Journal of Microbiology, Immunology and Infection 55 (2022) 69—79

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Original Article

D-mannose-sensitive pilus of *Acinetobacter baumannii* is linked to biofilm formation and adherence onto respiratory tract epithelial cells

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Received 12 November 2020; received in revised form 10 January 2021; accepted 26 January 2021
Available online 10 February 2021

KEYWORDS
Acinetobacter baumannii;
Pilus;
Biofilm;
Adherence;
Lectin;
Mannose sensitivity

Abstract *Background/Purpose: Acinetobacter baumannii* is an important nosocomial pathogen. To better understand the role of CsuA/BABCDE pilus of *A. baumannii* in virulence, bacterial biofilm formation, adherence and carbohydrate-mediated inhibition were conducted.
Methods: CsuA/BABCDE pilus-producing (abbreviated Csu pilus) operon of *A. baumannii* ATCC17978 was cloned for analysis of biofilm formation on an abiotic plastic plate, bacterial adherence to respiratory epithelial human A549 cells and carbohydrate-mediated inhibition. The carbohydrates used for inhibition of biofilm formation and adherence to A549 cells included monosaccharides, pyranosides, and mannose-polymers.
Results: The Csu pilus of *A. baumannii* ATCC17978 was cloned and expressed into a non-pilus-producing *Escherichia coli* JM109, and was knocked out as well. The recombinant Csu (rCsu) pilus on *E. coli* JM109/rCsu pilus-producing clone observed by both electro-microscopy and atomic force microscopy showed abundant, while Csu-knockout *A. baumannii* ATCC17978

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https://doi.org/10.1016/j.jmii.2021.01.008

mutant appeared less or no pilus production. The *E. coli* JM109/rCsu pilus-producing clone significantly increased biofilm formation and adherence to A549 cells; however, the Csu-knockout mutant dramatically lost biofilm-making ability but, in contrast, increased adherence. Moreover, both of biofilm formation and adherence could be significantly inhibited by D-mannose and methyl-α-D-mannopyranoside in Csu pilus-producing *E. coli* JM109, whereas in *A. baumannii* ATCC17978, high concentration of carbohydrates was required for the inhibition, suggesting that Csu pilus is sensitive to D-mannose.

Conclusion: This is the first study confirming that Csu pilus of *A. baumannii* belongs to mannose-sensitive type 1 pilus family and contributes to biofilm formation and bacterial adherence to human epithelial cells.

Introduction

Acinetobacter baumannii can survive in a wide range of environmental conditions, persist for extended periods of time on biotic or abiotic surfaces (i.e. biofilm formation), and is associated with nosocomial infections, such as ventilator- or catheter-associated infections.[1,2] Various clinical presentations caused by diverse *A. baumannii* include bacteremia, pneumonia, urinary tract infections (UTI), soft tissue infections and intra-abdominal infections.[3–8] Among them, pneumonia is the major clinical manifestation, especially in critical patients who need assisted ventilation.[7] *A. baumannii* has become the most common multidrug-resistant organism for hospital-acquired infections in intensive care units (ICUs) in Taiwan since 2007.[9]

As a resistant organism, few virulence genes of *A. baumannii* were reported, and little is known about the virulence mechanism to cause infections in humans.[10] It is important to understand how *A. baumannii* colonizes, adheres, and forms biofilm on epithelial cells. *A. baumannii* produces different types of pili, such as fimbrial CsuA/BABCDE-dependent pili (type IV secretion system) and non-fimbrial autotransporter adhesins (type V secretion system).[11–13] Recently, IS*Aba1* insertion at the upstream of *fimA* gene was found in two clinical isolates, suggesting the presence of IS*Aba1* could lead to overexpression of the FimA fimbrial (type 1 pilus, defined to be mannose-sensitive) protein.[14] The role of pili has been reported to not only promote adherence and biofilm formation, but the pilus adherence to host cell receptors may also induce inflammatory response, including the production of chemokines and cytokines.[15] This study aimed to study the characteristics of pilus-producing *A. baumannii* in biofilm formation and bacterial adherence onto host cells as well as sugar-mediated inhibition of biofilm formation and bacterial adherence.

Methods

Cloning, expression and mutagenesis of pilus genes

The Csu pilus-producing operon (namely *csuA/BABCDE*) in *A. baumannii* ATCC17978 was subsequently cloned into a low-copy number vector pK184 (kanamycin-resistant) (https://www.addgene.org/vector-database/3301/) with three-step cloning at the sites of *Sac*I, *Bam*HI, *Sal*I and *Pst*I (Table 1; Supplementary Fig. 1A), and expressed in a non-pilus-producing *Escherichia coli* JM109, which cellular surface is originally bald. The resulting strain was named JM109/pK184_*csuA/BABCDE*, also rCsu pilus-producing *E. coli* JM109. Gene expression inducer IPTG (isopropyl β-D-1-thiogalactopyranoside, a molecular mimic of allolactose) was supplemented to increase pilus production in recombinant *E. coli* JM109 clones in all following experiments. The lectin-like *csuE* and subclone $csuE_{sup}$ (highly hydrophilic and antigenic domain defined by the Protean software of DNASTAR LaserGene, Madison, WI, USA) were amplified by PCR using the primers as listed in Table 1. The resulting replicon was cloned into an expression vector pET29b (kanamycin-resistant) at the sites of *Nde*I and *Xho*I, confirmed by Sanger sequencing, and expressed in *E. coli* $BL21_{DE3}$ (Supplementary Fig. 1B). The expressed recombinant CsuE and $CsuE_{sub}$ (also namely rCsuE and $rCsuE_{sub}$) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and purified by Ni-NTA affinity purification system (QIAGEN) (Supplementary Fig. 2A and 2B). Because full-length *csuE* of *csuA/BABCDE* was expressed in an insoluble form in *E. coli* $BL21_{DE3}$, $rCsuE_{sub}$ was cloned and confirmed by Sanger sequencing. Purified recombinant $rCsuE_{sub}$ was used to raise polyclonal antibodies in rabbits (LTK BioLaboratories, Taoyuan, Taiwan) (Supplementary Fig. 2C).

