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Original article

SitA contributes to the virulence of *Klebsiella pneumoniae* in a mouse infection model

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Abstract

Klebsiella pneumoniae is an opportunistic pathogen, which causes a wide range of nosocomial infections. Recently, antibiotic resistance makes *K. pneumoniae* infection difficult to deal with. Investigation on virulence determinants of *K. pneumoniae* can provide more information about pathogenesis and unveil new targets for treatment or vaccine development. In this study, SitA, a Fur-regulated divalent cation transporter, was found significantly increased when *K. pneumoniae* was cultured in a nutrient-limited condition. A *sitA*-deletion strain (Δ*sitA*) was created to characterize the importance of SitA in virulence. Δ*sitA* showed higher sensitivity toward hydroperoxide than its parental strain. In a mouse intraperitoneal infection model, the survival rate of mice infected with Δ*sitA* strain increased greatly when compared with that of mice infected with the parental strain, suggesting that *sitA* deletion attenuates the bacterial virulence in vivo. To test whether Δ*sitA* strain is a potential vaccine candidate, mice were immunized with inactivated bacteria and then challenged with the wild-type strain. The results showed that using Δ*sitA* mutant protected mice better than using the wild-type strain or the capsule-negative congenic bacteria. In summary, SitA was found being important for the growth of *K. pneumoniae* in vivo and deleting *sitA* might be a potential approach to generate vaccines against *K. pneumoniae*.

Keywords: *Klebsiella pneumoniae*; Virulence determinant; SitA; Vaccination; Mouse intraperitoneal infection

1. Introduction

Klebsiella pneumoniae belongs to the *Klebsiella* genus of *Enterobacteriaceae* and is one of the microorganisms of human normal flora found on human mucosal surfaces [1]. It is usually harmless to immunocompetent individuals but opportunistic infections could occur to those who are immunocompromised. Therefore, immunodeficiency or underlying diseases such as diabetes, alcoholics and malignancy are risks of being infected by *K. pneumoniae* [2,3]. Infections caused by *K. pneumoniae* are commonly seen in hospital intensive care units and diseases frequently present as urinary tract infection, pneumonia, liver abscess, surgical site infection and bacteremia [1,2]. In pediatric wards, *K. pneumoniae* is a major cause of neonatal sepsis [1]. The mortality rate of bacteremia and pneumonia caused by *K. pneumoniae* can be higher than 50% [4,5]. Due to the fact that *K. pneumoniae* is commonly found in human intestinal tracts, patients' gastrointestinal tract could by itself serve as reservoirs for nosocomial transmission [6]. In intensive care units, high carrier rates of *Klebsiella* species

Abbreviations: cfu, colony-forming unit; CPS, capsule polysaccharide; 2-D, two-dimensional; DMEM, Dulbecco's Modified Eagle's Medium; Kp K2044, *Klebsiella pneumoniae* strain NTUH-K2044; LB broth, Luria–Bertani broth; OMVs, outer membrane vesicles; Q-TOF, quadrupole time-of-flight.

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among patients and personnel are reported [7] and the incidences of infection are proportional to the length of hospitalization [1].

Due to the production of β-lactamases, *K. pneumoniae* is frequently resistant to the first-line and the extended spectrum penicillins. Furthermore, the carbapenem resistant strains were terribly found [8]. What makes things worse is that *K. pneumoniae* also shows an increasing resistance to trimethoprim, sulfonamide, the first generation quinolones and aminoglycosides clinically [9]. For these frequent multi-drug resistances, infections of *K. pneumoniae* are recurrently difficult to manage. Restricted selection in antibiotic therapy has made developing vaccines against *K. pneumoniae* a compelling choice. Vaccines previously developed against *K. pneumoniae* could be divided into passive immunization and active immunization, including using whole-cell vaccine or subunit vaccine like capsular polysaccharide (CPS) and lipopolysaccharide (LPS). Immunization with attenuated whole cells could be the simplest approach to raise active immunity against *K. pneumoniae* infections. This approach would take full advantage of complete coverage of epitopes of *K. pneumoniae*, low manufacture cost and long preservation time. However, it is not practical and risky when attenuated live bacteria are applied to immunocompromised people.

So far, many virulence-associated factors, including the capsular polysaccharides, lipopolysaccharide, adhesins and iron-acquisition systems, have been suggested in *K. pneumoniae* infection [10,11]. Iron ion is an essential nutrient for all bacteria, and three iron-acquiring siderophore systems are commonly found in *Klebsiella*, including enterobactin, aerobactin, and the most virulence-related yersiniabactin [10]. The limitation of iron ions releases the repression of Fur protein, which regulates the siderophore systems as well as capsule synthesis [12]. During the *Klebsiella* infection, host's neutrophils and mucosal cells produce lipocalin 2 (Lcn2), which binds and prevents enterobactin from contacting its receptor, and the Lcn2-enterobactin complex stimulates interleukin 8 (IL-8) to promote inflammation [13].

Recently, outer membrane vesicles (OMVs) of *K. pneumoniae* were found inducing the innate immune response and lung pathology in neutropenic mice [14]. OMVs produced by Gram-negative bacteria contain immune-stimulating factors such as LPS, peptidoglycans, outer membrane porins, flagellins, and periplasmic proteins [14,15] and have been used as an effective vaccine against *Neisseria meningitidis* [16]. Thus, searching for novel virulence-associated factors of *K. pneumoniae* may provide insight into the pathogenesis of *K. pneumoniae* and offer new strategies to fight against the bacterial infection. In the present study, we characterized the *K. pneumoniae* proteins in a eukaryotic medium and found significantly increased expression of SitA protein in the culture supernatant. SitA protein, a Fur-regulated transporter for nutrient-immunity related ferrous and manganese ions in *Enterobacteriaceae* [17,18], was demonstrated to vary its expression levels with bacterial culture conditions. More importantly, we reported that protection against *K. pneumoniae* has been effective by vaccination with an UV-inactivated *sitA*-deleted mutant in a mouse model.

