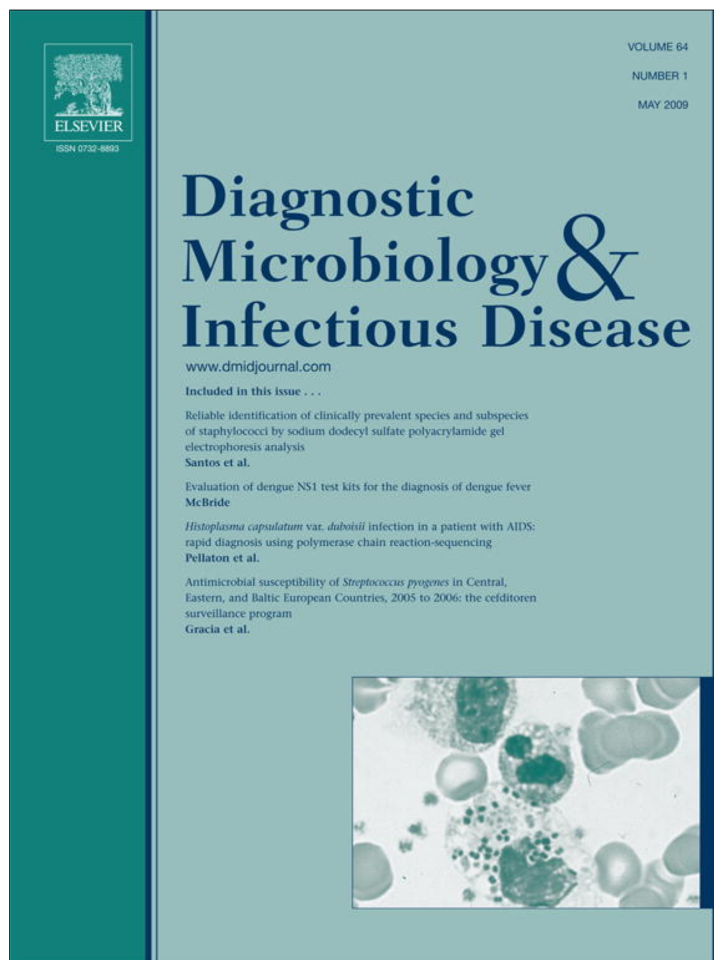
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Diagnostic Microbiology and Infectious Disease 64 (2009) 51 – 59

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

www.elsevier.com/locate/diagmicrobio

Antimicrobial Susceptibility Studies

Independent emergence of colistin-resistant *Acinetobacter* spp. isolates from Korea

Young Kyoung Park[a], Sook-In Jung[b], Kyong-Hwa Park[b], Hae Suk Cheong[c], Kyong Ran Peck[c], Jae-Hoon Song[c,d], Kwan Soo Ko[a,d,*]

[a]*Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon 440-746, South Korea*
[b]*Division of Infectious Disease, Chonnam National University Medical School, Gwangju 501-715, South Korea*
[c]*Division of Infectious Diseases, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea*
[d]*Asian-Pacific Research Foundation for Infectious Diseases (ARFID), Seoul 135-710, South Korea*

Received 1 September 2008; accepted 21 January 2009

Abstract

High colistin resistance rates in *Acinetobacter* spp. were recently reported in Korean hospitals (*J. Antimicrob. Chemother* 2007;60:1163). In this study, we investigated if colistin-resistant *Acinetobacter* spp. isolates from Korean hospitals disseminated clonally or emerged independently. Multilocus sequence typing (MLST) analysis was performed for 58 colistin-resistant *Acinetobacter* spp. isolates: 8 isolates of the *Acinetobacter baumannii* subgroup A, 16 isolates of the *A. baumannii* subgroup B, and 34 isolates of the genomic species 13TU. A phylogenetic tree inferred from concatenated sequences of 7 MLST loci showed a clear distinction among the 3 *Acinetobacter* groups. In the MLST analysis, most colistin-resistant *Acinetobacter* spp. isolates showed different allele profiles at the 7 loci; that is, they belonged to different clones. Despite the clear distinction between the 3 *Acinetobacter* groups, interrelationships among the 3 groups were not consistent within the gene trees. In addition, some isolates showed clustering incongruent with their species or group identities in some gene trees. MLST analysis indicated that most colistin-resistant *Acinetobacter* spp. isolates from Korean hospitals arose independently. Considering the increasing use of colistin and the high recombination rate of *Acinetobacter* spp., independent but frequent emergence of colistin resistance in *Acinetobacter* spp. isolates is of great concern.
© 2009 Elsevier Inc. All rights reserved.

