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在不同感染模式中的致病相關基因(2/2)

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Abstract

Klebsiella pneumoniae is one of the gram negative pathogens most commonly isolated from patients with community- or hospital-acquired infections. Little is known regarding the molecular basis of its pathogenesis and unique prevalence in Taiwan. We aim in this project to study the regulatory mechanism of stress-responsive genes and their functions in the physiology and virulence of *K. pneumoniae*. A promoter-probed (PP) genetic system that incorporates the use of a *gfp*-Tc tandem reporter and signature-tagged mutagenesis (STM) technique was established to identify genes required for bacterial survival *in vitro* and *in vivo*. 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. To identify genes required for conquering stressful conditions that *K. pneumoniae* must confront during infection, 60 mutant pools which contain mutants with no defects on displaying the mucoviscosity phenotype were screened upon a murine abscess model. Thirty-three independent mutants failed to be recovered from either the spleens or livers from infected mice. The mutants showing verified attenuation contained insertions in loci with a variety of putative functions, including transcriptional regulators, carbohydrate kinases, components of PTS system, membrane proteins, and hypothetical open reading frames. Determination of gene expression profiles of these novel virulence genes as well as characterization of their physiological roles in the pathogenesis of *K. pneumoniae* is now undertaken.

Background and significance:

Klebsiella pneumoniae, an important nosocomial pathogen, causes suppurative infection, pneumonia, urinary tract infection and septicemia, occurring particularly in immunocompromised individuals (4) and has a high fatality rate if untreated. In Taiwan, *Klebsiella* has been found to associate with liver abscess in patients with diabetes mellitus (5, 6, 14). Due to the opportunistic nature of the *Klebsiella* infection, the bacterium has drawn much less attention than other primary pathogens such as *Escherichia coli* and *Salmonella*. Nevertheless, with the extensive spread of antibiotic-resistant strains, especially of extended-spectrum β-lactamases (12)-producing strains, there has been renewed interest in *Klebsiella* infections. These multiple drug-resistant *Klebsiella* strains have become a major threat to the ever-increasing number of immunocompromised patients. Therefore, methods to prevent *Klebsiella* infections, to detect the infection rapidly, and treat the multiple-drug resistant strain-infected individuals effectively, are in urgent demands. All these technologies however, depend heavily on our understanding on the pathogenesis of the bacterium.

Several virulence factors have been found important in the pathogenesis of *K. pneumoniae*, including capsular polysaccharides (1), lipopolysaccharides (7), adhesins, iron acquisition systems, and several secreted toxins. The majority of clinical *K. pneumoniae* strains have well-defined capsular polysaccharides that provide the bacteria with the anti-phagocytotic ability and prevent the bacteria from killing by serum bactericidal factors (28). The O-antigen of LPS is responsible for serum resistance whereas the lipid A portion elicits septic shock (20, 21, 29). The ability of *K. pneumoniae* to colonize respiratory and urinary epithelium is due to the presence of adhesion molecules, including type 1 and 3 pili (25), as well as nonfimbrial adhesin CF29K and KPF28 (9-11). The aerobactin-mediated iron acquisition system has been shown to enhance the virulence of *K. pneumoniae* (8, 24). Secreted protein factors, such as cytotoxins (17, 19, 23), enterotoxins (13, 18, 22), and hemolysins (3) have also been associated with the virulence in *K. pneumoniae* sporadically. Despite these works, much of the pathogenesis events in *K. pneumoniae* are largely unknown.

The virulence genes of a pathogenic bacterium are commonly identified through studying transposon insertion mutants. However, by conventional

approaches, mutants those exhibited attenuated virulence are difficult to be selected positively from a mixed mutant pool. The limitation was overcome by the development of the signature-tagged mutagenesis (STM) technique (2, 15, 16, 27). STM technique (2) is initially generated as a genetic technique for large-scale analysis of bacterial genes involved in the infection of a host (26). Each transposon mutant in the STM library is pre-tagged with a unique short DNA sequence as an identifier. The tag allows identification of bacteria recovered from hosts infected with a mixed population of mutants and hence selects mutants with attenuated virulence. The utility of STM technique in various animal models has been demonstrated in several microbial pathogens (27). To apply this powerful tool to identify virulence genes in *K. pneumoniae*, we have constructed a novel promoter-probed STM system (PP-STM) that can simultaneously report the transcription level of the genetic region where the transposon inserted.

In this study, we used the well-constructed PP-STM system to positively select virulence-attenuated mutants in which genes inserted by transposon are required for *K. pneumoniae* to achieve a successful infection. A murine abscess model was established and used for STM-screening.

