科技部補助專題研究計畫成果報告
期末報告

探討第六型蛋白分泌系統在克雷白氏肺炎桿菌致病過程中扮演的角色

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 104-2320-B-040-021-
執 行 期 間 ： 104年08月01日至105年07月31日
執 行 單 位 ： 中山醫學大學醫學系

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中 華 民 國 105 年 10 月 31 日

中 文 摘 要 ： Klebsiella pneumoniae（克雷白氏肺炎桿菌）是世界性分布的致病菌，它的感染可引起多樣化的臨床表現。 自1980 年代開始，一種由單一致病菌引起的化膿性肝膿瘍被發現與K. pneumoniae 的感染由關。 儘管由K. pneumoniae 引起的感染症在臨床上的重要性已被許多流行病學的報告確立，目前對該菌感染的分子機轉方面的瞭解卻仍相當有限。 大部分T6SS的研究集中於霍亂弧菌與綠膿桿菌。目前並無關於K. pneumoniae T6SS功能的研究報告。 T6SS_I的重要基因的剔除造成K. pneumoniae明顯喪失對小鼠的致病力。 我們在本計畫的目標為瞭解探討T6SS_I是藉由何種分子機制參與在克雷白氏肺炎桿菌的致病過程。 本年度中，我們檢測T6SS_I中的icmF (tssM1) 藉由參與K. pneumoniae 與腹腔免疫細胞的交互作用進而決定細菌系統性擴散的能力。

中 文 關 鍵 詞 ： 克雷白氏肺炎桿菌，第六型分泌系統

英 文 摘 要 ： Klebsiella pneumoniae is a worldwide spread pathogen responsible for a broad spectrum of clinical syndromes. Since 1980s, a single pathogen-induced pyogenic liver abscess has been noticed to be predominantly mediated by the primary infection of K. pneumoniae. Despite the clinical significance of K. pneumoniae infections has been established, our knowledge regarding the molecular basis of how this bacterium causes an infection is rather restricted. So far, the T6SS is mostly characterized in Vibrio cholerae and Pseudomonas aeruginosa. The role of T6SS in K. pneumoniae has not yet been addressed. Mutants that had in-frame deletion of essential T6SS_I genes significantly attenuated K. pneumoniae virulence in mice. We aimed in this project to elucidate the molecular basis regarding the mechanism by which K. pneumoniae T6SS_I contributed to bacterial virulence. In this year, we determined the involvement of icmF (tssM1) in the interaction between K. pneumoniae CG43 and peritoneal immune cells that impacted the systemic dissemination for this bacterium.

英 文 關 鍵 詞 ： Klebsiella pneumoniae, T6SS

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, such as pneumonia, urinary tract infections, suppurative infections, bacteremia, meningitis, and sometimes life-threatening septicemia. Without immediate treatments, infections caused by this bacterium have a significantly high rate of mortality (*1*). In Gram-negative bacteria, protein secretion is a complex process that is carried out by proteinaceous machineries, known as protein secretion systems, in a controlled and efficient manner. At least six types of protein secretion system (T1SS-T6SS) have been identified. Among these, T6SS is the most recently characterized secretion system (*2, 3*). As analogous to the T3SS and T4SS, T6SS appears to constitute needle-like structures that span the bacterial cell wall and have the potential to translocate bacterial effector proteins directly into the target eukaryotic or bacterial cell. T6SS is widely distributed that has been predicted in > 25% of all sequenced Gram-negative bacteria.

During infections, the expression of T6SS encoding genes in pathogenic bacteria is activated and frequently regulated by environmental cues that mimic host conditions. Numerous reports implicate T6SS in virulence and/or interaction with host cells in multiple bacterial species, ranging from plat pathogens, animal pathogens, to human pathogens (*4*). However, the contributions of T6SSs to virulence are diverse. In *Burkholderia spp*. T6SS was essential for virulence and played a role in its intracellular life inside macrophages (*5, 6*). The subsequent infection by *B. mallei* was protected by immunization of animals with recombinant HCP proteins (*5*). T6SS endowed *Aeromonas hydrophila* with cytotoxicity toward macrophage and epithelial cell lines and full virulence in septicemia mouse model (*7*). Adhesion and invasion of human brain microvascular endothelial cells by K1 *E. coli* required the involvement of T6SS (*8*). *V. cholerae* employs T6SS to kill *Escherichia coli*, *Dictyostelium discoideum* amoebae, and macrophages. Except for *V. cholerae* T6SS, the underlying mechanism of the virulence requirement or T6SS-mediated interaction with eukaryotic cells is largely unknown. Based on its function, T6SS can be separated into four categories: (1) bacterial cell targeting, (2) eukaryotic cell targeting, (3) bacterial and eukaryotic cell targeting, and (4) other (such as conjugation, gene regulation, and

cellular adhesion).

