BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Inactivation of the virulence factors from 2,3-butanediol-producing *Klebsiella pneumoniae*

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Abstract The microbiological production of 2,3-butanediol (2,3-BDO) has attracted considerable attention as an alternative way to produce high-value chemicals from renewable sources. Among the number of 2,3-BDO-producing microorganisms, *Klebsiella pneumoniae* has been studied most extensively and is known to produce large quantity of 2,3-BDO from a range of substrates. On the other hand, the pathogenic characteristics of the bacteria have limited its industrial applications. In this study, two major virulence traits, outer core LPS and fimbriae, were removed through homologous recombination from 2,3-BDO-producing *K. pneumoniae* 2242 to expand its uses to the industrial scale. The *K. pneumoniae* 2242 Δ*wabG* mutant strain was found to have an impaired capsule, which significantly reduced its ability to bind to the mucous layer and evade the phagocytic activity of macrophage. The association with the human ileocecal epithelial cell, HCT-8, and the bladder epithelial cell, T-24, was also reduced dramatically in the *K. pneumoniae* 2242 Δ*fimA* mutant strain that was devoid of fimbriae. However, the growth rate and production yield for 2,3-BDO were unaffected. The *K. pneumoniae* strains developed in this study, which are devoid of the major virulence factors, have a high potential for the efficient and sustainable production of 2,3-BDO.

Keywords *Klebsiella pneumoniae* · 2,3-Butanediol · Virulence · Lipopolysaccharides · Capsule · Fimbriae

Introduction

Environmental pollution and the gradual exhaustion of natural resources have led scientists to look for renewable energy sources and alternative processes to produce useful chemicals from non-petroleum-based materials. The bulk chemical, 2,3-butanediol (2,3-BDO), is mostly derived from petroleum sources and can be converted to a number of useful derivatives, including butadiene, methyl ethyl ketone, and tetramethyl compounds, which have a wide range of applications in the chemical, food, cosmetic, and pharmaceutical industries (Perego et al. 2000; Xiu and Zeng 2008). The microbiological production of 2,3-BDO has attracted considerable attention in the chemical industry because it can provide an alternative way of producing these chemicals from renewable resources through an eco-friendly process (Celińska and Grajek 2009). Among the microorganisms that produce 2,3-BDO, *Bacillus polymyxa* (De Mas et al. 1988), *Enterobacter aerogenes* (Zeng et al. 1990), *Klebsiella oxytoca* (Afschar et al. 1993), and *Klebsiella pneumoniae* (Lee and Maddox 1986) have the potential for 2,3-BDO production on an industrial scale. The non-pathogenic *Saccharomyses cerevisiae* has also been reported to possess 2,3-BDO biosynthetic pathways which were further improved by in silico metabolic

engineering (Nan et al. 2014; Ng et al. 2012). In particular, *K. pneumoniae* can grow quickly in simple media with a wide variety of sugars. Together with the capability of producing significant amounts of 2,3-BDO, *K. pneumoniae* is considered the best candidate 2,3-BDO producer in industry (Zeng and Sabra 2011).

K. pneumoniae is an opportunistic pathogen that causes up to 10 % of all nosocomial bacterial infections (Jarvis et al. 1985; Spencer 1996; Strettoti et al. 1984). *K. pneumoniae* infections can occur at almost any body site; however, the urinary and respiratory tracts are the most frequent infection sites. *Klebsiella* is second only to *Escherichia coli* as a cause of nosocomial bacteremia (Yinnon et al. 1996). In particular, a respiratory tract infection by *K. pneumoniae* has a mortality rate of up to 50 % (Fresno et al. 2006).

The pathogenic factors of *Klebsiella* include capsular polysaccharide (CPS), lipopolysaccharide (LPS), and pili (fimbriae). *Klebsiella* develops prominent capsules composed of an acidic polysaccharide complex, which covers the bacterial surface and protects itself from phagocytic action and as well as killing by bactericidal serum factors (Podschun and Ullmann 1998; Strettoti et al. 1984). The size of the capsule, as well as the rate of CPS production, has been shown to influence its virulence considerably (Cryz et al. 1984; Highsmith 1985). In addition, the capsule plays an important role in the adhesion to the mucous membrane of epithelial cells. LPS is the main component of the outer membrane of *Klebsiella* spp., consisting of lipid A, core oligosaccharide, and O-antigen. Lipid A and core oligosaccharide are essential for the bacteria to spread through the circulatory system and cause sepsis (Williams and Tomas 1990). O-antigen, also called *O*-polysaccharide or O-side chain, is a glycosyl-repeating unit linked to the core polysaccharide of LPS. Gram-negative bacteria can avoid the defense system of the host by modification of these basal O-repeat unit structures (Raetz and Whitfield 2002). The adhesive feature of *Enterobacteriaceae* to the host cell is also represented by the types of fimbriae, also known as pili, which are non-flagellar filaments up to 10 μm long, consisting of globular protein subunits. *K. pneumoniae* has two predominant fimbrial types. Type 1 fimbriae are the best-studied bacterial adhesins, which bind to the D-mannose-containing trisaccharides of the host glycoproteins (Firon et al. 1984). Type 1 fimbriae also play an important role in the pathogenesis of urinary tract infections (Fader et al. 1982; Gerlach et al. 1989; Maayan et al. 1985). Type 3 fimbriae are known to adhere to the epithelia of the respiratory tract, uroepithelial cells, and endothelial cells (Hornick et al. 1988; Tarkkanen et al. 1997).

K. pneumoniae KCTC 2242 has been reported to produce large quantities of 2,3-BDO (Rim et al. 2012). To remove the major virulence factors from this industrially useful 2,3-BDO producer, a mutant strain that is devoid of fimbriae and outer core LPS was constructed by knocking out the *fimA* and *wabG*

genes, which encode major type 1 fimbrial subunit protein and glucosyltransferase, respectively. The *wabG* gene is involved in the synthesis of outer core LPS by attaching α-L-glycero-D-manno-heptopyranose II to the *O*-3 position of an α-D-galactopyranosyluronic acid. This study examined the virulence traits of the mutant strains, such as fimbriae, capsule, binding affinity to human epithelial cells and mucous layer, as well as their susceptibility to phagocytosis by human macrophage-like cells, in comparison to their parental strain. The influence of removing these virulence factors on 2,3-BDO production is also evaluated by a fermentation experiment.

Materials and methods

Bacterial strains, plasmids, and growth conditions

K. pneumoniae KCTC 2242 was obtained from the Korean Collection for Type Cultures (Daejeon, South Korea). *K. pneumoniae* KCTC 2242 Δ*wabG* mutant strain was constructed by Jung et al. (2013). The bacteria were grown in LB media (Difco, Lawrence, KS, USA) at 37 or 30 °C containing either chloramphenicol (20 μg/ml) or tetracycline (3 μg/ml). Homologous recombination was carried out using pRedET (Gene Bridge, Germany) and 707-FLPe (Gene Bridge, Germany) plasmids.

