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NOTES

Identification of Genes Induced In Vivo during *Klebsiella pneumoniae* CG43 Infection

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A novel in vivo expression technology (IVET) was performed to identify *Klebsiella pneumoniae* CG43 genes that are specifically expressed during infection of BALB/c mice. The IVET employed a UDP glucose pyrophosphorylase (*galU*)-deficient mutant of *K. pneumoniae* which is incapable of utilizing galactose and synthesizing capsular polysaccharide, as demonstrated by its low virulence to BALB/c mice and a white nonmucoid colony morphology on MacConkey-galactose agar. By using a functional *galU* gene as the reporter, an IVE promoter could render the *galU* mutant virulent while maintaining the white nonmucoid colony phenotype. A total of 20 distinct sequences were obtained through the in vivo selection. Five of them have been identified previously as virulence-associated genes in other pathogens, while another five with characterized functions are involved in regulation and transportation of nutrient uptake, biosynthesis of isoprenoids, and protein folding. No known functions have been attributed to the other 10 sequences. We have also demonstrated that 2 of the 20 IVE genes turn on under iron deprivation, whereas the expression of another five genes was found to be activated in the presence of paraquat, a superoxide generator.

Klebsiella pneumoniae is an important nosocomial pathogen that causes a wide range of infections, including pneumonia, bacteremia, urinary tract infections, and sometimes life-threatening septic shock. As an opportunistic pathogen, it primarily attacks immunocompromised individuals who are hospitalized and/or suffering from severe underlying diseases, such as diabetes mellitus, chronic alcoholism, or pulmonary obstruction (23). Many clinical strains of *K. pneumoniae* are highly resistant to antibiotics, indicating the relative ineffectiveness of current therapy.

During infections, bacterial pathogens must adapt to various changes in order to persist and proliferate in appropriate locations and to circumvent host defenses. It is reasonable to assume that the expression of many *K. pneumoniae* genes that participate in pathogenesis could be specifically induced within the host. Ideally, these in vivo-expressed (IVE) genes would serve as useful drug targets and vaccine candidates. Several approaches, including in vivo expression technology (IVET) (11, 15), comparative genomics (2), microarray DNA chips (7), signature-tagged mutagenesis (18, 26), differential display-PCR (1), and differential fluorescence detection (31), have allowed the identification of genes that are essential or specifically activated during infections. Nevertheless, none of these approaches has been applied to *K. pneumoniae*, primarily due to the limited number of mutants and genetic tools available for the bacterium.

IVE technology (IVET) is a powerful technique that has been used successfully for several important pathogens, including *Salmonella enterica* serovar Typhimurium (15, 16), *Yersinia enterocolitica* (33), *Staphylococcus aureus* (14), *Pseudomonas aeruginosa* (32), *Escherichia coli* (12), and *Actinobacillus pleuropneumoniae* (9). The original IVET, designed to identify promoters that turn on in vivo in *S. enterica*, used a tandem set of in vivo and in vitro promoterless genes as the reporter (15). There are now several different modifications of the IVET, such as the use of auxotrophic markers and antibiotic resistance genes, and induction of site-specific recombinase as the basis for the selection systems (12, 14, 32). In view of the limited genetic tools available for *K. pneumoniae*, we have designed a novel IVET selection system for this heavily encapsulated bacterium.

Rationale for the *galU*-based IVET selection system. The rationale for the IVET developed for *K. pneumoniae* is shown in Fig. 1. Instead of using a set of tandem reporter genes, our IVET system incorporates a copy of the promoterless *galU* gene as a dual-function reporter. The *galU* gene encodes the enzyme UDP glucose pyrophosphorylase, which regulates the supply of UDP galactose and UDP glucose, two major precursors for the biosynthesis of capsule polysaccharides (CPS) and lipopolysaccharides (LPS) in most enteric bacteria. A GalU$^-$ mutant of *K. pneumoniae* produces defective forms of CPS and LPS and hence loses virulence and the mucoid colony phenotype (5). In addition, the *K. pneumoniae* GalU$^-$ strain is incapable of fermenting galactose, a property that can be readily distinguished by using MacConkey–0.4% galactose agar (5). These unique properties make the *galU* gene an ideal reporter system for IVE gene selection. The bacterial strains which are able to survive in vivo selection while exhibiting a white nonmucoid colony phenotype on MacConkey-galactose agar

