Genomic analysis reveals high virulence and antibiotic resistance amongst phage susceptible \textit{Acinetobacter baumannii}

Udomluk Leungtongkam\textsuperscript{1}, Rapee Thummeepak\textsuperscript{1}, Thawatchai Kitti\textsuperscript{2}, Kannipa Tasanapak\textsuperscript{1}, Jintana Wongwigkarn\textsuperscript{3}, Kathryn M. Styles\textsuperscript{3}, Elizabeth M. H. Wellington\textsuperscript{3}, Andrew D. Millard\textsuperscript{4}, Antonia P. Sagona\textsuperscript{3} \& Sutthirat Sitthisak\textsuperscript{1,5}

In this study, we examined the association between antimicrobial resistance, CRISPR/Cas systems and virulence with phage susceptibility in \textit{Acinetobacter baumannii} and investigated draft genomes of phage susceptible multidrug resistant \textit{A. baumannii} strains from Thailand. We investigated 230 \textit{A. baumannii} strains using 17 lytic \textit{A. baumannii} phages and the phage susceptibility was 46.5\% (107/230). Phage susceptibility was also associated with resistance to numerous antibiotics (p-value < 0.05). We also found association between biofilm formation and the presence of \textit{ompA} gene among phage susceptible \textit{A. baumannii} strains (p-value < 0.05). \textit{A. baumannii} isolates carrying \textit{cas5} or combinations of two or three other \textit{cas} genes, showed a significant increase in phage resistance. Whole-genome sequences of seven phage susceptible \textit{A. baumannii} isolates revealed that six groups of antibiotic resistance genes were carried by all seven phage susceptible \textit{A. baumannii}. All strains carried biofilm associated genes and two strains harbored complete prophages, acquired copper tolerance genes, and CRISPR-associated (cas) genes. In conclusion, our data exhibits an association between virulence determinants and biofilm formation among phage susceptible \textit{A. baumannii} strains. These data help to understand the bacterial co-evolution with phages.

\textit{Acinetobacter baumannii} is a major cause of opportunistic infection, especially among immunocompromised patients. The emergence of multidrug-resistant \textit{A. baumannii} (MDR-AB) and even the extensively drug-resistant \textit{A. baumannii} (XDR-AB) has been increasing worldwide, and especially in Thailand and Nepal\textsuperscript{1-3}. Thus, alternative treatments against \textit{A. baumannii} infection is urgently needed. Bacteriophages (phages) are good candidates, specifically killing host bacteria resulting in minimal impact on bacterial normal flora and with no known critical side effects\textsuperscript{4}. To use phages for therapy, it is important to identify broad host range phages that kill the highest possible number of strains of bacterial species. In addition, it is crucial to understand the host-phage susceptibility mechanism. One of the important mechanisms impacting host range specificity is phage adsorption\textsuperscript{5}. This is a crucial step in the infection process, which represents the initial contact between virus and its host and requires phage receptors; outer membrane proteins (OMPs), lipopolysaccharides and teichoic acids\textsuperscript{5}. Several phages use the outer membrane protein OmpA as a receptor to infect Gram negative bacteria\textsuperscript{6,7}. Alterations to this molecule result in a decrease of bacterial virulence in phage resistance strains\textsuperscript{8}. Previous studies reported a positive correlation of antibiotic resistance in \textit{A. baumannii} with phage susceptibility\textsuperscript{9,10}. Phage susceptibility represents an evolutionary trade-off in \textit{A. baumannii} strains that were selected for antibiotic resistance, particularly in hospital environments with high antibiotic use\textsuperscript{9}. However, the mechanism and genetic basis of phage susceptibility in \textit{A. baumannii} is not completely understood. In this study, we aimed to determine the association between antimicrobial resistance and virulence with phage susceptibility in a large collection of \textit{A. baumannii}.
A. baumannii strains and to investigate draft genomes of phage susceptible of MDR-AB strains from Thailand to identify antibiotic resistance genes and virulence genes.

Results

Characterization and antimicrobial susceptibility profiles. Among 230 A. baumannii isolates, the resistance to various antibiotics was as follows: amikacin (53.47%), cefotaxime (80.43%), ceftazidime (83.04%), ceftriaxone (83.91%), cefepime (73.91%), ciprofloxacin (85.22%), gentamicin (63.48%), imipenem (82.17%), meropenem (81.74%), trimethoprim/sulfamethoxazole (65.65%), tetracycline (61.74%), cefoperazone/sulbactam (28.70%), piperacillin/tazobactam (28.70%), and colistin (12.17%).