Results and discussions:

Establishment of murine abscess model. Based on the observation that the invasive *K. pneumoniae* liver abscess has emerged in Taiwan in the past decade, we established a murine liver abscess model. Epidemiological studies have revealed that *K. pneumoniae* liver abscess infections are frequently preceded by gastrointestinal (GI) colonization and the GI tract is believed to be the most important reservoir for transmission of the bacteria into blood and other organ niches. Therefore, the bacterial suspension of 10^7 cfu of GFP-expressed *K. pneumoniae* CG43 was used to infect eight-week-old BALB/c mice through force-feeding route to establish a GI-induced bacteremia. At 48-h post-infection, the infected mice were sacrificed and the liver was harvested. Several abscess foci were observed on the surface of *K. pneumoniae* infected liver as shown in Fig. 1A. The infected liver was subsequently divided into two parts, one was homogenized for bacterial cfu counting and the other was frozen immediately with liquid N_2 for the preparation of cryostat sections. Three cryostat slices (5 μm) were observed directly with fluorescence microscope and other three were stained with hematoxyline-eosin. At 48 h after infection, several areas of inflammation and destruction were observed in the *K. pneumoniae* infected liver tissue (Fig. 1B (a)). Numerous CFP-expressed *K. pneumoniae* was shown as aggregates in the inflammatory areas of infected liver tissue (Fig. 1B (b, c)). The result indicates that the murine liver abscess model was successfully established in this study.

PP-STM screening for genes involved in formation of bacteremic abscesses. 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 (MP01-MP80), each containing 48 uniquely tagged mutants. Among these STM pools, mutants included within MP01-MP60 display no defects on expressing the mucoviscosity phenotype, and the mutants belonging to MP70-MP80 were mostly non-mucoid.

The hypermucoviscosity phenotype has been proved to be highly correlated with the development of *K. pneumoniae* liver abscess. In this study, we aimed to identify bacterial factors rather than the known hypermucoviscosity that would render *K. pneumoniae* capacity to penetrate GI-barrier, resist serum killing and result in liver abscess. Therefore, 100 μl of bacterial suspension of 10^8 cfu/ml of bacteria from each mutant pool (MP01 to MP60) was inoculated to 8-wk old Balb/c mice in triplicate by force-feeding route. The infected mice were sacrificed at 48-h postinfection and the livers and spleens were harvested and homogenized. Bacteria recovered in the liver or spleen homogenates were re-grown in LB containing 50 μg kanamycin per ml for 8 hr. Two microliters of the bacterial culture was used as a template for PCR-amplification of tags 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. 2. The potentially virulence-attenuated mutants were reassembled into new pools for another two rounds of selection. Following the third screen, 38 mutants were found to be stress attenuated. To identify the genes in which insertions attenuated bacterial virulence, 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 was used to probe 2-kb homologue fragment from the genomic sequence

database of *K. pneumoniae* MGH78578 provided in the Genomic Sequencing Center at Washington University, St. Louis, followed by blast search in the GenBank database for putative functions. As shown in Table 1, thirty-three independent mutants failed to be recovered from either the spleens or livers from infected mice. The mutants showing verified attenuation contained insertions in loci with a variety of putative functions, including transcriptional regulators, carbohydrate kinases, components of PTS system, membrane proteins, and hypothetical open reading frames. Determination of gene expression profiles of these novel virulence genes as well as characterization of their physiological roles in the pathogenesis of *K. pneumoniae* is now undertaken.

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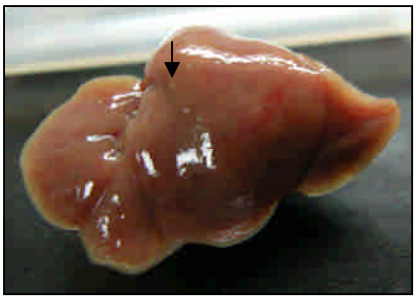
Table 1. Putative virulence genes involved in the *K. pneumoniae* bacteremic abscess.