Three distinguishable and conserved genetic loci, named T6SS locus I, II, and III, were identified harboring genes encoding for putative T6SS core components and effectors in sequenced *K. pneumoniae* genomes (*9*). *K. pneumoniae* CG43 has T6SS_I and T6SS_III in its genome. T6SS_I contains most of the 13 T6SS-core genes which are organized in 2 segments, the *vgrG* gene cluster and the *icmF* gene cluster. To determine whether T6SS_I was required for the *K. pneumoniae* virulence, in-frame deletion mutants for *hcp* (now named as *tssD*), *clpV* (*tssH*), *vgrG* (*tssI*), and *icmF1* (*tssM1*) of T6SS_I were generated. Loss of *tssM1* significantly attenuated *K. pneumoniae* cytotoxicity and the ability to develop a systemic dissemination in mice was also affected in Δ*tssM1*. Epidemiological observations indicate that patients who suffer from *K. pneumoniae* liver abscess are frequently have bacteremia and about 11-12% of these patients develop septic metastatic lesions, such as brain abscess, pyogenic meningitis, or endophthalmitis (*10, 11*). We aimed in this one-year project to explore the molecular basis regarding the mechanism by which *K. pneumoniae* T6SS_I contributes to virulence in mice.

Results and Discussion

T6SS_I in *K. pneumoniae*. Except for *K. pneumoniae* 342 (CP000964), almost all of the clinical strains of *K. pneumoniae* have only T6SS_I and T6SS_III. We extracted the sequences predicted as T6SS_I from *K. pneumoniae* 1084 (NC_018522), *K. pneumoniae* NTUH-K2044 (NC_012731), and *K. pneumoniae* CG43 (NC_022566). The T6SS-core genes which are organized in 2 segments, the *icmF* gene cluster (*icmF*/*impG*/*impH*/*sciN*/*impF*), and the *vgrG* gene cluster (*vgrG*/*clpV*/*hcp*/*ompA*/*dotU*/*impJ*/*impC*/*impB*).The 13 T6SS-core genes are conserved among these strains (marked in yellow region; Fig. 1A). Among the *K. pneumoniae* T6SS_I-core components, Hcp (Hemolysin co-regulated protein) and VgrG (valine-glycine repeat protein G) are the main extracellular components forming a needle-like injection device closely resembling the bacteriophage tail. ClpV and IcmF are ATPase in the cytoplasm that energizes the action of T6SS. Therefore, in-frame deletion mutants for *hcp* (now named as *tssD*), *clpV* (*tssH*), *vgrG* (*tssI*), and *icmF* (*tssM*) of T6SS_I were generated in CG43 (Fig. 1B) for the determination of the role

of this highly conserved secretion system in the pathogenesis of clinically hypervirulent *K. pneumoniae* strains.