2. Materials and methods

2.1. Bacterial strains

K. pneumoniae strains NTUH-K2044 (Kp K2044; K1 serotype), VGH525 (K2 serotype), VGH404 (K5 serotype), and VGH484 (K9 serotype) [19] were used in this study. Kp K2044, which caused liver abscess and meningitis in clinic and mortality in the mouse model, was used as a parental strain to create deletion mutant by allelic exchange inactivation (see below). A capsule-negative mutant (*wza*::Tn*5*) created from the same parental strain was used as a control when a comparison was needed [19]. *Escherichia coli* JM109 (New England Biolab) was used routinely during plasmid construction and *E. coli* S17-1 (λ*pir*) was used when two-step allelic exchange was engaged. All bacteria were routinely cultured in Luria–Bertani (LB) broth.

2.2. Two-dimensional (2-D) gel electrophoresis

Overnight-cultured Kp K2044 was 100-fold diluted into fresh LB broth and high glucose Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, cat. no. 12100), respectively, and then the cultures were agitated for 16 h at 37 °C. The culture supernatants were collected after removing bacteria by centrifugation at 30,000× *g* for 1 h at 4 °C. The extracellular proteins from the cleared media were concentrated by trichloroacetic acid precipitation, followed by dissolving in buffer containing 4 M urea and 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). After passing through a 10-kDa cut-off filter (Amicon), 100 μg each of the collected proteins was mixed with rehydration buffer containing 8 M urea, 2% CHAPS, 0.5% immobilized pH gradient buffer (Amersham Biosciences) and 0.002% bromophenol blue. The so prepared sample solution was applied to the immobilized pH gradient strip by performing strip rehydration. The first-dimensional isoelectric focusing was performed with the Ettan IPGphor II Isoelectric Focusing System (GE Healthcare), and the second-dimension SDS-PAGE was carried out on SE260 electrophoresis apparatus (Amersham Biosciences).

2.3. Q-TOF mass spectroscopy

Protein spots stained with Coomassie blue on 2-D gels were excised. After dehydration, the pieces of gel were soaked in digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$, 12 ng/μl trypsin) overnight at 37 °C. The peptide fragments eluted from the gel pieces were recovered in solution containing 5% trifluoroacetic acid and 50% acetonitrile. Analysis of the peptides was carried out with a quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, Milford, MA). Data from the mass profiling were used to search for protein candidates from the NR database with Mascot (http://www.matrix-science.com) [20].

2.4. Sequence alignment

To compare the *sitABCD* sequences among the publicized *K. pneumoniae* genomes, the *sitABCD* nucleotide sequence of Kp K2044 was used as the query sequence. On NCBI blastn, "nucleotide collection" was chosen as the database and "enterobacteria" was used as the organism. To align the SitA amino acid sequences obtained from *K. pneumoniae* and *Enterobacteriaceae* genomes, amino acid sequence of SitA (BAH64867) was put into the search query of NCBI tblastn and "nucleotide collection" was used as blast database whereas "enterobacteria" was used as the organism.

2.5. Expression of K. pneumoniae SitA protein

To construct plasmid pQE60-SitA to express His_{x6}-tagged SitA, DNA fragment containing *sitA* gene (GI:238896198) was amplified from Kp K2044 genomic DNA with primers sitAf (5′-CATGCCATGGCACACCATTGCGAGGGATCCT-3′) and sitAr (5′-GAAGATCTTGGCTGCTTCCTCATCCCGT-3′). After digestion with *Nco*I and *Bgl*II, the fragment was cloned into pQE60 (Qiagen) pretreated with the same enzymes.

E. coli JM109 was transformed with pQE60-SitA, and then the pQE60-SitA- transformed bacteria were cultured and induced with 1 mM IPTG for 3 h to express His_{x6}-tagged SitA protein. After disruption by passage through a French pressure cell (SLM Aminco, Rochester, N.Y.), His_{x6}-tag fusion protein in bacterial lysate was then purified with Profinity IMAC Ni-Charged Resin (Bio-Rad).

2.6. Immunoblotting with anti-SitA mouse antiserum

Nickel-affinity-column-purified SitA protein was electrophoresed on SDS-PAGE, extracted with Electro-Eluter (Bio-Rad) and used as antigen to immunize BALB/c mice. After several boosts, the anti-SitA antiserum was acquired and saved for immunoblotting analysis. SitA protein on blots was detected by anti-SitA antiserum, followed by reacting with a horseradish peroxidase-conjugated anti-mouse antibody (Sigma). The blot was exposed to X-ray film (FUJI) after a final development with Western Lightning® ECL substrate (PerkinElmer).

