行政院國家科學委員會專題研究計畫 成果報告

克雷白氏肺炎桿菌壓力反應性調控機轉與致病力之探討 (3/3)
研究成果報告(完整版)

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Abstract

Klebsiella pneumoniae is known to cause a wild spectrum of infections acquired in hospital or community settings.   Thus far, little is known regarding the molecular basis of its pathogenesis and unique prevalence in Taiwan.   We aim in this project to understand the regulatory role of stress responsiveness in the physiology and virulence of *K. pneumoniae*.   In the first part of this project, twenty-eight non-capsule related ORFs required for the persistence of *K. pneumoniae* during the development of pyogenic liver abscess in a mouse model were identified and characterized.   This part of work has been published in *Infection and Immunity* ***77:***2657-2671 (July, 2009).   The second part of this project focused on the regulatory role of Hfq, a conserved RNA-binding protein, in modulating *K. pneumoniae* responses under different stressful conditions.   The manuscript regarding part of the Hfq results is now under preparation.

Part I.   Identification of genes required for the development of *Klebsiella pneumoniae*-induced liver abscess in an oral infection model

Abstract

Klebsiella pneumoniae is the predominant pathogen of primary liver abscess. However, our knowledge regarding the molecular basis of how *K. pneumoniae* causes primary infection in the liver is limited.   We established an oral infection model that recapitulated characteristics of liver abscess, and conducted a genetic screen to identify *K. pneumoniae* genes required for the development of liver abscess in mice. Twenty-eight mutants with attenuated growth in liver or spleen were identified out of 2880 signature-tagged mutants that produced the wild type capsule, and genetic loci which were disrupted in these mutants were identified to encode products with roles in cellular metabolism, adhesion, transportation, gene regulation and unknown functions.   We further evaluated the virulence attenuation of these mutants in independent infection experiments and categorized them accordingly into three classes.   In particular, the class I and II mutant strains exhibited significantly reduced virulence in mice, and most of them were not detected in extraintestinal tissues at 48 h after oral inoculation.   Interestingly, the mutated loci of about one-third of the class I and II mutant strains encode proteins with regulatory functions, and the transcript abundances of many other genes identified from the same screen were markedly changed in these regulatory mutant strains, suggesting the requirement of genetic regulatory networks for *K. pneumoniae* to translocate across the intestinal barrier.   Furthermore, our finding that preimmunization with certain class I mutant strains protected mice against the wild type challenge implied a potential application of these strains in the prophylaxis of *K. pneumoniae* infections.

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Genetic Requirements for *Klebsiella pneumoniae*-Induced Liver Abscess in an Oral Infection Model[▽]

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Klebsiella pneumoniae* is the predominant pathogen of primary liver abscess. However, our knowledge regarding the molecular basis of how *K. pneumoniae* causes primary infection in the liver is limited. We established an oral infection model that recapitulated the characteristics of liver abscess and conducted a genetic screen to identify the *K. pneumoniae* genes required for the development of liver abscess in mice. Twenty-eight mutants with attenuated growth in liver or spleen samples out of 2,880 signature-tagged mutants that produced the wild-type capsule were identified, and genetic loci which were disrupted in these mutants were identified to encode products with roles in cellular metabolism, adhesion, transportation, gene regulation, and unknown functions. We further evaluated the virulence attenuation of these mutants in independent infection experiments and categorized them accordingly into three classes. In particular, the class I and II mutant strains exhibited significantly reduced virulence in mice, and most of these strains were not detected in extraintestinal tissues at 48 h after oral inoculation. Interestingly, the mutated loci of about one-third of the class I and II mutant strains encode proteins with regulatory functions, and the transcript abundances of many other genes identified in the same screen were markedly changed in these regulatory mutant strains, suggesting a requirement for genetic regulatory networks for translocation of *K. pneumoniae* across the intestinal barrier. Furthermore, our finding that preimmunization with certain class I mutant strains protected mice against challenge with the wild-type strain implied a potential application for these strains in prophylaxis against *K. pneumoniae* infections.

As a common pathogen for community-acquired and nosocomial infections, *Klebsiella pneumoniae* is responsible for a wide spectrum of clinical syndromes, including purulent infections, urinary tract infections, pneumonia, bacteremia, septicemia, and meningitis (34). Pyogenic liver abscess (PLA) is primarily a complication of intra-abdominal or biliary tract infections resulting from mixed infections with aerobic and anaerobic bacteria (28). A single-pathogen-induced form of PLA which is predominantly mediated by primary infection with *K. pneumoniae* has emerged in recent years (5, 10, 20, 29, 31, 33, 42). Different from the polymicrobial form of PLA, *K. pneumoniae*-induced liver abscess (KLA) is generically cryptogenic, without underlying hepatobiliary disorders, and is frequently complicated with septic metastatic lesions (5, 18, 25, 32, 42, 45). By virtue of its primary and invasive nature, KLA is considered one of the most severe infections caused by *K. pneumoniae* (19, 42). Epidemiological investigations have demonstrated that the occurrence of KLA significantly correlates with the prevalence of particular *K. pneumoniae* strains which

carry specific bacterial features, such as the K1 or K2 serotype and the hypermucoviscosity (HV) phenotype (6, 10, 13, 14, 46). Genetic loci that are involved in the biosynthesis of capsular polysaccharides and the production of siderophores, including a K1-specific gene for the HV phenotype (*magA*), a plasmid-borne gene for the HV phenotype (*rmpA*), a K1-specific gene for iron uptake (the *kfu* phosphotransferase gene), and three gene clusters encoding the TonB-dependent iron acquisition system (*iucABCD-iutA*, *iroA-iroNDCB*, and the *Yersinia* high-pathogenicity island), have been reported to contribute to the virulence of a K1 KLA-causing strain (13, 17, 26, 47). Despite the well-documented impact of KLA, our knowledge regarding the molecular basis of how *K. pneumoniae* causes an infection particularly in the liver is rather restricted.

Signature-tagged mutagenesis (STM) is a technique that permits screening of comparatively large pools of transpositional mutants in a single animal and has been successfully applied to identify virulence factors of a variety of pathogens (16, 36). While there are three reports for which the STM technique was used to investigate *K. pneumoniae* genes required for intestinal colonization, urinary tract infection, and pneumonia (24, 27, 41), no in vivo approach has been applied to identify the *K. pneumoniae* genes necessary for bacterial growth in the liver. In an effort to understand the molecular pathogenesis of KLA, we established an oral infection model that recapitulated the major characteristics of liver abscess and conducted an STM screen to identify the *K. pneumoniae* mu-

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tants which were attenuated in their growth in the liver. Because the role of capsule in bacterial pathogenesis has been well documented, only mutants that produced the wild-type capsule were selected for the STM screen. In this study, we identified 28 KLA-attenuated mutants which had transposon insertions in different genetic loci encoding both novel and previously known bacterial factors. We further evaluated the degree of virulence attenuation for each of these mutants in independent infection experiments and also characterized their roles in the pathogenesis of *K. pneumoniae*. Through this effort, we demonstrated the first use of an in vivo mutagenic screen approach to identify the genetic factors required for *K. pneumoniae* to cause liver abscess in an oral infection model.

MATERIALS AND METHODS

Bacterial strains. *K. pneumoniae* CG43, a K2 serotype strain, was clinically isolated from a patient with PLA. This strain was highly virulent to BALB/c mice, with an intraperitoneal 50% lethal dose of 10 CFU (7). Green fluorescent protein (GFP)-expressing *K. pneumoniae* was generated by transformation of pGFP*uv*-Tc, a tetracycline-resistant derivative of pGFP*uv* (Clontech).

Oral infection in mice. Male BALB/c mice were purchased from the National Laboratory Animal Center (Taiwan) at 6 weeks of age and allowed to acclimatize in the animal house of Chung Shan Medical University for 2 weeks before the experiments. Mice were starved of food for 16 h before inoculation. Twenty microliters of bacterial suspension containing 10^7 CFU of mid-log-phase *K. pneumoniae* (or GFP-expressing *K. pneumoniae*) was orally inoculated into mice by using a 21-gauge feeding needle. The mice were sacrificed at indicative time points, and intestine, cecum, colon, blood, liver, spleen, and kidney samples were retrieved. Serial dilutions of the tissue homogenates were cultured to enumerate bacterial counts. For histological examination, livers were fixed in 10% formaldehyde solution and processed for paraffin embedding, and 5-μm-thick sections were prepared and stained with hematoxylin and eosin. For detection of *K. pneumoniae*, the deparaffinized liver sections were hybridized for overnight at 4°C with rabbit polyclonal antibody against *K. pneumoniae* OmpA at 1:1,000, washed, incubated with peroxidase-conjugated goat antibody against rabbit (Chemicon) for 60 min at room temperature, and then examined after being incubated with chromogen-conjugated 3-amino-9-ethyl carbazole substrate. All animal experiments were performed according to guidance from the National Laboratory Animal Center, and the protocols were approved by the Institutional Animal Care and Use Committee.

Construction of an STM library. The mobilizable suicide vector pBSL180 (1) carrying an ATS Tn*10* transposase gene fused to a P_{tac} promoter, which drastically reduced the hot spot specificity and fully allowed randomness of Tn*10* transposon mutagenesis, was used as the backbone. Double-stranded 80-bp DNA signature tags, which were amplified with primers P2 (5′-TACCTACAACCTC AAGCT-3′) and P4 (5′-TACCCATTCTAACCAAGC-3′), with RT1 (a generous gift from D. W. Holden) (16) as the template, were introduced into the KpnI site of pBSL180. Based on the criteria that an efficient amplification and labeling of a unique tag and a lack of cross-hybridization to other tags, 48 uniquely tagged transposons were selected, sequence determined, and then transformed into *Escherichia coli* S17-1 λ-*pir* (39) via electroporation. Through 48 independent mating experiments, a total of 3,840 *K. pneumoniae* mutants were generated. Based on the colony morphology and tag uniqueness, these mutants were arrayed into 80 mutant pools (MPs), each containing 48 uniquely tagged mutants. Mutants that had no defects in producing the wild-type colony morphology were collected in MP01 to -60, while acapsulated mutants or mutants unable to display the HV phenotype (13) were collected in MP61 to -80. Randomness of mini-Tn*10* mutagenesis was verified by Southern analysis of 24 randomly selected mutants, with *nptII*, the kanamycin-resistant gene of mini-Tn*10*, as a probe. For preparation of 48 unique probes for hybridization, the inner diverse region within the 48 signature tags was individually amplified with p094 (5′-TACCTA CAACCTCAAGCT-3′) and p095 (5′-TACCCATTCTAACCAAGC-3′) (16), and the amplified DNA fragments were digested with HindIII, purified, and labeled with fluorescein-dUTP at the 3′ end by using a Gene Images 3′-oligonucleotide-labeling kit (Amersham Biosciences).

STM screen for mutants attenuated in the oral infection model. Three 8-week-old male BALB/c mice were orally inoculated with 20 μl of bacterial mixtures containing 10^7 CFU of signature-tagged mutants obtained from a particular MP (approximately 2×10^5 CFU per tagged mutant). At 48 h after oral inoculation,

livers and spleens were aseptically retrieved from the infected mice. To reduce bias between individual mice, the tissue homogenates obtained from mice infected with the single MP were combined and cultured with adequate dilutions on LB-kanamycin plates. For each of the recovered pools, at least 3,000 kanamycin-resistant colonies were scraped from the plates, resuspended in phosphate-buffered saline (PBS), and normalized for optical density at 600 nm. Bacterial genomic DNA from each pool was extracted and used as a template for amplification of the DNA tags in accordance with PCR conditions described previously (16). The amplified tag mixtures from both the inoculum and the recovered pools were spotted onto 48 separate Hybond N$^+$ membranes (Amersham Biosciences), which were then subjected to 48 hybridizations, one with each of the 48 tag-specific, fluorescein-labeled probes. Hybridization and detection were carried out using a CDP-STAR nucleic acid chemiluminescence kit (PerkinElmer). Hybridization signals were visually compared based on the results obtained upon 20 min of exposure to X-film (X-OMat; Kodak). Mutants that showed an eliminated signal in the recovered pool but not in the inoculum were selected and reassembled in new pools for another round of selection. Through selections, the secondary pools yielded 108 mutants which were attenuated for growth in the spleen, 84 mutants which were attenuated for growth in the liver, and 39 mutants which were attenuated for growth in both tissues.

Mapping of transposon insertion sites and DNA sequence analysis. Mutants that were not recovered from the liver or spleen were selected for Southern hybridization (37) to determine the number of transposition events. Thirty-six mutants which had only one mini-Tn*10* hit were identified as KLA attenuated. The DNA sequences flanking the mini-Tn*10* insertion site in the 36 mutants were cloned by ligation of PstI- or HindIII-restricted genomic DNA into a correspondingly linearized pUC18 vector. Plasmid DNA that contained various amounts of chromosomal DNA conjunct with the *nptII* locus of mini-Tn*10* was sequenced by Mission Technologies (Taipei, Taiwan) with primers M13-forward and M13-reverse and with primer p136 (5′-CTATCGCCTTCTTGACGAGT-3′), which is located outward relative to the *nptII* locus. Obtained sequences were used for querying the *K. pneumoniae* MGH78578 genome database (http://genome.wustl .edu/genome_index.cgi) and the contig database of *K. pneumoniae* CG43 (H.-L. Peng and S.-F. Tsai, unpublished results) to determine the identities of open reading frames (ORFs) containing mini-Tn*10* insertions. Sequence homology was determined by querying the public protein database with the BLAST search program (3) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the putative functional domains were analyzed with Pfam (http://www.sanger.ac.uk/Software/Pfam/).