Cell lines and culture conditions

The human ileocecal epithelial cell line, HCT-8, and the human bladder epithelial cell line, T-24, were provided by Korean cell line bank (Seoul, Korea). The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Welgene, Korea) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 mg/ml). The monocyte cell line, THP-1, was purchased from the American Type Culture collection (Manassas, VA, USA). The cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 % FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37 °C, 5 % CO_2. The differentiation of THP-1 into macrophage-like cells was induced by treating the cells with 100 nM phorbol myristate acetate (PMA) for 3 days (Gasser et al. 2003).

Construction of chloramphenicol-resistant cassette

The *fimA* gene of *K. pneumoniae* KCTC 2242 and *K. pneumoniae* KCTC 2242 Δ*wabG* was knocked out, as described earlier (Jung et al. 2013; Sawitzke et al. 2007). The target gene, *fimA*, was sequenced to design primer sets for homologous recombination. In this study, the chloramphenicol-resistant gene was used as a selection marker

to screen the Δ*fimA* mutant. A two-step PCR procedure was carried out to obtain a PCR fragment formed from a chloramphenicol-resistant cassette flanked by 34 bp of the flippase recognition site (FRT, 5′-GAA GTT CCT ATT CTC TAG AAA GTA TAG GAA CTT C-3′) and 50 bp of the region complementary to the target sequence (Link et al. 1997). An 891-bp PCR fragment containing the chloramphenicol-resistant cassette flanked by the 34 bp flippase recognition site was generated using the primer pair FRT-Cm/Forward (5′-GAA GTT CCT ATT CTC TAG AAA GTA TAG GAA CTT C*TG AGA CGT TGA TCG GCA CGT*-3′) and FRT-Cm/Reverse (5′-GAA GTT CCT ATA CTT TCT AGA GAA TAG GAA CTT C*AT TCA GGC GTA GCA CCA GGC*-3′). The 20 nucleotides homologous to the extremities of the chloramphenicol gene are indicated in italics. FRT was introduced to the cassette to remove the chloramphenicol selection marker from the chromosome after disrupting the target gene *fimA* by transforming the cells with the FLP expression plasmid. The PCR products (FRT-flanked chloramphenicol resistant gene, FCF) were used as a template for the PCR reaction with the primer pair containing 50 bp homology arms at their 5′ extremity corresponding to both ends of the target gene, *fimA*. The sequences of the primer pairs are as follows: *fimA*/forward (5′-*ATG AAA ATC AAA ACA CTG GCA ATG ATT GTT GTG TCA GCC CTG TCA CTG AGG* AAG TTC CTA TTC TCT AGA A-3′) and *fimA*/reverse (5′-*TTA CTC GTA CTG CAC TTT GAA CGT GGC ATC CGC GTT CGC TAT ACC AGC CG*G AAG TTC CTA TAC TTT CTA G-3′). The homologous overhangs are shown in italics. The final PCR product (AFCFA) contained chloramphenicol-resistant cassette flanked by FRT and 50 bp arms homologous to both ends of the *fimA* gene.

Site-specific recombination

K. pneumoniae 2242 was transformed with the pRedET expressing *gam*, *exo*, and *bet* genes. *K. pneumoniae* 2242 was cultured in fresh LB medium supplemented with 0.7 mM of EDTA. The cells were harvested when the optical density of the culture reached 0.2–0.3 at 600 nm (OD$_{600}$), followed by washing three times with deionized water containing 10 % glycerol. After mixing with 150 ng of the pRedET plasmid, the cells were electroporated with a 1.8-kV, 5-ms pulse. The electroporated cells were incubated at 30 °C with shaking for 1 h and then spread over the LB agar plate supplemented with tetracycline (3 μg/ml) followed by incubation overnight at 30 °C. The colonies were inoculated into fresh LB media and grown to an OD$_{600}$ of 0.3 at 37 °C. L-Arabinose was added to the culture to a final concentration of 1 % and incubated with shaking at 37 °C for 1.5 h. The cells were harvested and washed with pre-chilled dH$_2$O containing 10 % glycerol. Forty microliters of the cells resuspended in dH$_2$O was mixed with 150 ng of AFCFA and electroporated with a 1.8-kV, 5-ms

pulse. After incubating at 37 °C with shaking for 3 h, the electroporated cells were spread over a LB agar plate supplemented with chloramphenicol (20 μg/ml) and incubated overnight at 37 °C. The chloramphenicol-resistant colonies were selected and the *fimA*-deleted *Klebsiella* mutants were verified by colony PCR with the primer set: *fimA*-F (5′-ATG AAA ATC AAA ACA CTG GC-3′) and *fimA*-R (5′-TTA CTC GTA CTG CAC TTT GA-3′), which are complementary to the 20 bp region of each homologous arm. To remove the FRT-flanked chloramphenicol cassette from the chromosome of the mutant, the cells were transformed with the FLP expression plasmid (707-FLPe, Gene Bridges, Germany) by electroporation, as described above. The electroporated cells were grown in LB media at 30 °C with shaking for 1 h and then spread over a LB agar plate supplemented with tetracycline (3 μg/ml). After incubating overnight at 30 °C, a few colonies were chosen, inoculated into fresh 1 ml LB media, and incubated at 30 °C for 3 h, followed by additional incubation overnight at 37 °C. An aliquot of the culture was streaked on an antibiotic-free LB agar plate and incubated overnight at 37 °C. The removal of the FRT-flanked chloramphenicol cassette from the chromosome of the mutant was confirmed by colony PCR using the same primer set, *fimA*-F and *fimA*-R.

Atomic force microscopy

Overnight cultures of *K. pneumoniae* 2242 cells were harvested by centrifugation at 25 °C for 5 min followed by washing with distilled water. The cells were resuspended in distilled water to a final concentration of 10^8 CFU/ml. Five microliters of the resuspended cells was applied to a clean slide glass and dried at room temperature for imaging. The morphology of the cells and fimbriae was examined by atomic force microscopy (AFM, Park System XE-70) in non-contact mode.

Extraction and quantitation of the capsule polysaccharides

The capsular polysaccharides of *K. pneumoniae* 2242 were obtained using a slight modification of the method described elsewhere (Domenico et al. 1989). Briefly, 5000 μl of overnight bacterial cultures, corresponding to 10^9 CFU, was mixed with 100 μl of 1 % Zwittergent 3-14 detergent (Sigma-Aldrich, USA) in 100 mM citric acid pH 2.0 (Biosesang, Korea). After incubating the mixture at 50 °C for 30 min, the pellet was removed by centrifugation at 15,000 rpm for 5 min. Three hundred microliters of supernatant was transferred to a new tube, and absolute ethanol was added to a final concentration of 80 %. The mixture was precipitated at 4 °C for 1 h, followed by centrifugation at 4 °C, 15,000 rpm for 15 min. The pellet was dissolved in 200 μl of distilled water.