Phage susceptibility among MDR-AB, CR-AB, XDR-AB and non MDR-AB. We grouped the 17 phages (Table 1) into four clusters (I, II, III, and IV) depending on their ability to infect 230 A. baumannii isolates (Fig. 1). The vPhT19, vPhT29, and vPhT44 phages belonged to cluster I and infected approximately 1.01-3.48% of bacterial hosts. Cluster II contained 25 phages (vPhT02, vPhT04, and vPhT05), infecting approximately 1.30–3.48% of bacterial hosts. Cluster III contained five phages (vPhT05, vPhT09, and vPhT19), infecting approximately 1.30–3.48% of bacterial hosts. Cluster IV contained five phages (vPhT05, vPhT09, and vPhT19), infecting approximately 1.30–3.48% of bacterial hosts.

Association between antibiotics, drug resistance patterns, biofilm formation, REP-PCR typing, copper tolerance, and phage susceptibility. Comparisons of phage susceptibility between antibiotics and drug resistance patterns are shown in Table 2. Phage susceptibility was positively associated with resistance to amikacin, ciprofloxacin, cefotaxime, ceftazidime, cefoperazone/sulbactam, imipenem, meropenem, piperacillin/tazobactam, tetracycline, ceftriaxone, cefepime, and gentamicin (p-value < 0.05). We found the association of phage susceptibility and drug resistance across the MDR-AB, CR-AB, XDR-AB and non MDR-AB strains (p-value < 0.05). An analysis of phage susceptibility and biofilm formation also showed association (Table 3). The most common virulence gene associated with biofilm formation was detected in 94.39% of bacterial hosts.
Figure 1. UPGMA dendrogram based on phage susceptibility patterns among 230 A. baumannii clinical isolates. Tree branches represent three bacterial groups of phage susceptibility (Group 1: green, Group 2 red, Group 3 blue), phage susceptible is displayed as yes (black) and no (gray). Hospitals A-E located in Central, Lower Northern, Northern, East and Northern regions of Thailand, respectively. Hospital F, located in Nepal. Classifications of MDR-, CR-, XDR- and non MDR-AB were presented as yes (black) and no (gray). Antibiotic sensitivity is presented as resistant (green) and susceptible (gray). Antibiotics are abbreviated as follows: AK amikacin, CIP ciprofloxacin, SXT trimethoprim/sulfamethoxazole, CTX cefotaxime, CAZ ceftazidime, CSL cefoperazone/sulbactam, IPM imipenem, MEM meropenem, PIP piperacillin/tazobactam, TE tetracycline, CRO ceftiraxone, FEP cefepime, CN gentamicin.
bility and present of ompA gene (p-value < 0.05) (Table 3). A repetitive element palindromic-PCR (REP-PCR) analysis of all isolates characterized the phage susceptible A. baumannii into 15 REP-types, while phage resistant A. baumannii belonged to 22 REP-types. The majority REP-types that presented in bacteriophage susceptible strains were R16 (59/107, 55.14%), R4 (18/107, 16.82%) and R24 (9/107, 8.41%), while the majority REP-types that presented in phage resistant strains were R16 (28/123, 22.76%), R12 (23/123, 18.70%) and R1 (10/123, 8.13%) (Supplementary Table S2). Our results showed the relationships between phage susceptibility and the presence of copper tolerance phenotype and genotype (Table 3).

### Association between CRISPR-associated (cas) genes and phage susceptibility.

Across the 230 strains, amplicons of cas1 (506 bp), cas2 (196 bp) and cas3 (850 bp) genes were present in 32 (13.91%), 2 (0.87%) and 30 (13.04%) isolates, respectively. The cas5 gene was found in 34 (14.78%) isolates, while cas6 was detected in 7 (3.04%) isolates. The cas9 gene was not found among any of the A. baumannii isolates. Overall, a total of 44 of the 230 strains were found to contain at least one cas gene. The majority (31/44, 70.45%) of the cas positive strains were classified as phage resistant strains. The correlation between the presence of cas genes and phage susceptibility was statistically determined (p-value < 0.05), showing that only cas5 gene was associated with A. baumannii phage resistance (Table 4). Phage resistant A. baumannii isolates were more than twice as likely to carry two or more cas genes than phage susceptible isolates (resistant; 23/123 (18.70%), susceptible; 9/107 (8.41%)), (p-value < 0.05) (Table 4).