To knockout the pilus gene *csuA/B* in *A. baumannii* ATCC17978, double crossing-over recombination at the *csuA/B* with the replacement of kanamycin cassette was carried out according to the method described previously (Supplementary Fig. 1C).[16,17] The resulting CsuA/B knockout (ΔCsuA/B) mutant was named *A. baumannii* ATCC17978_ΔCsuA/B. The bacterial strains used in this study are listed Table 1.[15,18]

Electron microscope and atomic force microscope

Pili of the *A. baumannii* ATCC17978, bald *E. coli* JM109 (carrying vector pK184 alone) and the recombinant *E. coli* JM109/pK184_*csuA/BABCDE* were imaged by scanning electron microscope (SEM, Hitachi S-5000, Tokyo, Japan)

Table 1 Yeast, bacterial strains and primers used in this study are listed.

Microbe/primer	Characteristics	Remark
Candida sp.	Yeast, a clinical strain	This study
Acinetobacter baumannii		
ATCC17978	A blood isolate causing hospital-acquired infection, kanamycin-susceptible strain (Km^s)	Standard strain [15]
ATCC17978 Δ*csuA/B*	A ΔcsuA/B strain derived from strain ATCC17978	This study
MDRAB16	Clinical carbaperem-resistant strain, ST455, Km^r	[8,18]
Escherichia coli		
JM109	A strain without pilus expression (bald cellular surface); as a cloning strain	Standard strain
$BL21_{DE3}$	A recombinant protein expression strain	Standard strain
JM109/pK184	Vector pK184, a cloning vector, kanamycin-resistant (Km^r), alone in *E. coli* JM109	This study
JM109/rCsu pilus	pK184 carrying recombinant Csu (rCsu) pilus operon (csuA/BABCDE) in *E. coli* JM109; (Km^r)	This study
$BL21_{DE3}$/pET29b-rCsuE	Protein expression vector pET29b carrying recombinant csuE (rCsuE) in *E. coli* $BL21_{DE3}$, Km^r	This study
Primers		This study
Csu-Step-1-SacI	5′-caGAGCTCgtaggttatgaatatgaaaaacattcag	Three steps for cloning of full-length csu operon of *A. baumannii* TYTH-1 (ST455)
Csu-Step-1-BamHI	5′-taaattattatgccgaaggatcatgatg	
Csu-Step-2-BamHI	5′-cgaaatcaccaaaatggaGGATCC	
Csu-Step-2-SalI	5′-attgtaaagttggaaggtaagtcg	
Csu-Step-3-SalI	5′-catcggtaacaccttattattc	
Csu-Step-3-PstI	5′-caCTGCAGttaccaaagatgatgatcagttag	
CsuE-pET29b-NdeI	5′-ggaattcCATATGaatataaaaacaaaaaaattactcag	Intact csuE of *A. baumannii* TYTH-1 (ST455)
CsuE-pET29b-XhoI	5′-caccacCTCGAGaaactcgacttgtaccgtgaccgtatc	
subCsuE$_{sub}$-pET29b-NdeI	5′-ggaattcCATATGtatcgggttgagcaaatgtcaaattc	To subclone csuE$_{sup}$ with hydrophilic and antigenic region
subCsuE$_{sub}$-pET29b-XhoI	5′-caccacCTCGAGaatcgcaactgcgggtacagaatag	
Up_CsuA/B_F	5′-cgagtttgattcattttgttc	To clone a csuA/B-deleted (ΔcsuA/B) mutant of *A. baumannii* ATCC17978
CsuA/B_kan_rpF	5′-gtggtagtcaaactgaaggaaatatgagccatattcaacgggaaacg	
CsuA/B_kan_rpR	5′-cgtttcccgttgaatatggctcatatttccttcagtttgactaccac	
kan_CsuA/B_rpF	5′-catttgatgctcgatgagtttttctaaatataaaaaagaaataattaattgagt	
kan_CsuA/B_rpR	5′-ctgctactcaattaattatttctttttttatatttagaaaaactcatcgagcatcaaatg	
Dw_CsuA/B_R	5′-ctacaacattattattaacagaacaagaag	
Km_up	5′-ccggaattgccagctggg	Kanamycin-resistant gene
Km_dw	5′-ttcagaagaactcgtcaag	
CsuA/B_Sc_2F	5′-ctaggtcattcgacctaattaatg-3′	To confirm ΔcsuA/B mutant of *A. baumannii* ATCC17978
CsuA/B_Sc_R	5′-gaattaccattattaactaaactagc	

and transmission electron microscope (TEM, Hitachi H-7500, Tokyo, Japan) in Microscopy Core Laboratory, Chang Gung Memorial Hospital, LinKou branch. The standard procedures, including fixation, dehydration, and embedding, were performed for SEM according to manufacturer's instructions. To observe intact and fragile pili, bacterial sample was prepared under steady growth condition (without shaking) and no fixation for TEM. The images were recorded at magnifications of 10,000× ~150,000X. Atomic force microscope (AFM; Dimension Icon, Bruker, Billerica, MA, United States) supported by professor Long Hsu, Department of Electrophysics, National Chiao Tung University, Hsin-Chu, Taiwan using an SNL-W-D triangular cantilever probe (Bruker) with a nominal 0.06 N/m spring constant and 2-nm tip curvature radius was used to scan the surface properties of bacteria. The images were analyzed using NanoScope Analysis 1.4 software (Bruker).[17]

Biofilm formation, adherence, and sugar-mediated inhibition

Characteristics of pilus function, including biofilm formation, adherence and sugar-mediated inhibition of adherence, were analysed. Bacterial biofilm formation using the strain ATCC17978 was assayed by crystal violet staining on polystyrene ELISA plate as described previously.[19] ELISA-based method was used to detect bacterial adherence to A549 cells (adenocarcinomic human alveolar basal epithelial cells) with multiplicity of infection (MOI) of 100 using strain MDRAB16 as described previously.[8,20] Carbohydrates (Sigma, USA), including monosaccharides: D-fucose, L-fucose, D-mannose, L-mannose, D-glucose, and D-galactose; disaccharide: sucrose; pyranosides: methyl-α-L-fucopyranoside, methyl-β-L-fucopyranoside, and methyl-α-D-mannopyranoside; mannosylated (51 mannoside

molecules), through AI (amidino) linkage, bovine serum albumin (BSA) neoglycoprotein (D-mannose polymer Man_{51}-AI-BSA) and α-mannose-modified copolymer (S.T-4 copolymer) were used to compete recognition site of pili and inhibit bacterial adherence to A549 cells as described previously.[21,22]