Independent verification of virulence attenuation in KLA mutants. The degree of virulence attenuation of a single KLA-attenuated mutant was evaluated in independent infection experiments. Each of the virulence-attenuated (VA) strains was grown to mid-log phase in LB, and 20 μl of bacterial suspension (10^7 CFU) was used to challenge eight of 8-week-old BALB/c mice via the oral route. Small intestine, colon, spleen, liver, and blood samples were aseptically retrieved from three of the infected mice at 48 hours postinfection (hpi). Bacterial concentrations in these mouse tissues were determined by measuring viable counts on LB-kanamycin plates and were represented as numbers of CFU g^{-1} tissue or CFU ml^{-1} blood. The survival rate of the remaining five mice, which were infected with a single VA strain, was monitored daily for 2 weeks. Mortality rate and mean number of days to death (MDD) were determined by Kaplan-Meier analysis using Prism4 for Windows (GraphPad); P values of <0.05 were considered statistically significant. To further examine the virulence attenuation of the class III mutants, a competitive assay was performed as described previously (16). Twenty microliters of bacterial suspension containing equal amounts of the wild type (5×10^6 CFU) and a mutant strain (5×10^6 CFU) was used to orally challenge three of 8-week-old BALB/c mice. At 48 h after inoculation, viable counts of the wild-type and mutant strains in a particular mouse tissue were determined using LB agar with and without kanamycin, respectively. Competitive index (CI) values were calculated as described previously (16).

Detection of *kva* transcripts with RT-PCR and Northern blotting. Total RNA was isolated from bacterial cultures of wild-type *K. pneumoniae* and each of the regulatory *kva* mutant strains which were grown in regular LB medium for 1 h at 37°C with TRI reagent (Molecular Research Center). Contaminating DNA was eliminated with RQ1 RNase-free DNase (Promega). Individual total RNA samples were separately subjected to detect the transcript abundance of a particular *kva* gene by using a SuperScript III one-step reverse transcription-PCR (RT-PCR) kit (Invitrogen) as recommended by the manufacturer, with gene-specific primers. The primer sequences which were used for detecting various *kva* transcripts were as follows: for *galK*, ATGAGCGTATTTAACCCC and CACTCC GGATTCACTAAA; for *cbl*, GTGAACTTCCAGCAGCTG and CTGATAGT CGATTGCGGC; for *kva04*, ATGGCAATTAAATTAGAAATC and GCCAT GATTCACCCCCT; for *mrkC*, ATGAAGGGATTGCCGAAA and AACTAA TTCCCACGTTGC; for *kva07*, ATGCCAAACCGCCGCGTC and CAGAAA

AGTCGGGGTTGTG; for *kva08*, GTGGCGACTATGTACAAA and GAAGTC GACGTTGACGCC; for *kva09*, ATGCACAAATTTACTAAA and CGCCAG CTCTTCTTTGAA; for *kva10*, ATGCGGATGTATTTACC and ACCAATCA ATCCTTCAGT; for *kva11*, GTGAGTCAGGCACCCAGT and CTCGCGGG GGGAGGGCTC; for *kvgA*, ATGAACAGCAGTAACCAC and ACCGATAT TGTTATTGTTTG; for *kva13*, ATGAGTTCTCTGTATCAG and GCTA ACGGTTTCTGCTAC; for *moaR*, ATGTGTCATGTACCATCA and CAGAT GCAGCGTACGCTG; for *kva15*, ATGTTAAAGGGACGATTT and TAATG AACGTTCGGTCTG; for *kva17*, ATGTCACAAGCAATTCAA and ATCGTG GTGGCCGAAGGT; for *kvgS*, CTTGAATTAATTAATCAG and TTCCCTTG AGGTAAGCCC; for *kva19*, ATGACTAATTTTTTGTTCAATATA and GCG TTTGGCGAGCAGTTG; for *kva20*, GTGGGAAATTCCCGCGGA and ATT GTTACTCTCGCAACT; for *kva21*, ATGGCAAACCATCGTGGC and ACTT TCACGACTACCATGAC; for *kva22*, ATGTCGAATACAAGCTGG and TG CGCGGTCGGCATCAGC; for *fimC*, ATGATGAAGAAAATAATT and CTG CTGGGCGAAATCGCT; for *kva24*, ATGAACAGCAATAAAGCA and TGC CTGTTTCGCCTCGCT; for *kva25*, GAGAATCATCCTATTCCC and GATG AGAAAGAGATTGATATG; for *kva26*, ATGGCAGGCCAGACAGGC and ACGCCACTGGTACAGCAG; for *pagO*, ATGCGCAGAGTGACGATA and GGAAGTCTCAGTATGCTC; for *kva28*, ATGACGCGCCGTGCCATC and A AACTCTTCGCTATCCGC; for *kva29*, GTGACCACCAGCTGCAGC and CA GCGAATTCATAATGTT; for *galT*, CCAGGAATCCACCCTTAC and GCCC GTTATTAATCACGC; and for *kva33*, ATGTCCTTAATTAACACC and GA TCTTACCAACCAGATC. The reaction was carried out using a total volume of 50 μl at a final concentration of 10 ng/μl total RNA, with 0.2 μM sense and antisense primers. cDNA synthesis and predenaturation were performed using 1 cycle at 50°C for 40 min and 94°C for 2 min. PCR amplification was performed using 25 to 30 cycles at 94°C for 30 s, 52 to 58°C for 45 s, and 72°C for 2 min. RNA solution without reverse transcription was used as negative control, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which was amplified with primers (5′-GTGGAATATATGACTATC-3′ and 5′-TTTGGAGATGTG GGCAAT-3′), served as an internal control. The gene-specific RT-PCR products were visualized on 2% agarose gels stained with ethidium bromide, and the band intensities were quantified using AlphaEase FC software (version 3.2.1; Alpha Innotech). The final signal, obtained by averaging the results from two replicates for each RNA sample, was normalized to that of GAPDH. If the specific signal of a *kva* gene was not detected from the RNA samples isolated from mid-log-phase LB cultures, total RNA was prepared from stationary-phase LB cultures or mid-log-phase M9-glucose cultures and then subjected to another round of RT-PCR analysis. For Northern detection of the *ymdF* transcript, 20 μg of total RNA isolated from wild-type *K. pneumoniae* was glyoxal denatured, separated on a 2% agarose gel, and then transferred onto a BrightStar Plus nylon membrane (Ambion). After UV cross-linking, the membrane was blotted with ULTRAhyb hybridization buffer (Ambion) overnight at 42°C against a gene-specific biotin-labeled riboprobe, which was prepared using a BrightStar psoralen-biotin kit (Ambion). After a stringent wash, signals were detected with a BrightStar BioDetect kit (Ambion).

RESULTS

Oral infection model of KLA. Because the majority of *K. pneumoniae* infections are preceded by colonization of the patient's intestinal tract (30), we utilized an oral inoculation method to establish a mouse model that could recapitulate the characteristics of liver abscess. A K2 isolate from a KLA patient, *K. pneumoniae* CG43, was used to orally challenge 8-week-old BALB/c mice with a dose of 1×10^7 CFU, which was close to four times the oral 50% lethal dose of this isolate (2.6×10^6 CFU) (data not shown). Compared to the control group, *K. pneumoniae*-infected mice developed hepatic microabscess foci (Fig. 1A) concurrent with splenomegaly. The infected liver was infiltrated with numerous inflammatory cells, compared to what was found for the PBS-inoculated control mice (Fig. 1B, C, F, and G). At 72 hpi, the majority of the liver parenchyma became necrotic (Fig. 1D and H), and the *K. pneumoniae* which was given via the oral route was present in aggregates throughout the liver tissues, as detected by immunostaining with rabbit polyclonal antibodies against *K. pneu-*
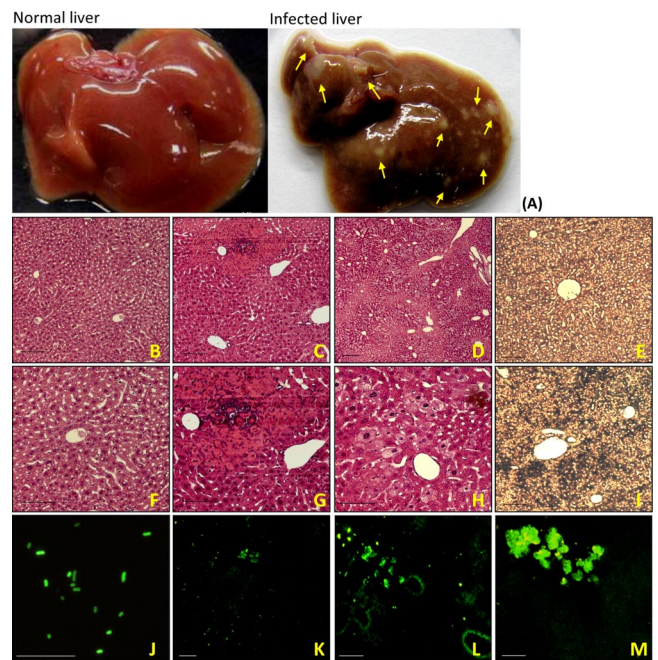

FIG. 1. Liver abscess developed in mice with *K. pneumoniae* infection. Livers were retrieved from BALB/c mice which were orally inoculated with PBS or 10^7 CFU of wild-type *K. pneumoniae*. As indicated with arrowheads in panel A, multiple microabscess foci developed in the infected liver by 72 hpi. Compared to what was found for the control liver retrieved from PBS-inoculated mice (B [magnification, ×200] and F [magnification, ×400]), histological examination of the livers of *K. pneumoniae*-infected mice showed infiltrates of inflammatory cells at 48 hpi (C [magnification, ×200] and G [magnification, ×400]) and necrosis of the liver parenchyma at 72 hpi (D [magnification, ×100 and H [magnification, ×400]). A high concentration of *K. pneumoniae* was detected in the infected liver at 72 hpi with anti-OmpA rabbit polyclonal sera (I) but not in the control liver (E). GFP-expressing *K. pneumoniae* (J [magnification, ×1,000]) was also used in the oral infection model for tracing its distribution. Liver cryosections from mice which were infected with 10^7 CFU of GFP-expressing *K. pneumoniae* were prepared at 24 (K), 48 (L), or 72 (M) hpi and were examined under a fluorescein isothiocyanate view. Scale bar, 50 μm.

moniae OmpA (Fig. 1I). Consistently, GFP-expressing *K. pneumoniae* was also noted to be distributed extensively in the liver after 48 hpi (Fig. 1L and M). To further characterize the progression of liver abscess through the oral infection, bacterial concentrations in different mouse tissues were examined at different time points after inoculation. As shown in Fig. 2, wild-type *K. pneumoniae* established the intestinal colonization within 12 h after oral inoculation and maintained its persistence throughout the entire course of infection. The bacterial concentration in the cecum and colon remained at a high level of 10^7 CFU g^{-1} tissue for 72 h after infection. In the liver, viable *K. pneumoniae* first appeared at 36 hpi and the bacterial amplification reached 1,000-fold at 72 hpi. The spleen was the first extraintestinal site where large numbers of viable bacteria were located at 24 hpi, and the bacterial burden increased and reached a peak concentration of 10^7 CFU g^{-1} tissue at 72 hpi. As indicated by the presence of viable bacteria in the bloodstream and kidneys, approximately 60% of the *K. pneumoniae*-infected mice developed septic metastases at 48 h following
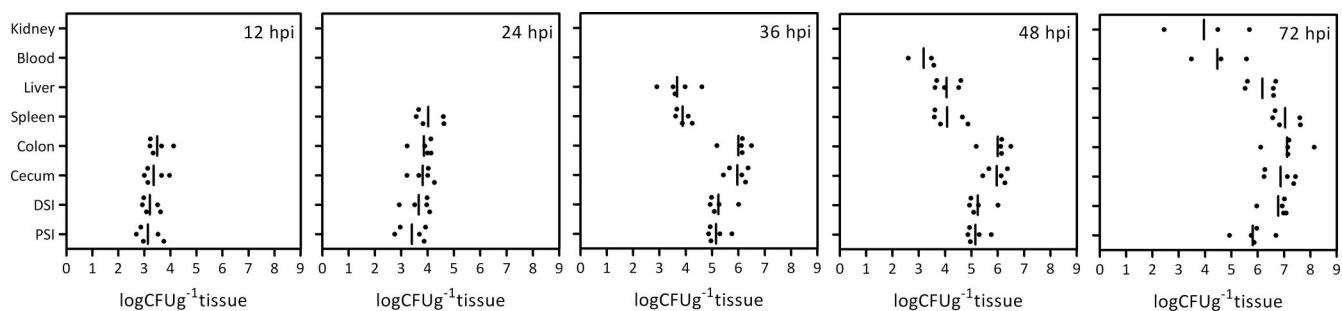
FIG. 2. Dynamics of wild-type *K. pneumoniae* growth in different mouse tissues after an oral inoculation. A single dose of 10^7 CFU of wild-type *K. pneumoniae* was used to infect BALB/c mice via the oral route. At 12, 24, 36, 48, and 72 hpi, samples from various sites in the infected mice, including proximal small intestine (PSI), distal small intestine (DSI), cecum, colon, spleen, liver, blood, and kidney samples, were retrieved and homogenized for measurements of viable bacterial counts, which are expressed as numbers of CFU g^{-1} tissue or CFU ml^{-1} blood. Five mice were examined at each time point. The limit of detection was approximately 50 CFU. Samples which yielded no colonies were not plotted. The bars indicate geometric means.

oral administration (Fig. 2), and more than 80% of mice died of septic shock within 1 week after oral infection. Taken together, these results demonstrate that the infected mice exhibited characteristics of bacteremic liver abscess within 2 days following inoculation with *K. pneumoniae* CG43. In addition, four stages of infection were observed to progress sequentially in our mouse model: intestinal colonization (by 12 hpi), extraintestinal dissemination (between 12 and 24 hpi), hepatic replication (after 36 hpi), and septic metastasis (after 48 hpi).

