Use of a *Dictyostelium* Model for Isolation of Genetic Loci Associated with Phagocytosis and Virulence in *Klebsiella pneumoniae*[▽]

Yi-Jiun Pan,[1] Tzu-Lung Lin,[1] Chun-Ru Hsu,[1] and Jin-Town Wang[1,2]*

Department of Microbiology, National Taiwan University College of Medicine, Taipei, Taiwan,[1] and Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan[2]

Phagocytosis resistance is an important virulence factor in *Klebsiella pneumoniae*. *Dictyostelium* has been used to study the interaction between phagocytes and bacteria because of its similarity to mammalian macrophages. In this study, we used a *Dictyostelium* model to investigate genes for resistance to phagocytosis in NTUH-K2044, a strain of *K. pneumoniae* causing pyogenic liver abscess that is highly resistant to phagocytosis. A total of 2,500 transposon mutants were screened by plaque assay, and 29 of them permitted phagocytosis by *Dictyostelium*. In the 29 mutants, six loci were identified; three were capsular synthesis genes. Of the other three, one was related to carnitine metabolism, one encoded a subunit of protease (*clpX*), and one encoded a lipopolysaccharide O-antigen transporter (*wzm*). Deletion and complementation of these genes showed that only Δ*clpX* and Δ*wzm* mutants became susceptible to *Dictyostelium* phagocytosis, and their complementation restored the phagocytosis resistance phenotype. These two mutants were also susceptible to phagocytosis by human neutrophils and revealed attenuated virulence in a mouse model, implying that they play important roles in the pathogenesis of *K. pneumoniae*. Furthermore, we demonstrated that *clpP*, which exists in an operon with *clpX*, was also involved in resistance to phagocytosis. The transcriptional profile of Δ*clpX* was examined by microarray analysis and revealed a 3-fold lower level of expression of capsular synthesis genes. Therefore, we have identified genes involved in resistance to phagocytosis in *K. pneumoniae* using *Dictyostelium*, and this model is useful to explore genes associated with resistance to phagocytosis in heavily encapsulated bacteria.

Phagocytes are a first line of defense against pathogenic organisms and play a key role in the host's innate immune response to bacterial infection. At the same time, bacteria that have developed resistance to phagocytosis or intracellular killing should be more virulent and more likely to succeed in establishing infection. Recently, a powerful model system involving *Dictyostelium discoideum* was introduced for studying the interactions between phagocytes and bacteria. The amoebal form of *D. discoideum* shares many unique traits with human phagocytes, especially the ability to ingest and kill bacteria. In addition, bacteria are thought to use similar mechanisms to resist both *Dictyostelium* in the environment and human phagocytes; therefore, *Dictyostelium* provides a useful system to study both cellular response and bacterial virulence. Many aspects of phagocytosis have been studied in *Dictyostelium*, such as the dynamics of the actin cytoskeleton, cellular adhesion, phagosome maturation, and intracellular killing (13, 33, 35). *Dictyostelium* has also been used to analyze virulence in different bacterial species, including extracellular and intracellular bacteria, such as *Pseudomonas* (11, 42), *Yersinia* (50), *Vibrio* (43, 44), *Legionella* (22, 25, 28), and *Mycobacterium* (20, 41). In addition, host genes required for the killing of a laboratory strain of *Klebsiella pneumoniae* by phagocytes were also identified with the *Dictyostelium* model (4).

Traditionally, *K. pneumoniae* is an opportunistic hospital-acquired pathogen which usually causes urinary tract infec-

tions, pneumonia, and septicemia (1, 40). Over the past 2 decades, community-acquired *K. pneumoniae* associated with pyogenic liver abscess has been found globally (9, 14, 26, 36, 37, 45, 51). This emerging disease is often complicated by septic meningitis and endophthalmitis. The capsule, allantoin metabolic genes, and the ferric iron uptake system were reported to be virulence factors that contribute to pathogenesis (6, 24, 32). The capsule was thought to play a crucial role in resistance to phagocytosis. Moreover, in a previous study we have identified genes determining resistance to phagocytosis and serum by screening of mucoviscosity and found that capsular mutants with the low-mucoviscosity phenotype became extremely sensitive to serum, susceptible to phagocytosis, and avirulent in mice (14). In the current study, we used the *Dictyostelium* model to screen for phagocytosis resistance genes in NTUH-K2044, a strain of *K. pneumoniae* isolated from a patient with pyogenic liver abscess (14).

MATERIALS AND METHODS

Transposon mutant library. A mutant library of strain NTUH-K2044 constructed by use of a randomly inserted mini-Tn*5* transposon was used in this study (14). The insertion sites of 30 randomly selected mutants were determined by semirandom PCR (8). The insertion sites of the mutants were mapped to 28 unique locations (26 mutants had unique insertion sites; the remaining four mutants had two insertion sites, with each two having the same insertion site). In the unique insertions, 23 (82%) were within the coding regions, while 5 (18%) were outsides the coding regions.

Plaque assay to screen for mutants susceptible to *Dictyostelium* phagocytosis. *D. discoideum* AX-2 cells were grown at 23°C in HL5 medium (10 g proteose peptone, 10 g glucose, 5 g yeast extract, 0.965 g $Na_2HPO_4 \cdot 7H_2O$, 0.485 g KH_2PO_4, and 0.03 g dihydrostreptomycin in a final volume of 1 liter, pH 6.5) (10). A transposon mutant library of *K. pneumoniae* strain NTUH-K2044 (14) was used for screening. To test *Dictyostelium* growth on *Klebsiella* mutant strains, log-phase cultured bacteria were plated on SM agar (2.31 g KH_2PO_4, 1 g