### Table 2. Association between antibiotics, drug resistance patterns and phage susceptibility. *p-values less than 0.05 were considered as a statistically significant difference (Fisher’s exact test). Bold font indicates statistically significant difference between two groups.

| Antibiotics/drug resistance patterns | No. of phage susceptible isolates | Drug resistant | Drug susceptible | p-values* |
|-------------------------------------|-----------------------------------|----------------|-----------------|-----------|
| Amikacin (AK)                       | 80/123 (65.04%)                  | 27/107 (25.23%)|                 | <0.001    |
| Ciprofloxacin (CIP)                 | 104/196 (53.06%)                 | 3/34 (8.82%)   |                 | <0.001    |
| Trimethoprim/sulfamethoxazole (SXT) | 75/151 (49.67%)                  | 32/79 (40.51%) |                 | 0.1859    |
| Colistin (CTX)                      | 96/185 (51.89%)                  | 11/45 (24.44%) |                 | 0.0009    |
| Ceftazidime (CAZ)                   | 100/191 (52.35%)                 | 7/39 (17.95%)  |                 | 0.001     |
| Ceferazime/sulbactam (CSL)          | 58/66 (87.57%)                   | 69/164 (42.07%)|                 | 0.0330    |
| Imipenem (IPM)                      | 103/189 (54.49%)                 | 4/41 (9.76%)   |                 | <0.001    |
| Meropenem (MEM)                     | 104/188 (55.32%)                 | 3/42 (7.14%)   |                 | <0.001    |
| Piperacillin/tazobactam (PIP)       | 101/190 (53.16%)                 | 6/40 (15.00%)  |                 | <0.001    |
| Tetracycline (TE)                   | 89/142 (62.67%)                  | 18/88 (20.45%) |                 | <0.001    |
| Ceftriaxone (CRO)                   | 101/193 (52.33%)                 | 6/57 (16.22%)  |                 | 0.0001    |
| Cefepime (FEP)                      | 89/170 (52.35%)                  | 18/60 (30.00%) |                 | 0.0028    |
| Gentamicin (CN)                     | 83/146 (56.85%)                  | 24/84 (28.57%) |                 | <0.001    |

| Drug resistance patterns            | No. of phage susceptible isolates | Drug resistant | Drug susceptible | p-values* |
|-------------------------------------|-----------------------------------|----------------|-----------------|-----------|
| MDR-AB                              | 105/199 (52.76%)                  | 2/31 (6.45%)   |                 | <0.001    |
| CR-AB                               | 103/192 (53.64%)                  | 4/38 (18.75%)  |                 | <0.001    |
| XDR-AB                              | 20/28 (71.43%)                    | 87/202 (43.07%)|                 | 0.0048    |
| Non MDR-AB                          | 2/28 (7.14%)                      | 105/202 (51.98%)|                | <0.001    |

### Table 3. Association between biofilm formation, ompA gene and copper tolerance among phage susceptible A. baumannii strains. *p-values less than 0.05 were considered as a statistically significant difference (Fisher’s exact test) and are shown in bold.

| Biofilm formation and copper tolerance | No. of phage susceptible isolates | Drug resistant | Drug susceptible | p-values* |
|----------------------------------------|-----------------------------------|----------------|-----------------|-----------|
| Biofilm formation                       |                                  |                |                 |           |
| Biofilm formation phenotype             | 88/173 (50.87%)                  | 19/57 (33.33%) |                 | 0.0214    |
| ompA gene                              | 101/198 (51.01%)                 | 6/32 (18.75%)  |                 | 0.0007    |
| Copper tolerance                       |                                  |                |                 |           |
| Copper tolerance phenotype              | 9/53 (16.98%)                    | 98/177 (53.37%)|                 | <0.001    |
| copRS gene                             | 9/46 (19.56%)                    | 98/184 (53.26%)|                 | <0.001    |
A. baumannii classified into cluster C. Other phage susceptible D (Fig. 2).