Agglutination assay

The anti-rCsuE$_{sub}$ polyclonal antibodies generated in rabbits were used for analysis of Western immunoblot and agglutination assay. The activity of D-mannose-recognizing lectin was tested by yeast-mediated agglutination because yeast commonly contains abundant D-mannose on the cell surface.[23] To perform agglutination assay, 20 μL of PBS (phosphate buffered saline, pH = 7.4) was added on a glass slide, followed by smearing a single colony of rCsu-producing *E. coli* JM109 on the slide. The smeared rCsu-producing *E. coli* JM109 was mixed with anti-rCsuE serum (1 μL), compared to mix with pre-immune serum (1 μL) as a negative control. Yeast *Candia lusitaniae* CL01 containing abundant D-mannose on yeast cell surface was used to test whether rCsu-producing *E. coli* JM109 contains D-mannose-recognition lectin-like domain.

Statistical analysis

Statistical analysis in individual triplicate experiments was performed using software Graphpad Prism® 7.03 (La Jolla, CA, USA). The significance was determined using Tukey's Multiple Comparison test (one-way ANOVA) and unpaired *t*-test. $P < 0.05$ was considered statistically significant.

Results

Morphology of Csu pilus

The morphology of rCsu pilus in rCsu pilus-producing *E. coli* JM109 with full-length Csu pilus operon *csuA/BABCDE* of *A. baumannii* imaged using TEM and AFM, compared to *A. baumannii* ATCC17978 is shown, respectively, while no pilus was shown on bald *E. coli* JM109/pK184 (Fig. 1A and B). The produced Pili were peritrichous, but too fragile to be seen with an intact image when bacteria sample was cultured under a shaking condition and prepared with a fixation treatment for SEM (data not shown). For better picturing for pili morphology, bacterium was cultured without shaking to prevent the breakage of pilus from bacterial surface. Therefore, bacterium was carefully prepared without fixation treatment for TEM; however, damaged and broken pili on bacterial cell surface were still observed in TEM image (Fig. 1A). The circumambient pili of *A. baumannii* ATCC17978 and the rCsu pilus-producing *E. coli* JM109 were more clearly observed by AFM than by TEM. In addition, the Δ*csuA/B* mutant of *A. baumannii* ATCC17978 imaged using AFM shows no or less pilus production, while abundant bundles of pili on the peritrichous surface of wild-type *A. baumannii* were observed (Fig. 1C).

Biofilm formation and sugar-mediated inhibition

To examine the role of Csu pili of *A. baumannii* in biofilm formation, the rCsu pilus-producing *E. coli* JM109 cloned in this study as shown in Supplementary Fig. 1A was assayed. We found that the biofilm formation ability in *A. baumannii* ATCC17978 was significantly greater than in the rCsu pilus-producing *E. coli* JM109 (Fig. 2A). In addition, the rCsu pilus-producing *E. coli* JM109 showed significant increase in biofilm formation upon induction with IPTG when compared to *E. coli* JM109/pK184 vector alone (Fig. 2A).

Type 1 pilus is known to be defined mannose-sensitive.[24] However, whether Csu pili of *A. baumannii* belongs to type 1 pilus was unknown before this study. Different concentrations of sugars (10, 50 and 250 mM), including D-glucose, D-mannose and methyl-α-D-mannopyranoside were used to test *A. baumannii* ATCC17978, ΔCsuA/B-pilus-knockout mutant of *A. baumannii* ATCC17978, rCsu pilus-producing *E. coli* JM109, and *E. coli* JM109/vector alone. The ΔCsuA/B mutant of *A. baumannii* exhibited a significant decrease of biofilm formation ($P < 0.0001$; *t*-test), compared to the wildtype *A. baumannii*, but no effect on biofilm formation of *A. baumannii* with the treatment of both 10- and 50-mM sugars except with 250-mM D-mannose and methyl-α- D-mannopyranoside was observed (Fig. 2B and C). Meanwhile, the bacterial densities showed no significant difference among those tested bacteria with different doses of sugar treatments on the moment of biofilm assay (Supplementary Fig. 3). Moreover, similar trends of biofilm reduction were observed in rCsu pilus-producing *E. coli* JM109 and *E. coli* JM109/vector alone with both supplement of D-mannose and methyl-α-D-mannopyranoside (Fig. 2C). In addition, 10 mM was an enough concentration for mannose or mannose-containing compound to significantly inhibit biofilm formation in Csu pilus-producing *E. coli* JM109, and the concentration of 10 mM was then used for other sugars in following experiments.

Adherence of Csu pili of *A. baumannii* to respiratory epithelial cells and sugar-mediated inhibition

The adherence of *A. baumannii* ATCC17978 ΔCsuA/B mutant to respiratory A549 cells significantly increase than that of wildtype; however, it was no significant difference between the supplement with and without 10-mM D-mannose (Fig. 3A). In addition, the rCsu pilus-producing *E. coli* JM109 showed a higher adherence to A549 cells than parental *E. coli* JM109, but it was much significantly less than *A. baumannii* ATCC17978 (Fig. 3B). In sugar-mediated inhibition assays of adherence, D-mannose but not sucrose significantly inhibited the adherence of the Csu pilus-producing *E. coli* JM109 to A549 cells (Fig. 3C). Moreover, 50% D-mannose-mediated inhibition concentration (IC_{50}) for the rCsu pilus adherence to A549 cells was determined to be 1.3 mM (Fig. 3D).