* Corresponding author. Mailing address: Department of Microbiology, National Taiwan University College of Medicine, 1, Sec 1, Jen-Ai Rd., Taipei 10051, Taiwan. Phone: 886-2-23123456, ext. 88292. Fax: 886-2-23948718. E-mail: wangjt@ntu.edu.tw.
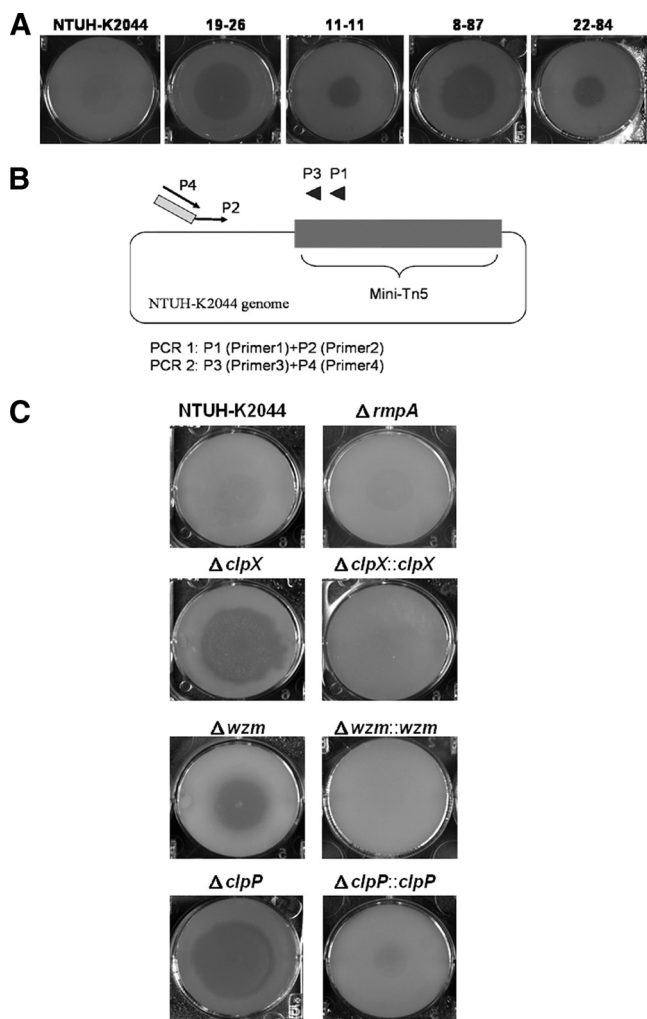
FIG. 1. Identification of genes for resistance to phagocytosis in *K. pneumoniae* NTUH-K2044 using *Dictyostelium*. (A) Screening of *K. pneumoniae* NTUH-K2044 mutant library by plaque assay. By plaque assay of the wild type, 4 of the 29 transposon mutants were shown to be permissive for *Dictyostelium* growth. These mutants, 19-26, 11-11, 8-87, and 22-84, were further identified as *wzc* (capsular synthesis gene), *wzm*, *clpX*, and *KP2125* insertional mutants, respectively. (B) Primers used for semirandom PCR. Primers P1 and P2 are used for the first PCR, and primers P3 and P4 are used for nested PCR. (C) Confirmation of the deletion mutants and complementation strains by plaque assay. Δ*clpX*, Δ*wzm*, and Δ*clpP* indicate deletion mutants of the *clpX*, *wzm*, and *clpP* genes, respectively; Δ*clpX*::*clpX*, Δ*wzm*::*wzm*, and Δ*clpP*::*clpP*, chromosome-complemented strains of the Δ*clpX*, Δ*wzm*, and Δ*clpP* mutants, respectively.

K$_2$HPO$_4$, 5 g glucose, 5 g Bacto peptone, and 5 g yeast extract in a final volume of 1 liter) (2). Five thousand *Dictyostelium* cells were added to the bacterial lawn, and the formation of phagocytic plaques was observed after 5 days at 23°C.

Identification of transposon mutants by semirandom PCR. Transposon insertional sites were determined by semirandom PCR (8) and DNA sequencing. Briefly, genomic DNA was extracted and subjected to PCR amplification with a transposon-specific primer (primer P1) and an anchored random primer (primer P2). The PCR conditions have been previously described (8). The products of the first PCR were used as the template for a second PCR with a nested transposon-specific primer (primer P3) and a primer (primer P4) with a sequence identical to the conserved portion of the P2 primer (Fig. 1B). Finally, the DNA sequence of the nested PCR product was resolved; and the transposon insertional site was defined accordingly. Furthermore, the results were confirmed by PCR with transposon-specific primers and primers for the disrupted genes.

Construction of unmarked deletion mutants of *K. pneumoniae*. Plasmid pKO3-Km was used to generate deletion mutants (29). The flanking regions of target genes *wzm*, *clpX*, and *clpP* were amplified with specific primers (Table 1) and cloned into the pKO3-Km plasmid. The resulting plasmid was used to generate deletion mutants in the NTUH-K2044 strain as previously described (24).

Construction of chromosomal complementation strains of *K. pneumoniae*. For chromosomal complementation, the intact *wzm*, *clpX*, and *clpP* genes with their promoters were amplified by PCR (Table 1) and cloned into the intergenic region of *pgpA* and *yajO* in a PKO3-Km-*pgpA*-*yajO* recombinant vector. The resulting plasmid was used to generate complemented strains as previously described (23).

Silver stain for lipopolysaccharide O antigen. Extracellular polysaccharides were isolated as previously reported (7). Samples were separated by SDS-PAGE. Gels were then placed in fixation solution (40% ethanol, 5% acetic acid) overnight at 4°C, and the fixer was replaced with oxidation solution (40% ethanol, 5% acetic acid, 0.7% periodic acid) for 5 min with gentle agitation. After oxidation, gels were washed three times in double-distilled H$_2$O (ddH$_2$O) for 15 min. The gels were then stained with freshly prepared staining reagent (9.3 ml 0.1 N NaOH, 0.66 ml NH$_4$OH, and 1.66 ml 20% AgNO$_3$ with ddH$_2$O added to a final volume of 50 ml) for 10 min and washed three times in ddH$_2$O for 15 min. For color development, developing solution (5 mg citric acid in 100 ml ddH$_2$O mixed with 50 μl 37% formaldehyde) was added for 10 to 20 min, and bands slowly appeared. Finally, the reaction was terminated by washing in stop solution (0.8% acetic acid).

Determination of transcriptional unit by RT-PCR. Total RNA was extracted from strain NTUH-K2044 as previously described (6, 24). Four micrograms of total RNA was reverse transcribed with 0.1 mmol of random hexamer primer (Roche, Indianapolis, IN) and 5 μl of reverse transcriptase II (Invitrogen, Carlsbad, CA) in a reaction mixture of 20 μl. Reaction mixtures without reverse transcriptase were used as negative controls. PCR was performed with 1 μl of each reverse transcription (RT) reaction mixture as template and subjected to the following program: 96°C for 3 min, followed by 30 cycles of 96°C for 30 s, 54°C for 15 s, and 72°C for 1 min.

Phagocytosis assay in *Dictyostelium*. Plasmid pCRII-TOPO with a gene encoding green fluorescence protein (GFP) was electroporated into wild-type and mutant strains. An inoculum containing 10^8 CFU of bacteria carrying this plasmid was incubated with 10^6 *Dictyostelium* cells at 23°C for 1 h. Cells were washed three times with phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde for 15 min, treated with 0.1% Triton X-100 for 1 min, and stained for 15 min with rhodamine-phalloidin, which was diluted 1:100 in PBS. After preparation, the cells were observed by confocal microscopy under ×630 image magnification, and the numbers of intracellular bacteria in five fields were counted (≈50 cells in each field). The sum of the intracellular bacteria was divided by the total number of cells in these fields and multiplied by 100, to calculate the number of intracellular bacteria in 100 cells.

Phagocytosis assay in human neutrophils. Human neutrophils were freshly isolated from peripheral blood donated by healthy volunteers (14). An inoculum containing 10^8 CFU of bacteria was opsonized with 25% normal human serum for 15 min on ice and incubated with 10^6 human neutrophils at 37°C for 15 min for observation of the cell-bacterium association or 45 min for phagocytosis. Cells were also stained with rhodamine-phalloidin for observation. The numbers of intracellular bacteria and cells were counted as described above.

Confocal microscopy. Images were capture by a Leica SP5 confocal microscope with a 488-nm argon laser and a 543-nm laser, which emitted the excitation wavelengths of GFP and rhodamine, respectively.

A serial confocal Z stack taken with a step of 0.5 μm was obtained by scanning a representative number of cells from top to bottom, and the images were combined for observation of cell-associated bacteria; a representative confocal section through the middle of the cell was shown for observation of intracellular bacteria.

Analysis of transcriptome of Δ*clpX* strain by microarray. Total RNA was isolated from log-phase cultured bacteria. A total of 40 μg RNA was reverse transcribed using random hexamers (Roche) and labeled for microarray hybridization (6, 24). Densitometry was performed and analyzed using Image J software, and the 23S rRNA signal was used as an internal standard for normalization.

