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Microbiological features and clinical impact of the type VI secretion system (T6SS) in *Acinetobacter baumannii* isolates causing bacteremia

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ABSTRACT

We investigated the genetic background and microbiological features of T6SS-positive *Acinetobacter baumannii* isolates and clinical impact of the T6SS in patients with *A. baumannii* bacteremia. One hundred and 62 *A. baumannii* isolates from patients with bacteremia in 2 tertiary-care hospitals in Korea were included in this study. Approximately one-third (51/162, 31.5%) of the *A. baumannii* clinical isolates possessed the *hcp* gene, and the *hcp*-positive isolates were found in several genotypes in multilocus sequence typing. The expression and secretion of Hcp protein varied among the clinical isolates. *A. baumannii* isolates with detectable Hcp secretion (T6SS+) could better outcompete *Escherichia coli* compared with T6SS- isolates, including *hcp*-negative and inactivated *hcp*-positive isolates. In addition, T6SS+ isolates showed higher biofilm-forming activity and better survival in the presence of normal human serum than the T6SS− isolates. T6SS+ isolates were more frequently detected in patients with catheter-related bloodstream infection, haematopoietic stem cell transplant recipients, and patients receiving immunosuppressive agents. However, T6SS was not a prognostic factor for mortality. Our results suggest that the T6SS of *A. baumannii* is associated with virulence and contributes to infections in immunocompromised patients and those with implanted medical devices.

Introduction

Protein secretion systems are characteristic components on the cell surface of gram-negative bacteria. In many bacteria, these systems export effector proteins, which are often mediators of virulence factors. The type VI secretion system (T6SS) has been recently shown to be important for microbial communication within human hosts and the environment.[1] These systems were first identified in *Vibrio cholerae* and *Pseudomonas aeruginosa*, and they were shown to play a role in the defense against eukaryotic hosts.[2,3] Since then, many bacterial species, including *P. aeruginosa*, *Pseudomonas syringae*, *Vibrio parahaemolyticus*, *V. cholerae*, *Burkholderia thailandensis*, *Serratia marcescens*, and *Citrobacter rodentium*, have been shown to use a T6SS to mediate interbacterial antagonism and increase their fitness in competition with other bacteria.[4]

The T6SS is structurally and functionally similar to a bacteriophage tail,[4,5] and it consists of 2 membrane-associated subunits with cytoplasmic elements, an anchoring assembly and an assembly with components that structurally resemble the bacteriophage sheath, tube, and tail spike proteins. These cooperate to translocate effector proteins across the envelope of the cell and inject them into the target cell membrane through a contractile phage tail-like apparatus.[5,6] The phage-like assembly has 3 components, hemolysin coregulated protein (Hcp), which forms a tubular structure that is secreted out of the cell, valine-glycine repeat G (VgrG), which forms the tip of this structure and has effector activity or promotes effector secretion, and TssB and TssC, which form a sheath that contracts to provide the energy for effector transport.[7] Since Hcp and VgrG are shed into the extracellular milieu by activation of the system, they serve as

molecular markers of a functional T6SS. Several studies have shown that the T6SS transports bacteriolytic effector proteins into target cells in a contact-dependent manner.[8-10] However, the role of T6SS beyond that of bacterial antagonism is unclear.

Recently, T6SS-encoding genes were identified in *Acinetobacter baumannii*,[11] and T6SS-positive *A. baumannii* strains were shown to have cytotoxic activity against competing bacteria.[12-15] However, the mechanisms of T6SS-dependent effectors, functions of the effectors within target cells, and regulatory systems in *A. baumannii* remain largely unknown. In some bacterial isolates, the T6SS was identified but was not activated. It was recently reported that the T6SS of *A. baumannii* was repressed by a repressor encoded by *tetR*, which is placed in a plasmid.[15]

In this study, we aimed to investigate the role of the T6SS in *A. baumannii* both in and around the human host. The function of T6SS was assessed by *in vitro* experiments, including competition, biofilm formation, and serum resistance assays. In addition, we conducted a retrospective study to determine its clinical impact in patients with *A. baumannii* bacteremia.

Materials and methods

Bacterial isolates

A total of 228 clinical isolates were collected from patients with *Acinetobacter* bacteremia during the period from January 2012 to December 2015 in 2 tertiary-care hospitals in South Korea, Samsung Medical Center (Seoul) and Samsung Changwon Hospital (Changwon). Using partial *rpoB* gene sequences (468 bp), *A. baumannii* was distinguished from other *Acinetobacter* species.[16,17] Of the 228 isolates, 162 (71.1%) were identified as *A. baumannii* and were analyzed in this study.

Antimicrobial susceptibility testing

In vitro antimicrobial susceptibility testing was performed for all *A. baumannii* isolates using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI).[18] Nine antimicrobial agents were tested, including imipenem, ceftazidime, ampicillin-sulbactam, gentamicin, piperacillin-tazobactam, ciprofloxacin, trimethoprim-sulfamethoxazole, colistin, and tigecycline. CLSI susceptibility interpretive criteria were used.[18] *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *P. aeruginosa* ATCC 27853 were used as control strains.