Other carbohydrates, including monosaccharides (D-fucose, L-fucose, L-mannose, D-glucose and D-galactose), pyranosides (methyl-α-L-fucose, methyl-β-L-fucose, and methyl-α-D-mannose) and polymers (neoglycoprotein Man_{51}-AI-BSA and α-Man-modified copolymer), were also

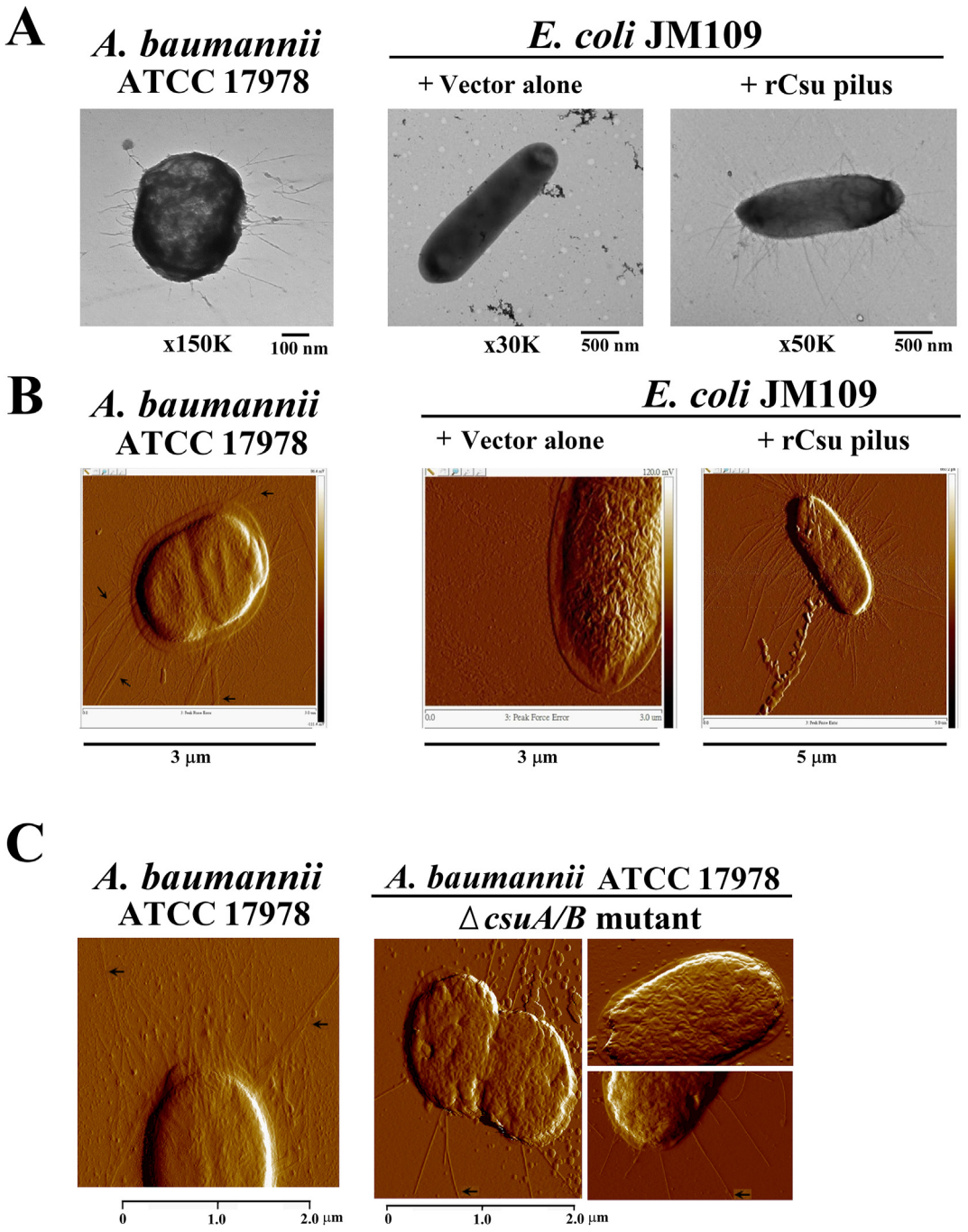

Figure 1. Pilus of *A. baumannii* ATCC17978 expressed in *E. coli* JM109. The morphology of *A. baumannii* ATCC17978 is shown using non-fixation TEM (A) and AFM (B and C). The image of pilus (hair-like) productions in *E. coli* JM109 carrying pilus-producing *csuA/BABCDE* in vector pK184 is demonstrated in non-fixation TEM (A) and AFM (B and C). The ΔCsuA/B mutant of *A. baumannii* ATCC17978 is pictured using AFM (C). Images are magnified ranging from 30K to 150K. The AFM scanning size is 3—5 nm x 3—5 nm with scan rates of 1Hz. The scale bar is indicated on each panel.

tested for inhibition of rCsu-pilus adherence to A549 cells using *E. coli* JM109 as an assay system. Among supplements with monosaccharides, D-mannose significantly inhibited rCsu pilus adherence to A549 cells, compared to those without sugar supplements, as shown in Fig. 4A—C. Among supplements with pyranosides, only methyl-α-D-mannopyranoside significantly inhibited the rCsu-pilus adherence to A549 cells (Fig. 4B). Among supplements with polymers, both Man_{51}-AI-BSA and α-mannose-modified copolymer showed slightly adherence inhibition in rCsu pilus adherence to A549 cells (Fig. 4C).

Agglutination of lectin-like Csu pilus

To characterize the lectin activity of rCsu pilus, rCsu pilus-producing *E. coli* JM109 was used as a target for agglutination assay, whereas rCsu pilus-producing *E. coli* JM109