In vivo screening for KLA-attenuated mutants. To understand the genetic basis of how *K. pneumoniae* acquired an invasive capacity to cause the liver infection, we conducted an in vivo mutagenic screen using the oral infection model. A total of 3,840 signature-tagged mini-Tn*10* mutants of *K. pneumoniae* CG43 were generated with an STM technique (16). Since the capsule is a well-documented virulence factor, in this study, in order to identify putative virulence factors, we subjected only mutants that were phenotypically similar to the parental strain when grown on M9-glucose minimal medium for in vivo selections. As depicted in Fig. 3A, 2, 880 mutants that produced the wild-type capsule were administered into BALB/c mice in pools of 48 mutants each and were examined for their growth in the liver and spleen at 48 h after oral inoculation. As determined by the lack of any hybridization signal on the output blot (Fig. 3B), 36 mutants which were not recovered from either the liver or the spleen were identified. No multiple hits occurred in these mutants, as confirmed by Southern blotting (data not shown). The nucleotide sequences of the regions flanking the transposon in the 36 KLA-attenuated mutants were determined and used as query sequences to search against the contig database of *K. pneumoniae* CG43 (H.-L. Peng and S.-F. Tsai, unpublished results) to determine the identities of the disrupted genetic loci. After the sequence analysis, these 36 mutants were found to result from 33 independent transposition events that occurred at one intergenic region, 3 promoter regions, and 29 different ORFs which encoded proteins with diverse functions. Subsequent to removal of sibling strains, 33 independent mutants, each carrying a transposon insertion at a different genetic locus, were later verified individually for their virulence attenuation.

Independent verification of virulence attenuation of KLA mutants. The bacterial virulence of each mutant strain was

evaluated in independent infection experiments and competitive infection experiments. Individual bacterial cultures obtained from each of the 33 mutant strains were used separately or mixed with the wild-type strain to challenge BALB/c mice via the oral route with a dose of 10^7 CFU, and the bacterial concentrations in different mouse tissues at 48 hpi as well as the mortality rates of infected mice observed for 2 weeks after infection were measured. According to these measurements, 28 out of the 33 mutant strains exhibit significant virulence attenuation. These 28 mutant strains were thus designated VA strains, and the ORFs that were inactivated in these strains were named *kva* (*k*lebsiella *v*irulence-*a*ssociated) genes (Table 1). The phenotypic characterization of these strains is presented in Table 2. No general growth defects resulted from mutations in these strains. Based on sequence analysis of the flanking region of the inserted transposon, the transposon insertion sites in 22 mutant strains were located either within monocistronic loci or in the last gene of polycistronic operons, whereas the transposon insertion sites in the remaining six mutants were located in front of more than one gene within polycistronic operons. The sequence analysis indicated that the disrupted polycistronic operons in these six mutant strains all contained groups of functionally related genes (Table 1). Therefore, although the mutations in these six mutant strains might cause polar effects, these insertions were nevertheless informative.

Based on the degree of their virulence attenuation, the 28 KLA-attenuated mutant strains were categorized into three classes. As shown in Fig. 4A, all mice infected with strains VA01, -05, -07, -12, -15, -18, -21, and -28 survived during the 1-month observation period. These strains were therefore regarded as class I mutants due to their loss of virulence in mice. Also different from wild-type *K. pneumoniae*, which killed mice in 8 days, with an MDD of 4, strains VA02, -04, -08, -10, -14, -19, -20, -22, -24, -25, -32, and -33 caused death in only 20 to 40% of the infected mice ($P < 0.05$; Kaplan-Meier analysis) and were therefore regarded as class II mutants. The class III mutants included the remaining seven VA strains, whose virulence to BALB/c mice did not significantly decrease in the independent infection experiments, but whose virulence attenuation was exhibited in the competitive infection experiments.

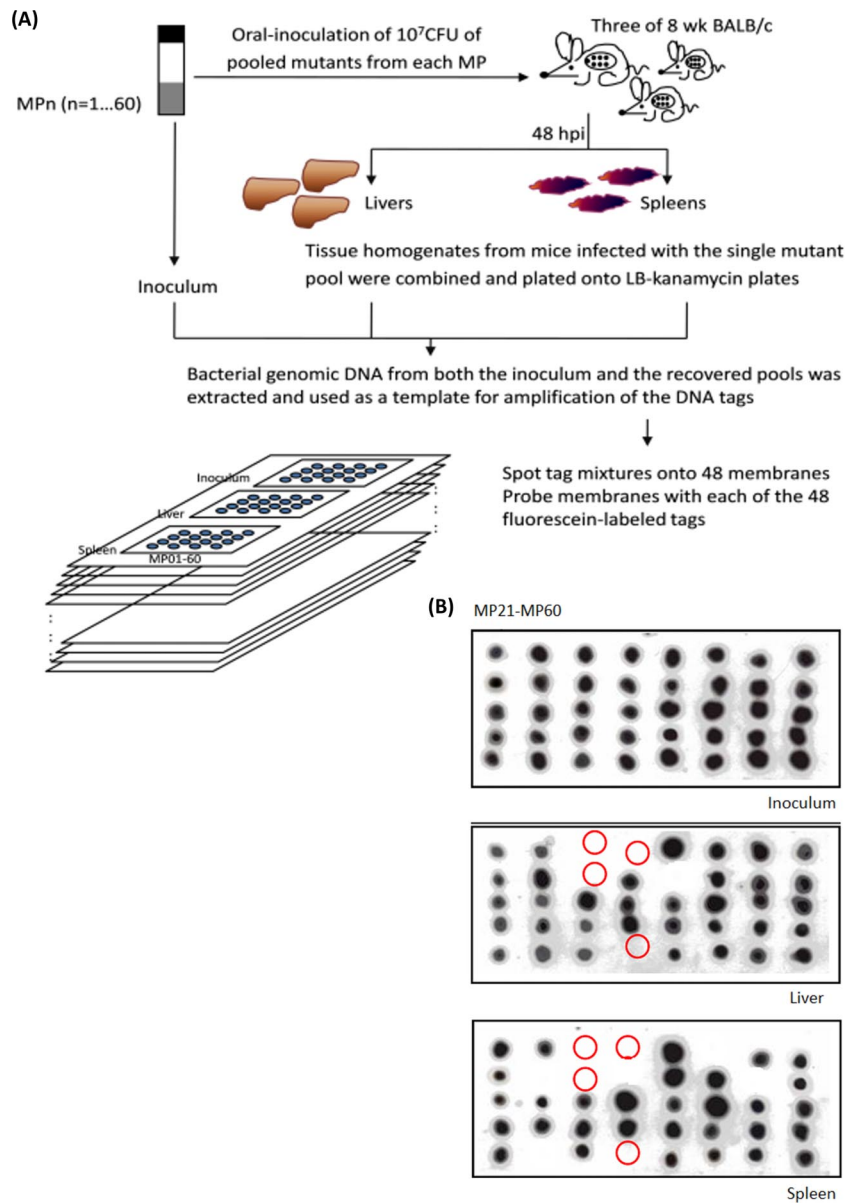As mentioned above, four separate stages of *K. pneumoniae*

FIG. 3. STM screen for KLA-attenuated mutants. (A) Schematic diagram showing the STM screen conducted in the oral infection model. A total of 2,880 mutants that produced the wild-type capsule were selected and arrayed in 60 MPs. Three 8-week-old BALB/c mice were orally administered 10^7 CFU of bacterial culture of a particular MP pool which contains 48 uniquely tagged mutants. Livers and spleens were harvested from these mice, adequate dilutions of tissue homogenates were plated onto kanamycin-LB agar, and at least 3,000 colonies for each output were pooled for the preparation of bacterial genomic DNA. The tag mixtures carried by the mutants in each of the inocula and the output pools were PCR amplified and spotted on 48 replica blots. Each unique fluorescein-labeled tag was subsequently used as a probe for hybridization to those blots, and the mutants whose associated tags were detected in the inocula but not in the pools recovered from either the liver or the spleen were identified as KLA attenuated, indicated by circles in panel B.

oral infection (intestinal colonization, extraintestinal dissemination, hepatic replication, and septic metastasis) were observed to develop sequentially in our mouse model. Because the 28 mutant strains were identified on the basis of their failure to be recovered from the liver or the spleen at 48 hpi, it is of interest to determine whether these attenuations resulted from the incapability of these mutants to achieve any particular infectious stage. Therefore, bacterial concentrations of each mutant strain in different mouse tissues were measured at 48 hpi in order to follow their distributions in the infected

mice. As shown in Fig. 4B, the class I and II mutants were present at significantly lower concentrations than the wild-type strain in the small intestines and colons of the infected mice. Apparently, most of the class I (VA05, -07, -14, -15, -21, and -28) and class II (VA10, -22, -25, and -32) mutant strains were not detected in samples from extraintestinal sites, including the spleen, liver, and bloodstream, whereas 60% of the mice which were infected with wild-type *K. pneumoniae* developed bacteremia at the same time point. On the other hand, when each of the class I mutant strains was inoculated into BALB/c mice via

TABLE 1. Genetic loci required for *K. pneumoniae* in the development of KLA

Function and mutant strain[a]	STM no.	Gene designation[b]	Similarity[c]			Function	Predicted polarity
			Gene	% Identity (% similarity)	Genus		
Cell metabolism							
VA01	E0505	*galK*			*Klebsiella*	Galactokinase	None
VA04	E1013	*kva04*	*proV*	92 (96)	*Salmonella*	Glycine betain/L-proline ABC transporter	None
VA09	G1201	*kva09*	*araF*	91 (96)	*Escherichia*	L-Arabinose binding periplasmic protein	None
VA17	E2605	*kva17*	*rhaB*	41 (59)	*Lactobacillus*	α-L-Rhamnosidase, putative	None
VA24	E2945	*kva24*	*gabD*	91 (95)	*Escherichia*	Succinate-semialdehyde dehydrogenase	*gabT* and *gabP*
VA29	C5243	*kva29*	*pgdH*	34 (54)	*Serratia*	D-3-Phosphoglycerate dehydrogenase	None
VA32	E5721	*galT*			*Klebsiella*	Galactose-1-phosphate uridylyltransferase	*galK*
VA33	A5901	*ahpC*	*ahpC*	99 (100)	*Salmonella*	Alkyl hydroperoxide reductase	None
Cell surface components and transporters							
VA05	D1112	*mrkC*			*Klebsiella*	Usher protein of type III fimbriae	*mrkD* and *mrkF*
VA08	E1205	*kva08*	*hgpA*	28 (46)	*Haemophilus*	Hemoglobulin binding protein	None
VA10	G1706	*kva10*	*pls*	29 (33)	*Streptococcus*	Surface large repetitive protein	None
VA13	G1815	*kva13*	*pteA*	39 (60)	*Bacillus*	Cellobiose-specific phosphotransferase IIA	None
VA22	F2730	*kva22*	*pteC*	55 (75)	*Bacillus*	Cellobiose-specific phosphotransferase IIC	None
VA23	A2805	*fimC*			*Klebsiella*	Chaperon protein of type I fimbriae	*fimD*
VA27	E5021	*pagO*			*Klebsiella*	PhoPQ-activated integral membrane protein	None
VA28	E5145	*kva28*	*uraA*	93 (97)	*Escherichia*	Uracil permease	None
Regulation							
VA02	F0514	*cbl*			*Klebsiella*	HTH-type transcriptional regulator Cbl[d]	None
VA12	E1813	*kvgA*	*evgA*	50 (66)	*Escherichia*	Response regulator of two component system	*kvgS*
VA14	F2014	*moaR*			*Klebsiella*	Monoamine regulon positive regulator	None
VA18	G2615	*kvgS*	*evgS*	52 (68)	*Escherichia*	Histidine kinase sensor	None
VA25	E3045	*kva25*	*csgD*	39 (62)	*Salmonella*	Response regulator for second curli	None
Hypothetical and others							
VA07	G1115	*kva7*				Hypothetical protein	None
VA11	D1812	*kva11*	*yfgG*	77 (91)	*Escherichia*	Hypothetical protein YfgG	None
VA15	E2145	*kva15*				Putative LuxR family transcription regulator	None
VA19	C2619	*kva19*				Putative UphA family transcription regulator	None
VA20	D2620	*kva20*	*int*	92 (96)	*Escherichia*	Putative integrase	None
VA21	C2635	*kva21*	*ymdF*	85 (94)	*Escherichia*	Conserved hypothetical protein YmdF	None
VA26	H4601	*kva26*	*lyxK*	31 (49)	*Pasteurella*	Putative L-xylulokinase	*pgdH*