The capsule polysaccharides were quantified by determining the concentration of uronic acid in the samples, using the

method described previously (Blumenkrantz and Asboe-Hansen [1973]). Two hundred microliters of the CPS sample was mixed with 1200 μl of 0.0125 M tetraborate (Sigma-Aldrich, USA) in absolute H_2SO_4 (Daejung, Korea). The samples were heated in boiling water for 5 min. After cooling at room temperature, 20 μl of 0.15 % 3-hydroxydiphenol (Sigma-Aldrich, USA) and 0.5 % NaOH were added and mixed thoroughly. The absorbance measurements were taken at 520 nm. Serially diluted solutions of D-mannuronic acid (A Johnson Matthey, Korea) were used for the standard curve.

Epithelial cell adherence assay

The cell adhesion assay was carried out using the method reported previously (Favre-Bonté et al. [1999]; Sahly et al. [2000]). The ileocecal epithelial cell line, HCT-8, and bladder epithelial cell line, T-24, were seeded at 10^4 cells/well in a 96-well tissue culture plate and grown with RPMI 1640 media (Welgene, Korea) supplemented with 10 % heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37 °C and 5 % CO_2 for 24 h. Resuspended 3×10^5 bacterial cells in RPMI 1640 media were introduced to each well and incubated at 37 °C for 3 h. The unbound bacterial cells were removed by washing the well three times with DPBS (Welgene, Korea). The cells were treated with 250 μl of 0.05 % Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS containing 20 % trypsin for cell lysis and releasing the bound *K. pneumoniae* to the media. The lysed samples were diluted serially and spread over a LB agar plate for colony counting. All experiments were carried out in triplicate.

Mucin binding assay

Mucin (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in an acetate buffer (pH 5.0) to a final concentration of 100 μg/ml. The surface of the 96-well plate was coated with mucin by filling each well with 100 μl of a mucin solution followed by incubation at 37 °C for 24 h (Ryan et al. [2001]). The unbound mucin was removed by washing the wells three times with PBS (pH 7.0) (Vishwanath and Ramphal [1984]). Overnight cultures of *K. pneumoniae* were harvested by centrifugation, washed three times with PBS, and resuspended in 0.1 M phosphate buffer at pH 8.5. Carboxyfluorescein diacetate (CFDA, Dojindo Molecular Technologies, USA) was added to a final concentration of 2 μg/ml, and the suspension was stirred at 37 °C in the dark for 30 min. The labeled bacteria were washed three times with PBS to remove the unconjugated fluorophore and resuspended in PBS. Cells 5×10^6 in PBS were introduced to each well and incubated at 37 °C for 1 h for adhesion. The wells were washed five times with PBS, and the fluorescence from the cells bound to the mucin surface was measured using a fluorescence spectrophotometer (Tecan, Infinite 200, Grodig, Austria) with an excitation and emission wavelength of 485 and 535 nm, respectively. The adhesion test was performed three times.

Phagocytosis assay

The phagocytosis assay was carried out by flow cytometry, as described earlier (Kabha et al. [1995]). The THP-1 cells were differentiated into macrophage-like cells by treating the cells with 100 nM phorbol myristate acetate (PMA) for 3 days (Gasser et al. [2003]). The differentiated macrophage-like cells were harvested from the cell culture flask, washed with DPBS, and suspended in RPMI 1640 medium to a final concentration of 2×10^5 cells/ml. The cells were incubated at 37 °C for 30 min in the presence of 5 % CO_2 with the CFDA-tagged *K. pneumoniae* 2242 wild type and its mutant counterpart to a final concentration of 2×10^6 CFU/ml. The ratio of macrophage-like cells to *K. pneumoniae* 2242 was set to 1:10. After incubation, the cells were washed three times with DPBS, resuspended in 200 μl PBS, and analyzed by flow cytometry (Guava easyCyte 8, EMD Millipore Co). Phagocytosis of *K. pneumoniae* to macrophage-like cells was determined by calculating the percentage of fluorescent macrophage-like cells from the entire macrophage-like cell population.

Fermentation of *K. pneumoniae* for 2,3-BDO production

The batch fermentation was carried out in a 2.5-l bioreactor (Kobiotech, Korea) in minimal medium (5 g/l yeast extract, 0.33 M glucose, 0.019 M K_2HPO_4, 0.083 M KH_2PO_4, 0.049 M $(NH_4)_2HPO_4$, 0.049 M $(NH_4)_2SO_4$, 1.01 mM $MgSO_4\cdot7H_2O$, 0.9 mM $FeSO_4\cdot7H_2O$, 3.5 μM $ZnSO_4\cdot 7H_2O$, 5.07 μM $MnSO_4\cdot7H_2O$, and 6.8 μM $CaCl_2\cdot2H_2O$) supplemented with 30 g/l glucose at 37 °C for 72 h. The pH of the minimal medium was set to 6.5 over the course of fermentation. The samples were withdrawn periodically and centrifuged at 5000 g for 10 min. The amount of *meso*-2,3-BDO, carbon source, and organic acids in the supernatants were analyzed by HPLC (Younglin, Korea) with an RI2414 detector (Waters Co, USA) and an Aminex HPX-87H organic acids column (300 mm×7.8 mm; Bio-Rad). Sulfuric acid (0.01 M) was used as the mobile phase at 60 °C at a flow rate of 0.6 ml/min. All solutions were filtered through a 0.2-μm membrane prior to use.

Results

Construction of *K. pneumoniae* Δ*fimA* and Δ*wabG*/Δ*fimA* mutants

K. pneumoniae 2242 expresses type 1 fimbriae which are encoded on a gene cluster containing all the genes necessary

for the fimbriae structure and assembly (Fig. 1). The expression of the major fimbrial subunit FimA protein in type 1 was regulated by the on-off switch of the promoter (Di Martino et al. 2003). Here, *fimA* gene, which encodes the major fimbrial subunit FimA protein in *K. pneumoniae* 2242, was knocked out. The resulting *K. pneumoniae* 2242 Δ*fimA* mutants were devoid of fimbriae, which are otherwise abundant in the wild-type strains. The red disruption system with chloramphenicol-resistant cassette flanked by 34-bp recognition site (FRT), together with 50-bp homologous regions complementary to the both ends of target gene was used, as shown in Fig. 2. After the transformation of *K. pneumoniae* 2242 with the chloramphenicol-resistant (Cm) cassettes, the Cm colonies were isolated. The PCR test using locus-specific primers showed that the middle region of the *fimA* gene of the isolate had been replaced with the FRT-flanked Cm cassette, which was reflected by the PCR product of 991 bp (Fig. 2). Upon elimination of the Cm cassette by transforming the host cell with the FLP expression plasmid, the resulting mutants gave the expected size of the PCR products (134 bp) in PCR analysis with the same locus-specific primers. The *fimA* gene of the *K. pneumoniae* 2242 Δ*wabG* mutant was also knocked out the same way as described above. These results together with the DNA sequencing data confirmed that the *fimA* gene of *K. pneumoniae* 2242 and *K. pneumoniae* 2242 Δ*wabG* had been successfully knocked out.

