulator but control a distinct set of K. pneumoniae genes from that in E. coli. To determine the regulatory mechanism by which RybB modulates gene expression in K. pneumoniae, in this one-year project, we generated rybB deletion mutants by using the allelic exchange technique. This mutant, named 岛rybB, significantly lost its virulence to mice. When compared with its parental strain and complement strain, bacterial tolerance to a high salt condition in vitro was affected in 岛rybB. Among the 31 RybB targeting candidates, we selected four genes, ompC, galU, hdeB, and KP1_0760, to validate their interaction with RybB by using a genetic approach. By comparison of GFP protein expression by Western blotting with specific antibodies in the presence and absence of arabinose induction, we observed that the overproduction of RybB resulted in 90%, 60%, 50%, and 40% decrease in GFP expression, respectively for gfp fusions of KP1_0760, HdeB, GalU, and OmpC, at 8 hours post-inoculation. How the RybB-mediated control of these

genes contributes to K. pneumoniae virulence and physiology is worth of further studies to elucidate.

英文關鍵詞： Klebsiella pneumoniae, small RNAs, RybB

Background and significance

Klebsiella pneumoniae is a Gram-negative enterobacterium ubiquitous in nature. As behaving like a saprophyte resided in nasopharyngeal and intestinal mucosa of humans, *K. pneumoniae* frequently involves in a wide range of clinical illnesses. Without immediate treatments, infections caused by this bacterium have a significantly high rate of mortality (*1*). Not solely confined inside the human host, *K. pneumoniae* has an immense capacity to adapt to various 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. Nevertheless, our knowledge about the regulatory mechanism by which this bacterium switches among different physiological programs that ensure its survival upon various conditions remains incompletely understood.

Until recently, global gene expression studies have mainly been focused on the transcriptional regulation exerted by DNA-binding proteins. With the identification of more and more sRNAs in bacteria, while functions of many of the RNA molecules are still not known, an increasing number of studies demonstrate that the RNA regulators behave as key effectors of bacterial cellular processes. Through rapid post-transcriptional adjustments, the regulatory sRNAs have advantages over protein regulators to rapidly promote bacterial adaptation to ever-changing environments (*3*). Genes subject to post-transcriptional control by sRNAs are involved in numerous cellular processes, such as iron homeostasis (*4*), outer membrane proteins (OMPs) biogenesis (*5*), sugar metabolism (*6*), quorum sensing (*7*) and various stress responses (*8*). Considering the potential impact on coordinating regulatory networks of stress adaptation and virulence gene expression, we pay particular attention on these RNA molecules. A mutant which has in-frame deletion on the gene encoding the RNA chaperone Hfq was therefore generated in *K. pneumoniae* to serve as a starting point for our study on small regulatory RNAs. The deletion of *hfq* significantly attenuated *K. pneumoniae* virulence in the KLA model and drastically deregulated the expression of almost a fifth of *K. pneumoniae* genes, as evident by microarray-based transcriptome analyses (*9*). As Hfq often acts in conjunction with sRNAs, it is likely that sRNAs play major roles in the control of numerous cellular processes in *K. pneumoniae*.

On the other hand, as it has been shown in *E. coli* that Hfq activities impact the regulation of both the stationary-phase sigma factor S (σ^S) and σ^E (*10*), a significant overlap between Hfq-regulon and σ^E-regulon in *K. pneumoniae* was revealed in our previous study (*9*). Overall, 13.6% (121/891) of Hfq-dependent genes belong to the σ^E-regulon. Of particular interest in the findings is the knock-in of σ^E significantly enhances the expression of eight sRNAs in *K. pneumoniae*. Among the 8 small RNAs, Sr0018, which is a homologue of *E. coli* RybB was up-regulated 306-fold by the overproduction of σ^E. Although σ^E is a transcriptional activator, 45% (149/333) of σ^E-dependent genes were negatively regulated in *K. pneumoniae*. Given RybB is a RNA regulator repressing gene expression at the post-transcriptional level; it is possible that the negative regulation of genes belonging to the σ^E-regulon in *K. pneumoniae* is mediated through the action of the σ^E-driven sRNAs. Previous studies of RybB in *E. coli* and *Salmonella* indicated that this sRNA repressed the synthesis of major OMPs by binding in the

5'-mRNA region (*11-13*). More than 20 non-OMP mRNAs were also found to be targeted by *E. coli* RybB (*14*). These findings suggest that RybB behaves like a global repressor in the post-transcriptional control of *E. coli* gene expression. To determine the regulatory impact of RybB on modulating *K. pneumoniae* gene expression, we performed a genome-wide transcriptome analysis to identify the potential mRNAs that were repressed by RybB. Upon a 10-min pulse-expression of RybB from the arabinose-inducible plasmids, *K. pneumoniae* genes with changes in mRNA abundance were identified by DNA microarray. By the transient expression of RybB, a total of 31 genes showed > 4-fold decrease in transcripts levels. The candidate genes identified in *K. pneumoniae*, except for *ompC*, were different from the set of *E. coli* targets for RybB (*14*). *K. pneumoniae* RybB has many candidate targets showing no envelope-associated functions, suggesting that the this sRNA act as a global regulator in *K. pneumoniae*, but control a distinct set of genes from that in *E. coli* or *Salmonella*.