Quantitative real-time reverse-transcription quantitative PCR (RT-qPCR) for quantification of relative RNA expression. RNA was reverse transcribed with random hexamers (Roche) and reverse transcriptase II (Invitrogen), according to the manufacturer's instruction. Amplification of the cDNAs was monitored using a SYBR green dye mixture in an ABI Prism 7900 thermocycler (Applied Biosystems, Foster City, CA). The relative quantities of RNA were analyzed by

TABLE 1. Primers used in this study

Primer name	Sequence	Position	Purpose (reference)
P1	GCGCGCTGCGCAGGGCTTTATTGATTCCATTTTTAC	EZ-Tn5 (KAN-2) transposon	Semirandom PCR (8)
P2	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	Randomly anchored on genome	Semirandom PCR (8)
P3	GTACCGAGCTCGAATTCGGC	EZ-Tn5 (KAN-2) transposon	Semirandom PCR (8)
P4	GGCCACGCGTCGACTAGTAC	Partially complementary to P2	Semirandom PCR (8)
22416F	CTTTGCCATACCTTCACGGT	Upstream of *wzm* gene	*wzm* mutant construct
wzt-R	TTAAGCCATTGCTTGCTTG	Downstream of *wzm* gene	*wzm* mutant construct
wzm-IR	AAATTATTCTGCAAATATCCACTGAATGAG	Upstream of *wzm* gene	*wzm* mutant construct
wzt-F	ATGCACCCAGTTATTAAC	*wzt*	*wzm* mutant construct
wzm-R	TTACAAGATCTCTGCAAATC	*wzm*	*wzm* complementation
clpX-750F	CGGTGAACGTTCTTTTG	Upstream of *clpX* gene	*clpX* mutant construct
clpX + 750R	TCAACCTGCAGCAGATC	*lon*	*clpX* mutant construct and junction PCR
clpX-F	TTAACCATTTCACTCAAGC	Downstream of *clpX* gene	*clpX* mutant construct
clpX-R	GAGTCCAAACCTCTTTTTAAG	Upstream of *clpX* gene	*clpX* mutant and *clpP* complementation
clpP-P	TTTCGCCGCTATCGTTTAAG	Upstream of *clpP* gene	*clpX* and *clpP* complementation
clpP5′R	TTCCGTCTCCTGGAGTAATTTT	Upstream of *clpP* gene	*clpX* complementation
clpP3′F	TGCCCAAGGCGCAAGGG	Downstream of *clpP* gene	*clpX* complementation
clpX-deR	TTATTCACCAGATGCCTG	*clpX*	*clpX* complementation
clpP-750F	AAATATGGAACGCGAGC	*tig*	*clpP* mutant construct and junction PCR
clpP + 750R	ATATAAACGATGCCGCG	*clpX*	*clpP* mutant construct
clpP-R-TUD	TCGTTCGCCACTGTATGAC	*clpP*	Junction PCR
clpX-R-TUD	CCGCCAACACCTTCTTC	*clpX*	Junction PCR
clpP-F-TUD	TTGACCCATCGTAATTG	*clpP*	Junction PCR
clpX-F-TUD	TCAGCAGGCATCTGGTG	*clpX*	Junction PCR
galF-145F	GCCGGGATCAAAGAGATTGTT	*galF*	Real-time PCR
galF-217R	AGGAGGTGTCGAAGTGGTTTTC	*galF*	Real-time PCR

normalizing the calculated threshold cycle (ΔC_T) of the detected gene to the C_T of 23S rRNA from the same sample and calculation of the fold change with the $\Delta\Delta C_T$ method (3).

Neutrophil killing assay. Human neutrophils were isolated as described above. An inoculum containing 10^3 CFU of bacteria was opsonized with 25% normal human serum for 15 min on ice and incubated with or without 10^5 human neutrophils in 1× PBS at 37°C for 45 min. Percent survival of wild-type and mutant strains was calculated on the basis of the viable counts relative to those for no-neutrophil controls.

Serum resistance assay. The serum resistance assay of *K. pneumoniae* strains was performed as previously described (14). Briefly, 2.5×10^4 CFU bacteria was mixed with serum from healthy human volunteers at a 1:3 volume ratio. The mixture was incubated at 37°C for 3 h, and bacterial numbers were determined.

Hypermucoviscosity test. The string test was performed as previously described (14). Briefly, the tested strains were inoculated on blood agar plates and incubated at 37°C overnight. A standard bacteriologic loop was used to stretch a mucoviscous string from the colony. A positive string test was defined by the formation of viscous strings >5 mm in length when a loop was used to stretch the colony. To further differentiate the subtle difference in the levels of capsule production in different strains, low-speed centrifugation was performed, since bacteria with thick and mucoid capsules were more difficult to pellet. Briefly, equal numbers of bacteria in 1.1 ml LB medium were centrifuged at $2,000 \times g$ for 5 min. After centrifugation, 1 ml of the supernatant was harvested and subjected to measurement of the optical density at 600 nm (OD_{600}).

Immunoblot. Capsule was extracted from wild-type and mutant strains with equal numbers of bacteria by the water-phenol extraction method (7). The capsule extracted from same amount of bacteria was serially diluted, subjected to immunoblotting (38), and detected with serotype K1-specific antiserum (Statens Serum Institute, Copenhagen, Denmark).

Animal inoculation. Five-week-old female BALB/cByl mice were intraperitoneally inoculated with 10^3 CFU of bacteria prepared from mid-log phase. Eight mice for each group were monitored for 30 days. The rates of survival of the different groups of mice were compared and analyzed with the Kaplan-Meier method. To determine the bacterial load *in vivo*, an inoculation dose of 10^3 CFU of wild-type or mutant bacteria was administered to each of four mice by intraperitoneal injection. Mice were killed at 24 h or 48 h after inoculation. Liver, spleen, and blood were harvested, and tissues were further homogenized for

quantitation of bacterial numbers. The number of bacteria detected in tissues was standardized per 0.1 g of wet organ weight.

Microarray data accession number. Microarray data for the transcriptome of ΔclpX have been deposited in the Center for Information Biology Gene Expression Database (http://cibex.nig.ac.jp) under accession no. CBX130.

RESULTS

Screening virulence genes essential for resistance to phagocytosis in *K. pneumoniae* using *Dictyostelium*. We had constructed a transposon mutant library of a *K. pneumoniae* strain (NTUH-K2044) causing pyogenic liver abscess (14). In the current study, we used a *Dictyostelium* system to investigate phagocytosis resistance genes in NTUH-K2044. A total of 2,500 transposon mutants were screened by plaque assay. Wild-type NTUH-K2044, which is resistant to *Dictyostelium* phagocytosis, does not permit *Dictyostelium* to form plaques on the bacterial lawn. In contrast, 29 transposon mutants permitted phagocytosis and showed clear plaques (Fig. 1A), implying that the genes disrupted by the transposon might be involved in resistance to phagocytosis. The locations of the inserted transposon, determined by semirandom PCR and DNA sequencing, are shown in Table 2. Twenty-one of these mutants had transposons inserted in the genes for capsule synthesis (mutants 3-1 to 3-16 were disrupted in the *wza* gene; mutants 3-18 and 3-23 were disrupted in *magA*; and mutants 3-22, 16-5, and 19-26 were disrupted in *wzc*). Six mutants were disrupted in a gene encoding the O-antigen synthesis transporter (*wzm*) (mutants 10-21, 11-11, 11-84, 12-28, 13-67, and 19-77). One mutant (mutant 22-84) was disrupted in a gene related to carnitine metabolism (*KP2125*), and for the remaining mutant (mutant 8-87),

