行政院國家科學委員會專題研究計畫　期中進度報告

克雷白氏肺炎桿菌 DNA adenine methylase (Dam) 與致病力相關性的研究(1/2)

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-002-178-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：國立臺灣大學醫學院內科

計畫主持人：方啟泰

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 6 月 2 日

(一) 中文摘要

克雷白氏肺炎桿菌是一種革蘭氏陰性的腸內菌。在台灣，克雷白氏肺炎桿菌是最重要的人類致病菌之一。常在有糖尿病的成人，甚至健康人，引起致命性的化膿性肝膿瘍、菌血症、轉移性眼內炎及腦膜炎，此外克雷白氏肺炎桿菌也是一種重要的院內感染病原菌，可造成肺炎，泌尿道感染。其致病力的調控機轉目前完全不清楚。去氧核醣核酸腺核? 甲基化? 可把細菌基因體上核酸序列 GATC 的 adenine 甲基化，從而控制細菌基因表現，在細菌基因調控機轉上扮演重要角色。在沙門氏桿菌-另一種革蘭氏陰性的腸內菌，去氧核醣核酸腺核? 甲基化? 在致病力調控機轉上的重要性已被證實，去氧核醣核酸腺核? 甲基化? 基因突變之沙門氏桿菌菌株，致病力顯著下降。但去氧核醣核酸腺核? 甲基化? 在克雷白氏肺炎桿菌致病力調控機轉的角色則尚未有人研究。本研究的目的即爲探討去氧核醣核酸腺核? 甲基化? 與克雷白氏肺炎桿菌致病力的相關性。我們目前已成功的選殖出克雷白氏肺炎桿菌的去氧核醣核酸腺核? 甲基化? 基因。先利用由 NCBI (National Center for Biotechnology Information) 網站所搜尋到的 *E. coli* K12 的去氧核醣核酸腺核? 甲基化? 基因序列爲模本，將其基因的 5'及 3'端序列設計爲引子對。以在臺灣已知爲高毒力的克雷白氏肺炎桿菌原始型菌株 NTUH-2044 的基因體 DNA 爲模板，用 PCR 的方式順利增幅出一段 669 個鹼基的開放讀架片段。將此開放讀架片段定序後，與 *E. coli* K12 的去氧核醣核酸腺核? 甲基化? 基因序列作比對，發現有 99 % 的相似度。選殖出克雷白氏肺炎桿菌菌株 NTUH-2044 的去氧核醣核酸腺核? 甲基化? 基因之後，我們進一步建構鑲嵌有 chloramphenicol acetyltransferase (CAT) cassette 的去氧核醣核酸核? 甲基化? 基因，以 conjugation 的方法送回 NTUH-2044，篩選出 4 株 dam^-基因突變菌株。測試突變菌株的血清抗性，與野生型比較發現有明顯的降低。未來的研究將進而以動物實驗測定其 50%致死量的變化，來量化去氧核醣核酸腺核? 甲基化? 基因與克雷白氏肺炎桿菌致病力的相關性。進一步，將用微陣列晶片技術，比較原始型菌株與突變型菌株在基因表現上的差異，來找出受到去氧核醣核酸腺核? 甲基化? 基因控制的下游基因群。最後，將以動物實驗測試去氧核醣核酸腺核? 甲基化? 基因突變菌株作爲疫苗菌株的可行性。

關鍵詞：克雷白氏肺炎桿菌， 致病力，去氧核醣核酸腺核? 甲基化?