Specific aims

Our goal is to determine the function of RybB in modulating *K. pneumoniae* gene expression and how RybB coordinate regulatory networks of stress adaptation and virulence gene expression by linking the σ^E circuit to other signaling pathways. The specific aims of the first year are listed as follows.

1.1 To generate the deletion mutant of *rybB*.

1.2 To characterize phenotypes of the *rybB* deletion mutants and examine their virulence in the KLA mouse model.

1.3 To validate the interaction between RybB with their mRNA targets in *K. pneumoniae*.

Results and Discussion

Generation of the *rybB* deletion mutant and knock-in strain. The *K. pneumoniae rybB* gene is located counterclockwise in the *ybfK*-KP1_1841 intergenic region as previously described in *E. coli* and *S.* Typhimurium. To get insights into how RybB contribute to the virulence potential of *K. pneumoniae*, the gene-specific deletion mutant of *rybB* was generated by an allelic-exchange technique (*15*). Briefly, the DNA fragments that flank the regions of *rybB* were PCR amplified and cloned into pKAS46. The resulting constructs carried on pKAS46 was mobilized into *K. pneumoniae* through conjugation from *E. coli* S17-1*λpir*. One of the kanamycin-resistant transconjugant of *K. pneumoniae* was picked and the overnight culture of this clone was spread on LB-streptomycin agar. After the occurrence of homologous recombination, the streptomycin-resistant but kanamycin-sensitive colonies were selected. The deletion of *rybB* was verified by PCR analysis. The entire region of *rybB* (93 bp) was synthesized and cloned into pBAD202 (Invitrogen). The resulting plasmid was named pYC458. By the induction of 0.2% of arabinose, overexpression of RybB was detected in the pYC458 knock-in strains.

Deletion of *rybB* attenuated *K. pneumoniae* virulence. To examine whether RybB is involved in regulating *K. pneumoniae* virulence, groups of 8-wk old BALB/c male mice were intraperitoneally injected with 10^4 CFU of the *rybB* deletion mutant, named Δ*rybB*, or its parental strain, CG43S. The

survival of infected mice was monitored daily for two weeks. As shown in Figure 1, 90% of the CG43S-infected mice died at day 5 post-injection, whereas only one of the ΔrybB-infected mice succumbed to the infection. This result suggested an involvement of RybB in a systemic *K. pneumoniae* infection.

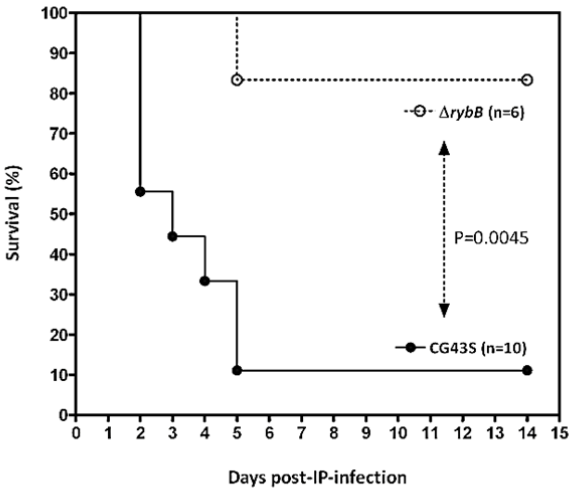

Figure 1. Loss of *rybB* attenuated *K. pneumoniae* virulence. Bacterial suspension containing 10^4 cfu of *K. pneumoniae* CG43S (solid line, n=10) or Δ*rybB* (slash line, n=6) was intraperitoneally injected into 8-wk old BALB/c male mice. Survival of the infected mice was monitored daily for two weeks. Kaplan-Meier analysis was used. *P* value of <0.05 indicates statistically significant.