TABLE 2. Transposon mutants permissive for *Dictyostelium* growth

Transposon mutant(s)	Disrupted gene name	Gene function	Reference or source
3-1 to 3-16	*wza*	Putative capsule polysaccharide export protein	14
3-18, 3-23	*magA*	Capsular polysaccharide synthesis	14
3-22, 16-5, 19-26	*wzc*	Tyrosine-protein kinase, capsular polysaccharide synthesis	14
10-21, 11-11, 11-84, 12-28, 13-67, 19-77	*wzm*	Lipopolysaccharide O-antigen transporter	This study
8-87	*clpX*	Protease subunit	This study
22-84	*KP2125*	Carnitine metabolism	This study

which created plaques comparable to those of the capsular mutants, the transposon was inserted in the gene encoding a protease subunit (*clpX*). Because the capsule has been well-known to play a role in resistance to phagocytosis, in the remainder of this study we focused on the three mutants that were not involved in capsular synthesis.

Two deletion mutants and complemented strains of noncapsular genes were generated and confirmed by plaque assay. The resistance to phagocytosis conferred by the three noncapsular synthesis genes was confirmed by construction of deletion mutants and complemented strains. The *wzm* and *clpX* deletion mutants showed defects in resistance to phagocytosis like the transposon mutants, and complementation of these strains restored the resistance to phagocytosis (Fig. 1C). In contrast, the *KP2125* deletion mutant was not susceptible to *Dictyostelium* phagocytosis, implying that there might be other mutations present in the transposon mutant (data not shown).

Observation of phagocytosis by confocal microscopy. We used confocal microscopy to observe phagocytosis of wild-type and mutant bacteria by *Dictyostelium* and to calculate the number of intracellular bacteria. The numbers of intracellular Δ*wzm* and Δ*clpX* mutants were significantly higher than the number of the wild-type strain after coculture of bacteria and *Dictyostelium* (Fig. 2A and B). These results indicated that the two mutants were more easily phagocytosed by *Dictyostelium* than the wild type was.

We also analyzed the susceptibilities of these mutants to phagocytosis by healthy human neutrophils by confocal microscopy. Compared to the level of association observed with wild-type strain NTUH-K2044, which is rarely associated with neutrophils, more association was observed between mutants and neutrophils with an incubation time of 15 min (Fig. 2C). Moreover, when the incubation time was extended to 45 min, the numbers of Δ*wzm* and Δ*clpX* mutants were significantly increased in the human neutrophils. However, wild-type strain NTUH-K2044 and complemented strains were rarely present inside the human neutrophils (Fig. 2D and E).

Neutrophil-mediated killing of Δ*wzm* and Δ*clpX* strains. Neutrophil-mediated killing of the Δ*wzm* and Δ*clpX* mutants was examined. Compared to the number of wild-type bacteria, the numbers of Δ*wzm* and Δ*clpX* mutants were significantly decreased after incubation with human neutrophils, and the complemented strains restored the resistance to neutrophil killing (Fig. 3).

Silver stain of *wzm* deletion mutant. The *wzm* gene is located in the *wb* gene cluster, which is responsible for O-antigen lipopolysaccharide synthesis and which has been reported to be an O-antigen transporter (12). Therefore, we further analyzed whether the Δ*wzm* mutant in *Klebsiella* had a defect in O-

antigen synthesis. The O antigen, revealed as a ladder form on silver staining, was absent in the Δ*wzm* mutant and restored in the complemented strain (Fig. 4).

Genetic organization of *Klebsiella clpX* and transcriptional unit determination. It has been documented that ClpX is a component of the ClpPX protease system and ClpX is responsible for substrate specificity (47). Moreover, in *Salmonella enterica* serovar Typhimurium, the *clpX* gene is located in an operon with *clpP*, and both of them are required for growth of *S. enterica* serovar Typhimurium in macrophages and virulence in mice (52). The genomic alignment surrounding *clpX* in *Klebsiella* was similar to the genetic organization of *S. enterica* serovar Typhimurium (Fig. 5). When RT-PCR was used to define the *clpX* transcriptional unit, mRNA was detected between *clpX* and *clpP* but not between *tig* and *clpP*, *clpX*, and *lon* (Fig. 5). The results indicated that *clpP* and *clpX* constitute a transcriptional unit.

Role of *clpP* in *K. pneumoniae* for phagocytosis resistance. To further explore whether *clpP* is important for resistance to phagocytosis, a *clpP* deletion mutant and the complemented strain were generated and subjected to a plaque assay with *Dictyostelium*. This mutant was susceptible to *Dictyostelium* phagocytosis (Fig. 1C), and it became more susceptible to phagocytosis by human neutrophils (Fig. 2D and E). These phenotypes were restored in the complemented strain (Fig. 1C; Fig. 2D and E).

Microarray analysis of Δ*clpX* mutant. To identify the genes affected by ClpX, we compared the transcriptome of the Δ*clpX* mutant with that of its parent strain, *K. pneumoniae* NTUH-K2044, by microarray analysis. Fold changes in transcription (increased and decreased) were ranked in duplicate experiments. Genes that were reproducibly represented in the top 5%, according to the level of decreased transcription, are shown in Table 3. No genes for which an increase in the level of transcription exceeded 0.3-fold were found. Among the genes with decreased transcription, the expression of the capsular synthesis gene was reduced in the Δ*clpX* mutant. We further validated this result by real-time RT-qPCR. Consistently, compared to the wild-type strain, the expression of a capsular synthesis gene, *galF*, was decreased approximately 3-fold in the Δ*clpX* mutant. The RNA level was 70% restored when the mutant was complemented with the wild-type *clpX* gene (for wild type and Δ*clpX* mutant, $P < 0.0001$; for Δ*clpX* and Δ*clpX*::*clpX*, $P = 0.0235$; paired t test for both comparisons) (Fig. 6).