[a] Twenty-eight KLA-attenuated mutant strains are shown and categorized by their putative functions. Class I mutant strains are indicated in bold.
[b] Known *K. pneumoniae* genes or ORFs identified as *kva* (*k*lebsiella *v*irulence *a*ssociated).
[c] Genes encoding proteins or hypothetical proteins in the public database with the highest similarity to the sequences identified. Identities and similarities were determined at the amino acid level over the entire ORF.
[d] HTH, helix-turn-helix DNA binding motif.

an intraperitoneal route with an inoculum of 10^2 or 10^5 CFU, as shown in Table 3, all of the strains exhibited virulence comparable with that of the wild-type strain. These results suggested that the attenuation of these mutant strains was restricted within the intestinal tract. Unlike the class I and II

mutants, most of the class III mutant strains generally caused the infected mice to develop KLA symptoms and were capable of propagating themselves to reach concentrations comparable to those of the wild-type strain in all the mouse tissues examined in the independent infection experiments (Fig. 4B). Nev-

TABLE 2. Phenotypic characterization of KLA-attenuated mutants[a]

Function and mutant strain	Gene designation	In vitro CI[b]		Uronic acid content[c]
		LB	M9-glucose	
Cell metabolism				
VA01	galK	1.037 ± 0.006	1.007 ± 0.038	18.9 ± 2.7
VA04	proV	1 ± 0.017	0.993 ± 0.051	14.5 ± 1.8
VA09	araF	1.017 ± 0.006	0.96 ± 0.02	14.2 ± 0.9
VA17	rhaB	1.063 ± 0.012	0.683 ± 0.081	13.1 ± 1.5
VA24	gabD	1.027 ± 0.025	0.93 ± 0.017	12.2 ± 1.0
VA29	pgdH	0.987 ± 0.006	0.967 ± 0.032	21.9 ± 2.1
VA32	galT	1 ± 0.01	0.947 ± 0.015	19.6 ± 2.7
VA33	ahpC	1.017 ± 0.025	0.937 ± 0.059	18.5 ± 3.8
Cell surface components and transporters				
VA05	mrkC	1.007 ± 0.006	0.94 ± 0.064	12.5 ± 0.9
VA08	hgpA	0.98 ± 0.015	0.95 ± 0.02	14.5 ± 1.4
VA10	pls	1.02 ± 0.026	1.013 ± 0.049	23.2 ± 3.8
VA13	pteA	0.983 ± 0.021	0.963 ± 0.025	13.3 ± 2.3
VA22	pteC	0.987 ± 0.025	0.963 ± 0.042	19.5 ± 1.2
VA23	fimC	1 ± 0.01	0.97 ± 0.026	24.5 ± 2.1
VA27	pagO	0.967 ± 0.012	0.917 ± 0.015	20.0 ± 1.5
VA28	uraA	0.997 ± 0.032	0.95 ± 0.03	12.5 ± 1.8
Regulation				
VA02	cbl	1.03 ± 0.02	1.027 ± 0.047	15.3 ± 0.9
VA12	kvgA	1.017 ± 0.012	0.983 ± 0.05	13.8 ± 1.5
VA14	moaR	1.03 ± 0.02	0.96 ± 0.046	13.4 ± 3.2
VA18	kvgS	1.003 ± 0.012	0.933 ± 0.023	15.0 ± 2.0
VA25	csgD	0.983 ± 0.021	0.957 ± 0.045	16.6 ± 2.0
Hypothetical and others				
VA07	Novel	0.997 ± 0.011	0.98 ± 0.026	15.0 ± 1.2
VA11	yfgG	1.02 ± 0.017	0.983 ± 0.059	19.0 ± 4.4
VA15	Novel	0.99 ± 0.017	0.97 ± 0.01	27.6 ± 7.3
VA19	Novel	0.977 ± 0.021	0.967 ± 0.015	17.6 ± 2.1
VA20	int	0.997 ± 0.035	0.937 ± 0.038	13.5 ± 1.0
VA21	ymdF	0.963 ± 0.021	0.99 ± 0.01	19.7 ± 2.9

[a] The HV phenotypes of the colonies were determined using the string-forming test with blood agar, as described previously (13). All strains showed positive results, defined as the formation of a $\geq$10-mm length of string, like that formed by the wild-type strain. Bacterial resistance to serum killing was determined as described previously (13). If the bacteria examined showed a reduction of 2 $\log_{10}$ in CFU after 30 min of incubation with nonimmune human serum at 37°C, the strain was defined as serum sensitive. If the viability of a VA mutant was comparable to that of the parental strain, the VA mutant was considered serum resistant. All strains were serum resistant.

[b] CI values were determined as described previously (16) and are shown as means $\pm$ standard deviations.

[c] The uronic acid content levels of the capsular polysaccharides were determined as described previously (24) and are expressed as means $\pm$ standard deviations (μg/10^{10} CFU). The value for the parental strain was 17.0 ± 2.2.

ertheless, in the competitive infection experiments, class III mutants exhibited significant disadvantages in comparison with the wild-type strain with respect to the spleen, the liver, or both organs (Table 4), suggesting that the mutations affected hepatosplenic colonization of *K. pneumoniae* after extraintestinal dissemination. Interestingly, strain VA17 (*rhaB*::Tn*10*) showed no defects in the competition assay, even though this strain was undetectable in the bloodstream when inoculated independently, suggesting that bacterial propagation in the blood might be affected by this mutation.

Protective immunization of the class I mutants against challenge with the wild-type strain. As revealed in the independent infection experiments, the class I mutants colonizing the intestine had only a limited ability to induce systemic spreading and were avirulent to BALB/c mice when given via the oral route. Because these strains still produced the wild-type capsule and retained their resistance to serum killing (Table 2), we were interested in knowing whether these avirulent yet immunogenic strains had the potential to serve as candidates for live

vaccines. To test this possibility, groups of BALB/c mice were orally administered each of the type I mutant strains with a single dose of 10^7 CFU. Six weeks later, these preimmunized mice, together with age-matched naïve mice, were challenged with 10^7 CFU of wild-type *K. pneumoniae*. As shown in Fig. 5A, immunization with any of the class I mutants provided mice with significant protection against wild-type *K. pneumoniae* infection, compared with what was found for the control group, in which all of the naïve mice died within 2 weeks. In particular, none of the mice that were orally administered a single dose of bacterial cultures from VA05, VA07, or VA28 succumbed to the subsequent challenge with the wild-type strain (Fig. 5B), and at 7 days postinfection, bacteria were cleared from all examined tissues obtained from these mice (data not shown). In addition, preimmunization with VA28 evoked increases in proinflammatory cytokine release, including gamma interferon, tumor necrosis factor alpha, and interleukin-10 (IL-10) (Fig. 5B) and stimulated the production of protective antibodies against the outer membrane constitu-
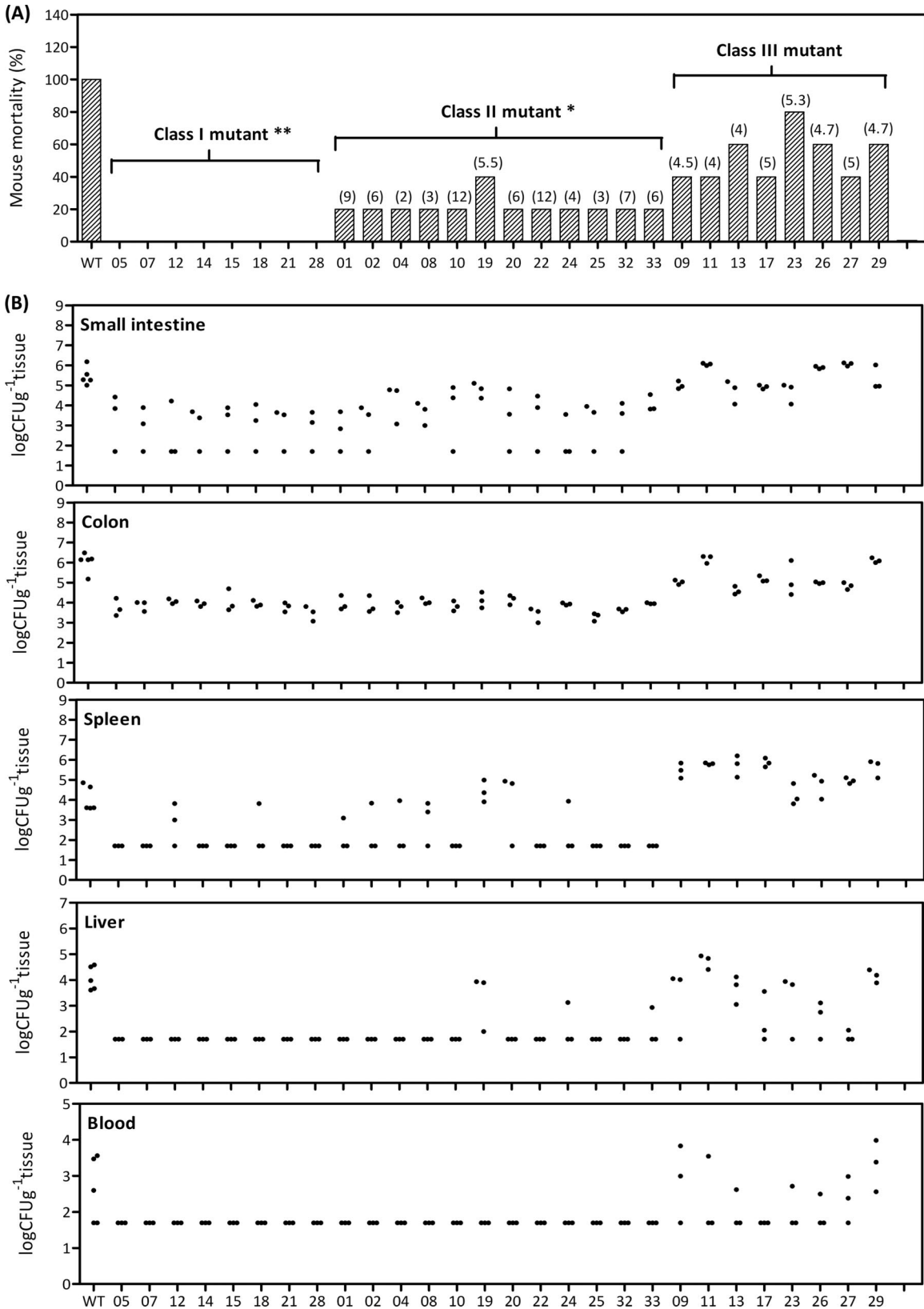
FIG. 4. Virulence attenuation in independent infection experiments. (A) Five BALB/c mice were orally administered 10^7 CFU of a single KLA-attenuated mutant strain. The survival rate of these mice was monitored daily for 2 weeks. MDD are shown in parentheses, and both mortality rate and MDD were determined by Kaplan-Meier analysis. Strains VA05, -07, -12, -14, -15, -18, -21, and -28, which were avirulent in mice during the observation period, were regarded as class I mutants. Strains VA01, -02, -04, -08, -10, -19, -20, -22, -24, -25, -32, and -33, which resulted

TABLE 3. Intraperitoneal virulence of class I mutant strains[a]

Strain	% Inoculum (MDD)	
	1×10^3 CFU	5×10^5 CFU
Wild type	0 (2)	0 (1)
Nonencapsulated mutant	100 (>14)	100 (>14)
VA05	0 (2)	0 (1)
VA07	0 (2.7)	0 (1)
VA12	0 (2)	0 (1)
VA14	0 (3.7)	0 (1)
VA15	0 (2.3)	0 (1)
VA18	0 (2.3)	0 (1)
VA21	0 (3)	0 (1)
VA28	0 (2.3)	0 (1)

[a] Three 8-week-old male BALB/c mice were challenged with a particular *K. pneumoniae* strain via an intraperitoneal route. The survival rate of the mice was monitored daily for 2 weeks, and survival rate and MDD were determined by Kaplan-Meier analysis using Prism4 for Windows (GraphPad).

ents as well as the capsular polysaccharides of *K. pneumoniae* (Fig. 5C). Together, these results indicated that these class I mutants could be utilized as oral-administered live vaccine candidates.

Molecular characterization of the class I mutants. Genetic loci which were transposon mutated in the eight class I mutant strains (VA05, -07, -12, -14, -15, -18, -21, and -28) encoded products with predicted roles in metabolism, adhesion, transportation, gene regulation, and unknown functions. As shown in Table 1, strain VA05 had an insertion in *mrkC*, along with a polar effect on *mrkD* and *mrkE*, which abrogated the assembly and anchorage of the type III fimbriae (2, 15). Strain VA28 was mutated in gene *uraA*, which encodes uracil permease. Meanwhile, a two-component system encoding gene cluster *kvgAS* (8, 21) was disrupted in strains VA12 (*kvgA*::Tn*10*) and VA18 (*kvgS*::Tn*10*). Gene *moaR*, which encodes a positive regulator of the monoamine regulon in *Klebsiella aerogenes*, was inactivated in VA14. The gene product of *kva15*, which was disrupted in VA15, had a predicted function as a transcriptional regulator of the LuxR family. A homologue of the conserved hypothetical protein YmdF was inactivated in VA21.