Characterization of fimbriae

The expression and morphological features of the fimbriae at *K. pneumoniae* 2242 and its mutant counterpart strains were examined by AFM (Fig. 3). The tapping mode AFM images showed that the rigid fimbriae radiated peritrichously from the surface of the *K. pneumoniae* 2242 wild type and Δ*wabG*

mutant strain. In contrast, the *K. pneumoniae* 2242 Δ*fimA* mutant strain was devoid of fimbriae, which shows that the *fimA* gene encoding the main structural protein of fimbriae had been knocked out successfully. The absence of fimbriae in the *K. pneumoniae* 2242 Δ*wabG*/Δ*fimA* double mutant strain was also observed. Interestingly, the expression level of fimbriae in the *K. pneumoniae* 2242 Δ*wabG* mutant strain was higher than that of its wild-type counterpart strain. The capsule and fimbriae are characteristic features of *K. pneumoniae* and are also involved in the pathogenicity and survival of the bacteria in the host tissues. Several studies have reported the inverse relationship between the capsule and fimbriae expression in *K. pneumoniae* (Matatov et al. 1999; Sahly et al. 2000; Schembri et al. 2005). Considering these reports, the relatively lower level of fimbriae expression in the wild-type strain compared to its Δ*wabG* mutant counterpart may be caused by the presence of a thick capsule layer on the surface of the bacteria.

Interactions with epithelial cells

The ability of wild-type *K. pneumoniae* 2242 and its mutant counterpart strains to interact with the human ileocecal epithelial cell line, HCT-8, and the bladder epithelial cell line, T-24, was examined because the bacterial association with the host cells is an important process in bacterial infections. As shown in Fig. 4, Δ*wabG* mutant strains revealed significantly increased association to both epithelial cell lines compared to the parent strain. This is in agreement with several studies showing that the presence of a capsule in *K. pneumoniae* reduces its adhesion ability to epithelial cells (Sahly et al. 2000). The masking of adhesion molecules on the bacterial surface by a capsule has been reported to be responsible for the reduced adhesion to epithelial cells. The expression of the

Fig. 1 Organization of the type 1 fimbriae and outer membrane in *K. pneumoniae* KCTC 2242. All genes involved in the synthesis of the type 1 fimbriae are placed in one operon
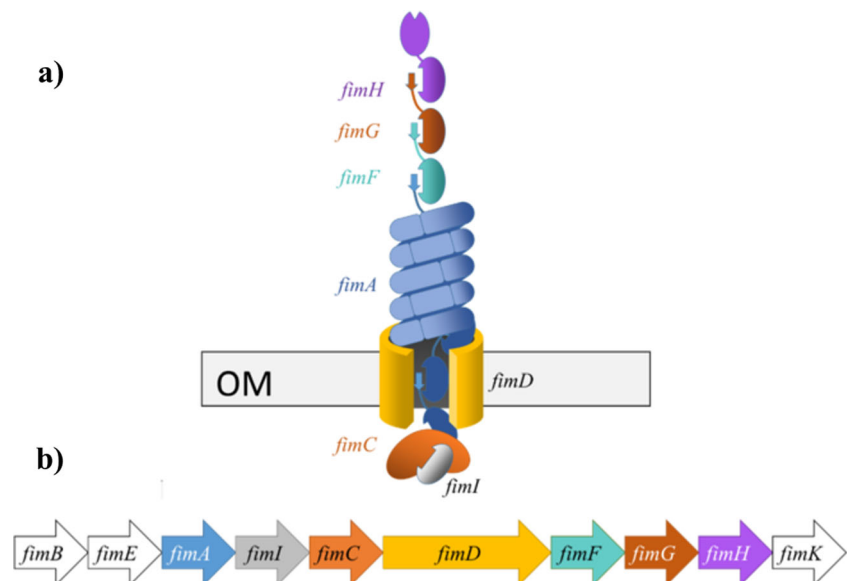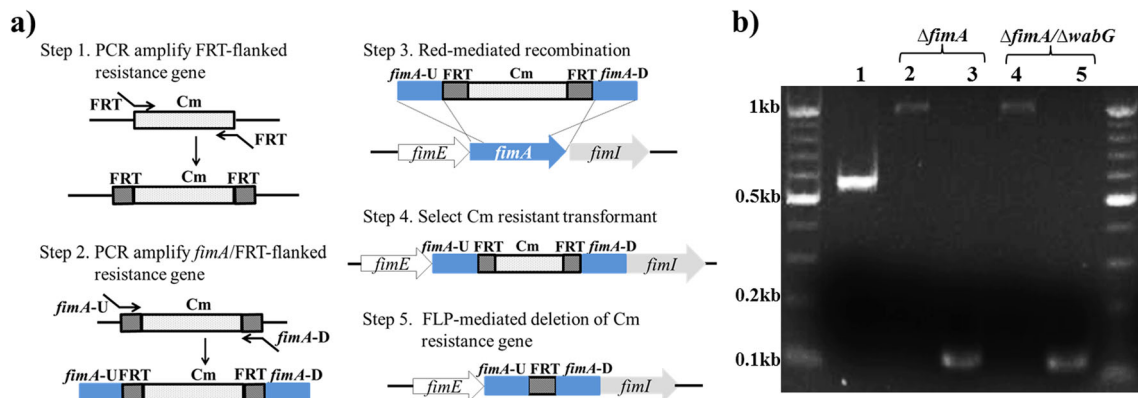
Fig. 2 Construction of *K. pneumoniae* KCTC 2242 Δ*fimA* mutant strain. **a** Diagram showing the generation of *fimA*/FRT-flanked chloramphenicol-resistant (Cm) cassette by PCR and subsequent homologous recombination using the cassette. The Cm cassette was removed by FLP helper plasmid. *fimA*-U and *fimA*-D refer to the homology extensions complementary to both extremities of *fimA* gene. **b** Agarose gel electrophoresis of PCR products amplified from the genomic DNA of *K. pneumoniae* 2242 and *K. pneumoniae* 2242 Δ*wabG* mutant before and after recombination, using a primer set that is complementary to both ends of *fimA* gene. The size of wild-type *fimA* gene is 549 bp (lane 1) whereas those after recombination and removal of Cm cassette were 991 bp (lane 2 and lane 4) and 134 bp (lanes 3 and 5), respectively

capsule was also found to downregulate CF29K adhesion at the transcriptional level in *K. pneumoniae* (Favre-Bonte et al. 1999). This study examined the role of fimbriae on the adhesion ability of *K. pneumoniae* 2242 to epithelial cells in vitro. Both Δ*fimA* and Δ*wabG*/Δ*fimA* mutants showed a more than fourfold and fivefold decrease in the association with the human ileocecal epithelial cell line, HCT-8. Their adhesion ability with the human bladder epithelial cell line, T-24, also decreased approximately twofold and fourfold, respectively, compared to their parent strain. These results suggest that the Δ*wabG* mutant lacking an outer core LPS and capsule has increased the adhesion to epithelial cells, which is in good agreement with previous studies (Struve and Krogfelt 2003). On the other hand, type 1 fimbriae are clearly related to the