Serum sensitivity of Δ*wzm* and Δ*clpX* mutants. After it was incubated with serum for 3 h, the numbers of CFU of the Δ*wzm* mutant decreased to about 1% of the initial inoculum,
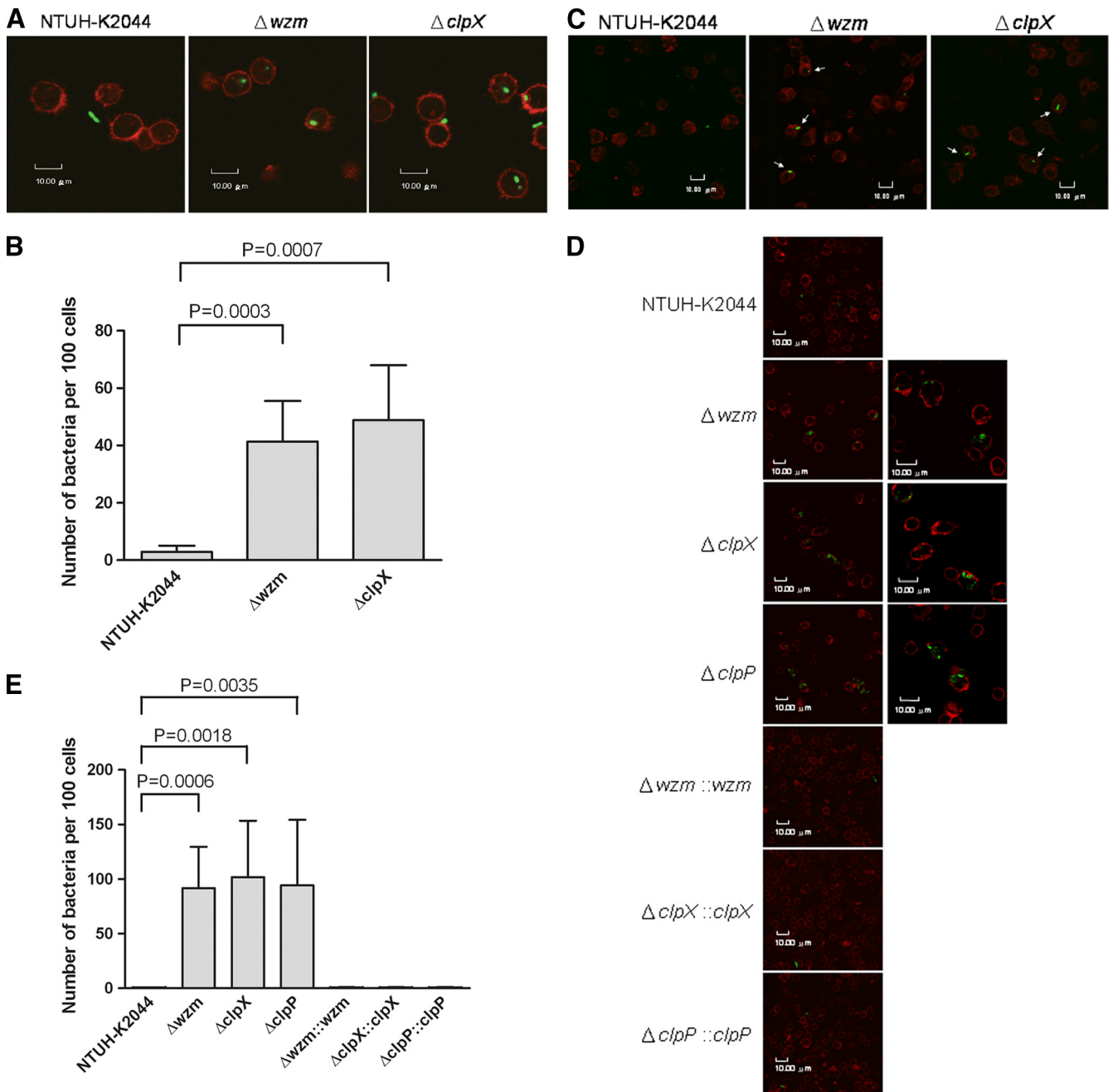
FIG. 2. Observation of phagocytosis using confocal microscopy. (A) Phagocytosis by *Dictyostelium*. The wild-type strain and *clpX* and *wzm* deletion mutants carrying the GFP plasmid were incubated with *Dictyostelium* and observed under a confocal microscope. (B) The numbers of bacteria of wild type and Δ*wzm* and Δ*clpX* mutants phagocytosed by *Dictyostelium* were counted under a confocal microscope. The *P* values for the Δ*wzm* and Δ*clpX* mutants were 0.0003 and 0.0007, respectively, by *t*-test analysis. (C) Observation of the association of bacteria and human neutrophils. Bacteria carrying the GFP plasmid were incubated with human neutrophils for 15 min and observed under a confocal microscope. Image magnifications, ×630 with a zoom-in factor of 2×. Serial confocal *Z* stacks taken with a step of 0.5 μm were obtained by scanning a representative number of cells from top to bottom, and the images were combined. Arrows denote cell-associated bacteria. (D) Determination of phagocytosis resistance of NTUH-K2044 and isogenic mutants by human neutrophils. Bacteria carrying the GFP plasmid were incubated with human neutrophils for 45 min and observed under a confocal microscope. A representative confocal section through the middle of the cell was shown for observation of intracellular bacteria. Image magnifications, ×630 with a zoom-in factor of 2× (left panels) and with a zoom-in factor of 4× (right panels). (E) Bacteria phagocytosed by human neutrophils were counted under a confocal microscope. For wild type and Δ*wzm* mutant, *P* = 0.0006; for wild type and Δ*clpX* mutant, *P* = 0.0018; for wild type and Δ*clpP* mutant, *P* = 0.0035; for Δ*wzm* mutant and Δ*wzm*::*wzm* strain, *P* = 0.0006; for Δ*clpX* mutant and Δ*clpX*::*clpX* strain, *P* = 0.0018; for Δ*clpP* mutant *and* Δ*clpP*::*clpP* strain, *P* = 0.0036 (all comparisons were by *t* test).
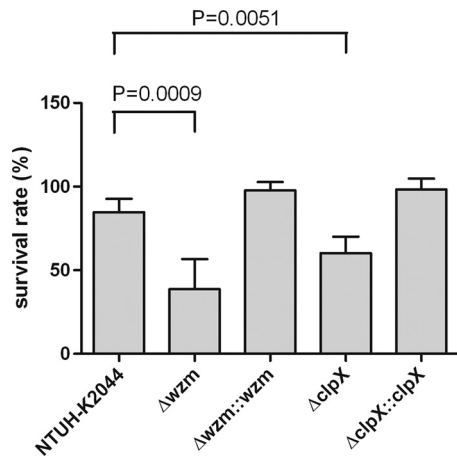
FIG. 3. Neutrophil-mediated killing of Δ*wzm* and Δ*clpX*. Bacterial susceptibilities to killing by human neutrophils of wild-type, Δ*wzm*, Δ*clpX*, and complemented strains after 45 min of incubation are presented. Survival rate indicates percent survival of wild-type or mutant strains calculated on the basis of viable counts relative to those for the no-neutrophil controls. For wild type and Δ*wzm* mutant, $P = 0.0009$; for wild type and Δ*clpX* mutant, $P = 0.0051$; for Δ*wzm* mutant and Δ*wzm*::*wzm* strain, $P = 0.0055$; fir Δ*clpX* mutant and Δ*clpX*::*clpX* strain, $P = 0.0049$ (all comparisons were by t test).

suggesting that the mutant became serum sensitive. However, the Δ*clpX* mutant was as resistant to serum as the wild type.