Multilocus sequence typing (MLST)

All *A. baumannii* clinical isolates were genotyped by MLST using the Oxford scheme as described previously.[19] The sequence of each allele was compared with sequences in PubMLST databases by BLAST, and sequence types (STs) were designated according to the allelic profiles. Newly identified allelic profiles and STs were submitted to the PubMLST database and approved (http://pubmlst.org/abaumannii). Based on the MLST data, the genetic relationships and clonal complexes (CCs) of the isolates were analyzed and represented in a minimum-spanning tree using the PHYLOViZ program.[20]

Detection of the hcp gene and quantitative reverse transcription PCR (qRT-PCR) to determine hcp expression levels

To identify strains containing T6SS, the *hcp* gene was amplified with the primers hcp-F (5′-TGCTGAGCGTG TTGAACATT-3′) and hcp-R (5′-ACGTTTATCGCCAT TTGCAC-3′). *hcp* expression levels were determined by qRT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems) with primers, hcp-QF (5′-TGCTGAGCGTG TTGAACATT-3′) and hcp-QR (5′-ACGTTTATCGCCA TTTGCAC-3′). These primers for the amplification and qRT-PCR were designed based on the sequences of A. baumannii strain ACICU (GenBank accession number CP000863), using Primer 3 online software. Expression of the *rpoB* gene was assessed in parallel to calculate the fold changes according to the threshold cycle (CT) method.[21] The experiments were repeated with 3 independent cultures, each tested in duplicate.

We assayed the presence of *tetR* in the isolates in which *hcp* did not express despite the presence of *hcp* gene using the primers tetR18-F (5′-ATGACTAAAGT-TATTTCAAAAAGAAAAACC-3′) and tetR18-R (5′-AG CCTCAAATGTCTGAATTAGACTC-3′) as described previously.[15] The presence of T6SS was confirmed by detection of another gene in T6SS, *tssM*, with primers tssM-F (5′-GCAAAGACGTCTACAACAGTTAGAC-3′) and tssM-R (5′-TTGCTGATCATCACGTTTACGAAC-3′), in addition to *hcp* gene.

Western blotting

Cloning and overexpression of histidine-tagged Hcp was performed as described previously,[12,13] with a few modifications. The *hcp* gene of *A. baumannii* ATCC 19606 was amplified using primers Hcp-(N)F

(5′-CCAACCATGGGCATGAAAGATATATACGTTG AGTT-3′) and Hcp-(H)R (5′-CCAAAAGCTTAGCTG CGTAAGAAGCTGTAT-3′) and cloned into the pET28a vector to generate a C-terminal histidine-tagged fusion of the Hcp protein. The resultant plasmid, pET28-Hcp (C-His), was electroporated into *Escherichia coli* BL21 cells. The histidine-tagged Hcp protein was purified with the QIAexpress Ni-NTA Fast Start Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified Hcp protein was sent to AbFrontier antibody services (Young In Frontier Co. Ltd., Seoul, Korea) to generate rabbit-derived polyclonal antibodies.

To prepare cell-free supernatants for the Hcp secretion assay, overnight bacterial cultures were inoculated 1:100 into 20 mL of fresh Luria-Bertani (LB) broth and grown to stationary phase. After approximately 4 h, bacteria were harvested by centrifugation, and the culture supernatants were collected and filtered through 0.22-μm syringe filters (Millipore Corporation, Billerica, MA, USA) to obtain cell-free supernatants. The cleared supernatants were concentrated by filtration through Amicon Ultra-15 10K centrifugal filters (Merck Millipore, Germany) according to the manufacturer's protocol. The proteins in the recovered concentrated supernatants were analyzed by SDS-PAGE. OD_{600}-normalized volumes of whole cells or supernatants were loaded onto 12% SDS-PAGE gels for separation, and the contents of the gels were transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 1 h and probed with polyclonal rabbit anti-Hcp (1:5000; Young In Frontier Co. Ltd.) and rabbit monoclonal anti-RNA polymerase β-subunit (1:2000; Abcam, Cambridge, UK) antibodies. A horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10000; Abcam) was used to visualize Hcp along with SuperSignal west Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific., Rockford, IL, USA) according to the manufacturer's instructions.

In vitro *competition assay*

The relative fitness of *A. baumannii* isolates against *E. coli* strain MG1655 was determined by calculating an *in vitro* competitive index (CI) using a described previously method[13] with some modifications. Overnight cultures of the *A. baumannii* isolates and the *E. coli* strain were inoculated to obtain a 0.5 McFarland standard and diluted 1:50 in 10 mL of LB broth. The diluted cultures were then mixed at a 2:1 predator:prey ratio and incubated at 37°C and 180 rpm for 20 h. The number of cells for each strain was determined by spreading serial 10-fold dilutions onto LB agar plates with or without 100 mg/L ampicillin. The CI was defined as the ratio of ampicillin-resistant CFUs (*A. baumannii*) to ampicillin-susceptible CFUs (*E. coli*). Eight independent competition experiments were performed.

Biofilm formation assay

To assess biofilm formation, 96-well microtiter plate assays were performed as described previously,[22] with minor modifications. Briefly, overnight cultures were normalized by the OD_{600} and diluted 1:100 in LB supplemented with 0.5% glucose. A 200-μL aliquot of this suspension was inoculated into the wells of a 96-well flat-bottom polystyrene plate and incubated for 18 h at 37°C. Sterile LB was added to one well, which served as a control. After incubation, planktonic bacteria were removed, and each well was gently washed and air-dried. Biofilm bacteria remaining in the well were stained with 0.5% crystal violet for 15 min and suspended in 200 μL of 95% ethanol. The OD_{600} was then measured with a microplate reader, yielding a measure of biofilm formation relative to the control. Experiments were performed with 3 independent cultures, each tested in duplicate.

Serum resistance assay

Serum resistance assays were performed as described previously,[23] with minor modifications. Briefly, overnight bacterial cultures were diluted 1:100 into 10 mL of fresh LB medium and incubated until the bacterial suspension reached an OD_{600} of 0.5. Then, a 1-mL aliquot of the culture was washed with phosphate-buffered saline (PBS) and resuspended in 1 mL of PBS. Next, 100 μL of the bacterial suspension was added and mixed with 300 μL of normal human serum (NHS). After mixing, the serum-bacteria suspensions were incubated at 37°C for 3 h. To calculate the serum bactericidal effect, a 100-μL aliquot was taken from each suspension before and after the 3-h incubation period, serially diluted, and plated. The serum bactericidal effect was expressed as the ratio of the CFUs in the serum-bacteria suspension to the CFUs in a bacterial suspension without NHS. All experiments were performed in triplicate, and results are expressed as percent survival.