Growth capacity affected in *K. pneumoniae* lacking *rybB*. To determine whether RybB was required for K. pneumoniae to cope with stresses that it may encounter inside the host, the growth of ΔrybB was characterized in regular cultivation and in various stressful conditions. As shown in Figure 2A, while ΔrybB (solid triangles) grew normally in LB medium (up left panel), the growth of *K. pneumoniae* was enhanced by over-expression of RybB from pYC458 (empty triangles vs. empty squares). In the presence of 0.7 M NaCl (middle left panel), the growth of ΔrybB lagged behind that of CG43S. The reduced capacity of ΔrybB to grow in the addition of 0.7 M NaCl was restored to the wild type level at 7 hours of recovery and even enhanced by the introduction of the rybB-complemeting plasmid (pYC458; empty triangles) when compared with the vector control (pBAD; empty squares). This result suggested that RybB may have a role in control of genes related to *K. pneumoniae* tolerance to a high salt condition. Although the production of capsular polysaccharides was not affected, the hypermucoviscosity phenotype was enhanced by the loss of rybB (Figure 2B).

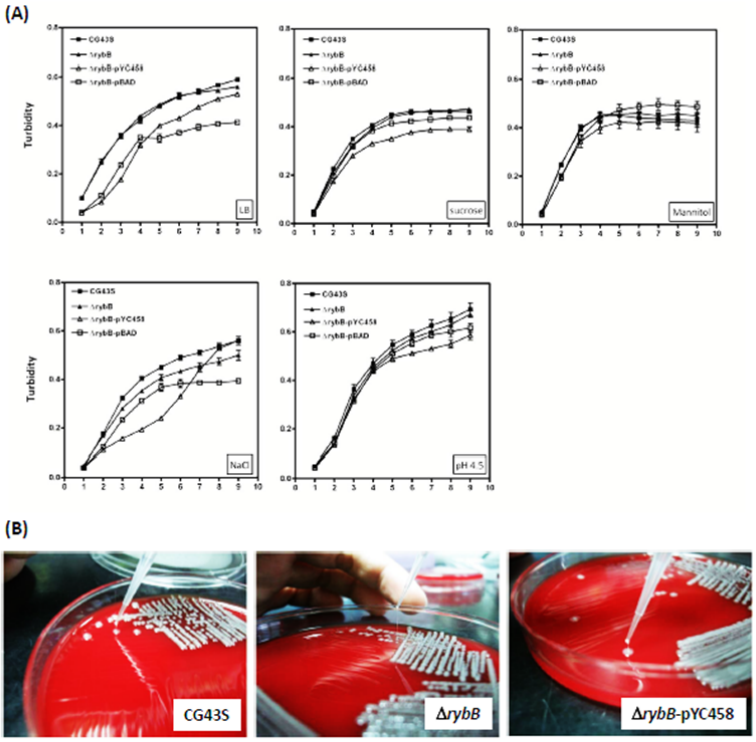

Figure 2. Effects of *rybB* deletion on phenotypic characteristics of *K. pneumoniae*. (A) Growth rate and capacity of K. pneumoniae CG43S (solid squares),ΔrybB (solid triangles), ΔrybB-pYC458 (empty triangles), and ΔrybB-pBAD vector (empty squares) under regular LB cultivation and stressful conditions, including 8% of sucrose, 2% of mannitol, 0.7 M NaCl, and pH4.5. (B) Hypermucoviscosity phenotype determined on blood agar.

Regulatory impact of RybB on the post-transcriptional control of *K. pneumoniae* gene expression. To determine the regulatory impact of RybB on modulating *K. pneumoniae* gene expression, we performed a genome-wide transcriptome analysis to identify the potential mRNAs that were repressed by RybB. Upon a 10-min pulse-expression of RybB from the arabinose-inducible plasmid pYC458, *K. pneumoniae* genes with changes in mRNA abundance were identified by DNA microarray. As shown in Table 1, by the transient expression of RybB, a total of 31 genes showed > 4-fold decrease in transcripts levels. The candidate genes identified in *K. pneumoniae*, except for *ompC*, were different from the set of *E. coli* targets for RybB (*14*). *K. pneumoniae* RybB has many candidate targets showing no envelope-associated functions, suggesting that RybB acts as a global regulator in *K. pneumoniae*, but controls unique sets of genes from that in *E. coli* or *Salmonella*. To formally consider a regulated mRNA as a direct sRNA target, we have to obtain evidence of post-transcriptional regulation for a specific sRNA-mRNA base-pairing. Therefore, a genetic approach using the target-GFP translational fusion (*16*) was used to validate the interaction between RybB and its potential target mRNAs in *K. pneumoniae* (Table 1). As shown in Figure 3A, the 5'-end UTR region of *ompC*, *galU*, *hdeB*, and *KP1_0760* was respectively cloned as a translational fusion to *gfp* in pXG10 (a gift from Dr. Jorg Vogel; Univ. Wurzburg, German). Each of the *gfp* translational fusions were separately cotransformed with pYC458 (BAD-RybB) into *E. coli* TOP10 cells. The effect of RybB on a target-*gfp* fusion was determined by comparison of GFP protein expression by Western blotting with specific antibodies in the presence and