During the development of liver abscess, the orally inoculated *K. pneumoniae* must circumvent a wide variety of stresses. We reasoned that the virulence attenuation of the class I mutants might be a consequence of reduced tolerance to the stress that bacteria encountered inside the host, such as gastric acids, bile insults, nutrient limitation, or reactive oxidative species inside the macrophages. Therefore, the bacterial growth of each class I mutant strain under different stressful conditions was measured. As shown in Fig. 6A, the growth capacities of strains VA12 (*kvgA*::Tn*10*) and VA21 (*ymdF*::Tn*10*) were significantly reduced upon exposure to H_2O_2. Because YmdF was predicted to function as a general stress protein in *E. coli*, we

were interested in knowing whether it played a role in the tolerance response to stress in *K. pneumoniae*. The transcript abundance of *ymdF* (*kva21*) under different stressful conditions was measured with Northern analyses. When wild-type *K. pneumoniae* grew in LB medium, the *ymdF* transcript level steadily increased and reached a peak at the stationary phase (Fig. 6B, lanes 9 to 12), while no *ymdF* transcripts were detected when the bacteria were incubated in M9-glucose medium (Fig. 6B, lanes 13 to 16). However, the amount of *ymdF* transcripts increased rapidly in *K. pneumoniae* at 6 min posttreatment with 20 mM H_2O_2 (Fig. 6B, lane 3), compared to that in *K. pneumoniae* which was cultured in LB medium for 1 h (Fig. 6B, lane 9). Similar significant increases in *ymdF* transcript levels could also be observed in *K. pneumoniae* after 15 min-treatments with different concentrations of sucrose (Fig. 6B, lanes 6 to 8). These results revealed an involvement of YmdF in the tolerance response to oxidative and osmotic stress of *K. pneumoniae*. Together with the results from independent infection experiments (Fig. 4B), it appeared that inactivation of *ymdF* attenuated the resistance of *K. pneumoniae* to stress and thus made *K. pneumoniae* incapable of translocation across the intestinal barrier.

Regulatory Kva in control of *kva* gene expression. Seven of the 28 *kva* genes identified in this study (*kva02*, -*12*, -*14*, -*15*, -*18*, -*19*, and -*25*) were found to encode proteins with regulatory functions (Table 1). These regulatory *kva* genes played indispensable roles in the pathogenesis of *K. pneumoniae* because all the mutant strains that carry transposon insertions in these genes exhibited significantly reduced virulence (Fig. 4). Bacterial virulence genes are generally controlled under a complicated regulatory network to ensure their expression at the right time and the right site during infection. Therefore, it was of interest to observe whether these seven regulatory *kva* genes modulated the expression of other *kva* genes to affect the virulence of *K. pneumoniae*. For this purpose, the transcript levels for all *kva* genes in wild-type *K. pneumoniae* and different regulatory *kva* mutant strains were determined by using semiquantitative RT-PCR with gene-specific primers. The transcripts of five *kva* genes (*kva04*, *kva22*, *kva24*, *kva26*, and *kva29*) were undetectable in wild-type *K. pneumoniae* grown under the in vitro conditions tested (LB for 1 h, LB for 4 h, M9-glucose for 1 h, and M9-glucose for 4 h), suggesting that these genes might be specifically expressed in vivo. After normalization to an internal control, the in vitro differences in the transcript levels of 23 *kva* genes were determined and are shown in Table 5. Remarkably, at least 10 of these *kva* genes exhibited >2-fold decreases in their transcript abundances in VA02 (*cbl*::Tn*10*), VA14 (*moaR*::Tn*10*), and VA15 (*kva15*::Tn*10*). The overlap of downregulated genes in the three mutant strains indicated that these downstream *kva* genes, together with *cbl*, *moaR*, and *kva15*, appeared to be part of the

in mortality rates of 20% to 40% in mice, had their virulence attenuated to a statistically significant level ($P < 0.05$) and were therefore categorized as class II mutants. The remaining VA strains, with no significantly attenuated virulence, were grouped as class III mutants. (B) Bacterial growth in different mouse tissues. Three BALB/c mice were orally administered 10^7 CFU of a single KLA-attenuated mutant strain. Small intestine, colon, spleen, liver, and blood samples were harvested at 48 hpi to enumerate bacterial concentrations, which were expressed as numbers of CFU g^{-1} tissue or CFU ml^{-1} blood. The limit of detection was approximately 50 CFU. Samples which yielded no colonies were plotted as having values of 50 CFU g^{-1} tissue or 50 CFU ml^{-1} blood.

TABLE 4. Competition assay for class III mutant strains

| Strain | CI (P)[a] | | No. of mice with KLA symptoms[b] |
	Liver	Spleen	
VA09 (*araF*::Tn*10*)	0.15 ± 0.003 (<0.05)	0.069 ± 0.004 (<0.05)	2
VA11 (*yfgG*::Tn*10*)	0.086 ± 0.024 (<0.05)	1.50 ± 0.16 (−)	1
VA13 (*pteA*::Tn*10*)	0.018 ± 0.04 (<0.05)	1.15 ± 0.14 (−)	1
VA17 (*rhaB*::Tn*10*)	2.12 ± 0.66 (−)	1.47 ± 0.09 (−)	1
VA23 (*fimC*::Tn*10*)	0.017 ± 0.008 (<0.05)	0.111 ± 0.043 (<0.05)	1
VA26 (*lyxK*::Tn*10*)	0.0014 ± 0.0004 (<0.05)	0.765 ± 0.053 (−)	0
VA27 (*pagO*::Tn*10*)	0.14 ± 0.0003 (<0.05)	0.0111 ± 0.067 (<0.05)	2
VA29 (*pgdH*::Tn*10*)	0.73 ± 0.005 (−)	0.005 ± 0.001 (<0.05)	3

[a] A mixture of equal amounts of the wild type (5×10^6 CFU) and a mutant strain (5×10^6 CFU) was orally inoculated into three 8-week-old BALB/c mice. CI values were calculated at 48 hpi as described previously (16) and are shown as means ± standard deviations. *P* values were determined using the Mann-Whitney test in the Prism4 software program (GraphPad). "−" indicates that the decrease in the CI value was not statistically significant.

[b] Development of KLA symptoms was determined by the formation of liver abscess foci at 48 h after an oral inoculation with 10^7 CFU of a particular class III mutant strain.

same regulatory network. In addition, there were several *kva* genes under the control of the KvgAS two-component system; a response regulator other than KvgA might be involved in transduction of the KvgS signal, since the *kva13* transcript was absent only in VA18 (*kvgS*::Tn*10*). The transcript abundances of a relatively small number of *kva* genes were affected in VA25 (*csgD*::Tn*10*) and VA19 (*kva19*::Tn*10*), indicating that the regulatory networks exerted by these two regulators were different from those for other regulatory *kva* genes, in which a large number of downstream genes were not recovered, due to an unsaturated search in this STM screen. On the other hand, the expression of *kva25* was upregulated in VA12 (*kvgA*::Tn*10*), and two- to fourfold increases in expression of *kva09* were also observed in some regulatory *kva* mutants. Most strikingly, the transcript levels of *mrkC* and *kva08* declined significantly in all of the regulatory mutants, suggesting that regulatory networks exerted by the Kva regulators might converge at these two effectors. These results indicated that the regulatory *kva* genes functioned coordinately to control the expression of a subset of genes, including downstream *kva* genes, which allowed *K. pneumoniae* to respond to the stress correctly and infect its hosts successfully.

DISCUSSION

K. pneumoniae is a worldwide-spread pathogen responsible for a broad spectrum of clinical syndromes. Since the 1980s, a single-pathogen-induced form of PLA, which is clinically and etiologically distinct from polymicrobial PLA, has been noticed to be predominantly mediated by primary infection with *K. pneumoniae* (20, 42). Although the clinical significance of *K. pneumoniae* in association with primary liver abscess has been established, we have limited knowledge regarding the molecular basis of how this bacterium causes an infection in the liver. The intestine is one of the major reservoirs of *K. pneumoniae*, but how the intestine-colonizing *K. pneumoniae* bacteria can acquire an ability to translocate across the intestinal barrier, disseminate systematically, and finally gain growth advantages within specific niches in the liver still remains elusive. We reasoned that a cohort of genes whose expression was coordinately regulated were necessary for *K. pneumoniae* to complete the process from colonization of the intestine to infection of the liver. To gain insights into the pathogenesis of KLA, in this

study, we established a mouse model and conducted a genetic screen for KLA-attenuated mutants. Although it had been shown that injecting 10^2 to 10^5 CFU of a K1-type *K. pneumonia* strain intraperitoneally or intravenously induced KLA symptoms in mice (13, 43), we decided to apply an oral inoculation method. This method comparatively provided a more relevant route in the pathogenesis of KLA, because epidemiological studies suggest that the majority of *K. pneumoniae* infections are preceded by colonization of the patient's gastrointestinal tract (30), and the corresponding immune responses are likely to be stimulated via gut-associated lymphoid tissues. By means of oral administration, infection of BALB/c mice with *K. pneumoniae* KLA isolates demonstrated significant similarities to the human KLA, including a dramatic rate of bacterial replication, infiltration of inflammatory cells, formation of microabscess foci in the liver, and lethality upon subsequent bacteremia and septicemia. The development of these KLA symptoms was consistently observed in mice challenged with 1×10^7 CFU of *K. pneumoniae* CG43 (K2-serotype) as well as in mice treated with two other K1 KLA isolates of *K. pneumoniae* (data not shown). Besides, this method also allowed us to monitor the progression of KLA in mice by sampling sites along the infectious route from the intestines to the liver. Interestingly, we observed that *K. pneumoniae* induced systemic infection in mice via the development of four sequential stages, including intestinal colonization, extraintestinal dissemination, hepatic replication, and septic metastasis. Different from the polymicrobial PLA, which was generically secondary to hepatobiliary infections, the propagation of *K. pneumoniae* from the intestine to the liver proceeded via the bloodstream, as indicated in our model in which the extraintestinal bacteria were first detected in the spleen at 24 h after oral inoculation. Taken together, the oral infection model established in this study could recapitulate the major characteristics of bacteremic liver abscess caused by *K. pneumoniae*.