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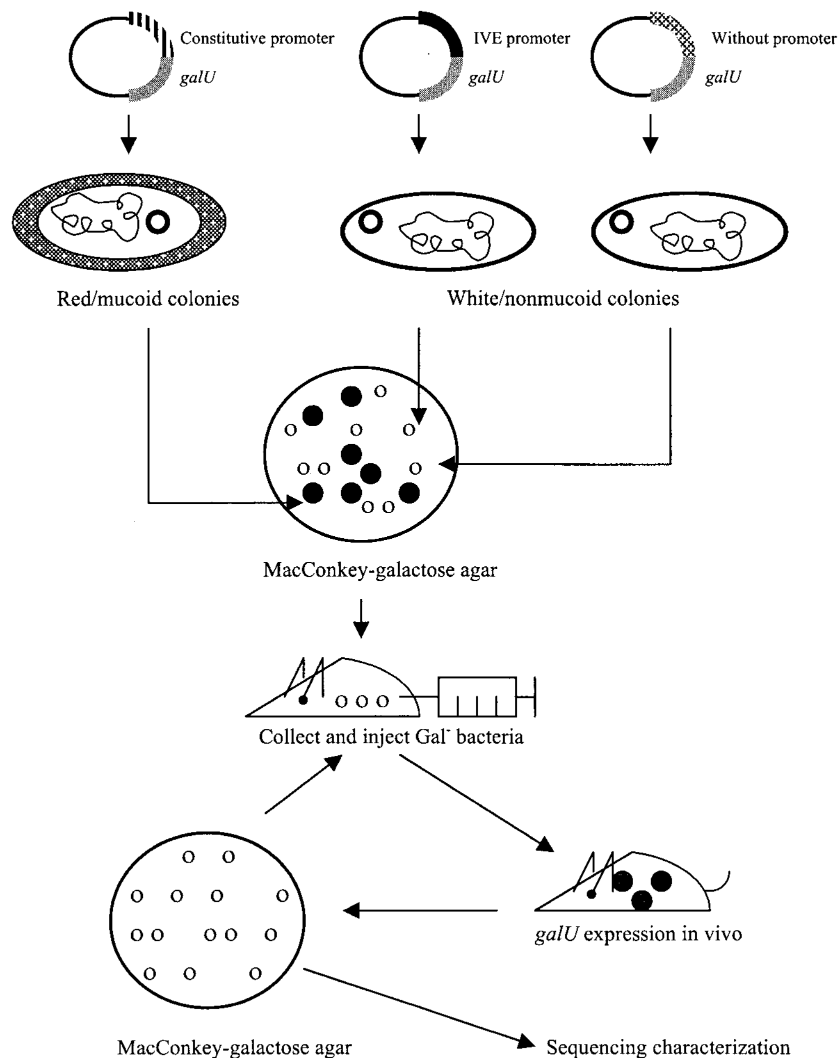

FIG. 1. Overall selection strategy for the *galU*-based IVET system.

would indicate that the DNA fragment upstream of the *galU* reporter contains an IVE promoter.