Table 1. RybB-targeted *K. pneumoniae* genes

Category	Gene/locus	Description	Fold change by the knock-in of RybB	
			Average	STD
Translation	rplA	50S ribosomal protein L1	-5.6	0.4
	rpsT	30S ribosomal protein S20	-4.5	0.3
	rpmI	50S ribosomal protein L35	-4.2	0.1
	rpsM	30S ribosomal protein S13	-5.0	0.2
	rpsQ	30S ribosomal protein S17	-5.0	0.3
	rplD	50S ribosomal protein L23	-4.8	0.2
	rpsJ	30S ribosomal protein S10	-4.3	0.2
	rpmG	50S ribosomal protein L33	-5.4	0.4
	tsf	elongation factor Ts	-4.5	0.3
	infA	translation initiation factor IF-1	-5.8	0.2
Envelope-associated	pulS	pullulanase-specific type II secretion system outer membrane lipoprotein	-4.5	0.3
	KP1_0760	putative PTS permease	-23.5	11.5
	KP1_0761	putative PTS permease	-16.1	4.4
	KP1_0762	putative PTS permease	-27.6	8.1
	KP1_0763	putative PTS permease	-21.2	3.8
	KP1_0764	putative glucosamine-fructose-6-phosphate aminotransferase	-16.1	4.4
	KP1_0765	putative glucosamine-fructose-6-phosphate aminotransferase	-10.0	1.2
	ompC	outer membrane porin protein C	-5.3	0.3
	wbbM	putative glycosyltransferase	-4.5	0.1
Metabolism	eutD	cobalamin adenosyltransferase in ethanolamine utilization	-9.6	3.8
	eutP	putative regulator of ethanolamine utilization	-5.8	0.4
	eutQ	putative ethanolamine utilization protein	-9.0	3.1
	suhB	inositol monophosphatase	-4.9	0.6
	galU	UTP--glucose-1-phosphate uridylyltransferase subunit GalU	-4.5	0.3
	glf	UDP-galactopyranose mutase	-4.5	0.4
Other	hdeB	acid-resistance protein	-5.0	0.5
	KP1_1106	hypothetical protein	-4.8	0.3
	KP1_1566	hypothetical protein	-4.4	0.3
	KP1_1624	hypothetical protein	-4.4	0.3
	KP1_2202	hypothetical protein	-5.1	0.6
	KP1_3089	hypothetical protein	-5.1	0.5

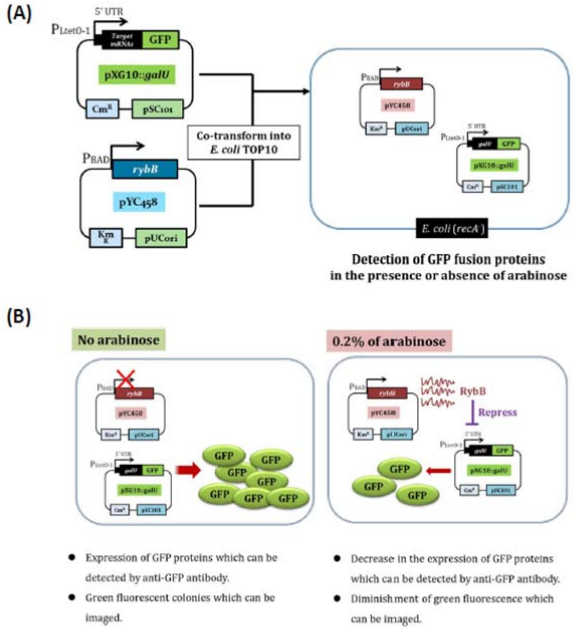

Figure 3. Validation of RybB : target mRNAs interaction by two plasmid system with target-GFP translational fusion. (A) Putative 5'-end UTR of RybB target genes is cloned as a translational fusion to gfp on a low-copy plasmid (pXG10). This construct is cotransformed with pYC458 into *E. coli* TOP10. (B) By comparison of GFP expression with Western blot analysis in the absence and presence of arabinose, the effect of RybB on its target mRNAs is determined.