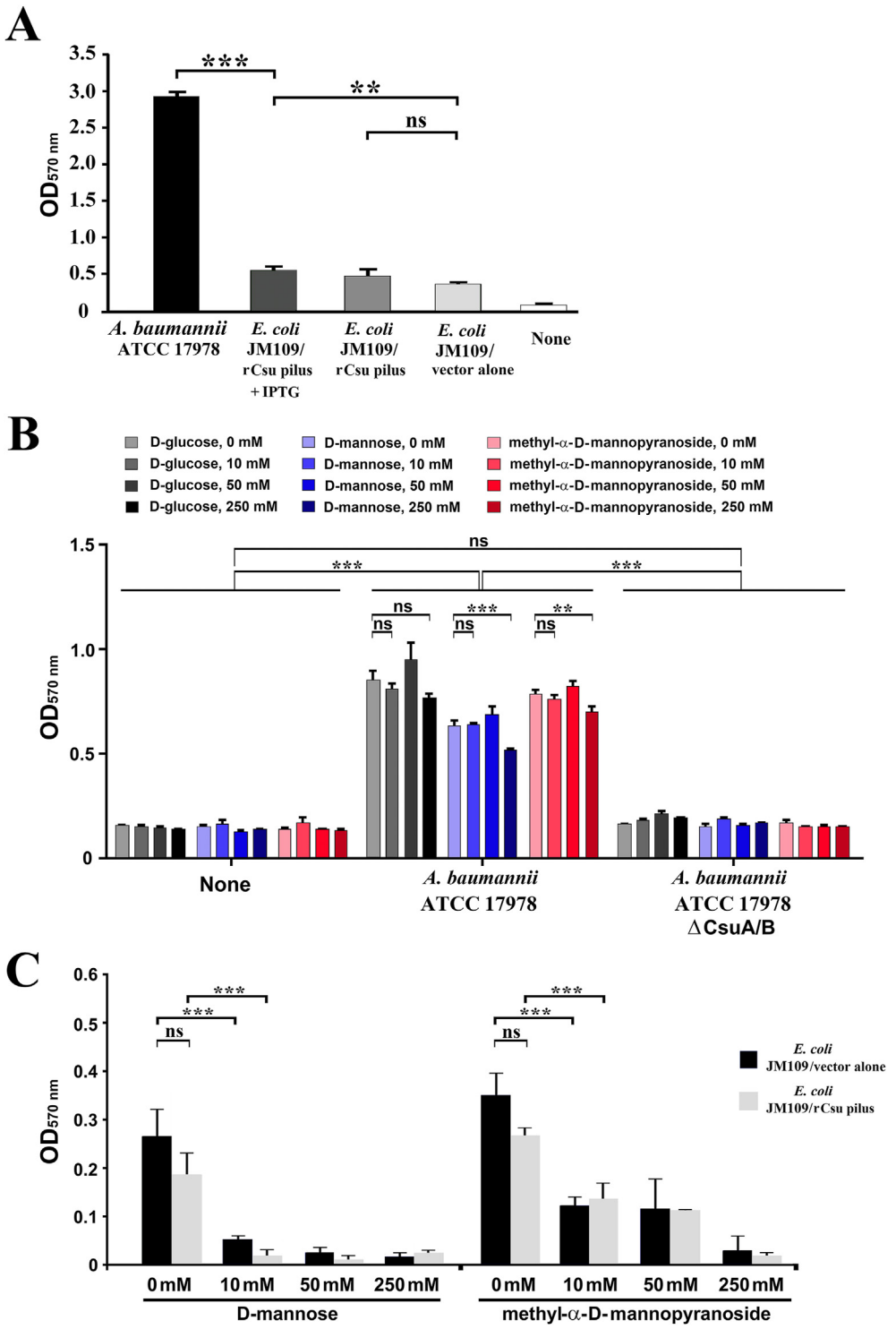

Figure 2. Formation and inhibition of biofilm. (A) Biofilm formation of *A. baumannii* ATCC17978, *A. baumannii* ATCC17978 ΔCsuA/B mutant, *E. coli* JM109, and Csu pilus-producing *E. coli* JM109 (carrying pK184-*csuA/BABCDE*) with or without IPTG under non-shaking growth condition. Serial doses (10, 50 and 250 mM) of sugars, including D-glucose, D-mannose and methyl-α-D-mannopyranoside, were tested for biofilm inhibition in (B) *A. baumannii* ATCC17978 and its ΔCsuA/B mutant, as well as in (C) *E. coli* JM109 and Csu pilus-producing *E. coli* JM109. None: no bacterial treatment. Significance **: $P < 0.01$, ***: $P < 0.001$; ns: no significance.

alone and pre-immune anti-serum were used as negative controls (Fig. 5A and B). Agglutination was observed both in the treatment of anti-CsuE polyclonal antibodies against rCsu pilus-producing *E. coli* and in the mixture of rCsu pilus-producing *E. coli* with D-mannose-abundant yeast *Candida* (Fig. 5C and D).