Keywords: *Acinetobacter*; Colistin resistance; Multilocus sequence typing (MLST)

1. Introduction

Although *Acinetobacter* spp. has long been regarded as rarely causing infections, its significance in nosocomial infections in immunocompromised patients and patients in intensive care units has been increasingly recognized. The emergence of multidrug-resistant (MDR) or pandrug-resistant (PDR) *Acinetobacter* spp. isolates has become a serious clinical concern worldwide (Perez et al., 2007). Few antimicrobial agents can be reliably used for effective therapy against MDR or PDR *Acinetobacter* infections. Although polymyxins such as polymyxin B and colistin (polymyxin E) have not typically been included in regimens to treat *Acinetobacter* infections because of their neurotoxicity and nephrotoxicity, they are now considered as one of the last resorts against MDR or PDR *Acinetobacter* infections (Falagas and Kasiakou, 2005; Li et al., 2006b). So far, colistin or polymyxin B resistance rates among *Acinetobacter* isolates are very low worldwide (Gales et al., 2006). However, some investigators have reported the emergence of heteroresistance and resistance to colistin after colistin treatment (Hawley et al., 2008; Li et al., 2006a). In addition, high resistance rates against polymyxin B and colistin among *Acinetobacter* isolates from Korea have been recently reported by us (Ko et al., 2007).

Although we previously reported high colistin resistance rates among *Acinetobacter* isolates from Korea, we did not

* Corresponding author. Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Jangan-gu, Suwon 440-746, South Korea. Tel.: +82-31-299-6223; fax: +82-31-299-6229.
E-mail address: ksko@med.skku.ac.kr (K.S. Ko).

0732-8893/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2009.01.012

Author's personal copy

investigate whether they emerged independently or disseminated clonally (Ko et al., 2007). It is important to understand the emergence pattern of antimicrobial-resistant pathogens because infection control strategies may differ with regard to their origin (Gardam et al., 2002). Because *Acinetobacter* infections are known to be nosocomial, it could be supposed that the high colistin resistance rates in *Acinetobacter* spp. isolates from Korean hospitals were due to clonal dissemination of specific and resistant clones. So far, a number of typing methods such as amplified fragment length polymorphism (AFLP) and pulsed-filed gel electrophoresis (PFGE) have been successfully applied for typing of *Acinetobacter baumannii* isolates (Towner et al., 2008; van Dessel et al., 2004). However, reproducibility and interlaboratory exchange of data for global epidemiologic analysis have been problematic. Multilocus sequence typing (MLST), a highly discriminative typing method of bacterial pathogens based on DNA sequences of several housekeeping gene fragments, has been applied successfully for molecular typing of many bacterial species (Maiden, 2006). For *A. baumannii*, it was recently developed and has been used in the investigation of spread and outbreak of *A. baumannii* (Nemec et al., 2008; Shelburne et al., 2008; Wisplinghoff et al., 2008). In this study, we applied the MLST in the investigation of the genetic divergence of colistin-resistant *A. baumannii* and genomic species 13TU (GS 13TU) because it can provide answers as to the relatedness with *Acinetobacter* isolates from other countries as well as their clonality.