Fig. 3 AFM images showing the presence or absence of fimbriae in *K. pneumoniae* KCTC 2242 wild strain (**a**), *K. pneumoniae* KCTC 2242 Δ*wabG* (**b**), *K. pneumoniae* KCTC 2242 Δ*fimA* (**c**), and *K. pneumoniae* KCTC 2242 Δ*wabG*/Δ*fimA* (**d**)
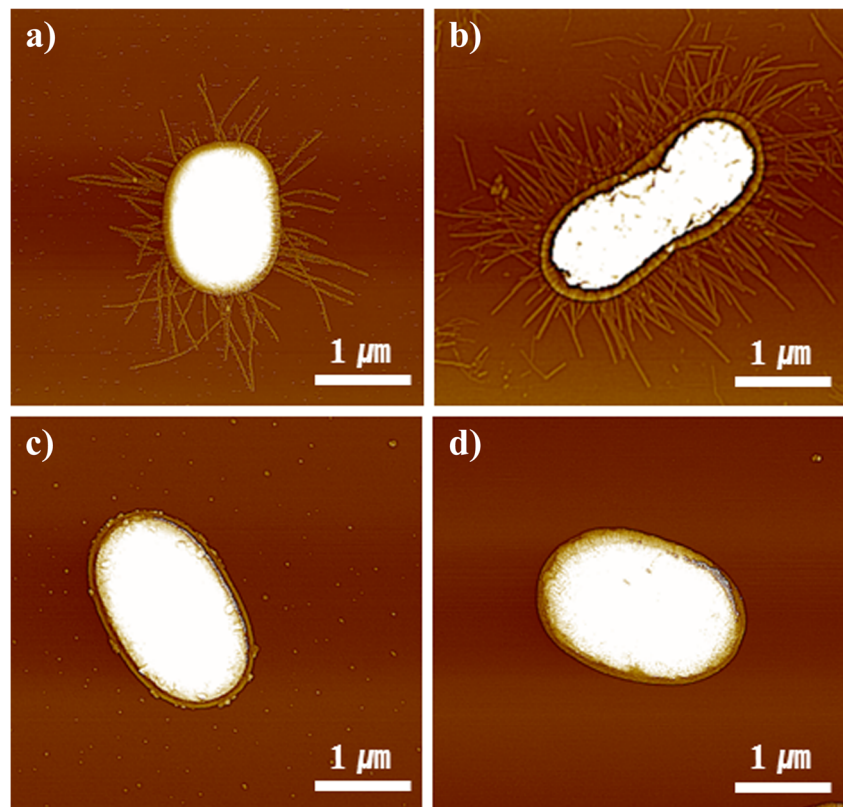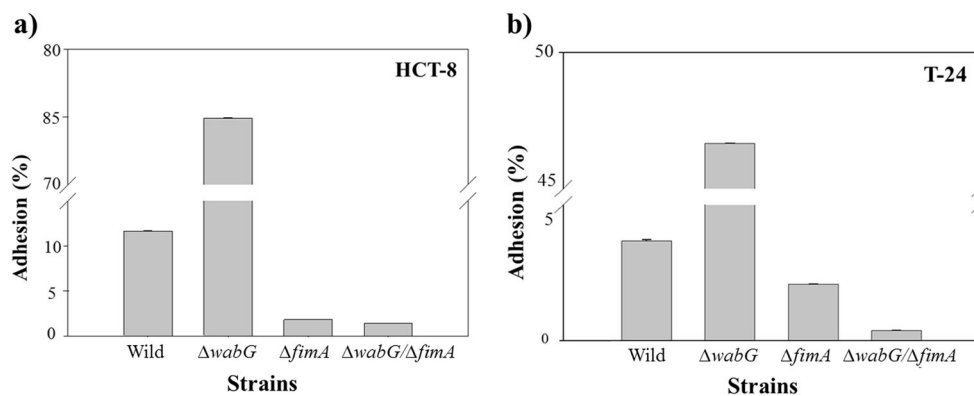
Fig. 4 Adhesion of
K. pneumoniae KCTC 2242 wild
strain and its mutant counterparts
to human ileocecal epithelial cell
HCT-8 (**a**) and human bladder
epithelial cell T-24 (**b**)

adhesion ability of *K. pneumoniae* 2242 to cultured epithelial
cells because the adhesion of the Δ*fimA* mutant to epithelial
cells was diminished greatly.

Interaction with mucin surface

A previous study reported that the ability of *K. pneumoniae* to
adhere to cultured epithelial cells is not always associated with
the infectivity or virulence of the bacteria because an in vivo
study using a mouse model showed no correlation with an
in vitro study (Shi et al. 2000). Therefore, this study investigat-
ed the adhesion or colonization ability of bacteria onto the
surface of the mucous layer. As a major component of mucous,
mucin is a family of glycoprotein that covers the luminal sur-
faces of the epithelial organs and serves as physical barrier
between the extracellular milieu and plasma membrane (Shi
et al. 2000). The association of bacteria with the mucous layer
on epithelial tissues increases the resistance to mucocilliary
clearance and facilitates the infection process. To test the adhe-
sion ability of bacteria onto the artificial mucous layer, a 96-
well plate coated with mucin was prepared. Each well was
filled with the same number (5×10^6) of CFDA-labeled
K. pneumoniae 2242. After 1 h incubation, the wells were
washed thoroughly with PBS and the fluorescence intensity
from each well was measured to determine the number of bac-
teria adhered to the mucin surface. The results showed that the

binding ability the *K. pneumoniae* 2242 Δ*wabG* mutant strain
to the mucin surface was significantly lower than its parental
strain (Fig. 5). The Δ*fimA* mutants showed slightly lower bind-
ing ability compared to the wild type but the degree of the
reduction was not significant. According to the results, the cap-
sule of *K. pneumoniae* 2242 appears to exert more pronounced
effect on binding to the mucin surface and resembles the mu-
cous layer of epithelial tissues. Favre-Bonté et al. examined the
role of *K. pneumoniae* capsular polysaccharides during the
colonization of the mouse intestine (Favre-Bonté et al. 1999).
They reported that the colonization ability of the bacteria de-
creased significantly in the isogenic capsule-defective mutant,
which is in good agreement with the present results. Most ep-
ithelial tissues, such as the large intestine or bladder, are cov-
ered with a mucous layer. Therefore, the binding characteristics
of *K. pneumoniae* to the mucous layer are more relevant to
measuring the virulence or pathogenicity of the bacteria.