2.7. Bacterial cell fractionation

To separate bacterial proteins into fractions, the method of Su et al. was slightly modified [21]. *K. pneumoniae* was inoculated into LB or DMEM medium and grown for 2 h or 16 h at 37 °C with agitation. To separate spent media and bacterial pellets, the bacterial cultures were centrifuged at 4 °C, 2180× *g*. The harvested supernatants were centrifuged two additional times at 5,581× *g* and 14,737× *g* at 4 °C sequentially. To avoid bacterial cell contamination, the supernatants were filtered with 0.45 μm filter, and then precipitated with trichloroacetic acid. The precipitated protein samples were dissolved in 100 mM Tris–HCl, pH 9.0 as the spent medium samples. The bacterial cell pellets were washed with 100 mM Tris–HCl, pH 7.4. The pellets were resuspended by adding 10 ml osmotic shock buffer A (20% sucrose, 100 mM Tris, pH 7.4). EDTA, at a final concentration of 1 mM, was subsequently added and then shaking at 4 °C for 10 min gently. The cells were then centrifuged at 2180× *g* for 10 min at 4 °C and then the pellets were resuspended by adding 10 ml osmotic shock buffer B (5 mM $MgSO_4$), which was followed by gentle shaking at 4 °C for 10 min. The supernatants were collected by centrifugation at 2180× *g* for 10 min at 4 °C. The resulting supernatants containing periplasmic proteins were centrifuged at 14,737× *g* for 20 min at 4 °C and concentrated using a Centricon filter (Millipore). The bacterial pellets were washed with 100 mM Tris–HCl, pH 7.4, resuspended in 6 ml lysis buffer (100 mM Tris–HCl, pH 7.4, 1.0 mM PMSF) and then were disrupted by the French pressure press. After centrifugation at 14,737× *g* for 20 min at 4 °C, the collected supernatants were spun in an ultracentrifuge at 80,000× *g* for 1 h at 4 °C. The resulting supernatants were spun again for another 30 min, filter concentrated and saved as the cytosolic fraction. The pellets from the first time ultracentrifugation were further washed with ddH_2O and centrifuged at 80,000× *g* for 30 min at 4 °C twice. The pellets were dissolved into 100 μl 0.1% SDS and this preparation was defined as the total-membrane fraction. The protein samples of different fractions were examined by SDS-PAGE followed by Western blot analysis. SitA was detected with anti-SitA antiserum prepared and described above.

2.8. Generation of the sitA deletion mutant of Kp K2044

Kp K2044 Δ*sitA* strain was constructed by using strategy of the allelic exchange [22]. In brief, the 5′ flanking region of *sitA* was PCR amplified with primers sitAF1f (5′-GCTCTAGACGAGCTTTGCCGCGCAAAAC-3′) and sitAF1r (5′-GGGGTACCAGGATCCCTCGCAATGGTGTGG-3′) by using Kp K2044 genomic DNA as the template, and similarly the 3′ flanking region of *sitA* was amplified with primers sitAF2f (5′-GGGGTACCATGCAGCGCAAGCGGGACTG-3′) and sitAF2r (5′-GGAATTCCAGGCCGAGCATATAGGCCCC-3′). The *Xba*I and *Kpn*I-restricted 5′ flanking PCR fragment, the *Kpn*I and *Eco*RI-restricted 3′ flanking PCR fragment and *Xba*I and *Eco*RI-digested pKAS46, which is a suicide vector [22], were three-way ligated and transformed into *E. coli* S17-1 (λ*pir*) to generate pKAS46-sitAF1F2. The plasmid was then transformed by conjugation into the wild-type Kp K2044 to perform allelic exchange. The strain with *sitA* deleted (Δ*sitA*) was selected and first confirmed by PCR; the disappearance of SitA was further verified by immunoblotting with anti-SitA antiserum.

2.9. Oxidative stress sensitivity assay

For H_2O_2 sensitivity assay, overnight cultures of bacteria were 10-fold diluted in fresh M9 minimum medium (22 mM $K_2H_2PO_4$, 48 mM $NaHPO_4$, 18.4 mM NH_4Cl, 0.8 mM $MgSO_4$, 8.55 mM NaCl, 0.45% glucose, 44 mM $NaHCO_3$, and 1 mM $CaCl_2$) and cultured until density reached the late-log

phase. The bacteria were then diluted to an equal density ($OD_{600} = 0.04$) in M9 or DMEM in the presence or absence of H_2O_2 (3 μg/ml in M9 and 21 μg/ml in DMEM, respectively). For tert-butyl hydroperoxide (TBHP) sensitivity assay, overnight cultures of bacteria were 100-fold diluted in fresh LB medium and cultured until density reached the late-log phase. The bacteria were then diluted in LB to a density at OD_{600} equal to 0.04 in the presence or absence of TBHP (0.4 mM). The bacterial growth was monitored every two hours with a spectrometer (Beckman DU-640).

2.10. Peritoneal infection of mice with K. pneumoniae

Overnight cultures of bacteria were refreshed for 2 h in LB with 1 mM EDTA, which is used to facilitate collecting bacterial pellets during centrifugation. After washing twice with sterile PBS by centrifugation, 5×10^4 colony-forming units (cfu) each were then added into 200 μl of PBS for inoculations. Eight-week-old BALB/c mice were intraperitoneally injected with the inoculums. The survival rates of mice were monitored for 14 d. Moribund mice were anesthetized and sacrificed prior to death. Mice were housed and monitored daily at the Animal Center of Yang-Ming according to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health). The animal studies were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University.

2.11. Mouse protection assay

Bacterial preparation was similar to that prepared for the infection except that 5×10^3 cfu each were added to 200 μl of sterile PBS and then exposed to UV (15 W) for 10 min. After UV-exposure, the so prepared inoculums were intraperitoneally injected into eight-week-old BALB/c mice individually. Two weeks after injection, the mice were challenged intraperitoneally with 200 μl of PBS containing appropriate amount of the wild-type Kp strains. The infected mice were then monitored daily for 14 d at the Animal Center of Yang-Ming according to the animal-care guideline.

2.12. Statistical analysis

To compare the mouse survival curves, Mantel–Cox log rank test, if not specifically stated, was applied by using GraphPad Prism 5. Among groups, differences were considered significant at $P < 0.05$.