Clinical data collection

We retrospectively reviewed the electronic medical records of patients with *A. baumannii* bacteremia. Patients under 18 y of age were excluded. Data were collected, including demographics, underlying diseases, comorbidities, acquisition of infection, antimicrobial regimens, and outcomes. The severity of illness was measured with the Pitt bacteremia score and Charlson

weighted index of comorbidity (WIC).[24,25] Drug resistance, multidrug-resistance (MDR), extensive drug resistance (XDR), and pandrug-resistance (PDR) were defined as described by Magiorakos et al.[26] Neutropenia was defined as an absolute neutrophil count of fewer than 500 neutrophils/mm^3. Acute kidney injury (AKI) was defined as an absolute increase in serum creatinine equal to or greater than 0.3 mg/dL or a percentage increase in serum creatinine equal to or greater than 50% from the baseline level within 48 h.[27] Hospital-acquired infection was defined as an infection that occurred more than 48 h after admission to the hospital.[28] The primary source of bacteremia was determined by the investigators based on medical records. Primary bacteremia was considered when no infection focus was diagnosed. All-cause mortality at 14 and 30 d was calculated from the day of blood sampling on which bacteremia was identified. The definition of appropriate antibiotics was an antimicrobial regimen that included at least one parenteral antibiotic active against *A. baumannii in vitro*. As an observational study, antimicrobial regimens were not standardized but selected by primary care physicians.

Statistical analysis

Student's *t*-test and one-way ANOVA were used to compare continuous variables. The χ^2 and Fisher's exact test were used to compare categorical variables. A stepwise logistic regression analysis was used to control for potential confounding factors. Variables with a *P* value less than 0.1 in the univariate analysis were included in the multivariate logistic regression model to control for confounding factors and identify independent risk factors for mortality. All *P* values were 2-tailed, and for all analyses, a *P* value less than 0.05 was considered statistically significant. Data were analyzed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

The prevalence of T6SS in A. baumannii *clinical isolates*

Among the 162 *A. baumannii* clinical isolates, 51 isolates (31.5%) contained an *hcp* gene. MDR and XDR were identified in 144 (88.9%) and 132 (81.5%) isolates, respectively (Table 1). One isolate was resistant to all antimicrobial agents tested in this study. The carbapenem resistance rate was 84.6% (137 isolates), of the resistant isolates, 99.6% (132/137) and 73.7% (101/137) were susceptible to colistin and tigecycline, respectively. The *hcp* gene was detected in 33.6% of carbapenem-resistant

Table 1. Antibiotic resistance rates of *A. baumannii* isolates according to the presence of the *hcp* gene.

	Number of resistant isolates (%)		
Antimicrobial agent	Total (n = 162)	hcp+ (n = 51)	hcp– (n = 111)
Imipenem	137 (84.6)	46 (90.2)	91 (82.0)
Ceftazidime	141 (87.0)	47 (92.2)	94 (84.7)
Ampicillin/sulbactam	138 (85.2)	46 (90.2)	92 (82.9)
Piperacillin/ tazobactam	140 (86.4)	46 (90.2)	94 (84.7)
Gentamicin	129 (79.6)	45 (88.2)	84 (75.7)
Ciprofloxacin	138 (85.2)	46 (90.2)	92 (82.9)
Trimethoprim/ sulfamethoxazole	143 (88.3)	48 (94.1)	95 (85.6)
Colistin	6 (3.7)	3 (5.9)	3 (2.7)
Tigecycline	15 (9.3)	3 (5.9)	12 (10.8)

A. baumannii (CRAB) isolates (46/137) and 34.8% of XDR isolates (36/132). Antimicrobial resistance rates in *hcp*-positive (*hcp*+) and *hcp*-negative (*hcp*–) isolates were not significantly different, although resistance rates to all antibiotics, except tigecycline, were slightly higher in *hcp*+ isolates (Table 1). *hcp*– isolates were also negative to *tssM* gene.

In the MLST, 35 distinct sequence types (STs) were identified among the 162 *A. baumannii* clinical isolates (Table 2 and Fig. 1). One hundred 32 isolates (81.5%) of 11 STs belonged to CC191, corresponding to global clone 2 (GC2), and all CC191 isolates were resistant to imipenem. Only 5 non-GC2 isolates (ST229 and ST491) were resistant to carbapenem; thus, 96.4% of carbapenem-resistant isolates belonged to GC2 (Table 2). There might be no correlation between the expression levels of *hcp* gene and ST Table S1.

The *hcp* gene was detected in *A. baumannii* isolates of 11 STs (Table 2), and in 41 of 132 *A. baumannii* GC2 isolates (31.1%), which is similar to the prevalence among all *A. baumannii* isolates (33.6%). However, the *hcp* gene was not distributed evenly across clones, instead was present in specific clones; all isolates of ST357 (11 isolates) and ST451 (13 isolates) were *hcp*+, and all isolates of ST208 except one (13 isolates) were *hcp*+ (Table 2; Fig. 1). All 4 isolates of ST229 in non-GC2 harbored the *hcp* gene. In contrast, all isolates of ST191 (66 isolates), ST368 (6 isolates), and ST784 (6 isolates) were *hcp*–. Only ST208 and ST369 included both *hcp*+ and *hcp*– isolates.

hcp *expression and secretion of Hcp protein*

To assess the expression of the *hcp* gene, qRT-PCR was performed on 51 *hcp*+ *A. baumannii* clinical isolates, and expression levels were compared with that of the reference strain ATCC 19606, which is known to express and secrete Hcp protein.[11,13] *hcp* transcript levels in the

Table 2. Genotypes and distribution of *A. baumannii* isolates.