Capsule production of Δ*wzm* and Δ*clpX* mutants. Although the Δ*wzm* and Δ*clpX* mutants were positive by the string test, which indicates their mucoid phenotype, the reduced production of capsule in these two mutants was evident by low-speed centrifugation. Compared to the wild-type strain, a capsular mutant (the Δ*magA* mutant) which lost its mucoviscosity, was

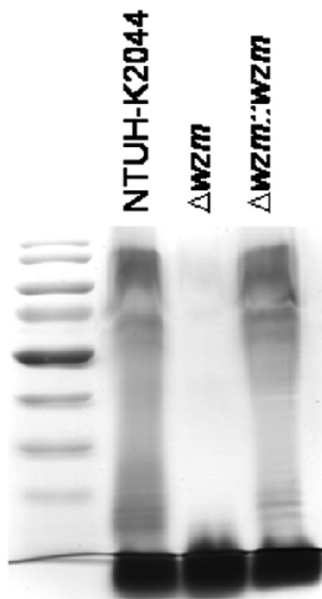FIG. 4. Effect of mutation in *wzm* gene on lipopolysaccharide O-antigen expression. Extracellular polysaccharides of the wild-type, Δ*wzm*, and Δ*wzm*::*wzm* strains were purified, and lipopolysaccharide O antigen was detected by silver stain.
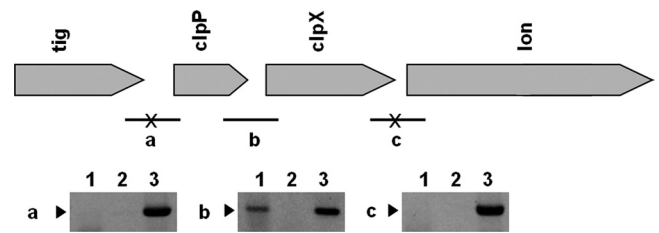

FIG. 5. Genetic organization of *clpX* and transcriptional unit *in K. pneumoniae* NTUH-K2044. (Top) Genetic organization. Transcriptional units of *clpX* in NTUH-K2044 were defined by RT-PCR. (a to c) The corresponding PCR products for primers located at each open reading frame (ORF) junction. (a) primers clpP-R-TUD and clpP-750F; (b) primers clpX-R-TUD and clpP-F-TUD; (c) primers clpX-F-TUD and clpX + 750R. Bar, RT-PCR-positive junction; bars with an X, RT-PCR-negative junctions. (Bottom) RT-PCR results. Lane 1, RT-PCR products of each junction; lane 2, RT-PCR without reverse transcriptase, as a negative control; lane 3, PCR with genomic DNA as a template, as a positive control. Arrowheads denote the expected sizes of the RT-PCR products.

easily pelleted and resulted in a tight pellet after centrifugation. The Δ*wzm* and Δ*clpX* mutants did not present tight pellets and were also more easily pelleted than the wild type, indicating that the two mutants were partially impaired in capsular production (Fig. 7). However, compared to the two mutants, a *rmpA* (regulator of capsule synthesis) deletion mutant whose mutation which resulted in an apparently lower level of capsule production ($P < 0.01$) appeared to be resistant to phagocytosis (Fig. 7 and Fig. 1C). Moreover, immunoblotting was performed to determine the reaction of serotype K1-specific antiserum and different dilutions of capsule extracts. As the results indicated, the capsular (Δ*magA*) mutant lost its antigenicity; in contrast, the Δ*wzm*, Δ*clpX*, and Δ*rmpA* mutants with mucoid phenotypes all preserved their antigenicity (Fig. 8); however, the signal intensity suggested that the Δ*rmpA* mutant produced a smaller capsule amount than the amounts produced by the Δ*wzm* and Δ*clpX* mutants.

Animal inoculation. Mice infected by an intraperitoneal inoculation with 10^3 CFU of the wild-type strain died within 3 days. In contrast, five out of eight mice (~60%) infected with the Δ*clpX* mutant and all of the mice infected with the Δ*wzm* mutant survived and appeared to be healthy after 30 days. Statistical analysis of survival rates revealed that both the Δ*clpX* and Δ*wzm* mutants were attenuated in virulence (Fig. 9). To further investigate the bacterial clearance *in vivo*, we examined the bacterial loads in liver, spleen, and blood of mice inoculated with 10^3 CFU of the wild-type, Δ*wzm*, or Δ*clpX* strain 24 h and 48 h after inoculation. The results indicated that Δ*wzm* mutants were rarely recovered from tissue at either 24 or 48 h, suggesting that Δ*wzm* mutants were rapidly cleared *in vivo*, and the Δ*clpX* group had significantly reduced colony counts in the liver and spleen compared with the wild-type group (Fig. 10A and B). Whereas bacteria were detected in the blood of only a few mice (at 24 h, one from the wild-type group with ~5×10^2 CFU per 100 μl and one from the Δ*clpX* group with ~2×10^2 CFU per 100 μl; at 48 h, one from the wild-type group with ~2×10^2 CFU per 100 μl). Therefore, the difference between the counts of mutants and the wild type in blood was not obvious at the two time points.

TABLE 3. Gene expression decreases in Δ*clpX* of *K. pneumoniae* NTUH-K2044

Putative function (gene name)	Avg fold decrease of RNA[a]
Putative inner membrane protein	114.0
Threonine dehydratase catabolic operon genes (*tdcDE*)	40.5
Putative transcriptional regulator	29.8
Putative transcription activator	26.5
Possible lipoprotein	24.6
Threonine dehydratase catabolic operon genes (*tdcCDE*)	22.8
Putative oxidoreductase (*aegA*)	22.3
DNA repair protein (*recO*)	21.6
Alcohol dehydrogenase	21.4
Threonine dehydratase catabolic operon genes (*tdcABC*)	19.8
Cadaverine/lysine decarboxylase operon genes (*cadBC*)	18.5
Superoxide dismutase (*sodB*)	17.3
Siroheme synthetase (*cysF*)	17.2
Permeases of the major facilitator superfamily	16.2
Outer membrane protein S1	16.0
Putative outer membrane protein	15.2
Putative ligase (*yjfG*)	14.8
Glyoxalase family protein	14.7
Transcriptional activator protein (LysR)	14.6
Ecotin precursor (*eco*)	14.3
Synthesis of vitamin B$_{12}$ adenosyl cobalamide precursor (*cbiKJHG*)	14.3
Tagatose-1-phosphate kinase (*tagK*)	14.0
Protein kinase	13.6
Putative sarcosine oxidase (*solA*)	13.5
Putative diguanylate cyclase/phosphodiesterase domain 1	13.3
Possible lipoprotein	13.2
Acid shock protein	13.1
CPS synthesis (*wzi*)	13.1
Lysine-accepting tRNA synthetase (*lysS*)	12.8
Sensor protein (*rcsC*)	12.5
Putative hydrolase (*yjcS*)	11.8
Transport of long-chain fatty acids (*fadL*)	11.5
Carbon starvation protein A (*cstA*)	11.4
Iron ABC transporter ATP-binding protein	11.3
Putative sulfatase (*yejM*)	11.2
Heat shock protein A (*hslT*)	11.1
O-antigen synthesis (*wzm*)	11.0
Paral putative membrane protein (*yhiI*)	10.8
Cold shock protein (*cspA*)	10.6
Outer membrane protein TolC precursor	10.6
Putative ferrous iron transport protein	10.5
Two-component system (*rstAB*)	9.9
Putative cytoplasmic protein	9.8
Putative protease (*yegQ*)	9.8
Adenylosuccinate synthetase (*purA*)	9.7
Putative lipase	9.3
Putative membrane protein	9.0
Putative membrane protein	9.0
Putative membrane protein	8.9
Putative UDP-glucose dehydrogenase (*ugd*)	8.5
Hypothetical protein	8.3

[a] The data represent averages of the fold decrease in the level of RNA expression from two independent experiments.