Construction of *galU*-based reporter gene system. Our IVET selection vector, designated pYC016 (Fig. 2), was constructed by using the mobilizable plasmid pSUP102 (27) as the backbone. Plasmid pYC016 was engineered sequentially in the order described as follows. Initially, a PCR-amplified copy of the *Pseudomonas aeruginosa* PAO1 *galU* gene coding sequence was inserted into the unique *ClaI* site of pSUP102. Subsequently, a nonessential *Bam*HI site in the plasmid was eliminated by end-filling with Klenow fragment of DNA polymerase I, followed by religation. Finally, a cassette from pKK232-8 (Amersham-Pharmacia, Piscataway, N.J.) that contains a transcriptional terminator, multiple cloning sites, and a set of three-way translational stop codons was inserted into a *Cla*I site of the vector. The main features of pYC016 include (i) a unique *Bam*HI site which is compatible with *Sau*3AI-digested chromosomal DNA fragments, (ii) use of the *galU* gene of *P. aeruginosa* PAO1, with the intention of minimizing the possibility of homologous recombination that could occur between the chromosomal and plasmid-borne *galU* genes in *K. pneumoniae*, (iii) the presence of translational stop codons in all three reading frames preceding the ribosome-binding site of the *galU* gene to ensure transcriptional fusions between inserted DNA fragments and the *galU* gene, and (iv) a transcriptional terminator, *rrnB* T_1 (4), upstream of the cloned chromosomal fragment to prevent transcription of the *galU* gene from a fortuitous plasmid promoter.

Construction of *K. pneumoniae* IVET library. Two isogenic *K. pneumoniae* strains were used in this study. *K. pneumoniae* CG43 is a clinical isolate which is highly virulent to laboratory mice, with a 50% lethal dose (LD_{50}) of 10 CFU (22). *K. pneumoniae* CG43-17 is a $GalU^-$ derivative of CG43, generated previously by Tn*5* insertion mutagenesis. The virulence of *K. pneumoniae* CG43-17 was found to be significantly attenuated (LD_{50}, ~10^6 CFU) (5). High-molecular-weight chromosomal DNA of *K. pneumoniae* CG43 was purified and partially digested with *Sau*3AI. Fragments ranging from 0.5 to 1 kb in

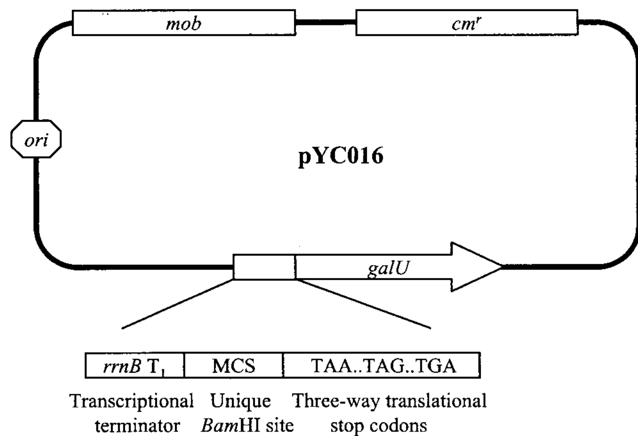

FIG. 2. Diagram of *K. pneumoniae* IVET vector pYC016, showing the locations of the transcriptional terminator (*rrnB* T_1), multiple cloning sites (MCS), three-way translational stop codons, and promoterless *galU* gene. Only the *Bam*HI site in the multiple cloning site is unique in the vector.

size were purified from agarose gels and ligated into the unique *Bam*HI site of pYC016. The ligation mixture was transformed into *E. coli* S17-1 (27) (*hsdR recA pro* RP4-2 [Tc::Mu; Km::Tn7]) and selected on Luria-Bertani (LB) agar supplemented with chloramphenicol (30 μg/ml). Approximately 5×10^5 of the transformants, 90% of which contain a plasmid with an insert, were obtained. These plasmids were then mobilized into *K. pneumoniae* CG43-17 by conjugation. The transconjugants were selected on MacConkey–0.4% galactose plates supplemented with kanamycin (75 μg/ml), chloramphenicol (30 μg/ml), and ampicillin (50 μg/ml). After an 8-h incubation at 37°C, red mucoid colonies were scored as Gal^+, whereas white nonmucoid colonies were scored as Gal^-. The red-white (Gal^+:Gal^-) ratio of the transconjugants was about 1:3.

Screening of *K. pneumoniae* IVE genes. A pilot study was performed with about 10^3 CFU of the IVE library. The study demonstrated that after infections in BALB/c mice and subsequent recovery from spleen, the ratio of red to white colonies shifted from the original 1:3 to 60:1, indicating that most of the Gal^- clones were eliminated effectively in vivo. To reduce the number of mice used in this study as well as to minimize undesired bacteria of the Gal^+ background, approximately 5×10^4 mostly white nonmucoid colonies were collected manually and separated into three batches for the experiments that followed.