Strain No.	NCBI Genebank homology putative function
VA01	galactokinase
VA02	HTH-type transcriptional regulator cbl
VA03	hypothetical glycosyltransferase
VA04	Glycine betain/L-proline transporter ATP-binding protein
VA05	Response regulator containing cheY-like reciever domian
VA06	Short chain dehydrogenase
VA07	Hypothetica protein
VA08	TonB-dependent ourter membrane heme receptor
VA09	L-arabinose binding periplasmic protein precursor
VA10	Surface adhesion protein
VA11	Putative inner membrane protein
VA12	Usher protein
VA13	PTS cellobiose-sepecific permease component II
VA14	Monoamine regulon positive regulator
VA15	Putative UphA family transcription regulator
VA16	Hypothetical sugar kinase
VA17	alpha-L-rhamnosidase
VA18	hypothetical glycosyltransferase
VA19	evgS precursor (629-1159)
VA20	Predict ORF
VA21	Putative prophage CDS-53 integrase
VA22	conserved hypothetical protein
VA23	PTS lichenan-specific enzyme IIC component
VA24	hypothetical fimbrial chaperon precursor
VA25	NAD-dependent aldehyde DHase
VA26	Putative response regulator for 2nd Curli (possible csgAB operon transcriptional regulator
VA27	carbonhydrate kinase containing FGGY domain
VA28	on pLVPK similar to phoPQ-activated CDS
VA29	Uracil permease
VA30	D-3-phosphoglycerate DHase
VA31	Nucleoside-specific channel forming protein tsx precursor
VA32	IGR of predict ORFs
VA33	Gal-1-P uridylyltransferase

(A)



(B)

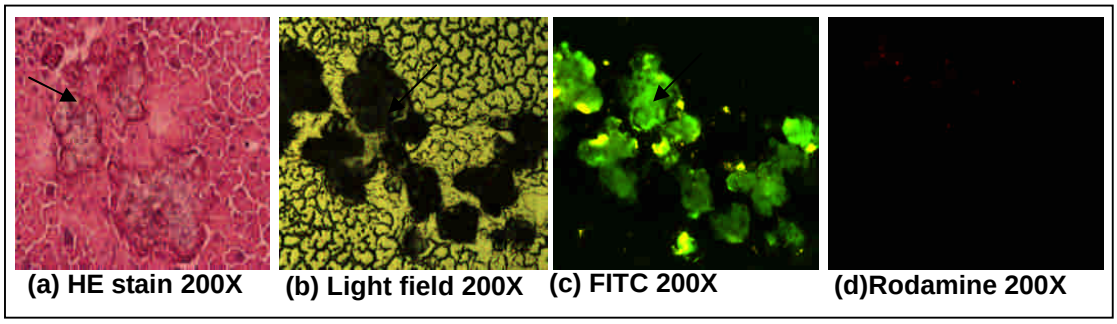


Figure. 1 (A) *K. pneumoniae* CG43 infected liver showing numerous abscess foci on the surface. (B) Histological examination of *K. pneumoniae* CG43 liver infection. At 48 h after infection, areas of inflammation and destruction of liver tissue was visible as indicated with arrow at (a). Numerous GFP-expressed *K. pneumoniae* was observed as aggregates in the inflammatory areas (b, c). (d) Negative control for GFP-expression.

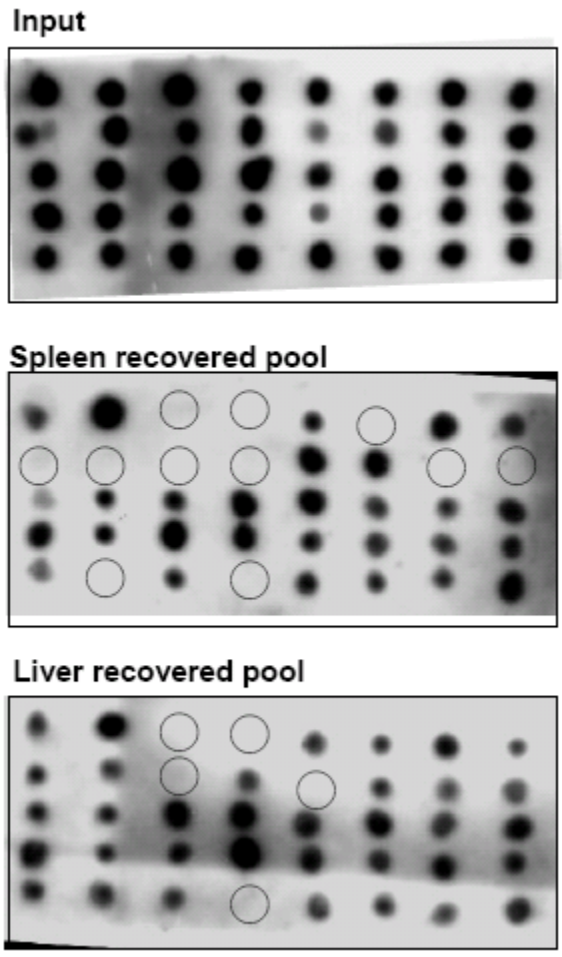


Figure 2. Representative result of PP-STM screening for mutants which failed to be recovered from the spleens or livers of *K. pneumoniae* infected mice.