Roles of capsule and fimbriae in phagocytosis
by macrophage-like cell

To examine the role of the capsule and fimbriae in bacterial
phagocytosis by macrophage-like cell, the degree of phagocy-
tosis of Δ*wabG* and Δ*fimA* mutants by differentiated THP-1
cells (dTHP-1) was compared with that of their parental strain.
THP-1 is a human monocytic leukemia cell line that can

Fig. 5 The amount of capsular
polysaccharides produced (**a**) and
the level of adhesion to mucin
surface (**b**) by *K. pneumoniae*
KCTC 2242 wild strain and its
isogenic mutant strains Δ*wabG*,
Δ*fimA*, and Δ*wabG*/Δ*fimA*
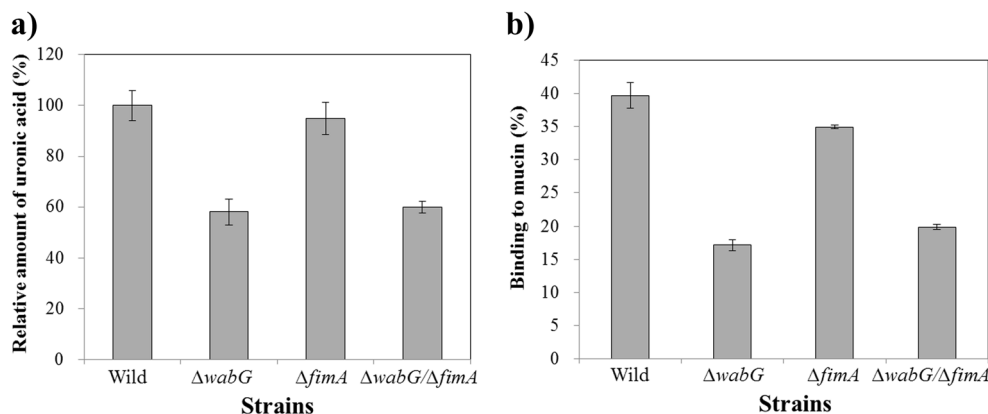
develop macrophage functions after the addition of stimulators. The dTHP-1 behaves more like native monocyte-derived macrophages (Auwerx 1991). Thus, the susceptibility of *K. pneumoniae* 2242 and its isogenic mutants, $\Delta wabG$ and $\Delta fimA$, to one of the human innate defense systems can be assessed using dTHP-1. The dTHP-1 cells were incubated with CFDA-labeled *K. pneumoniae* wild type and its isogenic mutant strains at a ratio of 1:10 for 30 min. As expected, the $\Delta wabG$ strain, which lacks outer core LPS with impaired capsule formation, showed higher phagocytosis by dTHP-1 (Fig. 6). After 30 min incubation, the ratio of the dTHP-1 containing $\Delta wabG$ mutant to the total dTHP-1 was 93.5 %, which is much higher than that containing its parental strain (47.2 %). The presence of a capsule is likely to influence the susceptibility of bacteria to phagocytosis by macrophage-like cells. The bacteria internalized within dTHP-1 were imaged by fluorescence microscopy (Fig. 6c). An aliquot of the

dTHP-1 cells incubated with the CFDA-labeled *K. pneumoniae* 2242 wild type and its isogenic mutant strains was taken before flow cytometry analysis and investigated by fluorescence microscopy. The number of wild-type bacteria in each dTHP-1 ranged from 0 to 5, whereas that of the $\Delta wabG$ mutant exceeded 10. Fluorescence microscopy also showed that the rate of internalization or phagocytosis of the $\Delta wabG$ mutant, whose outer core LPS is missing with impaired CPS formation, by dTHP-1 was much higher than that of its parental strain. The fimbriae, however, did not appear to influence the susceptibility or resistance of the bacteria to macrophage-like cells. The rate of internalization of the $\Delta fimA$ mutant by the dTHP-1 cells was similar to that of its parental strain. The *K. pneumoniae* 2242 $\Delta wabG/\Delta fimA$ double mutant presented similar characteristics to the $\Delta wabG$ mutant in terms of its susceptibility against macrophage-like cells. The results suggested that the outer core LPS or the amount of CPS is
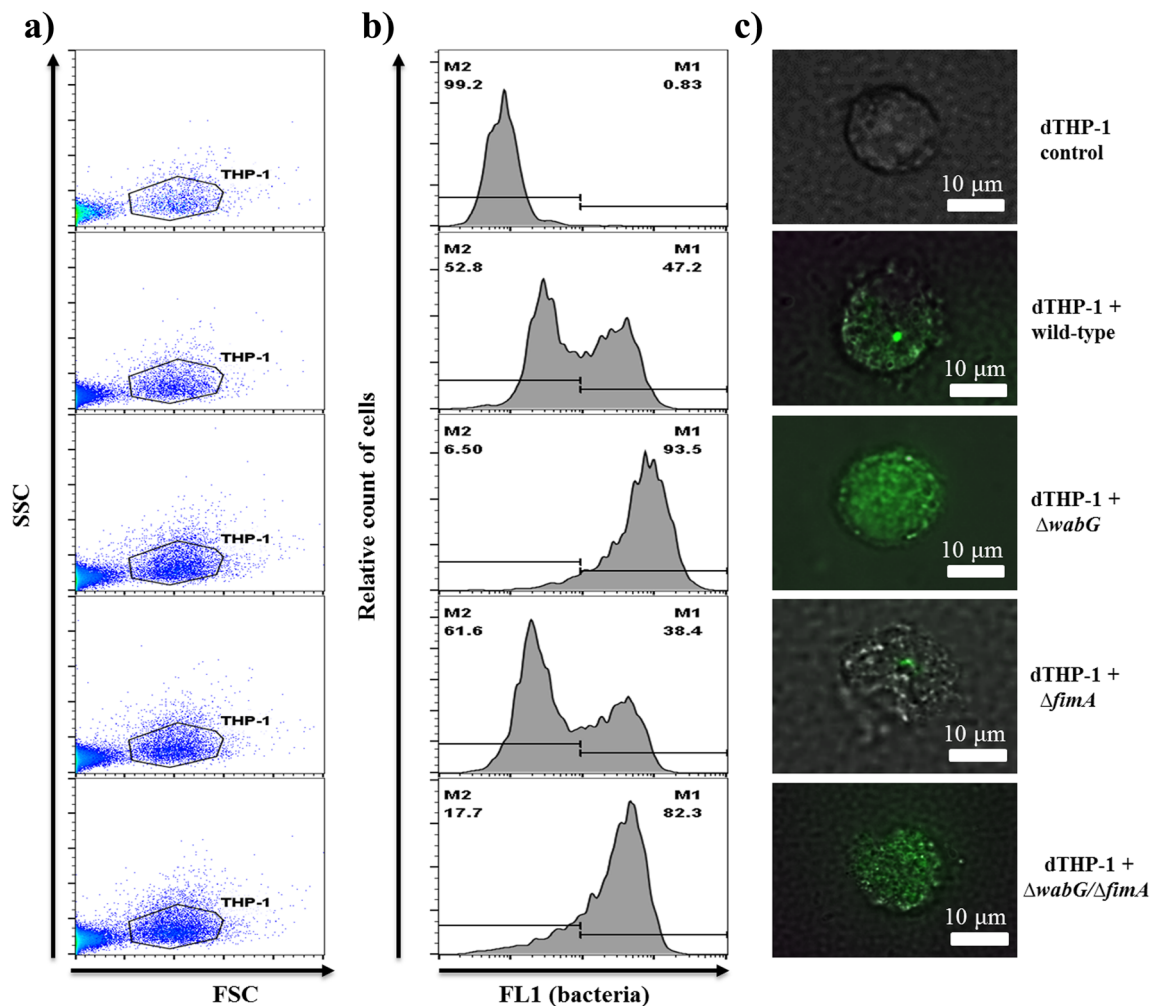

