行政院國家科學委員會專題研究計畫　成果報告

利用 PP-STM 轉位子突變系統搜尋克雷白氏肺炎菌 CG43 的壓力反應基因

計畫類別： 個別型計畫
計畫編號： NSC93-2320-B-040-013-
執行期間： 93 年 03 月 01 日至 93 年 07 月 31 日
執行單位： 中山醫學大學醫學研究所

計畫主持人： 賴怡琪

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報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中　華　民　國 93 年 11 月 4 日

行政院國家科學委員會補助專題研究計畫成果報告

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執行期間：93 年 3 月 1 日至　93 年 7 月 31 日

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執行單位：中山醫學大學

中　華　民　國　　93 年　　10 月　　31 日

行政院國家科學委員會專題研究計畫成果報告

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中文摘要

克雷白氏肺炎菌（*Klebsiella pneumoniae*）是一種造成人類感染的常駐性細菌，在台灣，其感染經常伴隨著廣泛的普遍性與高致死率。 為瞭解該菌伺機性感染行為背後的致病機轉，在本計畫中我們運用一個雙功能 PP-STM （promoter probed signature tagged mutagenesis）系統著手搜尋克雷白氏肺炎菌 CG43 的壓力反應基因。在二十個突變菌株庫中，總計有十六個突變株在三種不同的壓力條件：氧化壓力（paraquat），氧化壓力（H_2O_2），以及缺鐵壓力（2'-2'-dipyridyl）下篩選為抗壓缺損（stress-attenuated）菌株。 包括有：nitrate/nitrite transporter (STM06A8), glycosyltransferase for capsular synthesis (STMN1G6), Clp protease (STM01G2), two putative HTH transcriptional regulators (STMN1G5, STM37E5), 以及七個功能未知的基因。 基於 Clp proteolysis 在大腸桿菌細胞生理調控上的重要性，我們以 allelic exchange 的技術將 *clp* deletion 分別載入野生型與 Δ*lon* 的克雷白氏肺炎菌株後得到 X0117 (Δ*clp*) 與 LX0110 (Δ*clp*/Δ*lon*) 突變株。 為進一步瞭解 Clp proteolysis 在克雷白氏肺炎菌抗壓反應中扮演的調控角色為何，我們目前以蛋白質體學的方法進行分析中。

關鍵詞：克雷白氏肺炎菌，壓力反應，STM 轉位子突變系統

Abstract

Klebsiella pneumoniae is a nasty human commensal that frequently causes infections with a high prevalence and mortality rate in Taiwan. To understand underlying pathogenesis of the opportunistic behavior of this bacterium, in this project, we embarked on identifying stress-responsive genes of *K. pneumoniae* CG43 by using a bifunctional PP-STM system. Twenty mutant pools were screened upon three stressful conditions: oxidative stresses generated by addition of paraquat, or H_2O_2, and iron deprivation by 2'2-dipyridyl. Totally, 16 mutants were found to be stress attenuated. The predicted genes products of inserted sequences obtained from 13 of the stress-attenuated mutants include nitrate/nitrite transporter (STM06A8), glycosyltransferase for capsular synthesis (STMN1G6), Clp protease (STM01G2), two putative HTH transcriptional regulators (STMN1G5, STM37E5), and seven with unknown function. Based on the importance of Clp proteolysis on controlling a variety of cellular events in *Escherichia coli*, a *clp* deletion mutant strain, X0117, and a *clp*/*lon* double mutant stain, LX0110, were generated in the fresh genetic background of *K. pneumoniae* CG43 via an allelic exchange technique. To move further ahead, a

proteomics analysis is now undertaken to determine the underlying controlled panel of the Clp proteolysis in *K. pneumoniae*, on which a protective response may pivot for bacterial survival under stress.