(二) 英文摘要

Klebsiella pneumoniae, an enteric Gram-negative bacillus, is one of the most important human pathogen in Taiwan. *K. pneumoniae* causes life-threatening pyogenic liver abscess, bacteremia, septic endophthalmitis and meningitis in both diabetic persons and otherwise healthy people. *K. pneumoniae* is also an important pathogen of nosocomial pneumonia and urinary tract infection. The regulation of virulence gene expression in *K. pneumoniae* has been poorly understood. DNA adenine methylase (Dam) can add methyl group to the adenine of sequence GATC, and thus play an important role in the control of bacterial gene expression. In *Salmonella*, another enteric Gram- negative bacillus, the role of Dam in the regulation of virulence has been well documented. *dam*$^-$ *Salmonella* mutants significantly loss the virulence potential. The role of Dam in *K. pneumoniae* has not yet been studied. The purpose of this proposal is to investigate the relationship between Dam and bacterial virulence in *K. pneumoniae*. We have successfully cloned the *dam* gene in *K. pneumoniae*. We used the nucleotides sequence of dam gene in *E. coli* K12, available on NCBI website, to design PCR primers. Using designed primers, we successfully amplify a DNA fragment containing a 669 bps open reading frame from the genomic DNA of clinical invasive strain *K. pneumoniae* NTUH-2044. We further constructed *dam*$^-$ mutant by inserting chloramphenicol acetyltransferase (CAT) cassette into *dam* and sending the mutated *dam* into wild type NTUH-2044 for homologous recombination. Four *dam*$^-$ *K. pneumoniae* mutants were obtained, and all of them had much reduced serum resistance in comparison with the wild type. In the second year, animal study will be used to determine the difference in LD50 to quantify the correlation between Dam and bacterial virulence in *K. pneumoniae*. Microarray technique will be used to identify the downstream genes regulated by *dam*. Finally, the possibility of *dam*$^-$ *K. pneumoniae* mutants as vaccine strains will be explored through animal experiments.

Keywords*: Klebsiella pneumoniae*, virulence, DNA adenine methylase

(一) 前言

DNA 甲基化提供了細胞一套可以辨別外來 DNA 的機制，細菌的 DNA 甲基化的角色通常被認爲可保護自身的 DNA 免於受到限制內切? (restriction endonuclease) 的作用。一般而言，負責 DNA 甲基化的基因爲 DNA methyltransferases。而 DNA methyltransferases 主要又分爲 Dam(DNA adenine methyltransferase)以及 Dcm(DNA cytosine methyltransferase)，分別由 *dam* 及 *dcm* 基因所產生。在 *E. coli* 的 DNA 中 Dam 可以辨識 GATC 序列，並形成超過 99% 的 6-meAde。Dcm 則可辨認 CC(A/T)GG 序列，並形成全部的 5-meCyt。除了上述這個角色之外，在 *E. coli* 中的研究發現，鹼基被甲基化之後還有其他的生物功能。例如: 影響 DNA 的立體結構、dam-directed mismatch repair、改變 DNA 與其調控蛋白質之間的親合力、調控染色體的複製起始以及調控基因的表現。

由於 Dam 在細胞生理上扮演許多重要的角色，所以在 *dam* 突變菌株上可能會產生毒力減弱的現象。這種現象最早是在 *E. coli* 中發現，Dam 會調控 *E. coli* 的 pilus operons，而 pilus operons 對於 *E. coli* 引起的泌尿道感染及腹瀉扮演很重要的角色。之後，陸續發現 *Salmonella* 的 Dam^- 菌株變成無毒力，探究其原因可能是基因表現失去了控制，才導致毒力消失。而在 *Y. pseudotuberculosis* 及 *V. cholerae* 中 Dam 對其生存是必須的，但是在 Dam 過度表現的菌株中，其毒力也是大大地降低。由此可見，Dam 扮演著調控病原菌與寄主之間的相互作用，並對病原菌的毒力有所影響，但目前對 Dam 控制毒力的作用機制尚不清楚。

克雷白氏肺炎桿菌是一種革蘭氏陰性的腸內菌。在台灣，克雷白氏肺炎桿菌是最重要的人類致病菌之一。常在有糖尿病的成人，甚至健康人，引起致命性的化膿性肝膿瘍、菌血症、轉移性眼內炎及腦膜炎，此外克雷白氏肺炎桿菌也是一種重要的院內感染病原菌，可造成肺炎，泌尿道感染。其致病力的調控機轉目前完全不清楚。去氧核醣核酸腺核? 甲基化? 在克雷白氏肺炎桿菌致病力調控機轉的角色則尚未有人研究。本研究的目的即爲探討去氧核醣核酸腺核? 甲基化? 與克雷白氏肺炎桿菌致病力的相關性。