The genetic factor required for development of *K. pneumoniae*-induced bacteremic liver abscess was subsequently determined in this oral infection model. An STM technique was used as our in vivo screening approach to identify mutants which failed to replicate in either the spleen or the liver. A total of 33 independent strains were obtained from two rounds of in vivo selection, accounting for 1.1% of the total number of
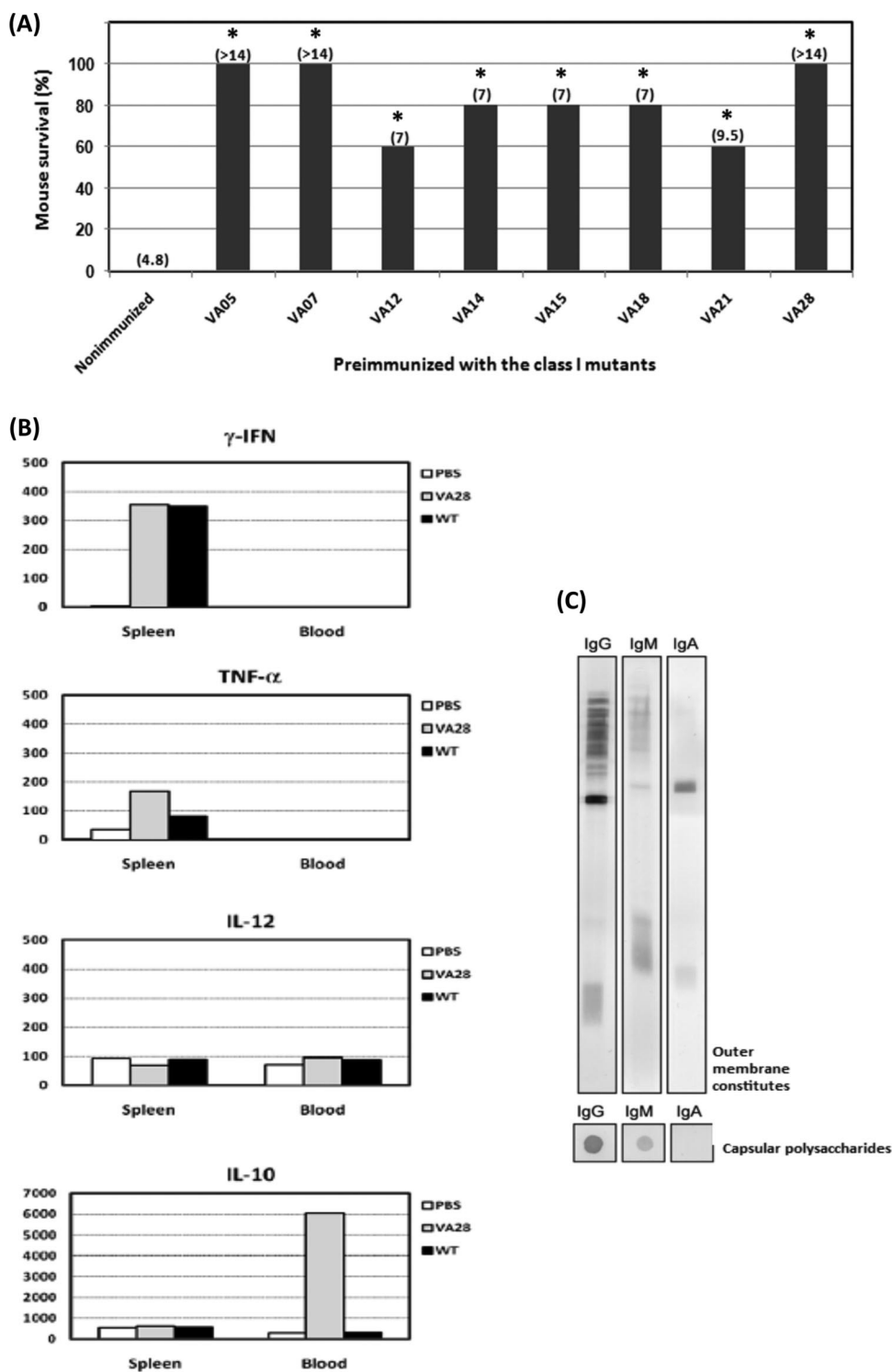
FIG. 5. Characterization of the avirulent yet immunogenic class I mutant strains. (A) Oral administration with the class I mutants allowed mice to survive challenge with wild-type *K. pneumoniae* infection. Five BALB/c mice were orally administered a single dose of 10^7 CFU of a particular class I mutant strain. The preimmunized mice, together with age-matched naïve mice, were challenged with 10^7 CFU of wild-type *K. pneumonia* 6 weeks later. The survival rate of the infected mice was monitored daily for 2 weeks. MDD are shown in parentheses, and both mortality rate and MDD were determined by Kaplan-Meier analysis. Sera from all mice were collected 1 day prior to challenge with the wild-type strain and were used to detect the production of *K. pneumoniae*-specific antibodies. $*$, $P < 0.05$; $**$, $P < 0.01$. Statistical significance was determined by comparison of survival curves by use of the log rank test (Kaplan-Meier analysis; Prism4). (B) Cytokine production upon oral administration with the class I mutant VA28. Sera and spleens were collected at 24 hpi and were analyzed for cytokine production by an enzyme-linked immunosorbent assay. The mean production levels of cytokines (gamma interferon, tumor necrosis factor alpha, IL-12, and IL-10) in five mice per group are shown in pg per 100 μg of total proteins. (C) Specific antibodies against the outer membrane constituents and capsular polysaccharides of *K. pneumoniae* were detected in pooled antisera from the five mice that were preimmunized with VA28.
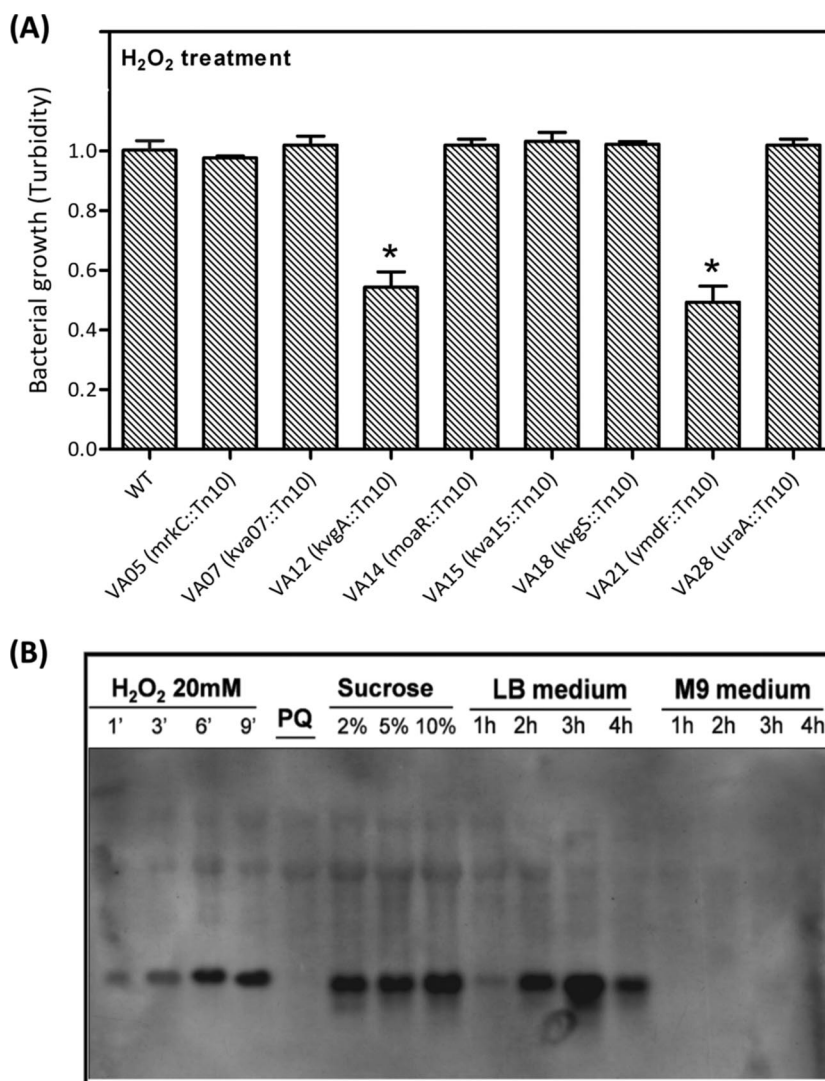
FIG. 6. Role for *K. pneumoniae* YmdF in resistance to oxidative stress. (A) Tolerance responses of class I mutants to various stresses. Bacterial cultures of the wild type and each of the class I mutants, which were grown in LB medium at 37°C, were adjusted to a turbidity value of 1 and were then subjected to treatment with 20 mM of H_2O_2 for 2 h at 37°C. The bacterial concentration upon oxidative stress was determined using a MicroScan turbidity meter (Dade Behring, CA). Results shown are means ± standard deviations for three independent experiments. * indicates statistical significance as determined by Student's *t* test ($P < 0.05$). (B) Northern blot analysis of *ymdF* transcripts. Total RNAs were isolated from wild-type *K. pneumoniae* grown in LB, in M9-glucose, and under various stress conditions. Twenty micrograms of total RNA from each sample was subjected to Northern blotting, and a biotin-labeled *ymdF* RNA probe was used for the hybridization. The expression levels of *ymdF* upon exposure to H_2O_2 for 1, 3, 6, 9 min; treatment with 15 mM paraquat (PQ); or treatment with different concentrations of sucrose for 15 min are shown in lanes 1 to 8, and the abundances of *ymdF* transcripts in *K. pneumoniae* grown in LB or M9-glucose medium is shown in lanes 9 to 16.

mutants. Because only mutants which were attenuated in both the liver and the spleen were identified, the recovery rate was lower than the 2 to 10% attenuation relative to that reported for other STM screens (38). In addition, the preselecting procedure in which only mutants producing the wild-type capsule were subjected to in vivo screening may also reduce the numbers of isolated mutants since capsule is a well-documented virulence determinant for *K. pneumoniae*. Capsule was reported to be indispensable for development of *K. pneumoniae*-induced systemic infection in a pneumonia model of C57B6 mice (24) and in a bacteremia model of BALB/c mice (7). Therefore, minimizing the number of mutants with insertions in the capsule polysaccharide synthesis locus would facilitate

identifying putative virulence factors which had not been previously characterized. Through this effort, though our screening of 2,880 mutants was not a saturating search, we identified a variety of genetic loci which encode both novel and previously known bacterial factors other than the capsule. None of these genes have been identified in the previous STM screens conducted in different infection models (24, 27, 41).

To further confirm whether the attenuated mutants had genuine defects in bacterial pathogenesis, we utilized independent infection experiments as the secondary screen. This approach was chosen instead of the conventional competition assay because possible bias might result from the interference of extracellular substances produced by the wild-type strain

TABLE 5. Comparison of *kva* gene transcript levels in the wild-type and regulatory *kva* mutant strains

Mutant class and gene	Fold difference[a]						
	VA02 (*cbl*::Tn*10*)	VA14 (*moaR*::Tn*10*)	VA15 (*kva15*::Tn*10*)	VA12 (*kvgA*::Tn*10*)	VA18 (*kvgS*::Tn*10*)	VA19 (*kva19*::Tn*10*)	VA25 (*csgD*::Tn*10*)
I							
mrkC	–	**0.320**	**0.280**	**0.353**	**0.429**	**0.418**	**0.305**
kva7	**0.274**	0.419	**0.336**	0.784	0.593	0.556	**0.477**
kvgA	0.553	1.112	0.537	–	0.612	1.005	0.537
moaR	0.912	–	0.812	0.717	0.898	0.888	0.832
kva15	**0.372**	0.673	–	0.721	**0.461**	0.829	**0.494**
kvgS	0.776	0.595	0.759	–	–	0.862	0.922
kva21	1.059	0.727	**0.316**	1.027	**0.433**	0.556	0.658
kva28	0.758	1.099	**0.269**	**0.313**	**0.346**	1.330	0.582
II							
galK	**0.362**	–	–	**0.394**	**0.500**	0.894	0.596
cbl[b]	–	–	0.850	–	0.900	0.950	0.588
kva8[c]	**0.092**	0.330	**0.151**	**0.281**	**0.265**	**0.254**	**0.178**
kva10	**0.371**	0.311	**0.374**	0.791	0.566	0.861	**0.417**
kva19	0.579	0.913	0.552	1.120	0.749	–	0.710
kva20	**0.434**	0.678	**0.455**	0.836	0.559	0.615	0.531
kva25	0.951	1.410	1.069	2.347	1.326	1.292	–
galT	**0.406**	–	0.508	**0.374**	**0.428**	0.706	0.642
III							
kva9[b]	1.789	–	2.596	1.982	2.579	4.561	2.667
kva11	**0.381**	0.470	**0.483**	0.936	0.597	0.712	0.534
kva13	0.522	0.119	**0.493**	0.903	–	1.194	0.731
kva17	–	–	**0.333**	0.743	0.857	0.724	0.819
fimC	0.989	0.108	0.742	1.688	1.161	1.581	1.097
pagO	0.628	1.377	1.014	1.214	0.930	1.195	0.977
ahpC	0.910	1.181	0.777	1.108	0.861	0.976	0.964

[a] Semiquantitative RT-PCR analysis was used to determine the relative transcript abundance of every *kva* gene in wild-type *K. pneumoniae* and each of the regulatory *kva* mutant strains. The relative transcript abundance of a particular *kva* gene in a bacterial strain was measured by quantification of the band intensity of the specific RT-PCR product, which was normalized to that of GAPDH. The level of each *kva* transcript from the wild-type strain was normalized to a value of 1. The transcripts of *kva04*, *kva22*, *kva24*, *kva26*, and *kva29*, which were not detected in wild-type *K. pneumoniae* grown under the in vitro conditions tested, are not shown. The differences in *kva* transcript levels between each of the regulatory mutant strains [VA02 (*cbl*::Tn*10*), VA12 (*evgA*::Tn*10*), VA14 (*moaR*::Tn*10*), VA15 (*kva15*::Tn*10*), VA18 (*evgS*::Tn*10*), VA19 (*kva19*::Tn*10*), and VA25 (*csgD*::Tn*10*)] are indicated (means of results from two independent determinations). The expression levels of specific *kva* genes which were downregulated in a particular regulatory *kva* mutant more than twofold are indicated in bold. "–" indicates that the transcripts of a specific *kva* gene were not detected under the in vitro conditions tested by two independent experiments.
[b] The transcripts of *cbl* and *kva9* were detected only in the stationary-phase LB cultures.
[c] The *kva08* transcripts were detected under the cultivation with M9-glucose medium.

during the in vivo competition. We categorized the 28 KLA-attenuated mutant strains into three classes according to the degrees of their virulence attenuation (Fig. 4A). When given via the oral route with an inoculum of 10^7 CFU, the class I and II mutant strains exhibited significantly reduced virulence to BALB/c mice ($P < 0.05$), and most of these strains were not detected in extraintestinal tissues at 48 hpi (Fig. 4B). Apparently, the virulence of these strains was attenuated, because they failed to reach the extraintestinal dissemination stage. This failure could reasonably be attributed to the inability of these mutants to translocate across the intestinal barrier or the loss of bacterial resistance to serum killing. Because all of the class I and II mutant strains were as resistant as the wild type to the killing of normal human sera (Table 2) and the class I mutant strains exhibited virulence comparable to that of the wild-type strain when they infected mice via an intraperitoneal route (Table 3), genetic loci which were mutated in these strains could reflect the genetic requirement for *K. pneumoniae* in dissemination beyond the intestine. While the class I and II mutant strains were restricted in the intestine, the retained ability of the class III mutants to translocate to distal sites allowed strains of this class to exhibit levels of virulence in mice

comparable to those for the wild type. The findings led us to reason that in the oral infection model, the ability of *K. pneumoniae* to progress from intestinal colonization to extraintestinal replication may determine its host lethality. Although no significant decreases in the mortality rates of those class III mutant-infected mice were observed at the statistical level, six of the class III mutant strains were significantly outcompeted by the wild-type strain in the spleen, the liver, or both organs (Table 4). Identification of genes which were disrupted in these class III mutants might provide information about certain limited resources in vivo for which *K. pneumoniae* had to compete with other bacteria. Interestingly, one class III mutant, VA17 (*rhaB*::Tn*10*), exhibited increased competitive colonization in the spleen and liver but caused death in 40% of the infected mice. The decreased virulence of VA17 might be due to the affected capacity of this strain to propagate in the bloodstream, as no colonies were yielded in the blood samples obtained from the VA17-infected mice at 48 hpi (Fig. 4B).