3. Results

3.1. Identification of potential virulence factors with 2-D gel electrophoresis

During infection, *K. pneumoniae* produces virulence factors to facilitate adaptation to the host environment and evading immune system. Among the virulence factors, some are extracellular components, such as OMVs and siderophores, which help *K. pneumoniae* in various aspects during infection [14,23]. To search for possible virulence factors critical in vivo but not affecting the bacterial growth in vitro, 2-D gel electrophoresis was used to analyze proteins of *K. pneumoniae* present only or increasingly detected in a mammalian cell culture condition. DMEM, which is used to culture most human cells, was used as a tool to identify differentially secreted proteins. We collected the spent media and precipitated the proteins from the media with trichloroacetic acid. Proteins in the precipitants were then separated with 2-D gel electrophoresis and compared. Our result indicated that patterns of the protein spots from the two preparations readily differed (comparing Fig. 1a and b). Several proteins are apparently more abundant when *K. pneumoniae* was cultured in DMEM than in LB. Among them, nine proteins (spots circled in Fig. 1b) were identified with quadrupole time-of-flight (Q-TOF) mass spectrometry. The protein identities (IDs) with predicted functions are listed in Table 1. Among them, two (IutA and SitA) are iron-acquisition-related proteins, five are glycolysis-related gene products (FbaA, GpmA, Pgk, Eno and GapA) and two are hypothetical proteins (YcdO and YncE).

3.2. SitA is critical for the survival of K. pneumoniae under oxidative stress

SitA is one component of SitABCD complex, which belongs to ATP-binding cassette type metal ion transporter family [24]. Highly conserved *sitABCD* operon (>97% identities by blastn) is located on the chromosomes of annotated *K. pneumoniae* genomes. The Kp K2044 SitA protein (BAH64867) shares extremely high identity (>95%) with all the predicted amino acid sequences of the above-mentioned *K. pneumoniae* SitA proteins, and shows more than 75% identity to predicted SitA homologues in *Enterobacteriaceae* by tblastn (Supplementary Table 1). The highly conserved coding sequence and a predicted Fur box sequence in the promoter

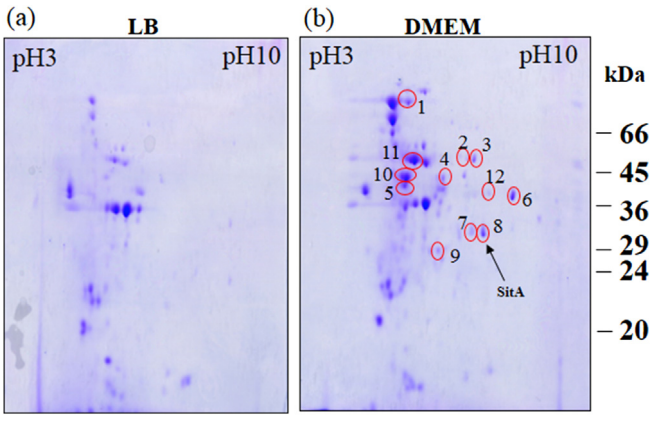

Fig. 1. Identification of increased proteins of *K. pneumoniae* in DMEM by proteomics. Proteins in the spent media of *K. pneumoniae* overnight cultured in LB (a) and DMEM (b) were resolved by 2-D gel electrophoresis, respectively. Nine apparently increased proteins from the DMEM preparation were identified with Q-TOF mass spectroscopy; proteins are numbered in accordance with those identities listed in Table 1.

Table 1
Identification of proteins of *K. pneumoniae* increasingly detected in DMEM.

#	Protein ID	Accession #	Function
1	IutA	BAH66193	Aerobactin-cloacin receptor
2	FbaA	ABR78751	Fructose-bisphosphate aldolase
3	YcdO	ABR76481	Hypothetical protein
4	YncE	ABR77339	Hypothetical protein
5	SitA	ABR78479	Iron transporter: fur regulated
6	GpmA	ABR76204	Phosphoglyceromutase
7	Pgk	ABR78752	Phosphoglycerate kinase
8	Eno	ABR78524	Phosphopyruvate hydratase
9	GapA	ABR76630	Glyceraldehyde-3-phosphate dehydrogenase 1

region (data not shown) suggest the possible role of SitA contributing to iron-acquisition in enterobacteria. To examine the results obtained from proteomic analysis of the culture media of strain K2044, more strains with different serotypes were examined for the increasing induction of SitA when in DMEM medium. Fig. 2a of the Western blot shows that several clinical *K. pneumoniae* isolates tested all yielded higher amounts of SitA when in DMEM than in LB. Similarly, increasing expression of SitA was seen when these different isolates were cultured in M9 as compared to that in LB (Fig. 2b). Therefore, these results substantiated the notion that increasingly expressed SitA is a general phenomenon when *K. pneumonia* is switched from LB media to DMEM or M9.