ST	Allelic profile[a]	Total (n = 162) (%)	hcp+ (n = 51) (%)	hcp–(n = 111) (%)	Imipenem resistance (%)
GC2		**132 (81.5)**	**41 (31.1)**	**91 (68.9)**	**132 (100)**
ST191	1–3–3–2–2–94–3	66 (40.7)		66 (100)	66 (100)
ST208	1–3–3–2–2–97–3	14 (8.6)	13 (92.9)	1 (7.1)	14 (100)
ST357	1–12–3–2–2–145–3	11 (6.8)	11 (100)		11 (100)
ST368	1–3–3–2–2–140–3	6 (3.7)		6 (100)	6 (100)
ST369	1–3–3–2–2–106–3	11 (6.8)	3 (27.3)	8 (72.7)	11 (100)
ST451	1–3–3–2–2–142–3	13 (8.0)	13 (100)		13 (100)
ST784	1–3–3–2–2–107–3	6 (3.7)		6 (100)	6 (100)
ST1114	1–12–3–2–2–79–3	1 (0.6)	1 (100)		1 (100)
ST1141	1–3–3–104–2–140–3	1 (0.6)		1 (100)	1 (100)
ST1144	1–3–3–102–2–94–3	2 (1.2)		2 (100)	2 (100)
ST1316	1–35–3–2–2–107–3	1 (0.6)		1 (100)	1 (100)
Others					
ST681	1–102–59–28–1–83–45	1 (0.6)		1 (100)	
ST734	1–1–5–59–28–4–157–45	1 (0.6)		1 (100)	
ST1319	1–15–59–28–4–269–45	1 (0.6)		1 (100)	
ST503	1–62–80–28–35–164–45	1 (0.6)		1 (100)	
ST1077	1–62–80–28–35–178–45	1 (0.6)		1 (100)	
ST435	1–54–80–28–48–127–45	1 (0.6)		1 (100)	
ST552	21–35–2–28–1–145–4	1 (0.6)		1 (100)	
ST1318	21–35–2–28–1–268–4	1 (0.6)		1 (100)	
ST711	21–12–2–28–1–109–4	1 (0.6)		1 (100)	
ST1262	21–35–2–28–22–23–4	1 (0.6)		1 (100)	
ST447	1–15–13–12–4–106–2	1 (0.6)		1 (100)	
ST1313	1–15–13–12–4–94–2	1 (0.6)		1 (100)	
ST1317	1–15–13–12–4–163–112	1 (0.6)		1 (100)	
ST1315	1–1–13–12–92–16–2	1 (0.6)		1 (100)	
ST751	33–31–2–28–1–83–5	1 (0.6)		1 (100)	
ST1181	33–31–2–28–1–144–5	1 (0.6)		1 (100)	
ST229	1–15–2–28–1–107–32	4 (2.5)	4 (100)		4 (100)
ST491	10–53–4–11–4–98–5	1 (0.6)	1 (100)		1 (100)
ST373	1–12–12–11–4–103–3	5 (3.1)		5 (100)	
ST613	15–48–58–42–36–54–41	2 (1.2)	2 (100)		
ST1131	60–17–140–1–4–103–107	1 (0.6)	1 (100)		
ST1312	1–53–135–12–1–110–110	1 (0.6)	1		
ST1314	21–15–2–28–35–193–111	1 (0.6)		1 (100)	
ST1320	1–102–80–85–28–214–113	1 (0.6)	1		

Note. [a]gltA-gyrB-gdhB-recA-cpn60-gpi-rpoD.

clinical isolates ranged from 0.0008- to 1.5-fold (mean, 0.18-fold) of the level in ATCC 19606 (Fig. 2). We identified 6 isolates with mRNA levels 100-fold lower than that of ATCC 19606. These isolates with low *hcp* expression that likely have non-functional T6SS belonged to ST369, ST229, and ST357 (Fig. 1).
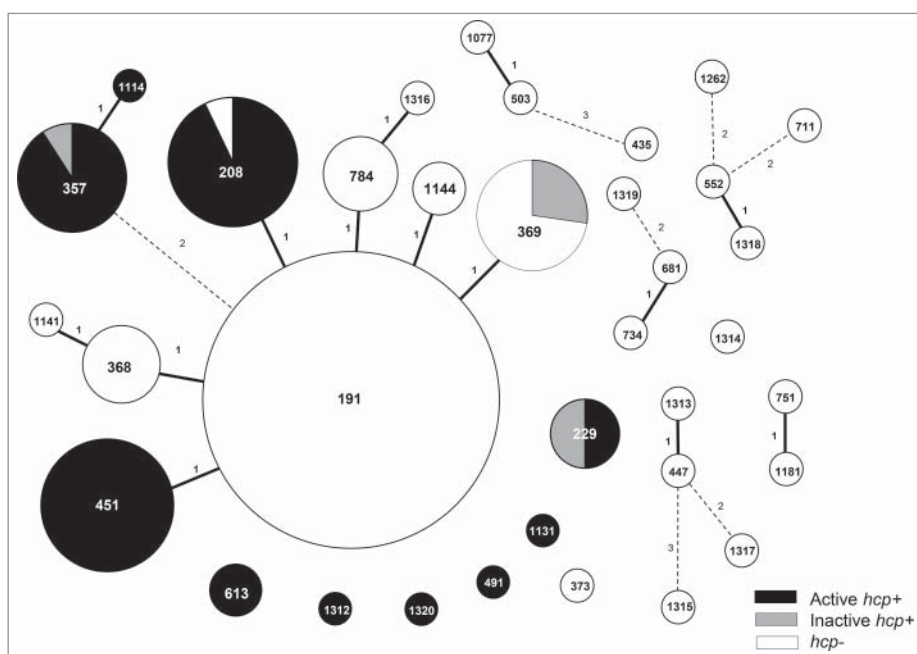
We assessed the secretion of Hcp protein in *hcp*+ isolates with a rabbit-derived polyclonal antibody generated against purified Hcp protein from reference strain ATCC 19606. For western blotting, we selected 5 isolates, 3 showing high *hcp* expression (B849, B1144, and 14AC-06; * in Fig. 2) and 2 showing *hcp* mRNA levels 100-fold lower than that of ATCC 19606 (B876 and 13AC-06; # in Fig. 2). Since RNA polymerase (RNAP) was used as a control, its detection in only whole cells suggested that the presence of Hcp in supernatants was due to exportation by the bacterium rather than cell lysis. Hcp was detected in whole cells and in concentrated culture supernatants of the 3 isolates showing high *hcp* expression, but not in the 2 isolates with low *hcp* expression (Fig. 3). These results showed that *A. baumannii* clinical