The creation of a whole-genome SNP-based phylogenetic tree carried by all phage susceptible A. baumannii and ompA, adeRS, csuE, gacS, csuCD, bap, and catB8 (catB8). Strain AB003, which is highly phage susceptible (9/17, 53%), carried antibiotic resistance genes in two groups: β-lactam and aminoglycoside. Tetracycline, β-lactam, aminoglycoside, and macrolide resistance genes were detected in most phage susceptible A. baumannii. Two strains (AB003 and AB053) carried one complete prophage each and had acquired copper tolerance genes. Two complete predicted prophages were detected (Table 5). CRISPR-associated (cas) genes were only detected in two strains, AB003 and AB329, when searched by CRISPRCasFinder.

| Characteristics | No. of isolates | Phage susceptible (n = 107) | Phage resistant (n = 123) | p-values* |
|-----------------|----------------|-----------------------------|---------------------------|-----------|
| cas1 positive   | 11/107 (10.28%) | 21/123 (17.07%)             | 0.1376                    |
| cas2 positive   | 0/107 (0.00%)  | 2/123 (1.67%)               | NC                        |
| cas3 positive   | 9/107 (8.41%)  | 21/123 (17.07%)             | 0.0517                    |
| cas4 positive   | 8/107 (7.47%)  | 26/123 (21.14%)             | 0.0036                    |
| cas5 positive   | 2/107 (1.87%)  | 5/123 (4.06%)               | NC                        |
| cas6 positive   | 0/107 (0.00%)  | 0/123 (0.00%)               | NC                        |
| cas9 positive   | 9/107 (8.41%)  | 23/123 (18.70%)             | 0.0245                    |
| Positive for two or three cas-types | 9/107 (8.41%) | 23/123 (18.70%) | 0.0245

Table 4. Comparisons of CRISPR-associated (cas) genes between phage susceptible and resistant A. baumannii. Bold font indicates statistically significant difference between two groups. NC not comparable. *p-values less than 0.05 were considered as statistically significant difference (Fisher’s exact test).

**Genomic sequence analysis of phage susceptible A. baumannii.** The whole genome of seven phage susceptible A. baumannii strains (AB003, AB053, AB089, AB135, AB140, AB229 and AB329) was sequenced and the result of the genomic sequence analyses are shown in Fig. 2 and Table 5. Antibiotic resistance genes carried by all phage susceptible A. baumannii strains were classified into six groups: sulphonamide (sul1, sul2), tetracycline (tet(B)), β-lactam (blaADE3, blaKX3, blacS4, blaCAR1), aminoglycoside (aac(6′)-Ib-3, adaA1, ampC, β-lactam, aminoglycoside, and macrolide resistance genes were detected in most phage susceptible A. baumannii (6/7). Multilocus sequence typing (MLST) types 1, 2, 98, and 129 (Pasteur) were investigated in phage susceptible A. baumannii. Two strains (AB003 and AB053) carried one complete prophage each and had acquired copper tolerance genes. Two complete predicted prophages contained genome size ranging from 31.3 to 40.6 kb and GC contents ranging from 39.10 to 40.01%. Incomplete prophage regions were detected in all seven strains. All seven strains carried biofilm associated genes including ompA, adeRS, csuE, gacS, csuCD, bap, and bfnS genes. Plasmid groups, GR1 (1/7), GR2 (7/7), GR6 (4/7), and pRAY (1/7) were detected (Table 5). CRISPR-associated (cas) genes were only detected in two strains, AB003 and AB329, when searched by CRISPRCasFinder. The creation of a whole-genome SNP-based phylogenetic tree using the seven A. baumannii genomes and 149 published A. baumannii genomes indicated that these bacteria are divided into four large clusters, which are designated as clusters A, B, C, and D (Fig. 2). Strain AB003 was classified into cluster C. Other phage susceptible A. baumannii strains from this study were grouped into cluster D (Fig. 2).

**Discussion**

In Thailand, the incidence of MDR-AB, CR-AB and XDR-AB infection has increased in the past decade. Tigecycline and colistin are still last resort drugs of choice for treatment of multidrug-resistant A. baumannii. Tigecycline and colistin are still last resort drugs of choice for treatment of multidrug-resistant A. baumannii. Tigecycline and colistin are still last resort drugs of choice for treatment of multidrug-resistant A. baumannii. Tigecycline and colistin are still last resort drugs of choice for treatment of multidrug-resistant A. baumannii. Tigecycline and colistin are still last resort drugs of choice for treatment of multidrug-resistant A. baumannii. Tigecycline and colistin are still last resort drugs of choice for treatment of multidrug-resistant A. baumannii. Tigecycline and colistin are still last resort drugs of choice for treatment of multidrug-resistant A. baumannii. Tigecycline and colistin are still last resort drugs of choice for treatment of multidrug-resistant A. baumannii.