（二）研究目的及文獻探討：

Klebsiella pneumoniae, an enteric Gram-negative bacillus, is one of the most important human pathogen in Taiwan. *K. pneumoniae* causes life-threatening pyogenic liver abscess, bacteremia, septic endophthalmitis and meningitis in both diabetic persons and otherwise healthy people [1–11]. After 1995, *K. pneumoniae* replaces *Streptococcus pneumoniae* as the leading pathogen of adult community-acquired bacterial meningitis [2, 3]. Other common syndrome include pneumonia in patients with poorly controlled diabetes mellitus or alcoholism, and febrile neutropenia in patients received anticancer chemotherapy [1, 12]. *K. pneumoniae* is also an important pathogen of nosocomial pneumonia, urinary tract infection, bloodstream and wound infections [12]. Despite of *in vitro* effective antibiotic therapy, there is significant morbidity and an at least 10% mortality in patients with *K. pneumoniae* pyogenic liver abscess [4–11]. For patients with *K. pneumoniae* meningitis, the mortality is as high as 30–40% despite *in vitro* effective antimicrobial therapy [2, 3]. Further study of the pathogenesis of invasive *K. pneumoniae* diseases in Taiwan is necessary in order to develop innovative approaches for early diagnosis, more effective therapy, and possible prevention.

Multiple bacterial virulent factors are probably involved during the dynamic process of invasive *K. pneumoniae* diseases, including factors promoting bacterial adhesion to epithelial surface, factors helping bacteria acquiring iron in tissue environment, factors protecting bacteria from attack of phagocytosis, complements and antibody, and factors that may directly damage host. Most previous studies on *K. pneumoniae* pathogenesis were directed to one or more of the specific virulent factors, such as polysaccharide capsule, lipopolysaccharide, adhesins, and iron siderophores [12–22]. Since these virulence factors must act synergistically to provide bacteria the best chance of successful infection, there must be some regulatory mechanism to coordinate their expression. However, little was known about the global control mechanism of virulence gene expression in *K. pneumoniae*.

DNA adenine methylase (Dam) plays a pivotal role in bacteria such as *Escherichia coli* and *Salmonella* – acting as a global regulator of gene expression and affecting a wide range of critical cellular functions, including DNA replication, DNA repair, transposition, and segregation of chromosomal DNA [23]. This extraordinary versatility stems from the inherent biochemical activity of Dam. Thus, by adding methyl groups to various sties along the cellular DNA, Dam alters interactions of a variety of regulatory proteins with their designated gene targets and in the process, effectively controls expression of those genes. In some cases, such changes modulate bacterial virulence. Studies of *E. coli pap* operon have highlighted

the biological importance of Dam mechanism [24]. *pap* operon encode pili that enable *E. coli* to adhere to the urogenital tract and establish kidney infections. Genes encoding Pap pili are reversibly switched between the unexpressed state and the expressed state, a process known as phase variation. This ON-OFF pilin switching leads the bacteria to attach and detach, enable them first to colonize the bladder and then the kidney, causing cystitis and pyelonephritis, respectively. Pap phase variation is principally controlled by the leucin-responsive regulatory protein (Lrp) and the state of methylation of two specific pap regulatory DNA sequence that reside within Lrp-binding sites. During the OFF-phase, $GATC^{prox}$ is unmethylated, then Lrp binds to pap promoter proximal site and blocks *pap* transcription. During the ON-phase, when $GATC^{prox}$ is methylated, Lrp binding is blocked and pili are produced.

Studies in *Salmonella*, another enteric rod, also pointed out the importance of Dam in the regulation of bacterial virulence [25, 26]. Many genetically determined virulence functions of this pathogen are induced during infections but are not expressed, or expression is relatively low, when the cells are growing in vitro– "On" in vivo, "OFF" in vitro. Because this response involves the coordinationed expression of may genes when cells are moved from host tissues to synthetic media, it suggests that the cells depend on global regulatory mechanism. Screening of mutants for the expression of in vivo induced (*ivi*) genes when cells are grown in vitro on syndthetic medium yielded cells with mutations that resulted in the loss of Dam activity. In turn, those changes derepress the activity of more than 35 distinct *ivi* genes in *Salmonella*, indicating that Dam is a global regulator of Salmonella gene expression. *Salmonella* strains that either lack Dam or overexpress it (Dam^{OP}) have severe virulence defects when tested in mice for their ability to cause typhoid fever. Mice survive and show no overt signs of disease when inoculated with 10,000-fold more Dam^{-} or Dam^{OP} cells than are needed of the wild-type (Dam^{+}) strain to kill 50% of the animals. Furthermore, inoculating mice with the Dam^{-} mutants protected them when they were subsequently exposed to the virulent wild-type strain. The effectiveness of these attenuated mutants is somewhat surprising since other highly attenuated mutants typically are cleared too rapidly from animals to elicit protective responses.