Keywords: *Klebsiella pneumoniae*, stress-responsive, Signature tagged mutagenesis

Background and specific aims:

Klebsiella pneumoniae causes suppurative infection, pneumonia, urinary tract infection and septicemia, occurring particularly in immunocompromised individuals (1) and has a high fatality rate if untreated. Although the steady and high frequency of *K. pneumoniae* isolation has been noted (2, 3, 7), little or no information is available on understanding the molecular virulence basis of its highly heterogeneous pathogenic behaviors of infection and its unusual prevalence in Taiwan. In order to analyze the virulence-associated genes of *K. pneumoniae* in a whole-genome scale, we have constructed a bifunctional PP-STM (Promoter Probed Signature Tagged Mutagenesis) system that allows attenuated mutants to be positively selected through the hybridization of a unique tag as well as simultaneously select for insertion downstream of an active promoter. As an opportunistic pathogen, on entering into the host from environment, *K. pneumoniae* must circumvent a variety of stressful situations, such as a temperature shift, the limitation of nutrients and iron, and reactive oxygen intermediates (ROI) produced by the immune cells. Therefore, a successful infection requires *K. pneumoniae* equipped themselves with a set of genes whose expression can render resistance to the stress encountered. The goal of this project is to identify and characterize *K. pneumoniae* genes that respond to stresses. The specific aims are to (1) screen of the PP-STM library constructed in *K. pneumoniae* CG43 for genes responding to stressful conditions, including iron-deprivation, nutrients limitation and oxidative stress; (2) characterize the stress-responsive genes.

Results and discussion:

We describe our progress in three parts.

(I) Establishment of PP-STM library in *K. pneumoniae* CG43. In order to select virulence-attenuated mutants positively, we developed a promoter-probed (PP) genetic system that incorporates the use of a *gfp*-Tc tandem reporter and signature-tagged mutagenesis (STM) to identify genes required for the survival of bacteria during infections. A promoterless copy of the green fluorescence protein encoding gene *gfp* and a tetracycline-resistant gene (Tc^r) was introduced into a mini-Tn*10* transposon as a promoter probe. Unique nucleotide sequence tags, which can be identified by hybridization, were subsequently inserted. For each targeting, the PP-STM system is allowed to monitor the disruption of phenotype by the insertion of transposon, the activation of promoter upon specific conditions by the selection of tetracycline resistance, and to measure the transcription level of gene using the GFP reporter activity. A total of 3840 PP-STM transposon mutants of *K. pneumoniae* CG43 were collected and arrayed in 80 pools, each containing 48 uniquely tagged mutants.

(II) Identification of stress-responsive genes. We have screened 20 mutant pools (MP01 to MP20) upon three stressful conditions: oxidative stresses generated by addition of paraquat, or H_2O_2, and iron deprivation by 2'2-dipyridyl. Each mutant pool, which contains 48 uniquely tagged mutants, was grown in regular LB medium

at 37°C for overnight and used to be inoculated into LB, LB with supplement of 250 μM paraquat (Sigma), LB with 500 μM H_2O_2 (RDH), and LB with 150 μM 2'2-dipyridyl (Sigma) at a dilution of 1:1000. After incubation at 37°C for 8 hours, 2 μl of bacterial culture of each mutant pool recovered from different selections were removed and used as a template for PCR-amplification of ST tag with primers p094 (5'-TAC CTA CAA CCT CAA GCT-3') and p095 (5'-TAC CCA TTC TAA CCA AGC-3'). The amplified PCR products were then spotted onto 48 membranes and followed by hybridization separately with 48 fluorescein-labeled ST-tag probes. Representative result of the hybridizations was shown in Fig. 1. The potentially stress-attenuated mutants were reassembled into new pools for another two rounds of selection. Following the second screen, 16 mutants were found to be stress attenuated. To identify the genes in which insertions attenuated bacterial survival under stress, DNA flanking the site of transposon insertion was cloned and sequenced. Genomic DNA of each attenuated mutant was purified with Wizard Genomic DNA Purification Kit and digested with a panel of restriction enzymes. The DNA fragments were ligated into linear pUC18 and the ligation mixture was used to transform *E. coli* DH5α to kanamycin resistance. Plasmids from kanamycin-resistant subclones were checked by restriction enzyme digestion and sequenced commercially by primer p136 (5'-CTA TCG CCT TCT TGA CGA GT-3'). The nucleotide sequence of the DNA flanking the insertion site obtained from 13 of 16 stress-attenuated mutants was used to search the GenBank database for homologous genes. The result was shown in Table 1.