Our findings that immunization of mice with a single dose of the class I mutants (VA05, VA07, or VA28) stimulated memory immune responses and provided complete protection against lethal *K. pneumoniae* challenge demonstrated a great

potential for the use of these mutants in prophylaxis against KLA disease. Previously, several purified components of *K. pneumoniae*, including capsular polysaccharides, lipopolysaccharides, and type III fimbriae (9, 12, 22, 44), as well as an acapsular *K. pneumoniae* mutant (23), had been used as immunizing agents against *K. pneumoniae* infection. However, the subunit vaccines required multiple parenteral injections and the use of immunostimulatory adjuvants, which sometimes stimulated only systemic humoral immune responses. On the other hand, acapsular mutants could not induce effective protective immunity, due to their lack of complete immunogenicity. Therefore, for vaccine development, the class I mutants identified here have several advantages. First, the failure of these strains to translocate across the intestinal barrier led them to trigger self-limited infection only in the intestine, without causing any symptoms of KLA disease. Not only systemic humoral immune responses but also the mucosal immunity was induced upon oral immunization with these strains. Second, in contrast to acapsular mutants, these strains carried intact capsular polysaccharide and lipopolysaccharide antigen and retained their resistance to host serum killing, which allowed the production of antibodies against *K. pneumoniae* cell surface components and the elicitation of immediate responses of the host's innate immunity (Fig. 5). Finally, the finding that a single dose of challenge with these strains via an oral route was enough to stimulate protective immune responses made these strains attractive for use as an orally administered vaccine. However, further studies are still required to evaluate the safety and efficacy of the application of these strains in prophylaxis against *K. pneumoniae* infections.

A glance at the genetic loci identified here (Table 1) suggests a requirement for cell surface components in the progression of KLA. Of particular interest is the identification of type I and III fimbriae: a transposon insertion in *fimC* with a polar effect on *fimD* that abrogated the chaperone-usher pathway in the biosynthesis of type I fimbriae was identified in strain VA23 (11), and the type III fimbria-encoding gene cluster *mrkABCDE* (2, 15) was disrupted in strain VA05 by a transposon insertion in *mrkC*. The inactivation of type I and type III fimbriae in VA23 and VA05, respectively, was ascertained by hemagglutination assays (data not shown). Though genetic loci encoding these two types of fimbriae were identified in the same screen, the type I and III fimbriae appeared to play different roles in the pathogenesis of *K. pneumoniae*. As indicated in the independent infection experiments (Fig. 4), the type I fimbria mutant VA23 (*fimC*::Tn*10*) exhibited the wild-type level of virulence and colonized all the mouse tissues examined, with its growth capacity unaffected. Consistent with this finding, it has recently been reported that the ability of *K. pneumoniae* with an isogenic type I fimbria mutation is not affected in colonizing the mouse intestine (40). The dispensability of the type I fimbriae was also shown in an intranasal infection model (4) in which a Tn*5* mutant of *fimA* still retained 75% mortality for mice. Nonetheless, the type I fimbria was not totally unnecessary for *K. pneumoniae*, as it was determined to be a virulence factor in a urinary tract infection model (40) and the growth of the type I fimbria mutant strain in the liver was significantly attenuated in the competition with the wild-type strain (Table 4). In contrast to type I fimbriae, type III fimbriae were crucial for *K. pneumoniae* virulence in

our oral infection model. As demonstrated in independent infection experiments, strain VA05 (*mrkC*::Tn*10*), whose ability to colonize the intestine was attenuated due to the inactivation of type III fimbriae, was unable to disseminate from the intestinal lumen to extraintestinal tissues and was consequently avirulent in mice (Fig. 4). The different requirements for type I and III fimbriae of *K. pneumoniae* in the progression of KLA will be further studied in the future.

Little is known about the regulatory cascades utilized by *K. pneumoniae* in the control of virulence gene expression so far. Interestingly, out of 28 *kva* genes identified in this study, there were 7 genes with regulatory functions. All the mutant strains featured insertions in these regulatory *kva* genes that were highly attenuated in mice, indicating a close correlation between these Kva regulators and bacterial virulence. The transcript abundances of quite a few *kva* genes were markedly changed in these regulatory *kva* mutants compared to that in the wild type, as revealed by semiquantitative RT-PCR (Table 5). The impact of the Kva regulators on *K. pneumoniae* virulence likely came from their transcriptional control of a panel of genes, some of which were identified in the same STM screen. By further comparing the *kva* gene expression profiles among the seven regulatory *kva* mutants, we found different subsets of *kva* genes with distinct expression patterns, which indicated the involvement of a number of regulatory networks controlling the virulence of *K. pneumoniae*. The fact that strains VA02, VA14, and VA15 shared the same set of down-regulated *kva* genes indicated that Cbl, MoaR, and Kva15 might serve in the same regulatory network, which was different from that controlled by the KvgAS-dependent signaling system. This was also reflected by the observation of outer membrane proteins in which the overlap of changes in the outer membrane protein profiles for VA02, VA14, and VA15 was distinct from that for VA12 and VA18 (data not shown). Because the screening in this study was not saturated, there may be other members in these regulatory networks that remain to be identified. It is of considerable interest that the expression levels of *mrkC* and *kva08* were downregulated in all the regulatory *kva* mutants. The convergence of Kva regulatory networks on the type III fimbria-encoding genes is reminiscent of the regulatory circuits controlling the expression of the pilus locus of *Streptococcus pneumoniae* (35). These results suggest that bacterial pili may function in parallel with other virulence mediators and that the regulation of pili is not assigned to a particular regulatory circuit. Although this work discloses only a part of the regulatory system controlling the expression of virulence factors required for *K. pneumoniae* to progress from intestinal colonization to the liver infection, our findings provide insights into the regulatory mechanism underlying the pathogenesis of this bacterium, which is far more complicated than previously thought. In this respect, we should view KLA as the outcome of a series of events orchestrated by a panel of virulence genes, and the expression of these genes is coordinately controlled when the bacteria are adapting inside the host.

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Part II.   The regulatory impact of Hfq-dependent srRNAs on physiological fitness and virulence potential in *Klebsiella pneumoniae*

Klebsiella pneumoniae adapts itself to various environments and is capable of causing a wild range of infections.   How *K. pneumoniae* can switch physiological programs to ensure its survival in a specific ecological niche is still a mystery. Recently, it has become clear that small non-coding RNAs are crucial regulators that modulate diverse cellular processes to enable prokaryotic cells to adjust physiological fitness to environmental changes.   Most small regulatory RNAs (srRNAs) exert their function in the post-transcriptional level via the binding of Hfq.   The RNA chaperone Hfq contributes to the fitness and virulence of several pathogens.   A number of Hfq-dependent srRNAs are proved to be important components of many regulatory circuits.   An *hfq* gene-knockout mutant was generated via an allelic exchange technique.   Proteomics comparison for the expression profiles of outer membrane proteins between the *hfq* mutant and wild type strain revealed that a variety of proteins were under control of Hfq via direct or indirect ways.   Characterization of the Hfq-deletion mutant indicated that Hfq affected the *K. pneumoniae* capsule in quantity and quality.   The major transcript of the K2 *cps* gene cluster was elevated by the deletion of *hfq*, suggesting that Hfq might have a negative regulatory role in control of the *cps* gene expression.   Consistently, the transcriptome analysis revealed a positive effect of the *hfq* deletion on the expression of most of these CPS regulatory genes.   The Hfq-dependent control of CPS biosynthesis in *K. pneumoniae* is different from that in *E. coli*.   We reason that the post-transcriptional control of CPS regulators may be mediated by a subset of small RNAs that are *K. pneumoniae*-specific.   Unraveling the molecular link between Hfq and the enhancement of CPS production is therefore warranted for further studies.

Introduction

As one of enteric bacteria, *Klebsiella pneumoniae* inhabits the intestinal tract of a variety of mammals, including the human.   Since the first identification of *Klebsiella* as a cause of pneumonia in 1882, the remarkable ability of *K. pneumoniae* to cause a wild range of human diseases, from urinary tract infections to life-threatening systemic infections (15), has attracted increasing attention to the pathogenesis of this bacterium.   *K. pneumoniae* has long been considered as an opportunistic pathogen because of its ubiquitous distribution and great capacity to adapt to diverse environments.   The physiological fitness of *K. pneumoniae* to specific ecological niches is not solely confined to the surroundings inside the animal hosts.   A recent report indicated that the nitrogen-fixing strain of *K. pneumoniae* was capable of colonizing the interior of plants and acted like an endophyte to promote the growth of plants (6).   It is interesting in knowing how *K. pneumoniae* can respond to environmental changes and thus adapts itself to a specific niche. Nevertheless, the molecular mechanism underlying the regulation of adaptive response that allows *K. pneumoniae* to switch among physiological programs to ensure its survival under different environments is still a mystery.

Over the past decade, it has become clear that small non-coding RNAs are crucial regulators that enable the eukaryotic or prokaryotic cells to adjust the physiology to environmental changes (8).   The small RNA molecules can rapidly In bacteria, functional small RNAs can be generated via processing or as primary transcripts which are generally untranslated and encoded in the intergenic regions of chromosomes (24).   Their synthesis is tightly regulated and is often induced by environmental cues or stressful conditions.   Small regulatory RNAs (srRNAs) directly affect the expression of effector and regulatory proteins.   Depending on the nature of the target molecules, srRNAs exert their function either by base-pairing

interactions with trans-encoded transcripts, or by forming ribonucleoprotein complexes with target proteins (16). The srRNAs that pair with target transcripts generally requires the RNA chaperone protein Hfq for facilitating the formation of srRNA-target RNA duplexes. Hfq was initially identified in *E. coli* as a protein required for bacteriophage Qβ RNA replication and was demonstrated to be an RNA-binding protein that forms homohexamers of ~12 kDa subunits (7). The importance of Hfq was unclear until the finding that an *hfq* insertion mutant in *E. coli* exhibited pleiotropic phenotypes affecting growth rate, cell morphology, and tolerance to stress conditions (22). Since the subsequent work revealed that Hfq is essential for the post-transcriptional control of *rpoS* transcript by srRNAs, an increasing number of srRNAs that associate with Hfq have been continually identified by systematic approaches in *E. coli* K12 (1).

Although most of these newly identified srRNA still remains functionally unknown, a potential role of the RNA-chaperone protein Hfq in the virulence of pathogenic bacteria has been addressed by recent studies. A *hfq* mutant of *Brucella abortus* had significant defects on its survival in macrophages and was consequently avirulent in a mouse model (17). Similarly, a *hfq* mutant that failed to colonize the suckling mouse intestine was demonstrated to be necessary for the virulence of *Vibrio cholerae* (4). Hfq also contributes to the pathogenesis of several common pathogens, including *Listeria monocytogens* (2), *Legionella pneumophila* (12), and *Pseudomonas aeruginosa* (20). The Hfq protein is structurally and functionally similar to eukaryotic Sm proteins and is highly conserved among *Enterobacteriaceae*. It is not surprising that loss of Hfq function significantly reduced the virulence of certain enteric bacteria, such as *Salmonella enterica* serovar Typhimurium (18), and uropathogenic *E. coli* (9). Although Hfq is being considered as the RNA chaperone that generally exerts it post-transcriptional function by facilitating the imperfect

antisense interactions of srRNAs and its target mRNAs, recent reports indicate that it also acts alone as a translational repressor of mRNA (23), modulates mRNA decay by stimulating polyadenylaion (13), and participates in tRNA biogenesis (11). Given that Hfq primarily acts in concert with srRNAs, the pleiotropic phenotypes resulted from the *hfq* mutation observed in the tested bacterial pathogens so far may be attributed, in part, to the loss of gene regulation by Hfq-dependent srRNAs.

Until recently, global gene expression studies have mainly been focused on the transcriptional regulation exerted by the specific action of DNA-binding proteins. With the identification of more and more srRNAs in bacteria, particularly in *E. coli*, while functions of many of these RNA molecules are still not known, an increasing number of studies demonstrate that the RNA regulators behave as key effectors of the bacterial adaptive responses, such as environmental signal recognition, stress response, and virulence control (16). Considering the impact of RNA regulators on the bacterial physiology and virulence, we pay particular attention to these molecules. A *hfq* deletion mutant was therefore generated in *K. pneumoniae* to serve as a starting point for our study on small regulatory RNAs. Through characterization of the hfq-deletion mutant, we demonstrated the involvement of Hfq in the adaptive responses and its possible role in coordinating regulatory circuits in *K. pneumoniae*.

Results and discussion

Construction of *K. pneumoniae hfq* deletion mutant. The *hfq* gene is located in clockwise orientation at bps 446148-446456 in the genome of *K. pneumoniae* strain NTHU-K2044 [unpublished result from Shih-Feng, Tsai (National Health Research Institute, Taiwan)]. As in *E. coli* K12, it is located in the *mutL-miaA-hfq-hflX-hflK-hflC* cluster of genes. The *K. pneumoniae hfq* gene shares 88% and 92% identity with *E. coli hfq* at the nucleotide and amino acid level, respectively. Based on the sequence, specific primers were designed to amplify the DNA fragments flanking the *hfq* coding region. The amplified DNA fragments together with a *cat* (chloramphenicol resistance) gene were cloned into the suicide vector pKAS46 (19). The resulting construct was mobilized to *K. pneumoniae* CG43S5 (a streptomycin-resistant derivative of CG43) via conjugation from *E. coli* S17-1 *λpir* (19). The replacement of entire *hfq* coding region by the *cat* marker was conducted by homologous recombination as described previously (10), and was verified by PCR and by Southern hybridization with a specific probe. To complement the *hfq* mutation, the arabinose inducible Hfq-expression plasmid pYC343 was generated by incorporation of the *hfq* coding region into pBAD202.