Mutations in Dam and other adenine methyltransferase from an even wider variety of microbial pathogens, including *Vibrio cholera, Yersinia pseudotuberculosis*, can attenuate virulence, suggesting that enzymes which methylate DNA are pivotal and might prove essential in bacterial pathogensis. However, the role of Dam in the virulence of *K. pneumoniae* has not yet been studied. The *dam*-equivalent gene in *K. pneumoniae* has not yet been sequenced and cloned, either. The purpose of this proposal is to investigate the relationship between

Dam and bacterial virulence in *K. pneumoniae*.

（三）研究方法：

Bacterial strains, plasmids, and culture conditions

Clinical isolates of *K. pneumoniae* were collected at National Taiwan University Hospital (NTUH) from 1993 through 2000. The identification of the *K. pneumoniae* and the antibiotic susceptibility testing were performed according to standard clinical microbiologic methods [27] The *K. pneumoniae* strains were stored at -80 ^{o}C before use. *K. pneumoniae* NTUH-2044 was selected as the wild-type strain used in the subsequent study. The *E. coli* strains and most plasmids will be used in this study has been previously described [28]. Suicide vector pUTKm1Δ*tnp*Δ*bla*Δ*mini-Tn5* [29, 30] has been constructed for the purpose of knockout. For general use, both *E. coli* and *K. pneumoniae* will be grown in Luria-Bertani (LB) broth or agar. When necessary, kanamycin (50 μg/ml) or chloramphenicol (50 μg/ml) will be added.

Cloning of Dam in *K. pneumoniae*

先利用由 NCBI (National Center for Biotechnology Information) 網站所搜尋到的 *E. coli* K12 的 *dam* 基因序列爲模本，將其基因的 5’及 3’端序列設計爲引子對。以在臺灣已知爲高毒力的克雷白氏肺炎桿菌原始型菌株 NTUH-2044 的基因體 DNA 爲模板，用 PCR 的方式增幅出克雷白氏肺炎桿菌帶有 *dam* 基因序列的 DNA 片段，再接到 pGEM T-easy vector 上，以利後續實驗的進行。

DNA sequencing and analysis

DNA sequencing will be performed by using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin- Elmer Applied Biosystems, Foster City, CA) on ABI PRISM 377 DNA Sequencer. Primers used for DNA sequencing will be purchased from Perkin-Elmer Biosystems. The nucleotide sequences and deduced amino acid sequences will be compared with those from nonredundant GenBank and SWISS-PROT databases by using computer software Sequencer 2.1.1 (Gene Codes Corp., Ann Arbor, MI) and the BLAST network service at the National Center for Biotechnology Information.

Knockout *dam* by homologous recombination

dam will be amplified by PCR , followed by TA cloning according to the recommended procedures provided by the manufacturer. A chloramphenicol acetyltransferase (CAT) cassette (gift from Dr. Diane Taylor) [33] will be inserted into *dam* on pGEM T-easy vector to construct *cat*:: *dam*. The *cat*:: *dam* fragment

will be further inserted into the *EcoR1* site on suicide vector pUTKm1Δ*tnp*Δ*bla*Δ*mini-Tn5*. The knockout experiment will be carried out by sending linear DNA *cat*:: *dam* or a suicide plasmid vector *cat*::*cepA*-pUTKm1Δ*tnp*Δ*bla*Δ*mini-Tn5* into *K. pneumoniae* NTUH-2044 by conjugation [28–30], respectively. After conjugation, the recombinant clones will be selected on LB agar supplemented with chloramphenicol 50 μg/ml and ampicillin 100μg/ml to remove the donor *E. coli* S17-1/*λpir*.