To test whether the increase of bacterial SitA expression in DMEM or M9 is due to a switch from a high level of Fe^{2+}/Mn^{2+} in LB to a low level of that in DMEM or none at all in M9, EDTA was added to the media to chelate the possible divalent metal ions. For a comparison, effect of supplying

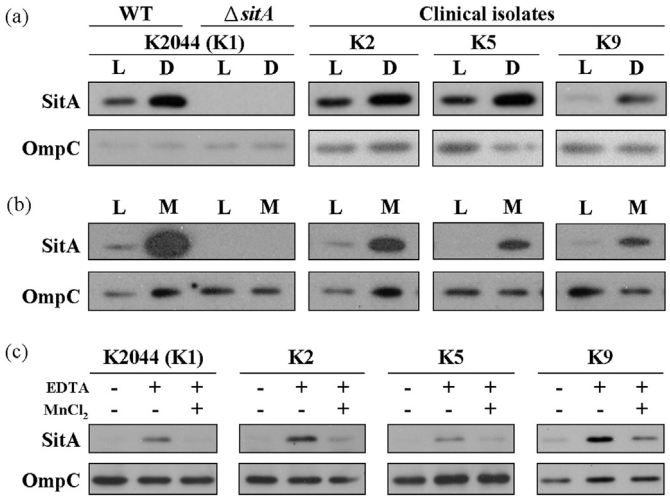

Fig. 2. SitA expression linked to medium differences but not to strain variations of *K. pneumoniae*. (a) Detection of SitA in the total lysates of serotype-different bacteria that were cultured in either LB (L) or DMEM (D). The bacterial lysates were analyzed with Western blotting by using anti-SitA antiserum. (b) SitA detected by Western blot analysis as in (a) except that a side-by-side comparison was made between LB and M9 (M). (c) SitA detection in the total bacterial lysates as in (a) while the same set of *K. pneumoniae* strains were cultured in LB, LB with 1 mM EDTA, and LB in the presence of 1 mM EDTA and 1 mM $MnCl_2$, respectively. OmpC was used to serve as a loading control in all samples.

extra Mn^{2+} to the media with supplemented EDTA was analyzed. The levels of SitA in bacteria were then examined by Western blot analysis using anti-SitA antiserum. Fig. 2c shows that in all strains of *K. pneumoniae*, addition of EDTA escalated the detected level of SitA and this elevation could be suppressed by supplying extra Mn^{2+} ions.

To consolidate the predication that SitA is a periplasmic cation-binding protein [18], we fractionated the total lysate of *K. pneumoniae*. Our fractionation result consistently indicated that the localization of *K. pneumoniae* SitA was in periplasm (Fig. 3a). When bacteria were cultivated for a longer period, SitA was also seen in cytosol and spent medium (Fig. 3b). This suggests that the occurrence of SitA in spent medium was likely due to a leakage of *K. pneumoniae* membrane or generated along with OMVs. The importance of SitA in pathogenesis could be expected since inactivation of its homologues in *Yersinia pestis* and *Salmonella enterica* Typhimurium resulted in reduced virulence in murine infection model [17,25]. Moreover, the transcriptome analysis in neonatal meningitis *E. coli* S88 showed that *sitA* was highly induced when growing ex vivo in human serum or urine, at a level ten-fold higher than that of *iutA* [26]. The importance of *sitA* is also evidenced by the consistent appearance of *sitABCD* operon in *K. pneumoniae* isolates, but *iucABCDiutA* is absent in some strains like MGH 78578 [27].

Since SitABCD has been shown to mediate transport of iron and manganese ions for H_2O_2 resistance in avian pathogenic *E. coli* [24] and highly induced as compared to *iutA* [26], we created a *sitA*-deleted mutant (Δ*sitA*) of *K. pneumoniae* and investigated the effect of *sitA* on the bacterial sensitivity to H_2O_2. In the presence of H_2O_2 (3 μg/ml), although a lagging period was seen within the first few hours, the growth of the parental strain in M9 reached to a level similar to that in the absence of H_2O_2 after cultivation for 6 h (Fig. 4a). On the other hand, the growth of the Δ*sitA* strain was totally abolished in the presence of the same concentration of H_2O_2 while the growth was equally well as that of the parental strain in the absence of H_2O_2 (Fig. 4a). Similarly, when *Klebsiella* strains were cultured in DMEM, which contains a trace amount of iron ions (0.248 μM $Fe(NO_3)_3$), high concentration of H_2O_2 (at 21 μg/ml) retarded the growth of Δ*sitA* strain severely but to a less extent with that of the parental strain (Fig. 4b). However, when *Klebsiella* strains were in nutrient-rich LB, no significant difference was seen between Δ*sitA* and parental strain toward the stress of H_2O_2 (data not shown). When a stronger oxidant, TBHP, was used in the same treatments, the parental strain revived after a lag period while the Δ*sitA* strain stayed unawake (Fig. 4c). Taken together, these observations strongly suggest that presences of both metal ions and SitA are highly crucial for the survival of *K. pneumoniae* when facing the stress of peroxide oxidation.

3.3. *SitA is required for the full virulence of* K. pneumoniae *in a mouse model*

To examine further whether deleting *sitA* could attenuate virulence of the bacteria, eight-week-old BALB/c mice were