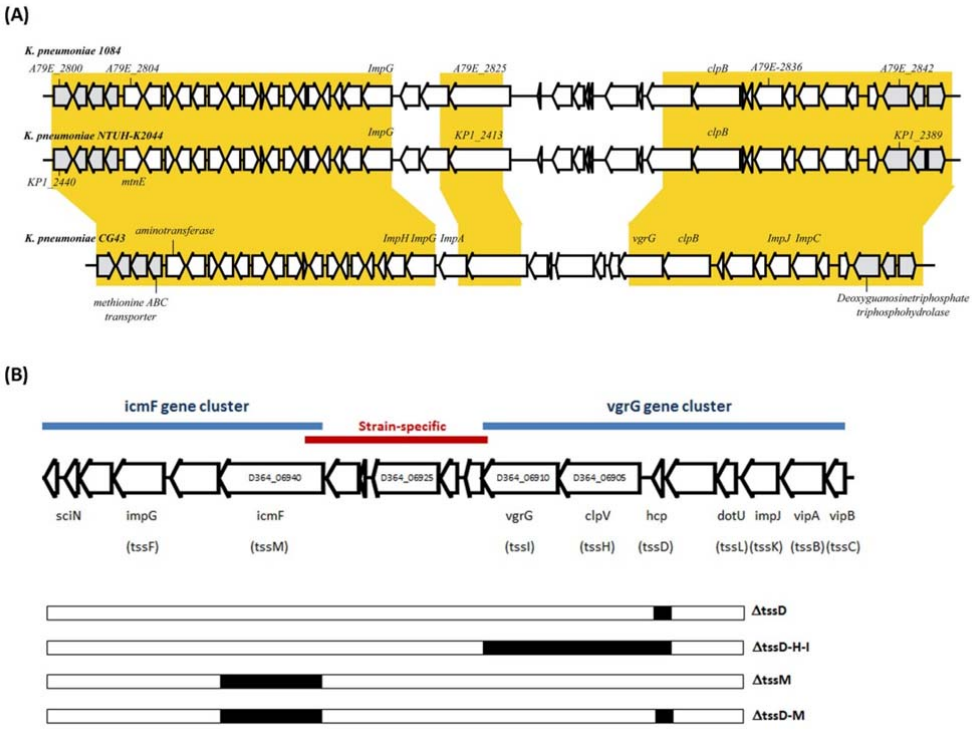

Figure 1. Schematic representation of T6SS I in *K. pneumoniae*. **(A)** Comparison of the T6SS (I) gene cluster predicted in the genome of *K. pneumoniae* 1084, NTUH-K2044, and CG43. **(B)** Arrows indicate the transcription direction. Gene-specific deletion mutants for T6SS I genes are shown in below. The region deleted in the genome of *K. pneumoniae* CG43.

Requirement of T6SS_I_icmF (tssM) for *K. pneumoniae* virulence to BALB/c mice. Comparative assessment of virulence for T6SS_I mutants was performed in the peritonitis mouse model. Groups of 8-9 wk-old male BALB/c mice were intraperitoneally inoculated with 10^4 CFU of *K. pneumoniae* CG43S or with the same inoculum of a particular T6SS_I mutant, including ΔtssD (*hcp* deletion mutant), ΔtssM1 (*icmF1* deletion mutant), ΔtssD-M1 (*hcp* and *icmF1* double deletion mutant), and ΔtssDHI (*hcp*, *vgrG*, and *clpV* triple deletion mutant). Survival of the infected mice was monitored for two weeks. The majority of mice which were infected with ΔtssM1, ΔtssD-M1, or ΔtssDHI, survived the experimental period (purple, brown, and green lines, respectively shown in Fig. 2A), which was significantly higher than the survival of wild type-infected mice (black-colored, Fig. 2A). All of the ΔtssD-infected

mice died at day 6 (red line, Fig. 2A). The attenuation on virulence was not related to growth defects, given the *in vitro* growth capacity for all the mutants were comparable to that of the wild type strain. This result suggested requirement of *tssM1*, *tssH*, and *tssI* for *K. pneumoniae* virulence. In contrast to tssM1, tssM3, the *icmF* gene in T6SS locus III was dispensable for *K. pneumoniae* virulence (blue line, Fig. 2A). Moreover, the requirement of tssM1 for *K. pneumoniae* virulence was also demonstrated in liver abscess mouse model. Through an intragastric route, 10^8 CFU of ΔtssM1 or wild type strain was used to challenge groups of BALB/c mice. At 48 hours post-inoculation, the average bacterial load of small intestines was $1\times\log_{10}$ decreased in the Δ*tssM1*-infected group when compared to the wild type group (Fig. 2B). When the wild type-infected mice had a bacterial burden approaching $10^{3\text{-}4}$ CFU in the liver and spleen, Δ*tssM1* was undetectable at these extra-intestinal organs for most of the mice infected (Fig. 2B). These results suggested the requirement of T6SS_I_icmF (tssM1) for *K. pneumoniae* CG43 virulence to BALB/c mice.