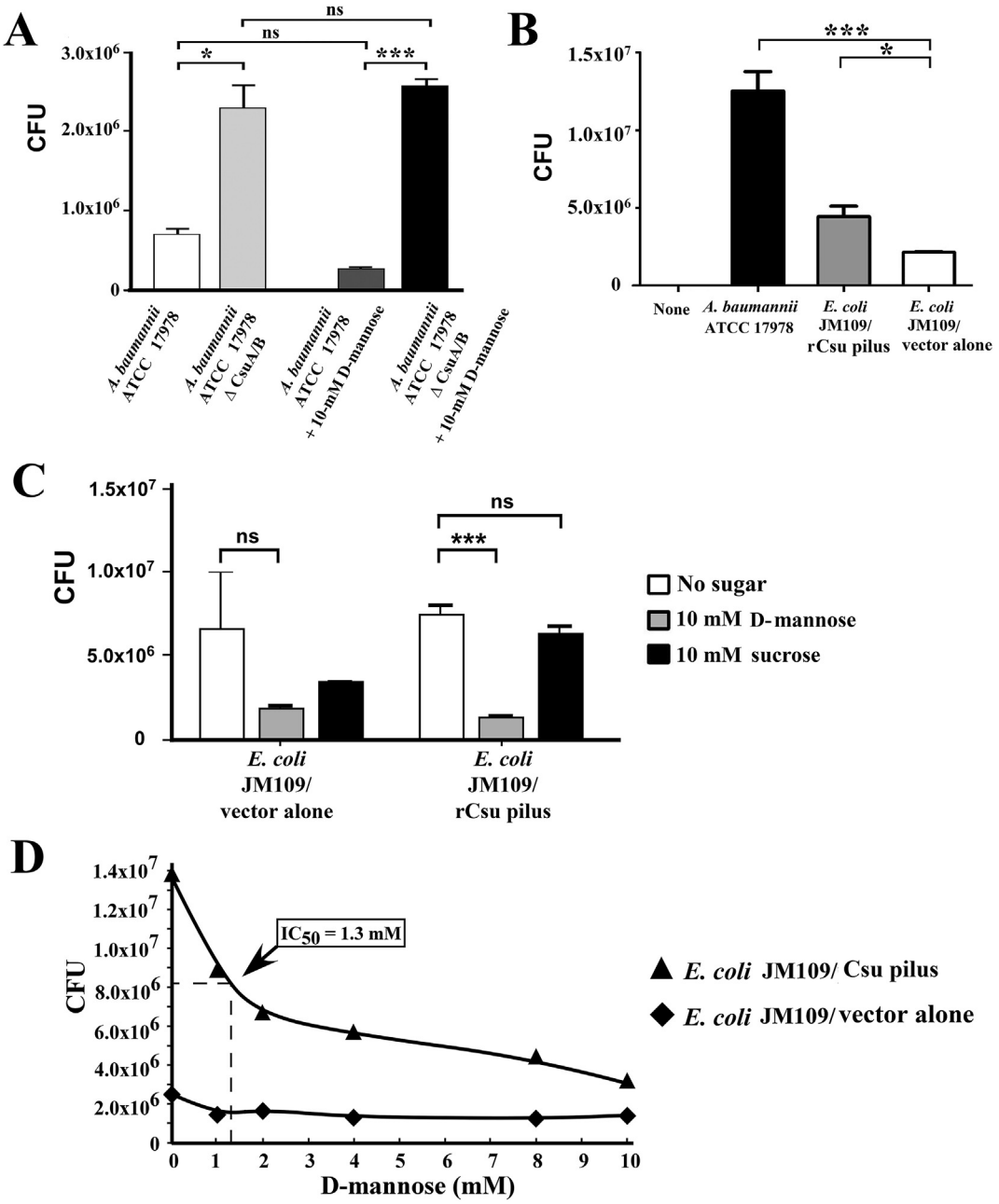

Figure 3. Adherence and mannose-mediated inhibition to respiratory epithelial A549 cells with MOI of 100. Comparisons of bacterial adherence (A) between *A. baumannii* ATCC17978 and its isogenic ΔCsuA/B mutant with or without supplement of 10-mM D-mannose and (B) among *A. baumannii* ATCC17978, *E. coli* JM109 and recombinant Csu-pilus-producing *E. coli* JM109. (C) Sugar-mediated inhibition of bacterial adherence to A549 cells between *E. coli* JM109 and recombinant Csu-pilus-producing *E. coli* JM109 using 10-mM D-mannose and 10-mM sucrose is compared. (D) Serial doses of D-mannose (1, 2, 4, 8, 10 mM) were tested, and 50% of inhibitory concentration (IC_{50}) was assessed. MOI is 100 (1.0×10^6 A549 cells). "ns": no significance. Significance *: P value < 0.05; ***: P value < 0.001. None: no bacterial treatment. CFU: colony formation unit.

Discussion

Carbohydrate specificity of pilus lectins, initiating bacterial adherence to respiratory epithelial cells, carbohydrate-mediated (including monosaccharides, pyranosides, and glycopolymers) inhibition of bacterial adherence that may potentially block bacterial infections, and pilus lectin-specific antibodies for theoretically developing specific diagnosis system to *A. baumannii* and blocking bacterial adherence/infection to host cells were performed in this study.

In the analysis of pili sequence in *A. baumannii* genome, only *csuA/BABCDE* and *fimA1-papCD-fimA* operons were found in strain ATCC17978. The identity of *csuA/BABCDE* operon between strain ATCC17978 and other multi-drug resistant strains, such as popular sequence