Serum resistant test

以 Podschun 等建立的方法來測試克雷白氏肺炎桿菌菌株的血清抗性強弱。血清的來源由沒有感染過克雷白氏肺炎桿菌的健康成人志願捐出。測試菌量爲 2.5×10^4 CFU/ml（colony-forming units）。將隔夜培養之克雷白氏肺炎桿菌菌液以 LB 肉湯稀釋 100 倍，在 37°C 搖晃培養 2–2.5 小時至菌液的吸光值在 600 nm（OD_{600}）爲 0.5。此時菌量約爲 10^8 CFU/ml。將 OD_{600} 0.5 的菌液以生理食鹽水稀釋 100 倍。此時取 25 μl 即爲所需之接種菌量。將此 25 μl 菌液與 75 μl 人類血清均勻混合後，於 37°C 培養箱中培養，並在 0、1、2 及 3 小時時，分別以序列稀釋法，將不同倍數的稀釋液（以生理食鹽水稀釋）均勻塗布於 LB 培養基上。隔夜培養後，計算培養基上菌落數目。克雷白氏肺炎桿菌之血清抗性以存活菌數百分比（survival ratio）來表示。

(四) 目前成果

1. 克雷白氏肺炎桿菌 *dam* gene 選殖:

我們已成功的利用由 NCBI (National Center for Biotechnology Information) 網站所搜尋到的 *E. coli* K12 的 *dam* 基因序列爲模本，將其基因的 5’及 3’端序列設計爲引子對。以在臺灣已知爲高毒力的克雷白氏肺炎桿菌原始型菌株 NTUH-2044 的基因體 DNA 爲模板，用 PCR 的方式順利增幅出一段 669 個鹼基的開放讀架片段。選殖出克雷白氏肺炎桿菌菌株 NTUH-2044 的 *dam* 基因。將此開放讀架片段定序後，與 *E. coli* K12 的 *dam* 基因序列作比對，發現有 99 % 的相似度。

2. *dam*$^-$突變菌株的建構:

建構鑲嵌有 chloramphenicol acetyltransferase (CAT) cassette 的去氧核醣核酸核? 甲基化? 基因，以 conjugation 的方法送回 NTUH-2044，篩選出 4 株 *dam*$^-$基因突變菌株。

Control

dam$^-$4

dam$^-$3

dam$^-$2

dam$^-$1

wt

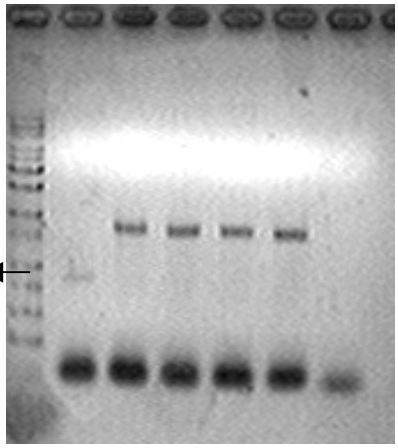

Fig1. 以 PCR 來檢測是否得到 *dam*$^-$ 的突變菌株，wt 爲 wild type NTUH-2044，而在突變菌株可檢測到鑲嵌有 chloramphenicol acetyltransferase (CAT) cassette 的去氧核醣核酸腺核? 甲基化? 基因。

3. *dam*$^-$突變菌株的血清抗性測試:

以 Podschun 等建立的方法來測試克雷白氏肺炎桿菌菌株的血清抗性強弱。血清的來源由沒有感染過克雷白氏肺炎桿菌的健康成人志願捐出。測試菌量爲 2.5 × 10^4 CFU/ml（colony-forming units）。將隔夜培養之克雷伯氏肺炎桿菌菌液以 LB 肉湯稀釋 100 倍，在 37°C 搖晃培養 2–2.5 小時至菌液的吸光值在 600 nm（OD_{600}）爲 0.5。此時菌量約爲 10^8 CFU/ml。將 OD_{600} 0.5 的菌液以生理食鹽水稀釋 100 倍。此時取 25 μl 即爲所需之接種菌量。將此 25 μl 菌液與 75 μl 人類血清均勻混合後，於 37°C 培養箱中培養，並在 0、1、2 及 3 小時時，分別以序列稀釋法，將不同倍數的稀釋液（以生理食鹽水稀釋）均勻塗布於 LB 培養基上。隔夜培養後，計算培養基上菌落數目。克雷白氏肺炎桿菌之血清抗性以存活菌數百分比（survival ratio）來表示。畫出其關係圖(Fig.2)，可明顯看出突變菌株的血清抗性下降。