(III) Characterization of stress-responsive genes. The predicted gene products of inserted sequences obtained from the 13 stress-attenuated mutants include nitrate/nitrite transporter (STM06A8), glycosyltransferase for capsular synthesis (STMN1G6), Clp protease (STM01G2), two putative HTH transcriptional regulators (STMN1G5, STM37E5), and seven with unknown functions. Bacterial cells lack ubiquitin but contain several proteolytic enzymes that require ATP for their function. ClpP protein is one of the most important proteases which is involved in protein catabolism under both optimal and stress conditions and participates within many crucial regulatory mechanisms. Stress induction of *E. coli* ClpP was first shown during heat shock (9). Clp protease activity increases during starvation and is directed against certain carbon starvation proteins as well as the starvation-mediated sigma factor, σ^s (4). Increased levels of σ^s trigger starvation-mediated differentiation, which involves more than 50 different genes (14). As for the *E. coli* proteins, ClpP synthesis in *Bacillus subtilis* increases during heat shock, but also during salt and oxidative stress, glucose and oxygen deprivation (16), and during ethanol and puromycin treatment (6). Insertional inactivation of the *clp* gene in *Lactococcus lactis* results in significant loss of cell viability, indicating a major role for ClpP proteolysis in basic cell metabolism (5). Similarly, loss of ClpP in *B. subtilis* causes pleiotrophic effects with distinct morphological and biochemical features. Cells of the *B. subtilis clpP* inactivation strain are highly filamentous and non-motile (11). Besides stress tolerance, ClpP proteolysis was found to modulate expression of the *ali* gene in *Yersinia enterocolitica* which can confer resistance to serum killing and the ability to attach and invade cells (12). Moreover, ClpP appears to play a critical role in the virulence of several bacteria. It was detected during a STM screen for new virulence genes in *Salmonella typhimurium* (8) and acts as a virulence factor by promoting the release of *Listeria* from

phagosomes of macrophages, thereby enhancing the intracellular survival of bacteria (13). Based on the importance of ClpP proteolysis reported in other bacteria, we decided to embark on characterizing the role of ClpP in *K. pneumoniae* from aspects of both physiology and pathogenesis.

Besides Clp, among five different ATP-dependent proteases identified in *E. coli*, Lon was the first to be identified and characterized as a serine protease which participates in the control of a variety of cellular processes such as protein quality control, UV tolerance, capsule synthesis, phage development, haeme and methionine biosynthesis, cell cycle and differentiation, ribosomal protein degradation after starvation. Initially, using an allelic exchange technique via the suicide vector pKAS46 (15), a *clpP* deletion mutation was introduced into fresh genetic backgrounds of the wild type strain *K. pneumoniae* CG43S5 and a *lon* gene deletion mutant, L2116, which was generated in CG43S5 and found to display extremely mucoid colony morphology and produce more capsular polysaccharides than the wild type strain when grown in M9 minimal medium (10). After verified by Southern blot analysis with a gene-specific probe, two gene-specific mutant strains – *K. pneumoniae* X0117 (Δ*clp*) and *K. pneumoniae* LX0310 (Δ*lon* Δ*clp*) were obtained. When compared to the parental strain CG43S5, the Δclp mutant X0117, which had slightly defects on the in-vitro growth in LB (Fig. 2), was significantly susceptible to H_2O_2 and bile salts (Fig. 3). The production of biofilm in the Δ*lon* or/and Δ*clp* mutant strains was slightly more than that in the wild type strain after a 48 h-incubation in the LB or M9 media at 30°C (Fig. 4), whereas only the Δ*lon* mutant L2116 produced significantly more capsular polysaccharides as shown in Fig. 5. In order to figure out the Lon and/or Clp proteolysis dependent regulatory pathway that controls the production of CPS, the formation of biofilms as well as the protective responses to stress, a proteomics analysis is now undertaken.

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Figures

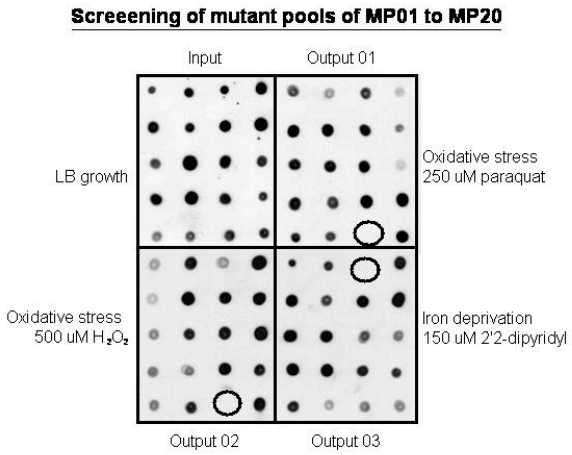

Figure 1. A representative result of PP-STM screening.