DISCUSSION

In this study, we used a model system incorporating wild-type *Dictyostelium* to investigate bacterial genes important for resistance to phagocytosis in a pathogenic clinical isolate of *K. pneumoniae* which is heavily encapsulated and highly resistant to phagocytosis. Moreover, we validated the results by testing the phagocytosis of the mutants by human neutrophils, and we examined the mutants' virulence *in vivo*. In our screening, capsular mutants that became permissive for phagocytosis were found, as predicted; but we also found two additional genes involved in resistance to phagocytosis: one was the lipopolysaccharide O-antigen transporter, and the other was a protease subunit. Our mutant library covered approximately 2,325 (2,500 × 93%) diverse mutants and displayed 7% redundancy. Of the 28 unique insertions from randomly selected
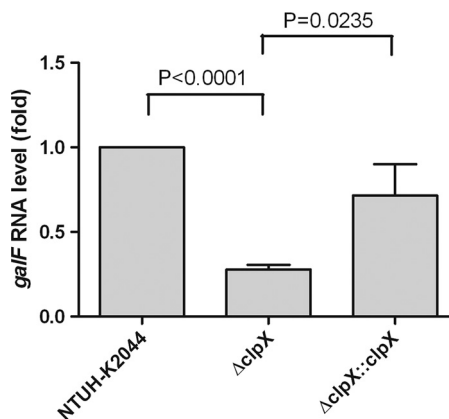

FIG. 6. Determination of RNA level of capsular synthesis gene by RT-qPCR. The transcriptional level of *galF*, a gene responsible for capsular synthesis, in the wild type, the Δ*clpX* mutant, and complemented strain Δ*clpX*::*clpX* was analyzed using real-time RT-qPCR. For wild type and Δ*clpX*, $P < 0.0001$; for Δ*clpX* mutant and Δ*clpX*::*clpX* strain, $P = 0.0235$ (both comparisons were by paired *t* test).

mutants, 23 (82%) were within the coding regions, while 5 (18%) were outside the coding regions. Accordingly, among these 2,500 transposon mutants there were 1,906 (2325 × 82%) mutants which were disrupted in different genes. However, the real coverage was difficult to estimate, since insertions close to an operon can cause phenotypic changes due to polar
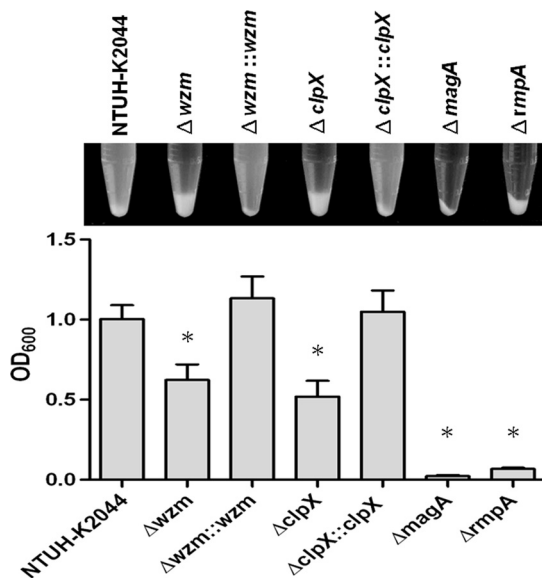

FIG. 7. Capsule production of Δ*wzm* and Δ*clpX* mutants. Capsule production of the wild-type, Δ*wzm* mutant, Δ*clpX* mutant, and complemented strains was assessed by low-speed centrifugation. The Δ*magA* mutant, a capsular mutant, was used as a control. Results for the Δ*rmpA* mutant, with mutation of a regulator of capsule synthesis, are also presented. OD$_{600}$ measurements of the supernatant were shown below. ζ, $P < 0.01$ compared to wild-type strain. For wild type and Δ*wzm* mutant, $P = 0.0073$; for wild type and Δ*clpX* mutant, $P = 0.0031$; for Δ*wzm* mutant and Δ*wzm*::*wzm* strain, $P = 0.0061$; for Δ*clpX* mutant and Δ*clpX*::*clpX* strain, $P = 0.0051$; for Δ*wzm* mutant and Δ*rmpA* mutant, $P = 0.0006$; for Δ*clpX* mutant and Δ*rmpA* mutant, $P = 0.0014$ (all comparisons were by *t* test).
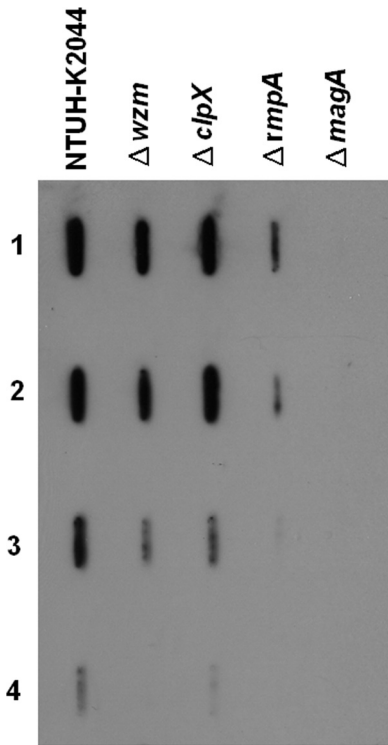
FIG. 8. Immunoblot. Capsule extracts from wild-type and mutant strains with equal numbers of bacteria were diluted 1/5, 1/10, 1/100, and 1/1,000 (rows 1 to 4, respectively) in water and detected with serotype K1-specific antiserum.

effects. On the other hand, essential genes, although they were less likely to be specific virulence genes, could not be identified by transposon mutagenesis. Therefore, there could be a few genes missed by screening of this library.

A *Dictyostelium* model has been used previously to study virulence in extracellular bacteria, such as *Yersinia* (50), *Pseudomonas* (2), and *Vibrio* (44), and in intracellular bacteria, such as *Legionella* (28, 48) and *Mycobacterium* (20, 41). The results of these investigations demonstrated that the *Dictyoste-*
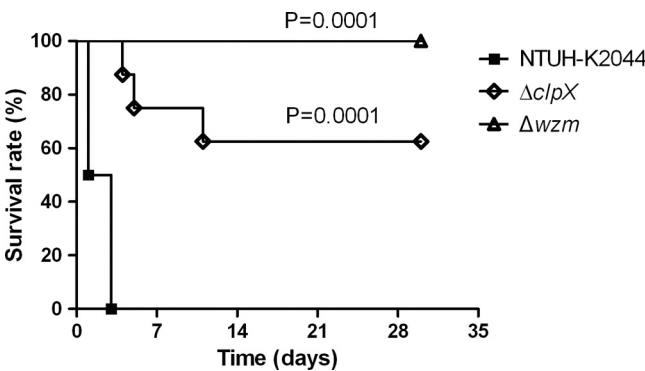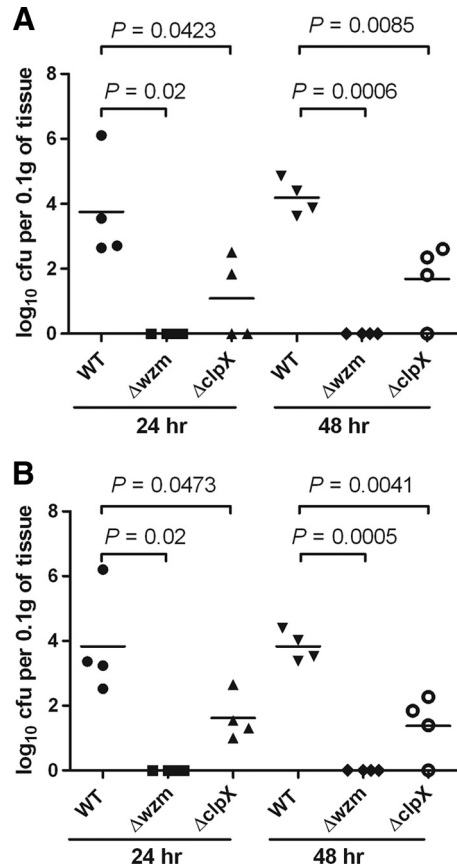