isolates with high *hcp* expression have an active T6SS (T6SS+), whereas isolates with low *hcp* expression have an inactive T6SS (T6SS–) under these conditions. Thus, we performed further analyses by classifying the isolates as T6SS+ and T6SS–.

Microbiological characteristics of T6SS+ isolates

To understand the microbiological characteristics of T6SS+ *A. baumannii* isolates, we selected 8 *A. baumannii* clinical isolates, 3 T6SS+ isolates (B849, B1144, and 14AC-06) and 5 T6SS- isolates including 2 inactive *hcp*+ (B876 and 13AC-06) and 3 *hcp*– isolates (AC12, B764, and 13AC-45; Table 3), and we performed competition, biofilm formation, and serum resistance assays using these isolates.

To determine whether T6SS+ isolates could better outcompete other bacteria, each *A. baumannii* isolate was mixed with an ampicillin-susceptible *E. coli* MG1655 strain at a 2:1 ratio and incubated. Then, surviving *A. baumannii* and *E. coli* MG1655 CFUs were

Figure 1. Diagram of the distribution of the *hcp* gene according to sequence type (ST). The numbers in the circles are the STs. The size of each circle represents the proportion of each ST, which are the marks within the circles. Solid line linkages indicate a single-locus variant, and dotted-line linkages indicate differences in 2 or 3 alleles.

counted by spotting serial dilutions on LB agar plates with and without ampicillin. T6SS+ isolates (mean CI, 4.15) caused a considerable reduction in *E. coli* MG1655 counts in competition assays compared with T6SS– isolates (mean CI, 0.9; $P = 0.006$; Fig. 4A).
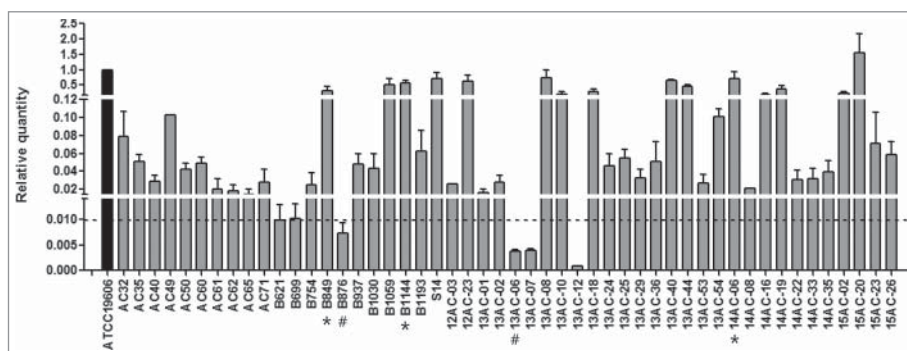
Relative biofilm-forming activity was estimated for T6SS+ and T6SS– strains and compared with that of ATCC 19606, and each isolate was tested in triplicate (Fig. 4B). The T6SS+ isolates formed significantly more biofilm mass than the T6SS– isolates (mean ± standard deviation [SD], 1.15 ± 0.51 vs. 0.54 ± 0.51; $P = 0.010$). The survival of *A. baumannii* in the presence of human serum also differed significantly among the 2 groups

(Fig. 4C). The T6SS+ isolates showed a mean survival rate of 67% after a 3-h incubation with normal human serum (NHS), and the survival rate of the T6SS– isolates was 26% ($P < 0.001$).
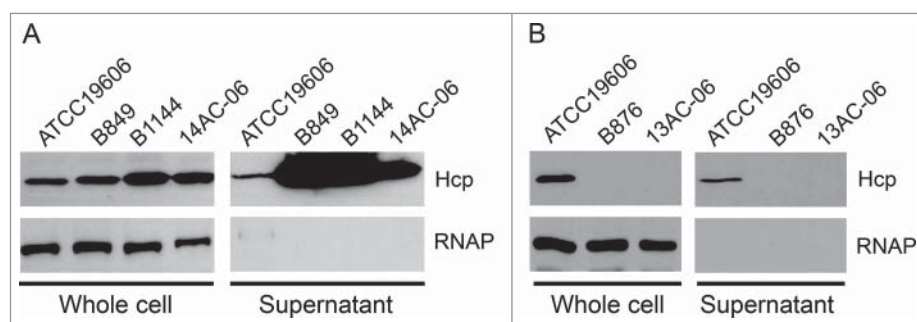
Among the T6SS– isolates, inactive *hcp+* and *hcp–* isolates showed no significant difference in terms of CI, biofilm formation, and serum resistance (Fig. S1).

Clinical impact of T6SS in patients with *A. baumannii* bacteremia

The clinical data analysis included 162 patients with *A. baumannii* bacteremia. Most patients (92.0%) had

Figure 2. Relative *hcp* transcript levels in *A. baumannii* isolates as measured by qRT PCR. *hcp* transcription levels are shown relative to that in *A. baumannii* ATCC 19606. The dashed line indicates 0.01-fold the expression levels of *hcp* in the reference strain, ATCC19606. The asterisk (*) and pound (#) symbols indicate the isolates with high and low *hcp* expression, respectively, that were used in the western blotting, competition, biofilm formation, and serum resistance assays.