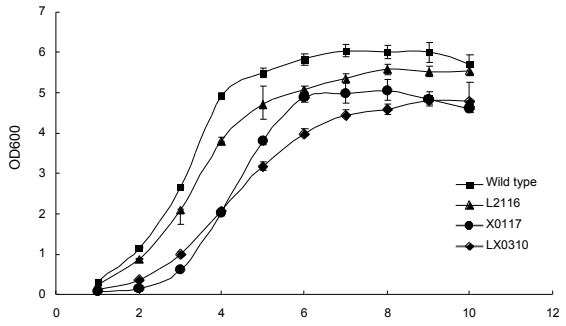

Figure 2. Growth curve.

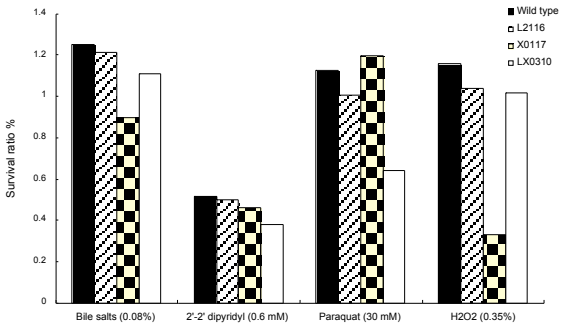

Figure 3. Survival assay under stresses.

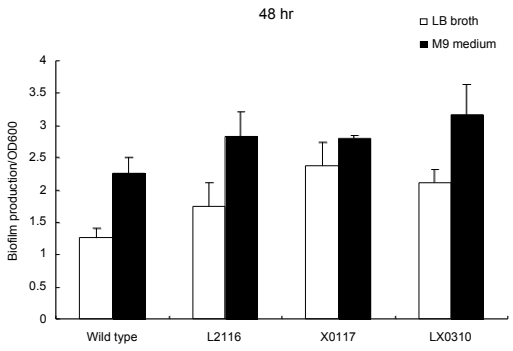

Figure 4. Biofilm formation.

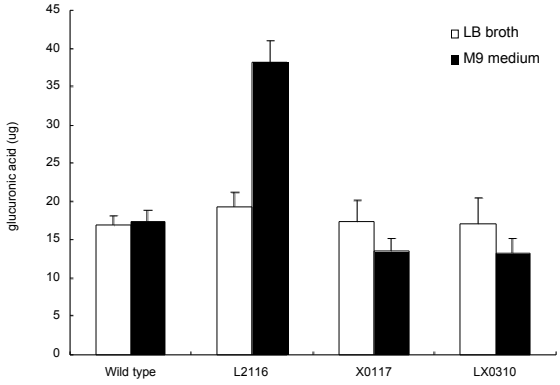

Figure 5. Production of capsular polysaccharides.

Table 1. Putative stress-associated genes of *K. pneumoniae* CG43.

Clone No.	Gene inserted (Homology; putative function)	Stress responded
STM06A8	*narU* (*S. typhimurium*) (83%) Nitrite assimilation; transport	Paraquat inducible
STMN1G5	*yfiE* (*E. coli*) (64%) Putative HTH transcriptional regulator	2'2-dipyridyl sensitive (non-mucoid)
STMN1G6	*orf14* (*K. pneumoniae*) (95%) Capsule synthesis	2'2-dipyridyl sensitive (non-mucoid)
STMN3G6	Novel sequence	2'2-dipyridyl sensitive (non-mucoid)
STM37E5	*yidZ* (*E. coli*) (76%) Putative HTH transcriptional regulator	Paraquat inducible
STM38E9	*ycaO* (*E. coli*) (72%) hypothetical protein	Paraquat inducible
STM05H3	Novel sequence	H_2O_2 and Paraquat sensitive
STM18G8	Novel sequence	H_2O_2 sensitive
STM23E6	Novel sequence	Paraquat sensitive
STM33E4	Novel sequence	Paraquat inducible
STMN4G12	Novel sequence	2'2-dipyridyl sensitive
STMN4A9	Novel sequence	2'2-dipyridyl sensitive
STM01G2	*clpX* (*E. coli*) (92%) Clp protease	H_2O_2 sensitive