Fig. 6 The susceptibility of *K. pneumoniae* 2242 and its isogeneic mutant strains to the phagocytic activity of dTHP-1 cells. Following coculture of dTHP-1 cells and bacteria, phagocytosis was assessed by flow cytometric methods. dTHP-1 cells were gated by FSC vs SSC scatter plot (**a**), and phagocytized bacteria were quantified in FL1 channel (**b**). The dTHP-1 cells after coculture with the bacteria were imaged by fluorescence microscopy (**c**)

important for the bacteria to exert a resistance to macrophage-like cells, whereas the fimbriae have little or no role.

Growth and 2,3-BDO production

Batch cultures were performed with an initial glucose concentration of 30 g/l. The production of 2,3-BDO together with lactate, acetate, and ethanol from a culture of the wild-type, Δ*wabG*, Δ*fimA*, and Δ*wabG*/Δ*fimA* double mutant strains was monitored. The growth of these four strains was monitored by measuring the optical density of the culture at 600 nm (OD_{600}) over the course of 48 h fermentation. As shown in Fig. 7a, the growth rate of the *K. pneumoniae* 2242 wild type and its isogenic mutants in the batch culture was similar. The OD_{600} of the

cultures reached 6~8 in 12 h and remained constant over the course fermentation. The overall production of 2,3-BDO for 48 h fermentation from the wild type, the Δ*wabG* mutant, Δ*fimA* mutant, and the Δ*wabG*/Δ*fimA* double mutant strains was 6.87, 6.51, 7.24, and 7.35 g/l, respectively. The production of 2,3-BDO was increased by approximately 5.38 and 6.98 % in the Δ*fimA* mutant and Δ*wabG*/Δ*fimA* double mutant strains, respectively. The amount of lactate, acetate, and ethanol from the four tested strains was similar. The Δ*wabG* mutant strain showed a slightly increased level of acetate production along with decreasing 2,3-BDO at later stage. Other than that, the characteristics of *K. pneumoniae* 2242 in growth and 2,3-BDO production were maintained after knocking out the representative virulence factors, such as the outer core LPS and fimbriae.

Fig. 7 Time profile of growth (**a**) and production of 2,3-BDO and other byproducts (**b**) by *K. pneumoniae* KCTC 2242 and its isogenic mutant strains in batch culture at 37 °C for 48 h
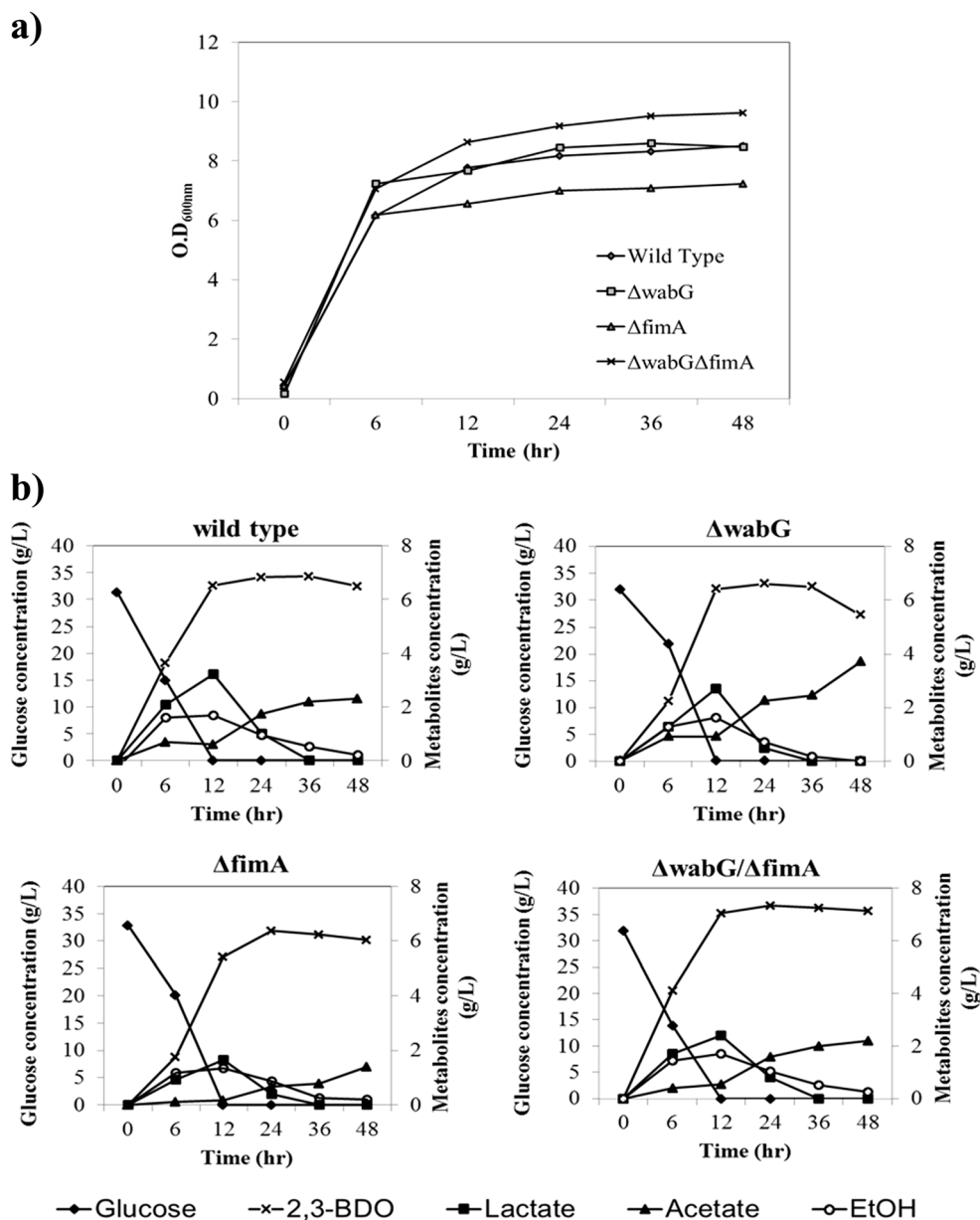
Discussion