Figure 3. Detection of Hcp, with RNA polymerase (RNAP) as a lysis control. (A) Three isolates showing high *hcp* transcript levels; (B) 2 isolates showing low *hcp* transcript levels, (i.e., less than 0.01-fold that of ATCC 19606). Immunoblots show Hcp in whole cells and concentrated culture supernatants prepared from cultures of clinical isolates.

hospital-acquired infections. More than half had bacteremic episodes in the ICU (61.1%) and one or more immunocompromising conditions (67.3%). Owing to the high rate of carbapenem resistance (88.7%), one-third of the patients (35.2%) were treated with appropriate antibiotics within 48 h.

Table 4 shows the clinical manifestations of patients infected with T6SS+ and T6SS– *A. baumannii* isolates. Among the patients, 45 (28.4%) had T6SS+ infections. Compared to patients with T6SS– infections, more patients with T6SS+ infections had haematopoietic stem cell transplantation (HSCT; 13.3% vs. 3.4%, $P = 0.029$), treatment with immunosuppressive agents (62.2% vs. 40.2%, $P = 0.014$), and catheter-related bloodstream infection (44.4% vs. 17.9%, $P = 0.001$). The rates of intra-abdominal infections and concurrent polymicrobial bacteremia did not differ between the 2 groups. There was also no difference in mortality rates between the T6SS+ and T6SS– groups.

Patients with T6SS+ CRAB infections also had higher frequencies of HSCT (15.0% vs. 3.1%, $P = 0.018$), treatment with immunosuppressive agents (60.0% vs. 40.2%, $P = 0.040$), and catheter-related bloodstream infection (50.0% vs. 21.6%, $P = 0.002$), as well as a lower frequency of intra-abdominal infection than patients with T6SS– CRAB infections (10.0% vs. 30.9%, $P = 0.036$; Table S2). There was no significant difference in the mortality rates between patients with T6SS+ and T6SS– CRAB infections. The variables associated with mortality of patients with *A. baumannii* bacteremia were also analyzed. In multivariate analysis, hematologic malignancy (OR, 2.945; 95% CI, 1.523–5.692; $P = 0.001$), acute kidney injury (OR, 2.977; 95% CI, 1.214–7.304; $P = 0.017$), higher Pitt bacteremia score (OR, 1.359; 95% CI, 1.130–1.635; $P = 0.001$), and pneumonia (OR, 2.441; 95% CI, 1.049–5.678; $P = 0.038$) were prognostic factors associated with 30-day mortality (Table S3). However, T6SS+ was not related to 30-day mortality.
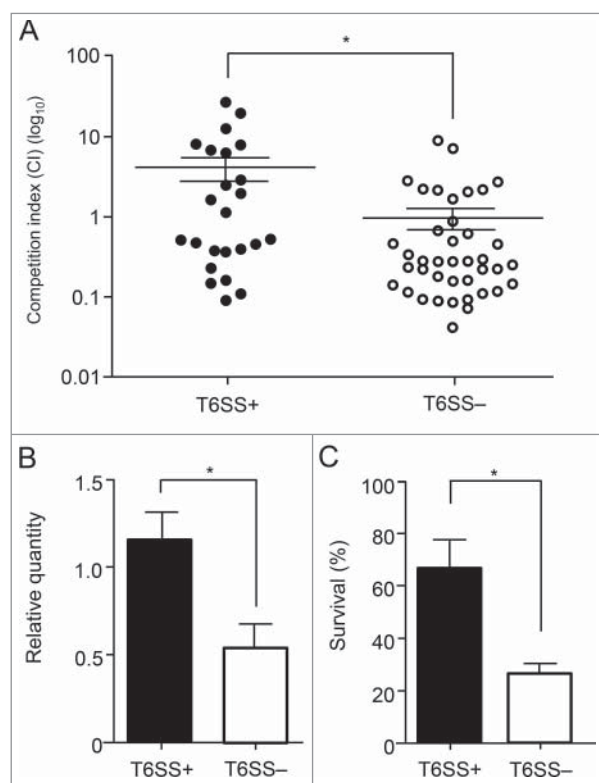
Discussion

A previous study reported that T6SS genes are conserved among several *A. baumannii* strains regardless of T6SS activity.[12] However, another comparative

Table 3. Profiles of *A. baumannii* isolates used in western blotting and analyses of microbiologic characteristics.

				MIC (μg/mL) of antimicrobial agents [b]								
Strain	Genotype	Date of isolation	Site of Infection[a]	IMI	CAZ	A/S	P/T	GEN	CIP	SXT	COL	TGC
T6SS+ (active *hcp*+)												
B849	ST357	2013 SEP 3	SSTI	>64	>64	64/32	<256/4	>64	>64	>32/608	2	1
B1144	ST357	2014 JUL 2	CRBSI	>64	>64	64/32	<256/4	>64	>64	>32/608	1	1
14AC-06	ST208	2014 JAN 9	VAP	>64	>64	>64/32	<256/4	>64	64	>32/608	1	1
T6SS– (inactive *hcp*+)												
B876	ST357	2013 SEP 14	CRBSI	64	>64	>64/32	<256/4	2	>64	>32/608	2	4
13AC-06	ST369	2013 MAR 3	CRBSI	>64	>64	64/32	<256/4	>64	64	>32/608	1	1
T6SS– (*hcp*–)												
AC12	ST191	2013 MAY 14	IAI	>64	>64	>64/32	<256/4	8	>64	>32/608	1	2
B764	ST191	2013 JUL 25	IAI	>64	>64	>64/32	<256/4	>64	>64	>32/608	1	4
13AC-45	ST369	2013 SEP 22	Primary bacteremia	>64	>64	>64/32	<256/4	>64	>64	>32/608	1	1