Ratio / Strain	0hr	1hr	2hr	3hr
KP wt	1	1.6	1.79	5.9
***dam*$^-$ 1**	1	0.81	0.63	0.91
***dam*$^-$ 2**	1	0.4	0.4	0.47
***dam*$^-$ 3**	1	0.87	1.07	0.75
***dam*$^-$ 4**	1	0.89	1.2	1.22

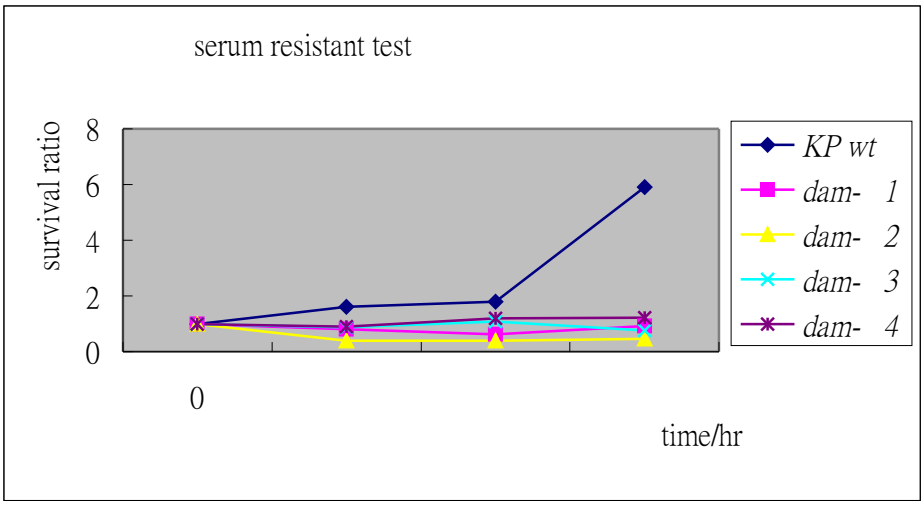

Fig.2 克雷白氏肺炎桿菌之血清抗性之存活菌數百分比，以 0 小時爲基準，及以此結果所得到的線性圖。由 serum resistant test 的結果發現，突變株對 serum 的抵抗能力下降。

(五) 討論

先前的研究指出 *dam* 會影響許多病原細菌的致病性(pathogenesis)及生長，而且 Dam 可以調控基因表現。因此，在 *dam* 突變後，*Klebsiella pneumoniae* 是否會產生毒力減弱的現象；以及哪些基因的表現發生改變，而這些基因及可能與 *Klebsiella pneumoniae* 的致病機轉或生長有關。所以本實驗則著重於研究 DNA adenine methyltransferase 對於克雷伯氏肺炎桿菌致病性產生的影響。

未來的研究將進而以動物實驗測定其 50%致死量的變化，來量化去氧核醣核酸腺核? 甲基化? 基因與克雷白氏肺炎桿菌致病力的相關性。進一步，將用微陣列晶片技術，比較原始型菌株與突變型菌株在基因表現上的差異，來找出受到去氧核醣核酸腺核? 甲基化? 基因控制的下游基因群。最後，將以動物實驗測試去氧核醣核酸腺核? 甲基化? 基因突變菌株作爲疫苗菌株的可行性。

(六)參考文獻

1. Eisenstein, B.I. & Zaleznik, D.F. Enterobacteriaceae. In: Mandell, G.L., Bennett, J.E. & Dolin, R. (eds) *Principles and Practice of Infectious Disease*, 5th ed. Philadelphia, Churchill-Livingstone, pp2294–2309. (2000)
2. Tang, L.M., Chen, S.T., Hsu, W.C. & Lyu, R.K. Acute bacterial meningitis in adults: a hospital-based epidemiological study. *Q. J. Med.* **92**, 719–725 (1999).
3. Fang, C.T. *et al.* Microbiological features of adult community-acquired bacterial meningitis in Taiwan. *J. Formos. Med. Assoc.* **99**, 300–304 (2000).
4. Fang, C.T., Chen, Y.C., Chang, S.C., Sau, W.Y. & Luh, K.T. *Klebsiella pneumoniae* meningitis: timing of antimicrobial therapy and prognosis. *Q. J.*

Med. **93**, 45–53 (2000).