Bacterial biofilm formation is one strategy mediating resistance against phage lysis. However, our study showed positive association between biofilm formation and phage susceptibility. Interestingly, some studies have shown that biofilm formation is one strategy mediating resistance against phage lysis.
also revealed that phages can induce and strengthen biofilms and it has been shown that biofilm composition and maturation had an impact on susceptibility towards phage infection15. Quorum sensing system, OmpA and OmpC are critical host factors for phage infection7,16,17. The receptor for *Shigella flexneri* phage Sf6 and *E. coli* phage M1 is the outer membrane protein A (OmpA). In this study, 94.39% (101/107) of phage susceptible strains *A. baumannii* harbored the *ompA* gene. We found positive association between the present of OmpA in phage susceptible strains (Table 3). These data imply that OmpA could also be a receptor for *A. baumannii* phage adsorption.

Bacteria have numerous strategies to prevent bacteriophage infection and cell lysis. The CRISPR/Cas systems are one of the phage immunity systems that are present in bacteria and enabling the organisms to respond to and eliminate invading genetic material18. In 2016 Lin et al. showed that *Klebsiella pneumoniae* isolated from the hospital environment have a decreased level of phage immunity and a reduction in CRISPR-Cas activity.

**Figure 2.** Whole-genome SNP-based phylogenetic tree. A phylogenetic tree base on WGS analysis showing the relationship between seven phage susceptible *A. baumannii* and 149 published *A. baumannii* genomes. Colours indicate four major clusters (A = green, B = purple, C = red, and D = blue). Seven phage susceptible *A. baumannii* strains are marked with dots.
| Strain ID/Genome characteristics | AB003 | AB053 | AB089 | AB135 | AB140 | AB229 | AB329 |
|---------------------------------|-------|-------|-------|-------|-------|-------|-------|
| Hospital/year isolated          | HE/2006 | HA/2013 | HA/2013 | HB/2014 | HB/2014 | HB/2014 | HD/2015 |
| Specimens                       | Sputum | Sputum | Sputum | Pus   | Sputum | Urine | Sputum |
| Phage susceptibility            | (9/17) | (2/17) | (3/17) | (3/17) | (4/17) | (3/17) | (6/17) |