Notes. [a]SSTI, skin and soft tissue infection; CRBSI, catheter-related bloodstream infection; VAP, ventilator-associated pneumonia; IAI, intra-abdominal infection
[b]MIC, minimum inhibitory concentration; IMI, imipenem; CAZ, ceftazidime; A/S, ampicillin-sulbactam; P/T, piperacillin-tazobactam; GEN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; COL, colistin; TGC, tigecycline

Figure 4. Results of competition, biofilm formation, and serum resistance assays. These results came from 3 T6SS+ and 2 T6SS- isolates. (A) Relative *in vitro* competition indexes (CIs) for *A. baumannii* isolates by the presence of T6SS (T6SS+ vs. T6SS−). The CI values shown are the number of *A. baumannii* divided by the number of *E. coli* MG1655. Each circle represents the CI of each *in vitro* replicate. (B) Relative quantity of biofilm in *A. baumannii* isolates compared with that of ATCC 19606. (C) Serum resistance in *A. baumannii* isolates of each T6SS+ and T6SS− replicate. Serum resistance as a viability ratio (colony forming units [CFUs] of serum-bacterial suspension/ CFUs of bacterial suspension without normal human serum). The error bars indicate the standard deviations. *, Statically significant difference ($P < 0.05$).

study, based on genome sequences, showed that the T6SS locus is only present in isolates of a particular clade.[29] In the present study, the *hcp* gene, an essential component of the T6SS, was detected in about one-third of *A. baumannii* isolates from bloodstream infections, which supports the latter study. In addition, our results indicate that the *hcp* gene (i.e., the T6SS locus) is present mainly in specific clones. Except ST208 and ST369, all isolates of a specific clone contained the *hcp* gene, and none of isolates of other clone contained the gene. Specifically, no isolates of ST191, a presumed ancestral clone of GC2, contained a T6SS. Thus, we speculated that the T6SS locus was incorporated independently into *A. baumannii* isolates during the divergence of ST191 into other clones. Sporadic T6SSs were found in minor clones of singletons, such as ST491, ST613, ST1131, ST1312, and ST1320, which also

suggests independent introduction. Further investigation based on whole genomes would verify it.

It has been reported that the T6SS is usually silenced and is only activated under stress responses, such as nutrient limitation, cell damage, and ecological competition [9]. In addition, a recent study reported that the T6SS of *A. baumannii* was activated by loss of a MDR plasmid containing its repressor, *tetR*.[15] When we assayed 51 *hcp*+ isolates for the presence of *tetR*, only 4 isolates (AC40, B621, B699, and B754) possessed *tetR*. All of the *tetR*-positive isolates belonged to ST229, and the T6SS in these isolates was either inactive or showed low activity since the *hcp* expression levels in these isolates were more than 30-fold lower than that in ATCC 19606. However, *tetR* was not detected in other *hcp*+ isolates with inactive T6SS. That is, inactivation of T6SS by a MDR plasmid containing *tetR* may be specific to a certain strain, and additional regulatory systems may be associated with the secretion of Hcp, as suggested by Repizo et al.[14] In addition, a trade-off between antibiotic resistance and T6SS was not identified in our study. Although it was recently shown that the T6SS leads to DNA release and induces horizontal gene transfer, which may contribute to the spread of antibiotic resistance in *V. cholera*,[30] we did not find any correlation between antibiotic resistance and T6SS in this study. Thus, the mechanisms underlying the repression of T6SS in *A. baumannii* should be investigated further.

In this study, we observed that T6SS+ isolates better outcompeted *E. coli* compared with T6SS–isolates, including both *hcp*– and inactivated *hcp*+ isolates. The competition index of 2 isolates containing inactivated *hcp* gene did not differ from that of the *hcp*– isolates. T6SS-mediated bacterial killing has been demonstrated in several bacterial species, including *A. baumannii*.[9,12,31-34] However, only a few studies included multiple clinical isolates. Our results, based on several T6SS+, inactivated *hcp*+, and *hcp*– clinical isolates, statistically support T6SS-mediated bacterial killing.

Our results also showed the biofilm-forming activity and resistance to human serum of T6SS+ *A. baumannii* isolates. The correlation between high biofilm-forming activity and high resistance to human serum in T6SS+ isolates has been well documented,[35] since growth in a biofilm allows bacteria to evade the host immune response.[36] Consistent with the increased biofilm formation in T6SS+ isolates, patients with T6SS+ isolates had significantly more frequent catheter-related bloodstream infections. *A. baumannii* controls biofilm formation in response to cell density through quorum sensing,[37] and T6SS is often induced under high cell density.[38,39] In addition, resistance to human serum *in vitro* might be responsible for the bacteremia in patients treated with

Table 4. Clinical characteristics of patients with T6SS+ *A. baumannii* bacteremia.[a]