FIG. 10. Bacterial clearance *in vivo*. An inoculation dose of 10^3 CFU of the wild-type (WT), Δwzm, or $\Delta clpX$ strain was administered to each of four mice by intraperitoneal injection. Bacterial levels in tissue were determined at 24 and 48 h. The number of $\log_{10}$ CFU was standardized per 0.1 g of wet organ weight. (A) Bacterial load in liver (at 24 h, for wild type and Δwzm mutant, $P = 0.02$; for wild type and $\Delta clpX$ mutant, $P = 0.0423$; at 48 h, for wild type and Δwzm mutant, $P = 0.0006$; for wild type and $\Delta clpX$ mutant, $P = 0.0085$; comparisons were by t test). (B) Bacterial load in spleen (at 24 h, for wild type and Δwzm mutant, $P = 0.02$; for wild type and $\Delta clpX$ mutant, $P = 0.0473$; at 48 h, for wild type and Δwzm mutant, $P = 0.0005$; for wild type and $\Delta clpX$ mutant, $P = 0.0041$; comparisons were by t test).

FIG. 9. *In vivo* virulence of $\Delta clpX$ and Δwzm mutants. Eight BALB/cByl mice in each group were infected with 10^3 CFU bacteria and monitored for 30 days. *x* axis, number of days after inoculation; *y* axis, survival rate of mice. Statistical analysis of the wild type and mutants was performed using the Kaplan-Meier method. For $\Delta clpX$, $P = 0.0001$; for Δwzm, $P = 0.0001$ (log-rank test for both comparisons).

lium model is useful for investigating both phagocytosis and bacterial virulence. This model was also used with *Klebsiella* in a previous study which focused on discovering host genes required for intracellular killing of a nonpathogenic laboratory strain of *K. pneumoniae* (4). Two *Dictyostelium* mutants, *phg1* and *kil1* mutants, were found to be incapable of growing on *K. pneumoniae* due to a defect in efficient intracellular killing. They also tried to identify bacterial genes for resistance to intracellular killing in a *Dictyostelium phg1* mutant. A total of 800 bacterial mutants were screened, and 6 of them were permissive for *Dictyostelium phg1* mutant growth. Genes for intracellular killing resistance were clustered into three categories: capsule or lipopolysaccharide biosynthesis-related genes, genes involved in amino acid biosynthesis, and DNA adenine methylase genes. The virulence of the mutants was assessed in a mouse pneumonia model, and for four of them, the result was a higher survival rate for mice compared with that achieved with the parent strain. In the current study, we

used a wild-type strain of *Dictyostelium* which is more similar to natural phagocytes than the mutants are to investigate bacterial genes important for resistance to phagocytosis in a pathogenic clinical isolate of *K. pneumoniae* that is highly resistant to phagocytosis.

Our observation that the lipopolysaccharide O-antigen transporter of *Klebsiella* is important for resistance to phagocytosis and intracellular killing is consistent with the results of the previous study in which a *Dictyostelium* model was used to identify host genes required for intracellular killing of a nonpathogenic laboratory strain of *Klebsiella* described above (4). The lipopolysaccharide O antigen of *Klebsiella* has also been implicated in resistance to killing by murine neutrophils in a previous report (31). In our study, we have proved that a similar phenomenon was operating in human neutrophils. Although the mechanism is still unknown, we conclude that the lipopolysaccharide biosynthetic genes in *Klebsiella* and intact lipopolysaccharide O antigen appear to be among the factors associated with resistance to phagocytosis and bacterial virulence *in vivo*. Besides, we also found that the Δ*wzm* mutant became sensitive to serum killing, which is consistent with the observation that O antigen is important for serum resistance (34). Therefore, the rapid clearance of the Δ*wzm* mutant from mice may have resulted from its susceptibility to serum.

Our findings with the Δ*clpP* and Δ*clpX* mutants suggest that the protease complex ClpPX is critical for resistance to phagocytosis in *K. pneumoniae*. ClpX was reported to be a component of the ClpPX protease system that accounts for substrate specificity (47). Previous reports also indicated that *clpP* was important for degradation of misfolded proteins and tolerance to stresses, such as heat shock, acid, and oxidative stress, in different bacteria (17, 19, 21, 46). In addition, *clpP* is required for the expression of the virulence factor listeriolysin O and plays a crucial role in virulence and intracellular survival of *Listeria monocytogenes* (18). Although the ClpPX protease has been studied in several bacteria, there are few reports of the study of ClpPX protease in *K. pneumoniae*. Furthermore, despite the reports that *clpP* is related to the intracellular survival of bacteria in macrophages (18, 27, 30, 52), there are no previous reports examining the relationship between resistance to phagocytosis and the ClpPX complex.

It has been reported that inactivation of *clpP* and *clpX* may alter the levels of transcription of some genes (15, 39, 46). We also identified several genes related to the cell surface, stress response, transport, and metabolism, the transcription of which was altered in Δ*clpX* mutants. Interestingly, the expression of capsular synthesis genes and capsule production was reduced in the Δ*clpX* mutant, indicating that ClpX could regulate capsule synthesis in *K. pneumoniae*. Although the exact mechanism still needs further studies, according to the results of microarray analysis, a two-component system, RcsBC, involved in *cps* gene expression, was also downregulated to a similar level as the *cps* genes in the Δ*clpX* mutant (Table 3). Therefore, ClpPX-mediated proteolysis was probably important for the normal existence of some mediator(s) which was involved in RNA expression of *rcsBC*, *cps* genes, and finally, capsule production. However, although disruption of *wzm* and *clpX* partially affected capsule production, a defect in the amount of capsule could not be the critical factor resulting in attenuation of phagocytosis resistance and virulence because a

rmpA mutant, which produced a smaller amount of capsule than the amounts produced by the Δ*wzm* and Δ*clpX* mutants, was not attenuated in phagocytosis resistance and *in vivo* virulence in mice (unpublished data).

According to the results of the microarray analysis, membrane proteins, proteases, and transcriptional factors were downregulated in the Δ*clpX* mutant. It has been documented that bacteria have different strategies to prevent phagocytosis. For example, the *Yersinia* type III secretion system injects effector protein to inhibit the host actin required for engulfment (49). Proteases and membrane or secreted proteins have also been found to interfere with deposition of opsonins or to directly inactivate antibody and complement in several bacterial species (5, 16). Although more experiments are still needed to reveal the mechanism of the resistance to phagocytosis contributed by *clpX*, our results provide some insight. In one hypothesis, the *K. pneumoniae* ClpPX protease system may be responsible for regulation of downstream genes, at least the capsular gene, resulting in resistance to phagocytosis by human neutrophils, and this could play some role in *K. pneumoniae* virulence.

In conclusion, we have identified capsular genes and two noncapsular virulence genes in *K. pneumoniae* that were related to resistance to phagocytosis in a *Dictyostelium* model, suggesting that the model is useful for evaluation of heavily encapsulated bacteria. Documentation of the role of *clpPX* in resistance to phagocytosis may help provide a further understanding of the virulence mechanism of *K. pneumoniae*.

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