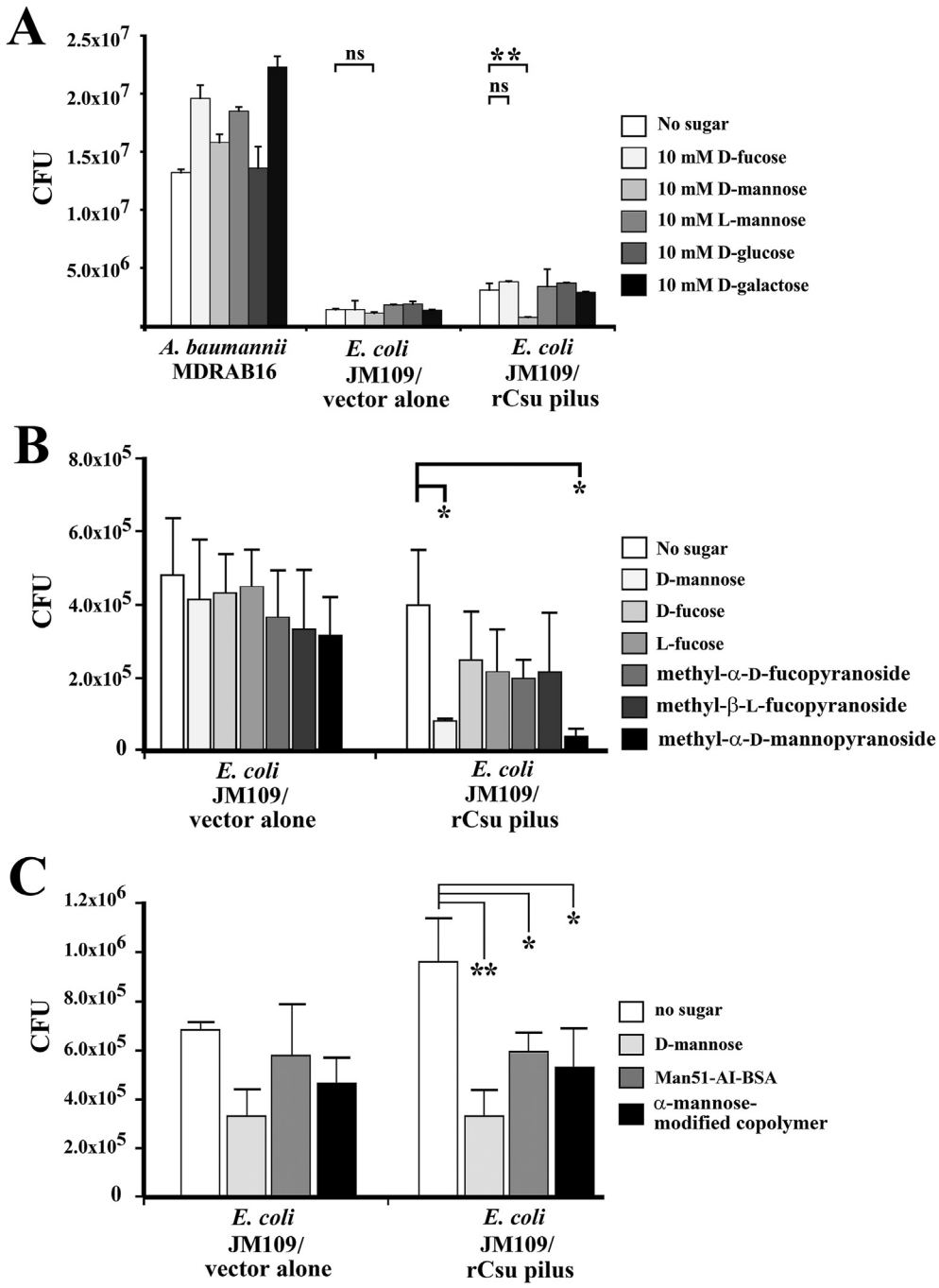

Figure 4. Bacterial adherence inhibition using different sugars. Sugars (10 mM/each) used for inhibition of adherence to respiratory epithelial A549 cells with MOI of 100 among *A. baumannii* MDRAB16, *E. coli* JM109, and recombinant Csu-pilus-producing *E. coli* JM109 were monosaccharides, including D-mannose, L-mannose, D-fucose, L-fucose, D-glucose and D-galactose (A), pyranosides, including methyl-α-D-fucopyranoside, methyl-β-L-fucopyranoside and methyl-α-D-mannopyranoside (B), and mannose-polymer, including Man_{51}-AI-BSA and α-D-mannose-modified copolymer (C). Significance *: *P* value < 0.05; **: $P < 0.01$; ns: no significant.

types ST208 and ST455,[15,18] is high (>97%). Similar to previous study using Csu knockout mutant of *A. baumannii* ATCC 19606,[11] the mutant of ATCC17978 showed reduced biofilm formation on abiotic surfaces and increased adherence to respiratory epithelial cells in this study. In addition, the mutant became bald on bacterial surface according to the images by AFM and EM, indicating that CsuA/BABCDE was the major pili on the surface of *A. baumannii*. As Csu pilus production is lost, some other adhesins (such as type 4 fimbrial pilus PilA and outer membrane protein OmpA) could in turn be exposed, and subsequently, contribute for more bacterial adherence to epithelial cells.[25,26] It is the reason why the adhesive fimbriae have been reported as a virulence factor associated with pathogenesis and involved in biofilm formation on abiotic surfaces.[27]

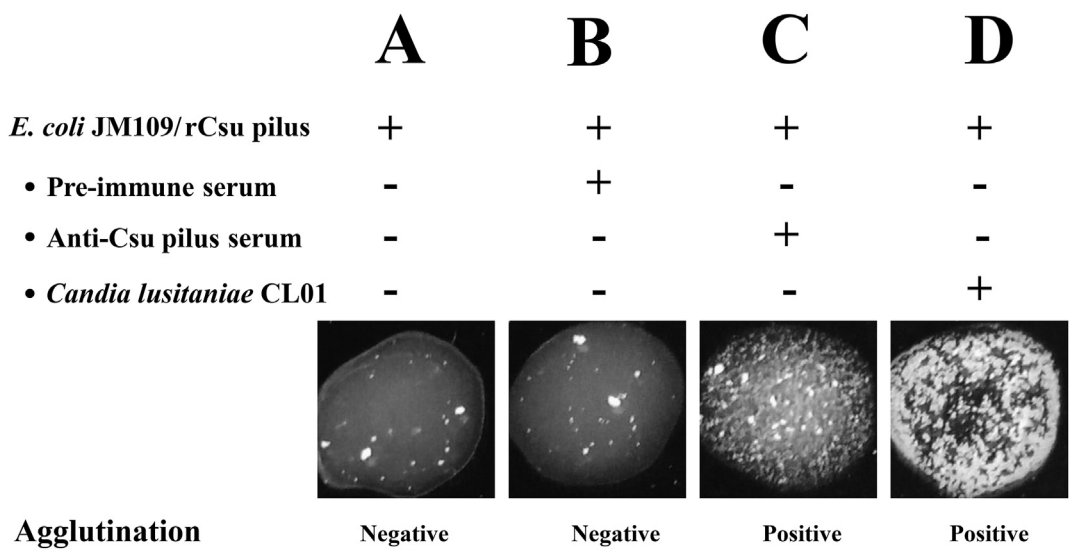

Figure 5. Agglutination assay. Agglutination of rCsu-pilus-producing *E. coli* JM109 (A) with pre-immune serum (B), anti-$CsuE_{sub}$ serum (C), and mannose-abundant *Candia lusitaniae* CL01 (D) is shown.

Given the analysis of SDS-PAGE, the production of cloned CsuE was less solubly expressed, and present in pellets. To solve the difficulty of recombinant protein solubility, antigenic and hydrophilic region of CsuE was subcloned, and $rCsuE_{sub}$ was then solubly well-expressed in *E. coli* $BL21_{DE3}$ and purified. The weak signal using anti-$rCsuE_{sub}$ antibodies against CsuE of *A. baumannii*, including two strains ATCC17978 and MDRAB16, was observed in Western blot analysis (Supplementary Fig. 2C). However, the fact that the native *A. baumannii*-produced Csu pili could not be recognized by the antibodies raised in rabbit using *E. coli* $BL21_{DE3}$-produced $rCsuE_{sub}$ as an antigen is still unknown. The reason could be because modifications on pilus protein occur during pilus protein production and assembling among various organisms are different. Thus, the antibodies raised by *E. coli*-producing $rCsuE_{A.\ baumannii}$ protein as an antigen could not recognize the CsuE of *A. baumannii*. If so, the recombinant $Csu_{A.\ baumannii}$ protein produced in *E. coli* could not be an idea vaccine candidate against pathogenic *A. baumannii*.