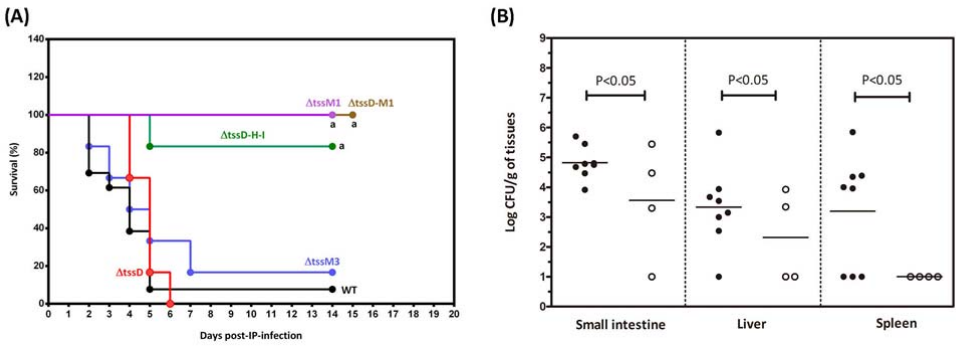

Figure 2. *In vivo* virulence assessment for T6SS mutants. (A) Survival rate of mice which were intraperitoneally infected with 10^4 CFU of *K. pneumoniae* CG43S (black) or with T6SS mutants, including ΔtssD (red), ΔtssD-M1 (brown), ΔtssM1 (purple), ΔtssDHI (green), and ΔtssM3 (blue). **(B)** Bacterial loads of wild type (solid circles) or ΔtssM1 (empty circles) in different mouse tissues at 48 hours post-intragastric inoculation with 10^8 CFU in BALB/c mice.

Involvement of T6SS_I_icmF in the interaction with peritoneal immune cells that contributed to the systemic dissemination of *K. pneumoniae* from peritoneal cavity. How T6SS_I contributed to *K. pneumoniae* virulence. To elucidate the possible underlying mechanism, we analyzed the interaction between *K. pneumoniae* and peritoneal immune cells. CG43S and Δ*tssM1* were respectively inoculated into the peritoneal cavity of BALB/c mice. At 3- and 24-hour post-inoculation (hpi), peritoneal fluids were collected for enumeration of bacterial loads and for the isolation of peritoneal immune cells. During this period (from 3 to 24 hpi), K.

pneumoniae CG43S, the parental strain, proliferated for approximately 10-fold from 10^5 CFU/ml in the peritoneal cavity (Fig. 3A). The number of Δ*tssM1* was reduced at the same timescale. Dissemination of *K. pneumoniae* CG43S and Δ*tssM1* into the blood was detected at both 3 and 24 hpi. However, the blood-carried Δ*tssM1* was totally cleared in two third of the inoculated mice at 24 hpi (Fig. 3B). The peritoneal cavity in mice contains different immune cell populations crucial for innate immune response against *K. pneumoniae*. To further analyze the possible interaction between *K. pneumoniae* and peritoneal cells, we collected peritoneal fluids from the infected mice and isolated lymphocytes and macrophages using Dynabeads Magnetic beads (ThermoFisher). The deletion of icmF (Δ*tssM*) significantly attenuated the capacity of *K. pneumoniae* CG43S in the recruitment of lymphocytes to the inflammatory site (Fig. 3C) and in the inactivation of macrophages (Fig. 3D). Moreover, a number of CG43S was found inside the peritoneal lymphocytes (Fig. 3C) and macrophages (Fig. 3D). This intracellular survival of *K. pneumoniae*, which was affected by the deletion