5. Wang, J.H. *et al.* Primary liver abscess due to *Klebsiella pneumoniae* in Taiwan. *Clin. Infect. Dis.* **26**, 1434–1438 (1998).
6. Yang, C.C., Chen, C.Y., Lin, X.Z., Chang, T.T., Shin, J.S. & Lin, C.Y. Pyogenic liver abscess in Taiwan: emphasis on gas-forming liver abscess in diabetics. *Am. J. Gastroenterol.* **88,** 1911–1915 (1993).
7. Chang, F.Y. & Chou, M.Y. Comparison of pyogenic liver abscesses caused by *Klebsiella pneumoniae* and non-*K. pneumoniae* pathogens. *J. Formosa. Med. Assoc.* **94**, 232–237 (1995).
8. Chang, F.Y., Chou, M.Y., Fan, R.L. & Shaio, M.F. A clinical study of *Klebsiella* liver abscesses. *J. Formosa. Med. Assoc.* **87,** 282–287 (1988).
9. Liu, Y.C., Cheng, D.L. & Lin, C.L. *Klebsiella pneumoniae* liver abscess associated with septic endopthalmitis. *Arch. Intern. Med.* **146**, 1913–1916 (1986).
10. Cheng, D.L., Liu, Y.C., Yen, M.Y., Liu, C.Y. & Wang, R.S. Septic metastatic lesions of pyogenic liver abscess: association with *Klebsiella pneumoniae* bacteremia in diabetic patients. *Arch. Intern. Med.* **151**, 1557–1559 (1991).
11. Chiu, C.T., Lin, D.Y. & Liaw, Y.F. Metastatic septic endopthalmitis in pyogenic liver abscess. *J. Clin. Gastroenterol.* **10**, 524–527 (1988).
12. R. Podschun and U.Ullmann. *Klebsiella spp* as nosocomial pathogen: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Review 1998;11:589-603.
13. Arakawa, Y., Wacharotayankun, R., Nagatsuka, T., Ito, H., Kato, N. & Ohta, M. Genomic organization of the *Klebsiella pneumoniae cps* region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. *J. Bacteriol.* **177**, 1788–1796 (1995).
14. Alvarez, D., Merino, S., Tomas, J.M., Benedi, V.J. & Alberti, S. Capsular polysaccharide is a major complement resistance factor in lipopolysaccharide O side chain-deficient *Klebsiella pneumoniae* clinical isolates. *Infect. Immun.* **68**, 953–955 (2000).
15. Williams, P., Lambert, P.A., Brown, M.R. & Jones, R.J. The role of the O and K antigens in determining the resistance of *Klebsiella aerogenes* to serum killing and phagocytosis. *J. Gen. Microbiol.* **129** (Pt 7), 2181–2191 (1983).
16. Tomas, J.M., Benedi, V.J., Ciurana, B. & Jofre, J. Role of capsule and O antigen in resistance of *Klebsiella pneumoniae* to serum bactericidal activity. *Infect. Immun.* **54**, 85–89 (1986).
17. Podschun, R., Sievers, D., Fischer, A. & Ullmann, U. Serotypes, hemagglutinins, siderophore synthesis, and serum resistance of *Klebsiella* isolates causing human

urinary tract infections. *J. Infect. Dis.* **168**,1415–1421 (1993).