Male BALB/c mice (6 to 8 weeks old) with an average weight of 25 g were obtained from the animal center of National Taiwan University and acclimatized in an animal house of our institute for 3 days. Exponential-phase *K. pneumoniae* was obtained by diluting an overnight broth culture 100-fold into warmed LB broth and shaking at 37°C until the optical density at 600 nm (OD_{600}) reached 0.3 to 0.4.

Typically 10^4 CFU were used to infect a BALB/c mouse. Prior to infection, bacteria were washed twice and resuspended in 200 μl of 1× phosphate-buffered saline (PBS), and the suspension was then injected intraperitoneally into a BALB/c mouse. The infected mice were sacrificed after 24 to 48 h, and the spleens were dissected, homogenized, serially diluted, and plated on MacConkey-galactose agar supplemented with appropriate antibiotics. The surviving white nonmucoid colonies were then collected for an additional two rounds of in vivo selection. In three independent experiments, each utilizing a different pool of white nonmucoid bacteria and three mice per round of selection, we arbitrarily picked 30 to 60 white nonmucoid colonies from the postselection pool of each infected mouse for plasmid DNA preparation and restriction endonuclease digestion analysis. A total of 20 distinct eletrophoretic patterns were observed, and these IVE clones were subjected to further characterization.

Verification of inducibility of *galU* fusion constructs in vivo and in vitro. The plasmid DNA was extracted from the 20 IVE clones and retransformed into *K. pneumoniae* CG43-17, and the transformants were tested individually for virulence in BALB/c. Approximately 1×10^6 to 5×10^7 CFU could be recovered from 1 g of spleen from the sick mice. The number is comparable to that of the wild-type *K. pneumoniae* CG43. In contrast, less than 10 CFU of *K. pneumoniae* CG43-17(pYC016) was observed under these conditions. Moreover, the use of a standard assay method (5) indicated that all these clones did not exhibit detectable UDP glucose pyrophosphorylase activity, confirming that the promoters were indeed turned off under in vitro growth conditions.

A serum susceptibility assay, which correlates LPS quantity in the gram-negative bacteria, was also performed. Less than 1% human serum was sufficient to achieve 50% killing of *K. pneumoniae* CG43-17, a mutant known to be incapable of synthesizing intact LPS (5). The *K. pneumoniae* CG43-17 IVE clones exhibited a similar behavior towards 1% human serum and were also killed efficiently. However, the concentration of human serum required to effectively kill the wild-type *K. pneumoniae* CG43 must exceed 50%. Together, these results indicated that the *galU* fusion clones were not expressed when grown in enriched medium but could be induced preferentially during infection of BALB/c mice.

Nucleotide sequence determination of IVE genes. DNA sequence determination was carried out by the PCR-mediated *Taq* DyeDeoxy Terminator Cycle sequencing kit on an Applied Biosystems model 373A DNA sequencer. The homology search of the GenBank/EMBL and SwissProt databases was performed using the BLAST programs provided by the National Center of Biotechnology Information through the Internet. The result of the sequence analysis is shown in Table 1.

Among the 20 IVE sequences, 5 have been shown to be genes essential for in vivo growth identified previously in other pathogens, demonstrating the effectiveness of the *galU*-based selection strategy. *iucA* and *fepA* are both involved in iron acquisition, and expression of these genes is known to be activated during infection (3, 8, 11, 17, 20, 31). The *ptfA* gene encodes a phosphotransfer system for fructose uptake. By using signature-tagged mutagenesis, it has been demonstrated that this gene is crucial for *Vibrio cholerae* to survive in the host (6). The gene product of *rbsR* is a repressor responsible for regulating the expression of *rbsC*, the ribose permease-encoding gene. The expression of *rbsR* was found to be essential for *Brucella melitensis* to survive in the host (13). *lysA* encodes diaminopimelate decarboxylase for lysine biosynthesis (28). It has been shown that in *Staphylococcus aureus*, *lysA* is prefer-