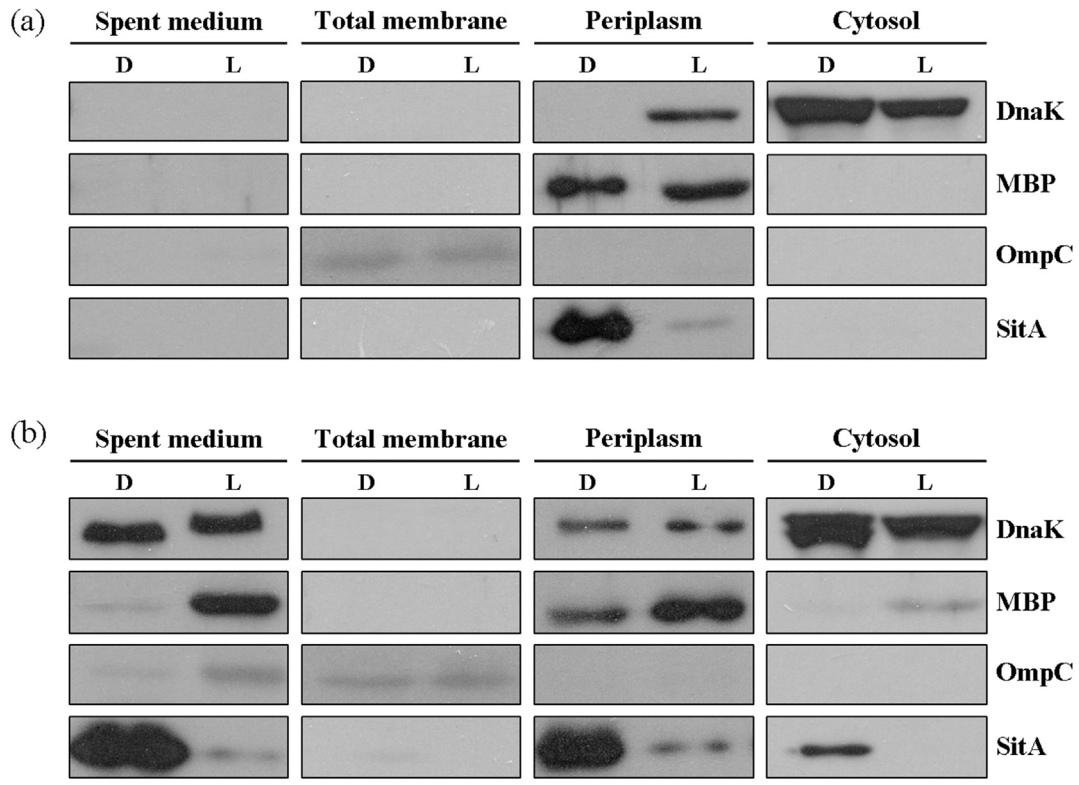

Fig. 3. Fractionation to localize SitA protein. Bacteria were cultured in LB (L) or DMEM (D) for 2 h (a) and 16 h (b), respectively. After collecting the spent media, bacteria were physically disrupted to prepare lysates that were subsequently separated into fractions of cytosol, periplasm and total membrane. Markers for individual fractions are: DnaK, cytosol; OmpC, total membrane; maltose-binding protein (MBP), periplasmic fraction.

infected intraperitoneally with 10^5 cfu of the Δ*sitA* strain. For a comparison, the parental wild-type strain and that of the capsule-negative mutant were included in parallel to examine the host survival rates. Shown in Fig. 5a, 80% of the wild-type *K. pneumoniae*-infected mice were killed at the third day after infection whereas 40% of the infected mice were killed by strain Δ*sitA* after infection for 14 days ($P = 0.0457$). And as expected, the capsule-negative mutant strain killed no mice through the infection course. Taking together, the above in vitro and in vivo experiments both support the notion that virulence of the Δ*sitA* strain is attenuated.

3.4. UV-inactivated ΔsitA strain protects mice against challenge

To test whether the attenuated strain could serve as a vaccine candidate to safeguard the high risk groups from contracting *K. pneumoniae*, we inactivated the Δ*sitA* strain with UV-exposure and then used it for testing with the mouse immunization/challenge model. For comparison, UV-inactivated strains of the wild-type and the capsule-negative were similarly prepared and, also, tested in parallel. Two weeks after injection, all the immunized mice were equally challenged with the wild-type strain. Fig. 5b shows that, 2 weeks after the challenge, 50% of the mice immunized with the inactivated capsule-negative mutant survived after the challenge whereas 80% of the mice immunized with the inactivated parental strain survived. In contrast, 100% of the mice immunized with the inactivated Δ*sitA* strain were protected from the challenge (Fig. 5b). These results indicated that immunization with the Δ*sitA* strain gave very satisfactory protection (Δ*sitA* vs. capsule-negative mutant, $P = 0.0114$) whereas the difference between Δ*sitA* and the wild-type strains were insignificant ($P = 0.1464$).

4. Discussion

K. pneumoniae is frequently seen in human gastrointestinal tract and respiratory tract as a commensal microorganism. This body-dwelling microorganism readily causes nosocomial infection in immunocompromised patients and newborns [28]. These colonization properties of *K. pneumoniae* elevate not only the risks for nosocomial infection [6] but also the incidence associated with the drug resistance of *Enterobacteriaceae* [29]. Due to the increasing difficulty in treatment of *K. pneumoniae* infection, to better characterize the pathogenesis of *K. pneumoniae* is becoming an urging need.

To search for novel virulence-associated factors, we have used Q-TOF mass spectroscopy to identify proteins increasingly detected in the supernatant when cultivation of *K. pneumoniae* was set in DMEM instead of LB broth. It is worth noting that DMEM is known to trigger the type three secretion system involved in the pathogenesis of enterohaemorrhagic and enteropathogenic *E. coli* [30]. Here, we have seen that the so-identified proteins include glycolysis-related enzymes