**Genome features**

| Number of assembled contigs | 57    | 142   | 174   | 99    | 77    | 333   | 256    |
|-----------------------------|-------|-------|-------|-------|-------|-------|--------|
| Average genome size (bp)    | 4,050,570 | 4,108,360 | 3,897,375 | 4,036,789 | 3,990,813 | 4,070,648 | 3,943,615 |
| GC content (%)              | 38.8  | 38.9  | 39.0  | 38.9  | 39.8  | 39.1  | 39.0   |
| Number of CDSs              | 3,965 | 4,059 | 3,808 | 3,955 | 3,875 | 4,134 | 3,923  |
| Resistance                  | ND    | ND    | ND    | ND    | ND    | ND    | ND     |
| Aminoglycoside resistance   | ND    | ND    | ND    | ND    | ND    | ND    | ND     |
| Sulfonamide resistance      | ND    | sul1, sul1 | sul2 | sul2 | ND    | sul2 | sul2   |
| Tetracycline resistance     | ND    | tet(B) | tet(B) | tet(B) | tet(B) | tet(B) | tet(B) |
| Betalactam resistance       | blaADC-25, blaBGLA-25 | blaBGLA-25, blaBGLA-25, | blaBGLA-25, | blaBGLA-25, | blaBGLA-25, | blaBGLA-25, | blaBGLA-25, |
| Aminoglycoside resistance   | aph(3')-Ia | aac(6')-Ib, aac(3')-Ib, | aph(3')-Ia, aph(6')-Id, | aph(3')-Ia, aph(6')-Id, | aph(3')-Ia, aph(6')-Id, | aph(3')-Ia, aph(6')-Id, | aph(3')-Ia, aph(6')-Id, |
| Phenylic resistance         | ND    | mph(E), mtr(E) | mph(E), mtr(E) | mph(E), mtr(E) | mph(E), mtr(E) | mph(E), mtr(E) | mph(E), mtr(E) |
| Virulence genes             | entE, zur | entE, zur | entE, zur | entE, zur | entE, zur | entE, zur | entE, zur |
| Iron acquisition            | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS |
| Biofilm formation           | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS | ompA, adeRS, cdu, gacS, gacS, cadCD, bfmS |
| Type V, VI and IV secretion systems | hac, traU, traC | hac, hac | hac, traU, traC | hac, traU, traC | hac, traU, traC | hac, traU, traC | hac, traU, traC |
| Copper tolerance            | copRS5/tolerance | copRS5/tolerance | copRS5/tolerance | copRS5/tolerance | copRS5/tolerance | copRS5/tolerance | copRS5/tolerance |
| Number of prophages (complete/incomplete) | 9 (1/8) | 8 (1/7) | 4 (0/4) | 10 (0/10) | 5 (0/5) | 8 (0/8) | 6 (0/6) |
| Plasmid replicon typing (GE) | GR2, GR6 | GR1, GR2, pRAY | GR2 | GR2, GR6 | GR2, GR6 | GR2, GR6 | GR2 |
| CRISP-associated (cas) genes | CAS-1, cas3, cas2, cas1; | Negative | Negative | Negative | Negative | Negative | CAS-1, cas3, cas2, cas1; |
| Accession nos/ Bioproject     | PRJEB32181 | JACBCNM0000000000 | JABCN1000000000 | JABCN1000000000 | JABCN1000000000 | JABCN1000000000 | JABCN1000000000 |

Table 5. Genome features, acquired antimicrobial resistance genes, putative prophages, copper tolerance, virulence genes, and CRISP-associated (cas) genes identified in whole genome sequences of phage susceptible A. baumannii. ND not detected.

compared to strains isolated from outside the hospital environment19. PCR with cas-specific primers showed that 19% of the A. baumannii isolates had a CRISP-Cas system. The majority (70.5%) of the cas positive strains were classified as phage resistant strains. The positive correlation between the presence of cas5 or combinations of two or three different cas genes and phage resistant strains was statistically determined (p-value < 0.05). This data supports a hypothesis that CRISP-Cas systems are one of the important phage resistance mechanisms in A. baumannii. We analyzed whole genome sequence of Acinetobacter spp. deposited on National Center for Biotechnology Information (NCBI) database showed that these contain six cas genes including cas1, cas2, cas3, cas5, cas6 and cas920,21. Cas1 and Cas2 proteins recognize invading phage nucleic acids and insert them into the CRISP-Cas array as a spacer in order to transcribe into pre-CRISP-RNA (crRNA)22, whereas, the Cas5 protein has RNase activity, resulting in inhibition of the transcription machinery in phages19. The cas6 gene encodes a type I-F CRISP-associated endoribonuclease Cas6/Csy4 protein, which has an important role in the cleavage
of the repeat sequences to yield mature CRISPR-RNAs (crRNAs) that complementary pair with invading phage nucleic acid bases, causing destruction of the target phage DNA