absence of arabinose induction (Figure 3B). In 2, 4, 6, and 8 hours after recovery in LB supplemented with 0.2% of arabinose, the level of GFP protein translated from 5'-end UTR of KP1_0760 was significantly reduced when compared with that obtained from bacterial cultures in the absence of arabinose (Figure 4A and 4B). We observed that the overproduction of RybB resulted in 60%, 50%, and 40% decrease in GFP expression, respectively for *gfp* fusions of HdeB, GalU, and OmpC, at 8 hours post-inoculation (Figure 4B). Together with the transcriptome result, our finding suggested that *K. pneumoniae* RybB negatively regulated the expression of *ompC*, *hdeB*, *galU*, and KP1_0760, by blocking the ribosome binding at 5'-end UTR and also increased RNA instability. By comparing the list of RybB targets in *E. coli*, except *ompC*, *hdeB*, *galU*, and KP1_0760 were *K. pneumoniae*-specific. How the RybB-mediated control of these genes contributes to *K. pneumoniae* virulence and physiology is worth of further studies to elucidate.

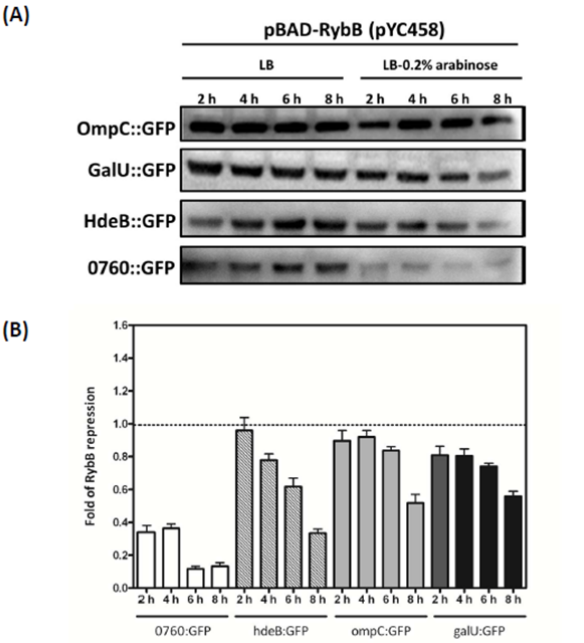

Figure 4. Arabinose-induced RybB inhibit the expression of OmpC:GFP, GalU:GFP, HdeB:GFP, and 0760:GFP translation fusions. Bacterial lysates were obtained from cultures recovered in the absence or the presence of 0.2% of arabinose in 2, 4, 6, and 8 hours. Thirty micrograms of total proteins were subjected to Western blotting analyses with anti-GFP antibodies. A representative result from three times of independent experiments is shown in (A). Band intensity for the expression level of GFP was determined by Densitometry calculation. Fold of RybB repression was determined by intensity comparison between LB and LB-0.2% of arabinose in the same time point. Data shown in (B) are means ± SEM.

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科技部補助計畫衍生研發成果推廣資料表

日期:2014/03/31

科技部補助計畫	計畫名稱: 探討 Sigma E-dependent small RNA RybB 與 MicA 在克雷白氏肺炎桿菌的生理與致病上的後轉錄調控機制
	計畫主持人: 賴怡琪
	計畫編號: 101-2320-B-040-013- 學門領域: 微生物及免疫學

無研發成果推廣資料

101 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：101-2320-B-040-013-
計畫名稱：探討 Sigma E-dependent small RNA RybB 與 MicA 在克雷白氏肺炎桿菌的生理與致病上的後轉錄調控機制	

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	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%		
國外	論文著作	期刊論文	1	1	100%	篇	Activation of IFN-γ/STAT/IRF-1 in Hepatic Responses to Klebsiella pneumoniae Infection. PLoS ONE, 2013
		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	

科技部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)

RybB, one of the sigma E-dependent sRNAs, plays important roles in regulation of K. pneumoniae physiology and virulence. Loss of rybB significantly attenuated K. pneumoniae virulence in murine models. The in vitro growth of K. pneumoniae was enhanced by over-expression of rybB. Among the RybB-regulated target genes, we validated the interaction between RybB and galU, KP1_0760, and hdeB by using the GFP-based two-plasmid system. In addition to affect the RNA stability of its target genes, RybB negatively regulated the translation of these genes through blocking the ribosome binding manner. Taken together, this study demonstrated that RybB was a virulence gene and the RybB regulon of K. pneumoniae was different from that of E. coli. The results provide us insights into how an opportunistic pathogen as K. pneumoniae adapts itself to the host milieu by rapidly fine-tuning virulence-associated genes by small RNA molecules. In the near future, some of K. pneumoniae-specific regulators of the RybB circuit may serve as an ideal chemical scaffold for discovery of novel antimicrobial drugs.