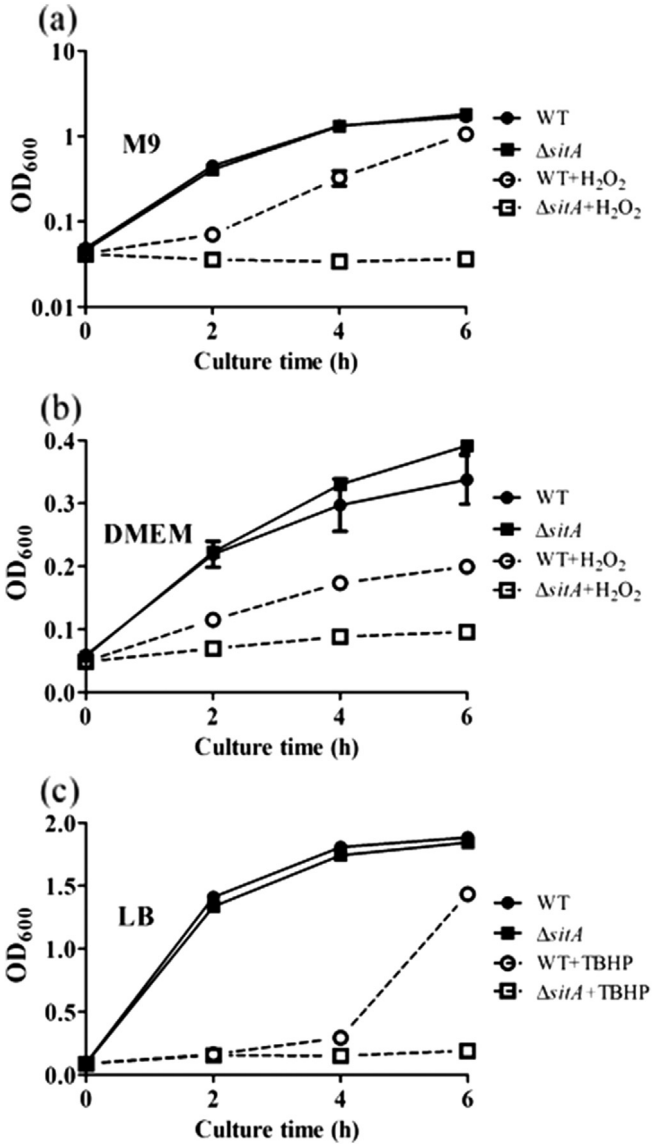

Fig. 4. SitA critical for *K. pneumoniae* to resolve the oxidative stress in culture. (a) Growth curves of bacteria cultured in M9 medium with or without H_2O_2 (3 μg/ml). Turbidity (OD_{600}) of bacterial culture was measured every two hours. (b) Growth of bacteria monitored as in (a) except that medium used was DMEM, with or without H_2O_2 (21 μg/ml). (c) Bacterial growth comparison as in (a) except that LB was used and TBHP was supplemented at 0.4 mM. Repeated experiments gave similar observations.

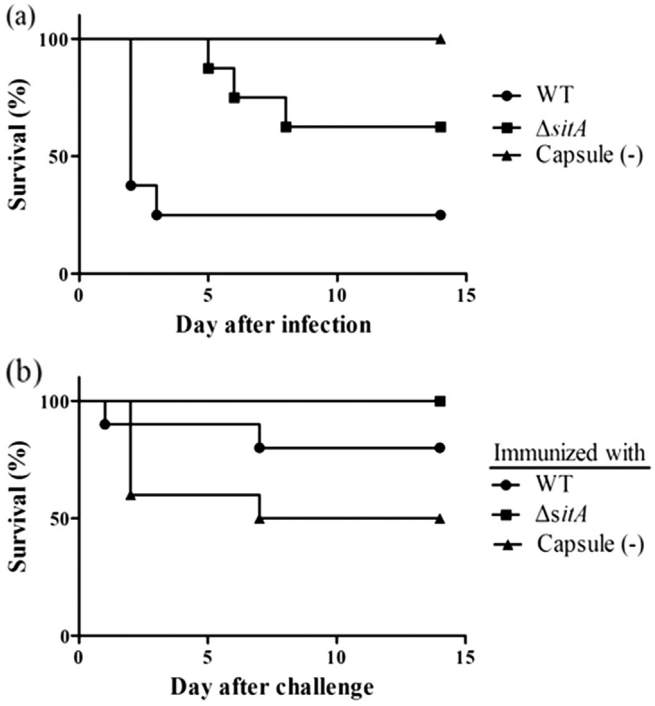

Fig. 5. Δ*sitA* is attenuated and offers protection against Kp K2044 challenge in a mouse model. (a) Survival of mice after challenge. Eight-week-old BALB/c mice were infected intraperitoneally with 10^5 cfu of different strains of bacteria and then monitored daily for 14 d. Number of mice used: wild-type (WT), 8; the Δ*sitA* strain, 8; the capsule-negative mutant, 6. (b) Protective efficacy of immunization with various UV-inactivated strains against *K. pneumoniae* challenge. Eight-week-old BALB/c mice were first immunized intraperitoneally with 5×10^3 cfu of UV-inactivated bacteria. After two weeks, the immunized mice of different groups were challenged with 1×10^4 cfu of the wild-type (WT) strain and monitored daily thereafter. Ten mice each were used in the individual groups.

(FbaA, GpmA, Pgk, Eno, and GapA) and iron acquisition-associated proteins (IutA and SitA). While the real reasons of detecting these protein fluctuations remains unknown, one simple speculation is that the amount of glucose in DMEM (5.5 mM) is higher than that in LB broth (<0.1 mM fermentable sugar equivalents [31]), so that glycolysis-related proteins are involved. On the other hand, the increasingly detected IutA and SitA proteins are possibly due to less iron ions in DMEM. The fact that M9 with an absence of iron and manganese ions stimulated the expression of SitA equally well or even better than DMEM (Fig. 2) fully supports this notion.

The iron ion uptake systems in *K. pneumoniae* can be divided into TonB dependent, including *fhu*, *iut*, *fep*, *fec*, *iro* and *fyu* systems, and TonB independent, such as *kfu*, *feo* and *sit* systems [27]. Instead of being outer membrane proteins like IutA, SitA located in periplasm [27]. Many divalent metal acquiring systems may help bacteria to compete the important nutrient immunity ions [32]. Whether these proteins are leaked out or secreted via OMVs into the media remains to be explored. In the present study, SitA, a divalent ion transporter component is proved to be required for *K. pneumoniae* infection in the mouse model. This finding provides us a target to attenuate *K. pneumoniae* genetically. Furthermore, we have proved that the Δ*sitA* strain does reduce the virulence, as shown in the mouse infection model (Fig. 5a). Previously, Hsieh et al. had demonstrated that *tonB* mutant inhibited several iron transporter systems and significantly decreased the mortality in mouse infection model [27], which supports our findings.