Pilus structure is too fragile to maintain its intact morphology using regular standard procedure for SEM and TEM except no agitation for bacterial growth culture and no fixation and dehydration for EM sample preparation. In terms of the facilitation of pilus sampling and processing in this study, AFM seems better than EM. The reason to explain why pilus is fragile is because pilus units are noncovalently assembled. Interestingly, abundant Csu pili are produced, assembled and formed significant bundles on the surface of *A. baumannii*, but not so much observed in the rCsu-producing *E. coli* clone in our AFM assay. If, therefore, would like to affect the equilibrium of bacterial attachment (or recognition) to host, bacterium-to-bacterium aggregation, microcolony formation and biofilm maturation, more mannose (as high as 250 mM) for neutralizing the interactions of Csu-pili and its free-formed Csu units with abiotic surfaces or with *A. baumannii* itself could be required, as seen in the inhibition of biofilm formation.

This is the first study to verify the carbohydrate target specifically recognized by *A. baumannii* Csu pilus which was cloned and expressed from *A. baumannii* into pilus-free *E. coli* JM109. The adherence of rCsu pilus-producing *E. coli* strain to A549 cells could be inhibited by mannose-related carbohydrates, including D-mannose, methyl-α-D-mannose and D-mannose polymers Man_{51}-AI-BSA, and α-mannose-modified copolymer. Also, rCsu pilus contains mannose-recognition lectin domain because rCsu pilus-producing *E. coli* JM109 formed agglutination with abundant mannose-containing *Candida* yeast.[23] Given the finding in this study, we suggested that the mannose-sensitive Csu pilus is one of type 1 pili rather than mannose-resistant P pili according to the definition of Sarowar et al.,[27] although high similarity of Csu pilus operon sequence to P pilus was observed.

Interestingly, in our experimental conditions, D-mannose and other tested monosaccharides could slightly but not significantly inhibit or even increase wild-type *A. baumannii* ATCC17978 adherence to A549 cells. This could be explained by the fact that not just mannose-sensitive Csu pili are present on the surface of *A. baumannii*, but other mannose-resistant adhesins or adhesive fimbria, including OmpA, TonB-dependent copper receptor and 34-kDa fibronectin-bound outer membrane protein reported for adherence to A549 cells,[26] could also synergistically contribute to the adherence and biofilm formation. For example, the adhesin could be OmpA that has been reported as an extracellular matrix fibronectin-binding protein.[26] On the other hand, monosaccharides could serve as a nutrient source or a stimulant, and in turn promote bacterial adherence as well as biofilm formation via unknown mechanism. In addition, Cevahir et al. (2008) have ever shown that the presence of mannose could not affect *A. baumannii*-mediated agglutination to human erythrocytes.[28] Since cell surface of A549 and *A. baumannii* exhibit glycan heterogeneity,[29] in addition to mannose, Csu pilus may also bind to other carbohydrates (such as fucose) present on A549 cells and *A. baumannii* cells with low specificity. Further investigations need to be tested

for identifying the role of Csu pilus in adaptation to host niches and the inhibitory potency of polyvalent mannose derivatives in wild-type *A. baumannii* ATCC17978 adherence to A549 cells.

The prevalence of carbapenem resistance among UTI samples reduces the therapeutic options.[7] One of the most effective drugs to treat UTI is colistin; however, its systemic application is associated with nephrotoxicity and neurotoxicity, and the number of cases treated topically to reduce the toxicity is low.[6] Therefore, new strategies targeting the bacterial fimbria to treat UTI are being developed using mannoside-based antiadhesives, including indolinylphenyl mannoside and D-mannose.[30,31] A recent study reported a successful practical prophylaxis using D-mannose to reduce the risk of recurrent UTI patients by 53% in a cohort of complex paediatric urology patients, suggesting D-mannose could be also a potential drug for UTI treatments.[32]

The WHO developed a global priority pathogens list of antibiotic-resistant bacteria to help in prioritizing the research and development of new and effective antibiotic treatments or control strategies.[33] Carbapenem-resistant *A. baumannii* is one of the top three critical MDR bacteria shown in the list that requires immediate attention. The prevalence of *A. baumannii*-mediated UTI and pneumonia are more than 200 and 800 cases per annum in Chang Gung Memorial Hospital, Taoyuan, Taiwan, respectively (Supplementary Table 1). Abundant pili on surface of *A. baumannii* contributing for adherence to epithelial cells of urinary tract and respiratory tract and biofilm formation are ideal targets for developing control strategies against its infections. At least four distinct assembly pathways for pilus formation are known, including type 1 and type P pili (chaperone-usher pathway), curli pili (extracellular nucleation-precipitation pathway), CS1 pili (alternate chaperone pathway), and type IV pili (general secretion pathway).[34] The limitation of the study is that we did not examine the adhesins of wild-type *A. baumannii* and mutants to uroepithelial cells.

In conclusion, this study suggests that Csu pilus of *A. baumannii* is a D-mannose-sensitive type 1 pilus. This is the first study to characterize the Csu pilus of *A. baumannii* related to pilus morphology, biofilm formation on abiotic surface, adherence to respiratory epithelial cells. Carbohydrate D-mannose could be significantly antagonistic against *A. baumannii* adherence to epithelial cells, but could not prevent biofilm formation of Csu pilus-carrying *E. coli* recombinants and wild-type *A. baumannii*. The Csu pilus could be an ideal target to develop new drugs or diagnostic methods for infection control and antimicrobial therapy, especially for difficult-to-treat carbapenem-resistant *A. baumannii* infections.

Conflicts of interest

All authors declare no competing interests.

Acknowledgements

The study was financially supported by grants CMRPG3 G0981-2 and CMRPG3J1851-2 from Chang Gung Memorial Hospital, Taiwan, and grant 105-2627-M-182A-001 from the Ministry of Science and Technology, Taiwan. We thank Dr. Yuan-Chuan Lee, Department of Biology, Johns Hopkins University, Baltimore, MD, USA, for gifting D-mannose polymer Man51-AI-BSA and Dr. Koji Matsuoka, Division of Material Science, Graduate School of Science and Engineering, Saitama University, Sakura, Saitama, Japan, for gifting S.T-4 copolymer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.01.008.