Hfq affected the *K. pneumoniae* capsule in quantity and quality. Clinically isolated *K. pneumoniae* usually produces large amounts of capsular polysaccharides (CPS) as reflected by the formation of glistening mucoid colonies with viscid consistency. The degree of mucoidy has been shown to positively correlate with the establishment of *K. pneumoniae* infection. The mucoid capsule is well known to act as the protection for *K. pneumoniae* to avoid complement-mediated serum killing and phagocytosis. Interestingly, the *hfq* deletion mutant H0201 exhibited a drastically mucoid phenotype on either LB or M9 agar. We quantified the CPS amount by measuring the uronic acid content and found that the mutant strain

H0201 produced four times more CPS than its parental strain.   Complementation of the 0.02% of arabinose induced Hfq by pYC343 restored nearly the wild type level of CPS production and mucoviscosity in H0201 (Table 1).   This result indicated that Hfq might have a modulating role in the regulation of CPS biosynthesis through either a direct or indirect manner.

Hfq affected the outer membrane profiles.   As one of *Enterobacteriaceae*, *K. pneumoniae* has its outer membrane (OM) as a selective barrier to allow bacterial survival under diverse environments.   The studies in *E. coli* and *Salmonella* have demonstrated that the OM composition of these enteric bacteria is fine-tuned by at least eight srRNAs (InvR, MicA, MicC, MicF, OmrAB, RseX and RybB) at the post-transcriptional level.   These srRNAs exert their functions under a variety of growth and stress conditions and the mode of their action is Hfq-dependent. Similarly, the expression profiles of outer membrane proteins (OMP) markedly changed in the *hfq* deletion mutant when compared to its parental strain (Fig. 1).   In accordance with the observation in *E. coli* that OmpA was negatively regulated by MicA, the protein level of OmpA decreased in the *hfq* deletion mutant of *K. pneumoniae* and the reduction was restored to the wild type level in the complement strain (Fig. 1, lower panel).

An involvement of Hfq in the envelope stress response.   To identify the envelope components that were controlled by Hfq-dependent regulation, we compared the two-dimensional profiles of outer membrane enriched proteins extracted from CG43S5 and H0201 (Fig. 2).   One of the most interesting after determination of the amino acid sequence of proteins that showed significantly different expression in the *hfq* deletion mutant was the *htrA*-encoding protein. HtrA is a heat-shock induced serine protease that is activated in the periplasm of *E. coli.*   Homologues of HtrA have been described in diverse bacteria.   The pivotal

role of HtrA is to degrade misfolded proteins accumulated in the periplasm.   The

expression of *htrA* is regulated by a complex set of signal transduction pathways,

which includes an alternative sigma factor, RpoE, an anti-sigma factor, RseA, a

two-component regulatory system, CpxRA, and two phosphoprotein phosphatases,

PrpA and PrpB.   Mutations in the *htrA* genes of *Salmonella*, *Brucella* and *Yersinia*

cause decreased survival in mice and/or macrophages (14).   Besides, a recent

report demonstrated that the disruption of *htrA* gene in a virulent strain of *K.*

pneumonia reduced the production of capsule and the tolerance to stress and thus

attenuated bacterial virulence in a mouse model (3).   Given that the production of

capsular polysaccharides significantly increased after the deletion of *hfq*, we

reasoned that this effect on CPS might be due to an elevated level of HtrA that was

probably induced by extracytoplasmic stress.   Two Hfq-dependent srRNAs, MicA

and RybB, have been demonstrated to inhibit the expression of OmpA, OmpC, and

OmpW; therefore, the *hfq* deletion might accumulate misfolded outer membrane

proteins and thus induce the envelope stress.   A function link between Hfq and the

envelope stress is revealed by recent studies that a reciprocal interaction may exist

between the Hfq expression and the RpoE activation.   RpoE has been well studied

for its role in combating the envelope stress.   In *E. coli*, RpoE regulates the

expression of at least 100 genes and was found to be negatively regulated by the

srRNA RybB (21).   Loss of Hfq caused strong activation of RpoE due to diminished

RybB activity coupled with increased envelope stress in *Salmonella* (5) and *Vibrio*

cholerae (4).   Because the spectrum and severity of mutant phenotypes observed

upon the deletion of *hfq* varies significantly among the different pathogens so far, it

was not surprising that we observed that the protein level of RpoE was significantly

downregulated in the hfq deletion mutant of *K. pneumonia* (Fig. 3A).   The

Hfq-dependent regulation of RpoE in *K. pneumonia* was similar to that in

uropathogenic *E. coli* (UPEC), as a recent study demonstrated that a *hfq* deletion mutant of UPEC had significant phenotypic overlap with a *rpoE* deletion mutant (9). Because the RpoE transcript increased in the *hfq* deletion mutant (Fig. 3B), we reasoned that Hfq might exert its positive control of RpoE expression through certain srRNAs different from RybB at the post-transcriptional level.

Determination of the involvement of small regulatory RNAs in tolerance response of *K. pneumonia* to stress. Approximately 80 srRNAs are known in *E. coli* thus far. A few dozen more were discovered in other bacteria, such as *Bacillus subtilis*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*. The majority of the srRNA genes were discovered as a result of systematic genome-wide screens that were based on: (i) computational prediction; (ii) direct detection with microarray; (iii) direct isolation with shotgun cloning; and (iv) co-purification with RNA-binding proteins (1). To identify small regulatory RNA candidates of *K. pneumonia*, we used the computational approach that relies on the nucleotide sequence conservativity to search for homologues of *E. coli* srRNAs in the genome of *K. pneumoniae* strain NTHU-K2044 [unpublished result from Shih-Feng, Tsai (National Health Research Institute, Taiwan)]. As shown in Table 2, we identified 47 srRNAs. Based on primary sequences of these srRNAs, we performed a small scale microarray analysis to determine their transcriptional levels in different stressful conditions. As shown in Fig. 4A, when bacteria were treated with H_2O_2, the expression of eight srRNAs (Ffs, IsrR-2, MicF, OxyS, RybB, RygA, RyhB, and TisAB) was upregulated for than four folds, while three srRNAs (DsrA, RyhB, and MicF) were significantly upregulated upon exposure to the hyperosmotic stress generated by addition of 10% sucrose. The activation of MicF and OxyS upon these stresses was ascertained by means of Northern blotting with gene-specific biotin-labeled RNA probes (Fig. 4B). The transcriptional level of thirty-two srRNAs

identified here was affected by the deletion of *hfq* (Table 2 and Fig. 4C), suggesting that these srRNA might exert their function in a Hfq-dependent manner.   Owing to the fact that the computational approach only discovered the *E. coli* srRNA homologues in our preliminary study, there would be a certain number of *K. pneumoniae*-specific srRNAs remained to be identified.

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Hfq regulates polyadenylation dependent mRNA decay in Escherichia coli. Mol Microbiol **54:**905-920.

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Table 1. Effect of Hfq on CPS production and the display of mucoviscosity phenotype

Strain	CPS production (ug uronic acids/10^{10} cfu)	Mucoviscosity phenotype[b]
CG43	17.16 ± 2.22	++
CG43-H0201 (Δhfq)	79.12 ± 14.68	++++
CG43-H0201 [pYC343][a]	27.99 ± 5.41	+

[a]Expression of wild type Hfq protein could be driven by addition of 0.2% of arabinose in this strain.
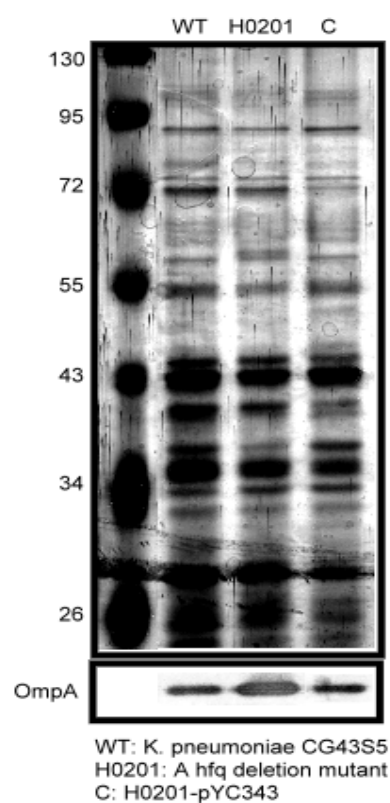[b]This phenotype is determined using a string-forming test on LB plates.

Figure 1. Comparison of outer membrane proteins displayed in between *K. pneumoniae* CG43S5, the hfq deletion mutant H0201, and a complement strain H0201-pYC343.
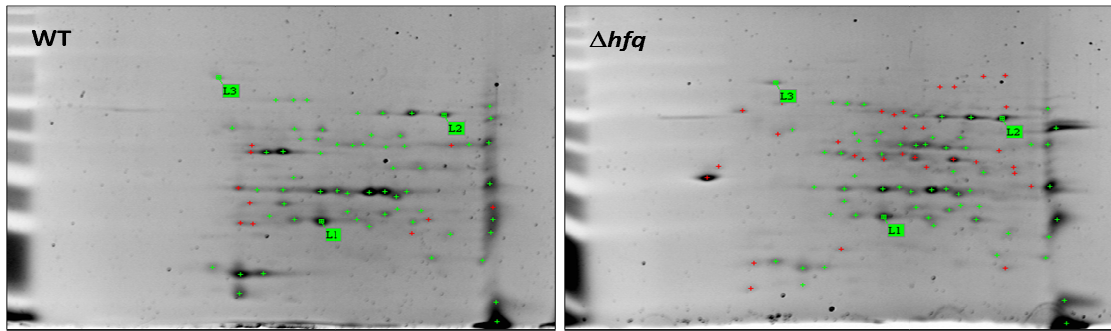
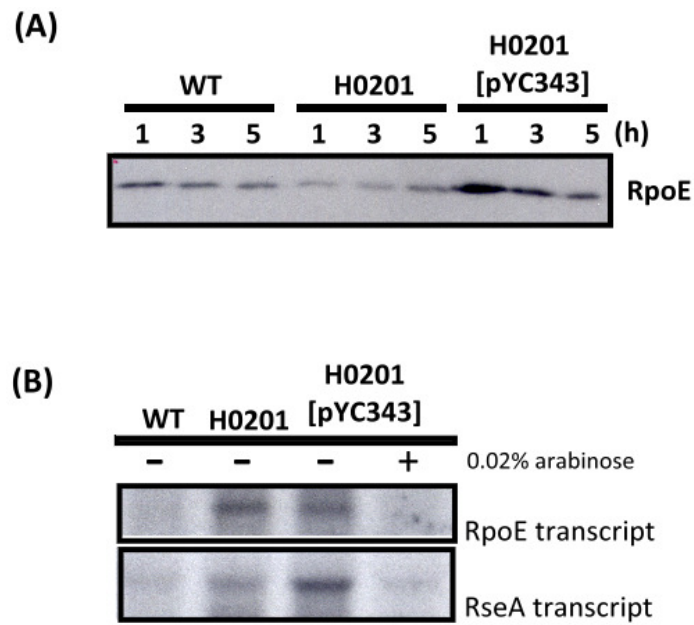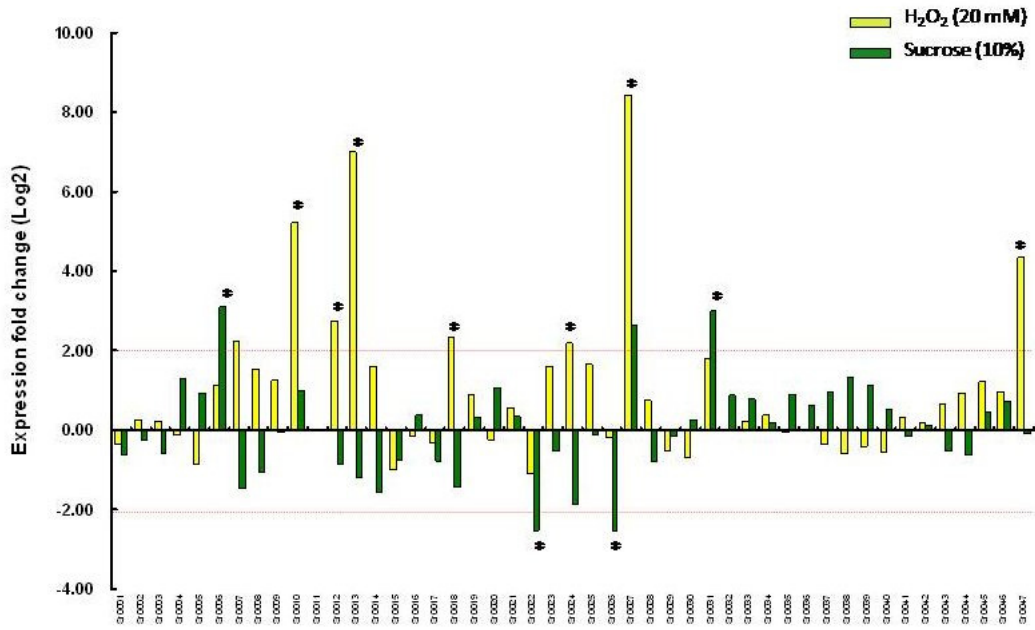**Figure 2.** Proteomics analysis of the wild type and a *hfq* deletion mutant of *K. pneumoniae.*

(A)

	WT			H0201			H0201 [pYC343]			
	1	3	5	1	3	5	1	3	5	(h)

RpoE

(B)

	WT	H0201	H0201 [pYC343]		
	−	−	−	+	0.02% arabinose

RpoE transcript

RseA transcript

Fig. 3

(A)

Fig. 4

(B)

Fig. 4

(C)

Fig. 4