Investigation on the virulence-associated genes cannot only provide better understanding to the pathogenesis of bacteria but also offer more targets for attenuating bacteria. In host environment, bacterial pathogens utilize divalent ion transporters such as SitA and MntH to acquire iron and manganese ions [24]. Both ions are important co-factors for bacterial metabolic enzymes and can help bacteria to survive under oxidative stress in nutrient-limiting environment. MntH is a

broad distributed membrane protein, belonging to the NRAMP family, in both Gram-positive and Gram-negative bacteria, including *K. pneumoniae*. *mntH* is regulated by both Fur and MntR repressors and the encoded transporter prefers to transport manganese ions [33]. MntH was known to respond to reactive oxygen species in *E. coli* and *S. enterica* Typhimurium [24,34]. SitA transporter belongs to the ATP-binding cassette family and contributes to the iron and manganese transport in *Y. pestis* and *S. enterica* [24,35]. The contribution of SitABCD and MntH to virulence has been demonstrated in avian pathogenic *E. coli* [24]. Δ*mntH* and Δ*sitABCD* mutants were both attenuated in the coinfection assay with the parental APEC. However, Δ*sitABCD* showed a further reduced competitive index as compared to that of Δ*mntH*, suggesting that *sitABCD* may be worth of interest [24]. Moreover, SitA has been found contributing to the intracellular survival of *Shigella flexneri* in mammalian cell lines [36]. Although *K. pneumoniae* is regarded as an extracellular pathogen, previous study revealed its ability to invade and persist in cultured epithelial cell lines [37]. However, the role of SitA related to the persistence of *K. pneumoniae* in host cells remains to be confirmed.

Enhancing immunity against *K. pneumoniae* by vaccination provides a promising alternative towards disease prevention. Passive immunization is reasonably effective and safe in treatment of *K. pneumoniae* infection in both immunocompetent and immunocompromised patients. The passively administered anti-CPS antibody was found effectively to prevent mice from *K. pneumoniae* − caused burn wound sepsis [38]. For LPS, an O1-antigen specific monoclonal antibody was proved effective against *K. pneumoniae* sepsis in a mouse model [39]. However, passively administered anti-CPS antibody fails to reduce the incidences and severity of *Klebsiella* infection significantly in intensive care units [40]. Meanwhile, multiple serotypes of CPS or LPS antigens in *K. pneumoniae* make the preparation of all-serotype antisera a huge challenge. As to immunization, whole organisms as well as subunit vaccines could be considered for antigen preparations [41,42]. Surface components of *K. pneumoniae* are theoretically safe and, for this reason, outer membrane proteins and fimbriae proteins have been tested and shown to induce host specific antibodies and proved to be effective without the need of additional adjuvants in the mouse model [43,44]. With the advantage of being highly immunogenic and nontoxic, capsule antigen has been proved to be protective against intraperitoneal infection and burn wound sepsis of *K. pneumoniae* in mice [45]. The benefit of capsule carried by the bacteria in protecting mice from the challenge of *K. pneumoniae* was revealed in our data (Fig. 5b). Also, the titer (IgM) of mouse sera against CPS increased in the one-shot immunization with the inactivated Δ*sitA* strain (Supplementary Fig. 1). Interestingly, but not surprisingly, we have seen that immunization with the inactivated Kp K2044 Δ*sitA* strain also provided some protection against strain of K2 (Supplementary Fig. 2), a result presumably due to a degree of cross-reactivity.

By controlling iron uptake systems, generation of *K. pneumoniae* vaccine candidates has been explored previously, including using polysaccharide-iron-regulated cell surface protein or *tonB* mutant [27,46]. These experimental candidates strengthened the benefit on developing *K. pneumoniae* vaccine by disturbing metal ion acquisition. Unfortunately, the *tonB* deletion in *K. pneumoniae* retarded the growth and disturbed several environment sensing systems, so that it might require special cultivation condition as exampled in *Haemophilus influenzae* [47]. Nevertheless, to develop an applicable vaccine against *K. pneumoniae*, both immunogenicity and safety shall be both taken into consideration. To improve the safety for vaccine immunization, we have demonstrated that a simple UV exposure is sufficient to inactivate most (if not all) of the *K. pneumoniae* Δ*sitA* strain. And after immunization, the mice could be protected from the *K. pneumoniae* challenge (Fig. 5b). Apparently, the protection efficiencies, as revealed from the survival rates, between the immunization with the UV-inactivated wild-type and that with the UV-inactivated Δ*sitA* strains differed insignificantly ($P = 0.1464$). This minute difference probably hints a slightly higher benefit of using Δ*sitA* strain than using the wild-type strain. The good protection efficiency of Δ*sitA* could be probably attributed to increased expression of outer membrane proteins under reduced intake of iron or manganese condition [48]. Increased outer membrane proteins were proved immunogenic and have been applied to conjugate with additional antigens to generate vaccine in a mouse peritonitis model [49]. On the other hand, a threat of incomplete inactivation when embracing the wild-type bacteria should never be underestimated. In this regard, incomplete inactivation of the attenuated Δ*sitA* would become readily less risky. In conclusion, deletion at *sitA* followed by a brief bacterial inactivation may provide safe and protective vaccines for those individuals risking for *K. pneumoniae* infection.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2013.10.019.

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