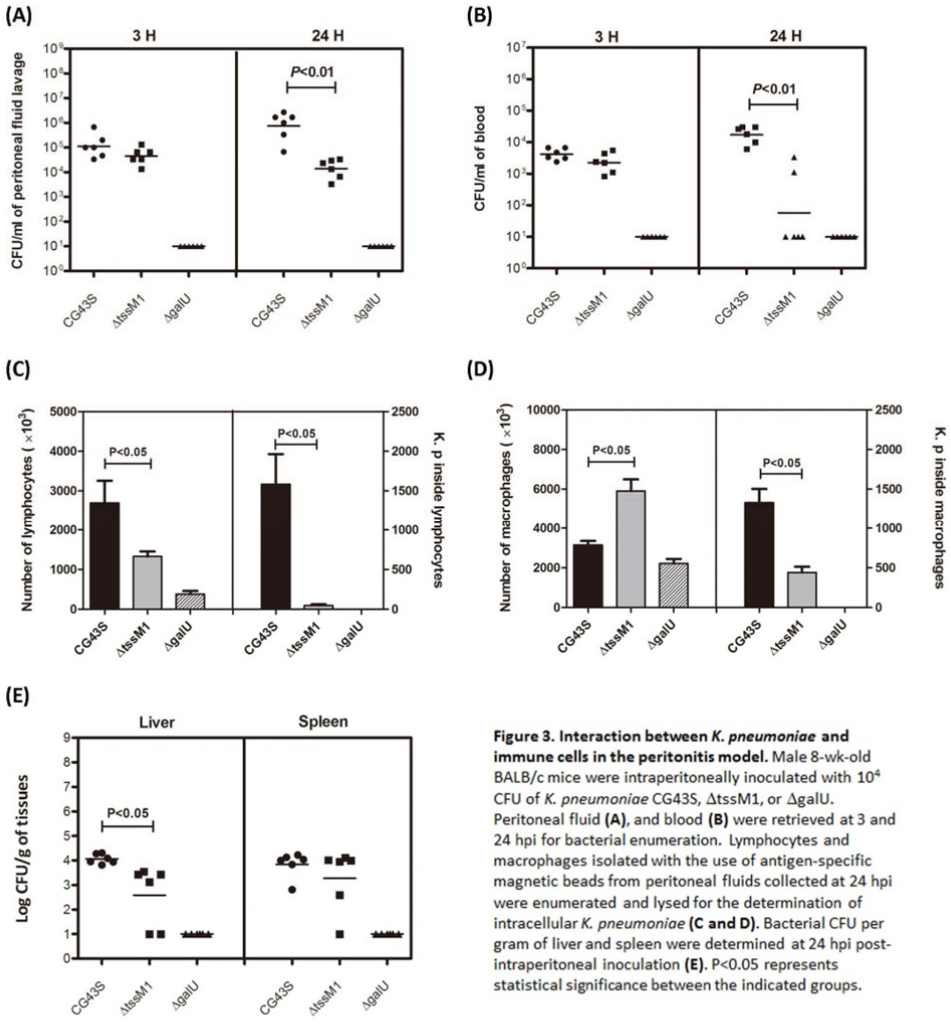

Figure 3. Interaction between *K. pneumoniae* and immune cells in the peritonitis model. Male 8-wk-old BALB/c mice were intraperitoneally inoculated with 10^4 CFU of *K. pneumoniae* CG43S, ΔtssM1, or ΔgalU. Peritoneal fluid **(A)**, and blood **(B)** were retrieved at 3 and 24 hpi for bacterial enumeration. Lymphocytes and macrophages isolated with the use of antigen-specific magnetic beads from peritoneal fluids collected at 24 hpi were enumerated and lysed for the determination of intracellular *K. pneumoniae* **(C and D)**. Bacterial CFU per gram of liver and spleen were determined at 24 hpi post-intraperitoneal inoculation **(E)**. P<0.05 represents statistical significance between the indicated groups.

of *icmF* (Δ*tssM*), may contribute to bacterial dissemination to the liver (Fig. 3E). To further explore the interaction between *K. pneumoniae* and immune cells, we are now using the cell models to dissect the pathogenesis mechanism.

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科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/31

科技部補助計畫	計畫名稱: 探討第六型蛋白分泌系統在克雷白氏肺炎桿菌致病過程中扮演的角色
	計畫主持人: 賴怡琪
	計畫編號: 104-2320-B-040-021- 學門領域: 微生物免疫

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：賴怡琪	**計畫編號：**104-2320-B-040-021-
計畫名稱：探討第六型蛋白分泌系統在克雷白氏肺炎桿菌致病過程中扮演的角色	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		

	技術移轉	件數	0	件	
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參與計畫人力	本國籍	大專生	0	人次	
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		專任助理	1		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
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。）					

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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（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）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（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
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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）
   本計畫確認克雷白氏肺炎桿菌CG43的第一套第六型分泌系統在該菌引起小鼠感染的不可或缺性，同時亦在腹腔感染的模式中發現該分泌系統與克雷白氏肺炎桿菌與免疫性細胞交互作用相關，這個發現除幫助我們進一步了解克雷白氏桿菌由腹腔擴散至肝臟與脾臟的機制外，也暗示著部分細菌可能可以藉由特定免疫細胞的攜帶進而擴散，值得我們設計新的實驗進一步探討。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
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   說明：（以150字為限）