TABLE 1. *K. pneumoniae* IVE genes identified in this study

Predicted function	Accession no.	Homologous gene (accession no.)	% Identity (length of comparable amino acid sequence, no. of residues)	Induction by		Predicted protein
				2′,2′-Dipyridyl	Paraquat	
Iron acquisition						
	AJ277397	*E. coli iucA* (1073533)	86[a]	+	−	Aerobactin biosynthesis
	AJ292298	*E. coli fepA* (P05825)	59 (263)	+	−	Ferrienterobactin receptor
Transport/binding proteins						
	AJ292299	*E. coli ptfA* (P24217)	91 (60)	−	−	Phosphotransferase system
	AJ292304	*E. coli uup* (P43672)	91 (40)	−	−	ATP-binding cassette transporter
Regulatory proteins						
	AJ292310	*E. coli tdcA* (P11036)	78 (175)	−	−	Tdc operon transcriptional activator
	AJ292311	*H. influenzae rbsR* (P44329)	38 (94)	−	−	Ribose operon repressor
Amino acid biosynthesis						
	AJ277396	*E. coli lysA* (P455170)	85 (69)	−	+	Diaminopimelate decarboxylase
Isoprenoid biosynthesis						
	AJ292312	*E. coli yaeM* (P45568)	71 (115)	−	−	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
DNA metabolism						
	AJ292307	*E. coli gyrA* (P09097)	99 (136)	−	−	DNA gyrase subunit A
Protein folding						
	AJ292309	*S. enterica ppiA* (P20753)	87 (124)	−	−	Peptidylprolyl *cis-trans* isomerase A
Unknown/hypothetical						
	AJ292305	*E. coli yjjB* (P18389)	57 (73)	−	−	Protein P-14
	AJ292308	*E. coli ydgH* (P76177)	62 (158)	−	−	Protein YdgH precursor
	AJ292300	*E. coli yjjZ* (P55914)	51 (47)	−	−	Hypothetical 8.7-kDa protein
	AJ292303	*E. coli yhgI* (P46846)	79 (191)	−	−	Protein YhgI
	AJ292306	*E. coli yfjB* (P37768)	90 (43)	−	+	Hypothetical 32.6-kDa protein
	AJ292313	*E. coli yfaE* (P37910)	85 (81)	−	−	Hypothetical 9.3-kDa protein
	AJ292315	*E. coli yjcC* (P32701)	43 (116)	−	+	Hypothetical 60.8-kDa protein
	AJ292301	KPN_CONTIG 880[b]	99[a]	−	+	Unknown
	AJ292302	KPN_CONTIG 765[b]	86[a]	−	+	Unknown
	AJ292314	Novel sequence		−	−	Unknown

[a] Indicates DNA sequence homology.
[b] Data obtained from the Genomic Sequencing Center, Washington University, St. Louis, Mo.

entially expressed during infection, presumably due to limited supply of lysine in the host (18).

The other five *K. pneumoniae* IVE genes found in this study that have been characterized in other organisms include *uup* (25), an ATP-binding cassette type transporter encoding gene; *yaeM* (29), which encodes 1-deoxy-D-xylulose 5-phosphate reductoisomerase, which is responsible for terpenoid synthesis; *gyrA* (19), DNA gyrase subunit A; *tdcA* (10), the product of which is a transcription activator for the *tdc* operon, which encodes a system involved in threonine and serine metabolism and transport during anaerobic growth; and *ppiA* (30), peptidylprolyl *cis-trans* isomerase A. Among the remaining 10 sequences of unknown function, 7 matched the *Escherichia coli* hypothetical protein-encoding genes. Two sequences were found in the genome database of *K. pneumoniae* MGH 78578, established in the Genomic Sequencing Center at Washington University, St. Louis, Mo., and one was a novel sequence.