Phage resistance may be related to lower virulence, making the resistant bacteria less virulent than non-phage-resistant strains. We detected antibiotic resistance, CRISPR-associated (cas) genes and virulence genes among A. baumannii isolates using PCR and found association between phage susceptibility with antibiotic resistance, CRISPR-associated (cas) genes and virulence genes. Whole genome sequencing of virulence genes involving biofilm formation revealed that the seven phage susceptible A. baumannii in this study harbored various virulence genes linked to the ability to form biofilms, iron acquisition and bacterial secretion systems. cas genes (cas1, cas3-cas2, and cas6) were found in two of seven the phage susceptible A. baumannii genomes. Antibiotic resistance genes involved sulphonamide, tetracycline, β-lactam, aminoglycoside, macrolide and phenicol resistance were found in seven phage susceptible A. baumannii genomes. In a previous study, we identified the acquired copper tolerance genes, copRS that correspond to copper toxicity in the genome of A. baumannii. These genes were identified in two of the seven phage susceptible strains and both strains also showed the copper tolerance phenotype. The correlation between phage susceptibility and regulating heavy metal toxicity is in agreement with the findings of Zhang et al., where plasmid-borne cadmium resistant determinants were associated with the susceptibility of Listeria monocytogenes to phages. Antibiotic resistance genes and heavy metal tolerance genes can be disseminated within the microbial population by horizontal gene transfer mechanisms using plasmids and phages. Among 19 groups of plasmids identified in A. baumannii, in this study, we found plasmids GR1, GR2, GR6, and pRAY in phage susceptible A. baumannii. Plasmid GR6, linked to the dissemination via horizontal gene transfer by conjugation was detected in four strains of phage susceptible A. baumannii. In contrast, phages are important genetic vehicles for transferring genetic information between bacteria via transduction. All seven phage susceptible A. baumannii strains in our analysis carried prophage associated sequences on their chromosomes. However, only two strains harbored complete prophages. Propagates are directly related to genome diversity, evolution and strains variation as well as an association with the presence of antibiotic resistance genes and virulence genes. In addition, prophages are responsible for gene disruption or translocation to phenotypic changes in their host and can introduction of pathogenicity determinants that contribute positively to bacterial fitness.

An analysis of the clonal relationship of the seven phage susceptible A. baumannii genomes from this study with 149 previously published A. baumannii genomes (Fig. 2) revealed that strain AB003, isolated from hospital HE, was closely related to strains AB0057 (CP001182), NCTC13421 (LS483472), A85 (CP021782) and USA15 (CP020595). The three strains isolated from hospital HB (AB140, AB229 and AB135) all belonged to the same ST type and are very closely related with Malaysian and Chinese isolates AC30 (CP007577), AC29 (CP007535), XH731 (CP021321) and Aba (CP030083). The presence of closely related bacterial strains collected from Thailand, Malaysia and China might be descriptive that these organisms shared ancestry and emerged at the same time, caused by rapid dissemination of genetic material.

In conclusion, we found that phage susceptibility was associated with antibiotic resistance and virulence among A. baumannii strains. Moreover, in silico analysis showed that seven strains of phage susceptible A. baumannii carried at least six antibiotic resistance genes which included sulphonamide, tetracycline, β-lactam, aminoglycoside, macrolide, phenicol and several virulence genes involved biofilm formation. Thus, the data from this study can be used as essential information about phage therapy in the future.

Materials and methods

Bacteria and phages used in this study. We used 230 A. baumannii isolates collected from inpatient units of five hospitals in Thailand (HA-HE) and one hospital in Nepal (HF) as described by Niumsup et al., Joshi et al., and Leungtongkam et al. (Supplementary Table S1). Seventeen A. baumannii phages used in this study were isolated from wastewater treatment plants of two hospitals (HE and HG) in Phitsanulok province, Thailand. A. baumannii strains used as the host for phage propagation are shown in Table 1 of this study. The protocol was approved by Naresuan University Institutional Biosafety Committee (NUIBC) (No. NUIBC GM 62-06-22).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2017) with fifteen antibiotics: amikacin, cefotaxime, cephalotaxime, cefoperazone/sulbactam, cefazidime, ceftriaxone, ciprofloxacin, colistin, gentamicin, imipenem, meropenem, piperacillin/tazobactam, tetracycline, tigecycline and trimethoprim/sulfamethoxazole. Escherichia coli ATCC 25922 was used as a quality control strain. All isolates were defined as being MDR-AB, when there was resistance to more than three antibiotic classes, as carbapenem-resistant A. baumannii (CR-AB), when there was resistance to carbapenems and as XDR-AB, when there was resistance to all antimicrobial agents tested except the polymyxin colistin, and tigecycline. Non MDR-AB classified as bacteria that non-resistant or less resistant than the three antibiotic group.

Phages susceptibility of A. baumannii. Assessment of phage susceptibility was determined by spot test on all A. baumannii isolates. A colony of bacteria was suspended in 0.85% (w/v) NaCl to the equivalent of a 0.5 McFarland standard (1 × 108 CFU/ml). The suspensions were swabbed on to Trypticase Soy Agar (TSA). Phage suspensions (2 µl at 1 × 108 PFU/ml) were dropped into the bacterial lawn. Then, plates were incubated at 37 °C for 8 h. The result of spot test being clearance at the location of phage inoculation when the host was sensitive to phage (Supplementary Fig. S1). All experiments were performed in duplicate.
Biofilm formation, detection of ompA gene, repetitive element palindromic-PCR (REP-PCR) and copper tolerance. The genetic diversity among phage susceptible *A. baumannii* isolates was studied using REP-PCR typing as described previously by Leungtongkam et al. Detection of *ompA* gene and biofilm formation were performed as described by Thummeepak et al. The copper tolerance phenotype and copper-related genes (copRS) studies were conducted by using Minimum Inhibitory Concentration (MIC) and PCR methods as described previously.