	Total(n = 162)	T6SS+(n = 45, 28.4%)	T6SS−(n = 117, 71.3%)	*P*-value
Age, years	62.2 ± 14.7	61.6 ± 12.5	62.5 ± 15.5	0.710
Male gender	94 (58.0)	27 (60.0)	67 (57.3)	0.859
Underlying disease				
Immunocompromised status	109 (67.3)	35 (77.8)	74 (63.2)	0.093
Solid tumor	47 (29.0)	11 (24.1)	36 (30.8)	0.449
Hematologic malignancy	32 (19.8)	10 (22.2)	22 (18.8)	0.662
HSCT	**10 (6.2)**	**6 (13.3)**	**4 (3.4)**	**0.029[c]**
Immunosuppressive agents	**75 (46.3)**	**28 (62.2)**	**47 (40.2)**	**0.014**
Neutropenia	32 (19.8)	8 (17.8)	24 (20.5)	0.827
Chronic renal disease	52 (32.1)	14 (31.1)	38 (32.5)	1.000
Hemodialysis	17 (10.5)	8 (17.8)	9 (7.7)	0.084[c]
Congestive heart failure	23 (14.2)	4 (8.9)	19 (16.2)	0.317
Liver cirrhosis	18 (11.1)	5 (11.1)	13 (11.1)	1.000
Diabetes mellitus	52 (32.1)	14 (31.1)	38 (32.5)	1.000
Comorbid conditions				
Septic shock	80 (49.4)	21 (46.7)	59 (50.4)	0.727
Acute kidney injury	68 (42.0)	16 (35.6)	52 (44.4)	0.375
Mechanical ventilation care [b]	81 (50.0)	15 (33.3)	53 (45.3)	0.214
Surgery within 30 d	45 (27.8)	12 (26.7)	33 (28.2)	1.000
Pitt bacteremia score	3.3 ± 2.9	3.1 ± 2.6	3.4 ± 3.0	0.515
Charlson WIC	3.2 ± 2.6	3.4 ± 2.9	3.1 ± 2.4	0.525
Site of infection				
Pneumonia	59 (36.4)	12 (26.7)	47 (40.2)	0.145
CRBSI	**41 (25.3)**	**20 (44.4)**	**21 (17.9)**	**0.001**
Intra-abdominal infection	34 (21.0)	4 (8.9)	30 (25.6)	0.303
Primary bacteremia	17 (10.5)	4 (8.9)	13 (11.1)	0.782[c]
Skin and soft tissue infection	8 (4.9)	4 (8.9)	4 (3.4)	0.219[c]
Urinary tract infection	3 (1.9)	1 (2.2)	2 (1.7)	1.000
Polymicrobial bacteremia	32 (19.8)	9 (20.0)	23 (19.7)	1.000
Bacteremic episodes in ICU	99 (61.1)	29 (64.4)	70 (59.8)	0.719
Hospital-acquired infection	149 (92.0)	44 (97.8)	105 (89.7)	0.114[c]
Appropriate antibiotics ≤ 48 h	57 (35.2)	16 (35.6)	41 (35.0)	1.000
Antibiotic resistance				
MDR	144 (88.9)	41 (91.1)	103 (88.0)	0.781
CR	137 (84.6)	40 (88.9)	97 (82.9)	0.468
XDR	132 (81.5)	40 (88.9)	92 (78.6)	0.176
30-day mortality	94 (58.0)	25 (55.6)	69 (59.0)	0.725

Notes. [a]Data are expressed as no. (%) of patients, unless otherwise indicated. Continuous variables expressed as mean and standard deviation. Bold indicates *P* < 0.05. HSCT, haematopoietic stem cell transplantation; WIC, weighted index of comorbidity; CRBSI, catheter-related bloodstream infection; ICU, intensive care unit; MDR, multidrug-resistant; CR, carbapenem-resistant; XDR, extensively drug-resistant; [b]Mechanical ventilation care before bacteremia onset; [c]Fisher's exact test

immunosuppressive agents who had compromised immune systems.[40] These findings point to the potential roles of T6SS beyond antagonism, which could be used for defense when the cells acquire spatial segregation after clonal expansion.[4] Our results suggest that T6SS are beneficial for host colonization and survival in *A. baumannii*.

However, it is not clear whether the high biofilm-forming activity and serum resistance of T6SS+ isolates is due to the function of the T6SS itself. Several studies have demonstrated that the T6SS functions in biofilm-forming activity in several bacterial species;[41-44] however, some bacteria do not require the T6SS for biofilm formation.[45] The *A. baumannii* reference strain ATCC 17978 has a functional T6SS, but does not form biofilms.[13,14] Repizo et al.[14] showed that the T6SS of an environmental *A. baumannii* isolate, DSM30011, was not required for biofilm formation through experiments with a *tssM*-deletion mutant. Thus, it is unclear whether the high biofilm-

forming activity of T6SS+ isolates in this study was due to the presence of the T6SS or other features. In addition, the possibility that the high proportion of catheter-related bloodstream infection in the T6SS+ patient group may be due to the enhanced characteristics of certain clones rather than the T6SS itself cannot be excluded. Higher biofilm formation in a certain *A. baumannii* clone was previously reported.[46] However, we also found that the differences in clinical characteristics, such a higher rate of immunosuppression and a lower rate of pneumonia and intra-abdominal infection in T6SS+ cases. High rate of immunosuppression in T6SS+ cases may be partially due to high resistance to human serum. Clinically, pneumonia and intra-abdominal infections often include diverse bacterial species.[47] However, our study included only bacteremic patients rather than patients with chronic infections who were likely to be colonized by mature communities of *A. baumannii*. Moreover, T6SS was less frequent in cases of

polymicrobial bacteremia or ventilation. In addition, the 30-day mortality rates in patients with *A. baumannii* bloodstream infections was higher in the T6SS+ group; however, the difference was not significant (35.3% vs. 28.7%, $P = 0.396$). T6SS was not a prognostic factor for mortality in patients with *A. baumannii* bacteremia. Thus, additional studies should be performed to determine if the T6SS itself is involved in the virulence of bacteria, including *A. baumannii*.

One-third of the *A. baumannii* clinical isolates in this study contained T6SS. *A. baumannii* isolates with a functional T6SS exhibited the capability to outcompete *E. coli*, biofilm-forming activity, and increased resistance to human serum *in vitro*. These phenotypes were also in agreement with the clinical features of T6SS+ in patients with *A. baumannii* bacteremia, including catheter-related bloodstream infections, receiving immunosuppressive agents, and HSCT recipients. However, it is unclear if these characteristics are due to T6SS itself.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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