18. Fung, C.P. *et al.* A 5-year study of the seroepidemiology of *Klebsiella pneumoniae*: high prevalence of capsular serotype K1 in Taiwan and implication for vaccine efficacy. *J. Infect. Dis.* **181**, 2075–2079 (2000).
19. Cryz, S.J. Jr, Mortimer, P.M., Mansfield, V. & Germanier, R. Seroepidemiology of *Klebsiella* bacteremic isolates and implications for vaccine development. *J. Clin. Microbiol.* **23**, 687–690 (1986).
20. Merino S, Altarriba M, Izquierdo L, Nogueras MM, Regue M & Tomas JM. Cloning and sequencing of the *Klebsiella pneumoniae* O5 wb gene cluster and its role in pathogenesis. *Infect. Immun.* **68**, 2435–2440 (2000).
21. Old, D.C., A. Tavendale, & B.W. Senior. A comparative study of the type-3 fimbriae of *Klebsiella* species. *J. Med. Microbiol.* **20**, 203–214 (1985)
22. de Lorenzo, V., & Martinez, J. L. Aerobactin production as a virulence factor: a reevaluation. *Eur. J. Clin Microbiol. Infect. Dis.* **7**, 621–629 (1988).
23. Reisenauer, A.L.S. Kahng, S. McCollum, and L. Shapiro. 1999. Bacterial DNA methylation: a cell cycle regulator? J. Bacteriol. 181: 5135–5139.
24. Braaten, B.A., Nou, L.S., Kaltenbach, and D.A. Low. 1994. Methylation patterns in *pap* regulatory DNA control pyelonephritis-associated pili phase variation in *E. coli*. Cell 76;577–88.
25. Douglas M. Heithoff, Robert L. Sinsheimer, David A. Low, Michael J. Mahan. An essential role for DNA adenine methylation in bacterial virulence. Science 1999;**284**:967-970.
26. Garcia del Portillo, F., M. G. Pucciarelli, and J. Casadesus. DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. Proc. Natl. Acad. Sci. USA 1999 96:11578-11583.
27. Abbott, S. *Klebsiella, Enterobacter, Citrobacter and Serratia*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, (eds). *Manual of Clinical Microbiology*. 7th ed. Washington D.C., ASM Press, pp475–482 (1999).
28. Chang, K. C., S. W. Ho, J. C. Yang, and J. T. Wang. 1997. Isolation of a genetic locus associated with metronidazole resistance in *Helicobacter pylori*. Biochem. Biophys. Res. Commun. **236:**785–788.
29. Herrero, M., Lorenzo, V., & Timmis K.N. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**, 6557–6567 (1990).
30. Lorenzo, V., Herrero, M., Jakubzik, U., & Timmis K.N. Mini-Tn5 transposon derivatives for insertion mutagenesis, promotor probing, and chromosomal

insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**, 6568–6572 (1990).
31. Sambrook, J., Fritsch, E.F. & Maniatis, T. (eds) *Molecular cloning: a laboratory manual* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1981).
32. Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse. 1988. Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res. 16:7583–7600.
33. Wang, Y., and D. E. Taylor. 1990. Chloramphenicol resistance in *Campylobacter coli*: nucleotide sequence, expression, and cloning vector construction. Gene **94:**23–28.
34. Brordovsky M. and McIninch J. GeneMark: parallel gene recognition for both DNA strands. Computers & Chemistry, 19:123–133 (1993)
35. Lukashin, AV & Borodovsky M. Genekark.hmm: new solutions for gene finding. Nucleic Acids Res, 26: 1107–1115.

（七）計畫成果自評

完成本計畫第一年預定之進度:

1. 選殖到克雷白氏肺炎桿菌菌株 NTUH-2044 的 *dam* 基因並完成定序的工作,dam 基因長度爲 669 個鹼基。與 *E. coli* K12 的 *dam* 基因序列作比對，發現有 99 % 的相似度。

2. 建構鑲嵌有 chloramphenicol acetyltransferase (CAT) cassette 的去氧核醣核酸核? 甲基化? 基因，以 conjugation 的方法送回 NTUH-2044，篩選出 4 株 dam^- 基因突變菌株。

3. 測試克雷白氏肺炎桿菌之去氧核醣核酸腺核? 甲基化? 基因突變的菌株的血清抗性可明顯看出突變菌株的血清抗性下降。因此，在 *dam* 突變後，克雷白氏肺炎桿菌是否會產生毒力減弱的現象；以及哪些基因的表現發生改變，而這些基因及可能與克雷白氏肺炎桿菌的致病機轉有關。有待進一步的研究。

目前初步的研究結果尚未完備，*dam* 基因在克雷白氏肺炎桿菌的致病機轉上，扮演何種角色及其相關性，都有待進一步的研究來釐清。未來的研究將進而以動物實驗，來量化去氧核醣核酸腺核? 甲基化? 基因與克雷白氏肺炎桿菌致病力的相關性。進一步，將用微陣列晶片技術，比較原始型菌株與突變型菌株在基因表現上的差異，來找出受到去氧核醣核酸腺核? 甲基化? 基因控制的下游基因群。最後，將以動物實驗測試去氧核醣核酸腺核? 甲基化? 基因突變菌株作爲疫苗菌株的可行性。