Detection of CRISPR-associated (cas) genes. The bacterial protein sequences and GenBank nucleotide sequences of well-identified *cas* genes, detected in six example *Acinetobacter* spp., were used as templates to design the specific primers using Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Supplementary Table S3). The genomic DNA of all isolates in this study was extracted by a previously described boiling method for use as a template in a multiplex-PCR method with *cas* specific primers (Supplementary Table S3) to detect CRISPR-associated (cas) genes. Conditions for multiplex-1 were the following: 95 °C for 5 min and then 35 cycles at 94 °C for 30 s, at 62 °C for 40 s, and 72 °C for 50 s, followed by a final extension at 72 °C for 5 min. Multiplex-2 condition was one cycle of 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 50 s and finally, one cycle of 72 °C for 5 min. The PCR product was visualized by agarose gel electrophoresis, stained with ethidium bromide in a UV transilluminator.

Whole genome sequencing of *A. baumannii*. We selected seven phage susceptible *A. baumannii* strains (Group1 and Group2) that were susceptible to phages from all four clusters, namely AB003 (cluster III), AB053 (cluster IV), AB089 (cluster I), AB135 (cluster I), AB140 (cluster II), AB229 (cluster I) and AB329 (cluster IV) to study the whole genome sequence. Genomic DNA of *A. baumannii* strains were isolated using HiYield Genomic DNA Mini Kit (RBC Bioscience, New Taipei, Taiwan). Extracted DNA was quantified using a Qubit DNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA). Genomes were sequenced by the Illumina MiSeq platform (250 bp paired end). DNA libraries were constructed by Nextera XT DNA Library Preparation Kit according to the manufacturers’ instructions. Reads were trimmed and assembled by using Sickle v1.33 and SPAdes genome assembler v3.6.036 with default settings, respectively. After assembled contigs, annotation was conducted with RAST pipeline using default parameters. Single nucleotide polymorphisms (SNPs) phylogenetic analysis was conducted by using CSI Phylogeny v1.4 with default options (with reference strain SDF, NC_010400.1). Phylogenetic tree image was visualized and edited by Figtree v1.4.4 (https://tree.bio.ed.ac.uk/software/figtree/). Antibiotic resistance genes in draft genomes were detected by ResFinder tool. CRISPR-associated (cas) genes within bacterial genomes were detected by using CRISPRCasFinder programs and the prediction of prophage regions genomes were performed by using PHASTER. Propage hits with status “questionable” were scored as incomplete prophages. The virulence genes were examined by BlastN algorithm using collection of virulence genes as queries.

Statistical analysis. The Fisher’s exact test was used to analyze phage susceptibility associated with the drug resistance patterns, biofilm formation, *ompA* gene, copper tolerance and CRISPR-associated (cas) genes. Data with a *p*-value <0.05 were classed as statistically significant.

Nucleotide sequence accession number. The nucleotide sequences of seven *A. baumannii* have been deposited to the GenBank database under accession numbers PRJEB32181 (AB003), JABCNM000000000 (AB053), JABCNJ000000000 (AB089), JABCNK000000000 (AB135), JABCNI000000000 (AB140), JABCNL000000000 (AB229), and JABCNH000000000 (AB329).

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Author contributions

S.S., K.T., and J.W. conceived and designed the experiments; U.L., R.T., and T.K. conducted the experiments; U.L., R.T., S.S. A.P.S., K.M.S., and A.D.M. interpretation of data; R.T., U.L., A.D.M., S.S. performed the data and bioinformatic analysis; U.L. and S.S. wrote the manuscript; S.S., A.P.S., K.M.S., A.D.M., and E.M.W edited the manuscript, funding acquisition was done by A.P.S and S.S. with E.M.W and A.D.M. as collaborators; All authors read and approved the manuscript.

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Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to S.S.

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