Inducibility of IVE promoters under iron deprivation and oxidative stress. On entering the host, bacterial pathogens must circumvent iron deprivation and the attack of reactive oxygen species produced by the immune cells. Therefore, it is reasonable to assume that some of the IVE promoters identified in this study may encode a product that assists the bacteria in countering these stresses. To verify the possibility, the IVE clones were spotted individually on MacConkey-galactose plate containing either 200 μM 2′,2′-dipyridyl, an iron chelator, or 10 μM paraquat, a superoxide generator. The phenotype of the colonies after overnight incubation at 37°C was then examined. Two IVE clones, AJ277397 and AJ292298, representing the promoters of two iron acquisition genes, *iucA* and *fepA*, respectively, were found to display the red mucoid colony phenotype in the presence of 2′,2′-dipyridyl that reflects the activation of the promoters under iron deprivation conditions (Fig. 3).

Paraquat was found to activate five IVE clones, including *lysA* (AJ277396) and four carrying genes of no known function (AJ292306, AJ292315, AJ292301, and AJ292302). Our result indicates that many genes are likely to be turned on to counter oxidative stress during infection in the host. The number may be more, since it has been shown that *E. coli* responds to the redox stress imposed by paraquat by activating the synthesis of as many as 80 polypeptides (21). The identification of *lysA* as a superoxide-inducible gene is intriguing, although we do not have a good explanation for this finding yet.

In summary, we have constructed a novel IVET for identification of virulence-associated genes in gram-negative bacteria. By using this system, we have successfully identified 20 IVE genes in *K. pneumoniae*. In addition to being used in IVE gene identification, the convenient red-white selection on MacConkey-galactose plates provided by the *galU* reporter system

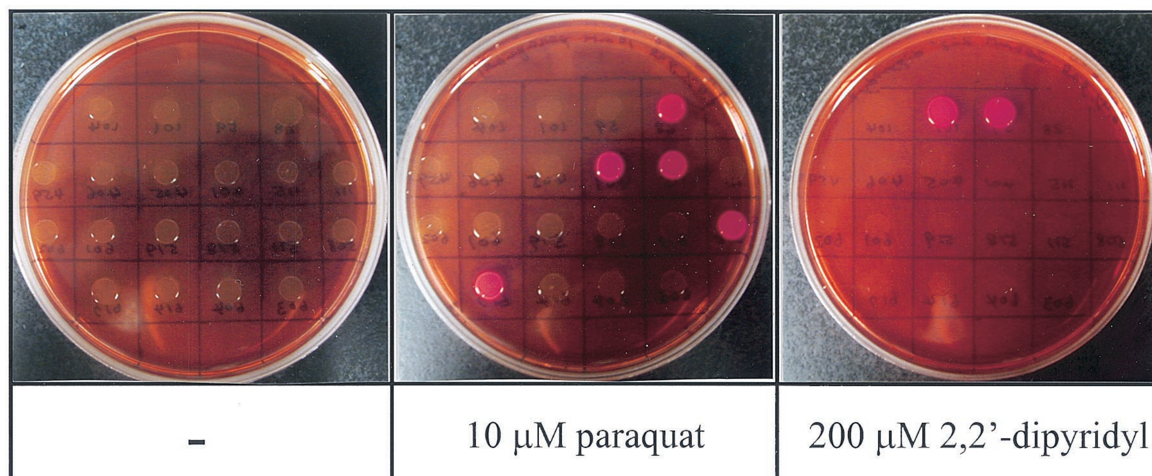

FIG. 3. Phenotypes of IVE clones on MacConkey-galactose agar containing 10 μM paraquat (A) or 100 μM 2′,2′-dipyridyl (B). The bright red color of the colonies indicates expression of the *galU* reporter gene.

might be applied in identifying genes specifically expressed under certain growth conditions, such as iron deprivation and oxidative stress. Like many other IVET, the procedure demands that the reporter gene be expressed throughout the course of an infection. Therefore, this strategy may be unable to identify certain IVE genes that are expressed only during a specific stage of infection. However, this drawback can be complemented by screening the IVE library in multiple animal infection models, as demonstrated in *Streptococcus pneumoniae* with signature-tagged mutagenesis (24).

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