The two most prominent virulence factors associated with pathogenic *K. pneumoniae* are the fimbriae and capsule, which are responsible for the adherence to epithelia as well as evading the host defense system (Tarkkanen et al. 1992; Williams and Tomas 1990). In this study, strains devoid of fimbriae and outer core lipopolysaccharide were constructed by homologous recombination, and their roles in the virulence were investigated. AFM showed that the Δ*fimA* mutant completely lost its ability to express fimbriae, whereas its parental strain possessed a number of fimbriae expressed radially from the surface of the cell membrane (Fig. 3). Interestingly, the level fimbriae expression in the Δ*wabG* mutant was higher than that of its parental strain. The outer core LPS was reported to be essential for the formation of a capsule around the cell (Jung et al. 2013). As shown in Fig. 5, the amount of capsular polysaccharides in Δ*wabG* mutant is approximately a half that of its parental strain. The thick capsule layer in the wild-type strain may impede the full expression of fimbriae, whereas the expression of fimbriae is less inhibited in the Δ*wabG* mutant having a partially impaired form of capsule. This is in a good agreement with a previous study, which reported an inverse relationship between the capsule synthesis and type 1 fimbriae expression (Schembri et al. 2005). On the other hand, the amount of capsular polysaccharides in the Δ*fimA* mutant was similar to that of the wild-type strain.

The ability to associate with the host cells is another important characteristic of pathogenic bacteria. The binding characteristics of *K. pneumoniae* KCTC 2242 and its isogenic mutants to the human ileocecal epithelial cell line, HCT-8, and the bladder cell line, T-24, were tested (Fig. 4). The ability of the Δ*wabG* mutant strain to associate with the human epithelial cell lines was significantly higher than that of its parental counterpart (Fig. 4). Several researchers have also reported an increased association of the Δ*wabG* mutant strain having a partially impaired capsule toward human epithelial cells in vitro (Favre-Bonté et al. 1999; Sahly et al. 2000; Struve and Krogfelt 2003). On the other hand, the association of the Δ*fimA* and Δ*wabG*/Δ*fimA* mutants to the same cell lines decreased dramatically, suggesting that the fimbriae are responsible for the interaction between the bacteria and host cell in vitro. In addition, an infection takes place mostly in the epithelia covered with a thick layer of mucous. This is why an in vitro test sometimes produces inconsistent results when investigating the pathogen's colonization ability in the host tissues.

Epithelial cells have a mucous layer to protect the gut, bladder, and respiratory tract as a barrier to prevent pneumococcal colonization (Nelson et al. 2007; Snyder and Walker 1987). The study using a mucous-producing cell line, HT-29-Rev MTX 10^{-6}, showed that the capsule is required for the

initial steps of colonization by binding to the mucous layer (Schild et al. 2005). As predicted, the binding ability of the Δ*wabG* mutant strains to the mucous layer was significantly lower than that of the wild type, whereas the binding ability of *K. pneumoniae* to the mucous layer was not affected significantly by the presence or absence of fimbriae (Fig. 5b). The binding ability of *K. pneumoniae* 2242 wild type and its isogenic mutants are closely related to the amount of capsular polysaccharides that each strain possesses (Fig. 5). In other words, the more capsular polysaccharides that the bacteria possess, the more the bacteria can associate with the mucous layer. These results show that the fimbriae are responsible for the direct association of the bacteria with epithelial cells, whereas the capsule is required for the bacteria to interact with the mucous layer as the first step of colonization and subsequent infection process.

K. pneumoniae has developed survival strategies to resist the host defense system, such as macrophage-mediated phagocytosis. Therefore, this study investigated the susceptibility of *K. pneumoniae* 2242 wild type and its isogenic mutant strains to macrophage-like cell dTHP-1. As shown in Fig. 6, the susceptibility of the Δ*wabG* mutants to the macrophage-like cell was approximately two times higher than that of its parental strain. On the other hand, the phagocytic rate of the Δ*fimA* mutant strain was similar to that of wild type. This suggests that the outer core LPS and capsular polysaccharide on the surface of the bacteria is the key factor that confers the bacteria the ability to resist the host immune system. The role of fimbriae on the resistance of the bacteria to phagocytic activity by macrophage-like cells is insignificant.

Cellular components, such as LPS, capsule, and fimbriae are sometimes associated with the cellular integrity and growth ability of the bacteria, particularly when it comes to industrial applications. Therefore, this study examined the growth and production ability of 2,3-BDO. The growth rate of the Δ*wabG* mutant strain is comparable to that of the wild type. Interestingly, the growth rate the Δ*wabG*/Δ*fimA* double mutant was highest among the four tested strains. On the other hand, all the strains reached an optimal density of 6~8 at 600 nm within 12 h and remained constant over the course of fermentation. The correlation between OD and cell mass was not notably changed after knocking out the genes that are responsible for the synthesis of outer core LPS and fimbriae, suggesting that the OD represent actual cell mass for the four strains (Fig. S1). The overall production of 2,3-BDO from the four strains was similar to one another. The yield of 2,3-BDO from the Δ*wabG*/Δ*fimA* double mutant strain was slightly higher than that of its parental strain. The production patterns of the byproducts, such as lactate, acetate, and ethanol, are also relatively unaffected by knocking out the virulence-associated genes. The production of the outer core LPS, capsule, and fimbriae is essential for the bacteria to achieve its virulence but they also requires high-energy expenditure for

K. pneumoniae. Therefore, the strains developed in this study will have high potential as an efficient 2,3-BDO producer on an industrial scale. In conclusion, two major virulence factors, outer core LPS and fimbriae, were removed from *K. pneumoniae* KCTC 2242, which has a high potential in the industrial production of 2,3-BDO. The ability to produce 2,3-BDO was unaffected by removing these pathogenic factors. This non-virulent *K. pneumoniae* 2242 strain is suitable for industrial applications and may contribute to the sustainable production of high-value chemicals from renewable resources.

Conflict of interest The authors declare that they have no competing interests.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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