2. Materials and methods

2.1. Bacterial isolates

A total of 58 clinical isolates of colistin-resistant *Acinetobacter* spp., collected from 2 tertiary-care hospitals in Korea, were included in the study: 26 isolates from Samsung Medical Center, Seoul, Korea, and 32 isolates from Chonnam National University Hospital, Gwangju, Korea, all of which were included and characterized in a previous study (colistin MIC values, ≥4 mg/L) (Ko et al., 2007). Colistin susceptibility was tested using the broth microdilution method according to the Clinical and Laboratory Standards Institute (2007) guidelines.

2.2. Acinetobacter spp. identification

Species identification was performed via partial *rpoB* gene sequencing (Ko et al., 2007; La Scola et al., 2006). In a previous article, we classified the species into 3 categories, *A. baumannii* subgroups I, II, and III (Ko et al., 2007). Later, the *A. baumannii* subgroup II was ascertained to belong to the *Acinetobacter* GS 13TU based on amplified ribosomal DNA restriction analysis (ARDRA) and *gyrB*-based multiplex polymerase chain reaction method (Higgins et al., 2007). Thus, we reclassified *A. baumannii* subgroup I, including the type strain and reference strains of European clones I, II, and III, as *A. baumannii* subgroup A (*Aba* A), and the *A. baumannii* subgroup III was reclassified as *A. baumannii* subgroup B (*Aba* B). Two subgroups of *A. baumannii* showed distinct clustering and exhibited *rpoB* sequence divergence of less than 1.5% within each subgroup but 6.3% to 7.5% divergence between 2 subgroups. Based on *rpoB* gene sequences, 8 of the isolates belonged to *Aba* A and 16 isolates belonged to *Aba* B. The other 34 isolates were GS 13TU.

2.3. MLST analysis

The 7 gene fragments of the MLST scheme in *Acinetobacter* spp. were amplified as described (Bartual et al., 2005), with the addition of the following modified primer sets: *gpi*-F2 (5′-AAT ACC GTG GTG CTA CG-3′), *gpi*-R2 (5′-TTC AGG AGC AAT CCC CCA CT-3′); *rpoD*-F2 (5′-CGA ATY GCA TTG CAA AAC G-3′), *rpoD*-R2 (5′-CNG CAA TYT TTT GYT GGA A-3′); *gyrB*-R2 (5′-GAC TAT GAA GGY GGT TTA TC-3′). We unambiguously determined the sequences of 7 housekeeping genes from all 58 colistin-resistant isolates. In addition, gene sequences from 2 colistin-resistant *Acinetobacter* isolates (S117-1 and S133), which showed a high degree of divergence from the other 58 isolates in the sequences of MLST loci, were used as outgroups in phylogenetic analyses. Based on the determined sequences, we constructed 7 gene trees from each loci of the MLST using the neighbor-joining method. In addition, a phylogenetic tree was generated from concatenated sequences of the 7 housekeeping genes using the same method. Bootstrap values were evaluated with 1000 replications to determine the robustness of the branches in the phylogenetic tree inferred from these concatenated sequences.

3. Results and discussion

Fig. 1 showed the phylogenetic clustering inferred from concatenated sequences of 7 gene fragments. It indicated a clear distinction between the 3 groups, *Aba* A, *Aba* B, and GS 13TU, with robust bootstrap support. Most colistin-resistant *Acinetobacter* spp. isolates belonged to the different clones; that is, most did not have identical sequences at the 7 loci. With respect to the sequence type (ST) in the MLST analysis, 46 STs were found among the 58 colistin-resistant *Acinetobacter* spp. isolates in this study. Only 2 *A. baumannii* isolates, S011 and S034, showed previously identified ST, ST22. PFGE also indicated that colistin-resistant *Acinetobacter* isolates belonged to different clones (data not shown). It suggested that most colistin-resistant *Acinetobacter* spp. isolates in Korean hospitals emerged independently and not by clonal spreading of and individual bacterial clone. Although all 8 colistin-resistant isolates of *Aba* A were collected from the Samsung Medical Center, they showed sequence divergence from each other, except for 2 isolates (S011 and S034). That is, they belonged to different STs or clones. This situation is different from the previously characterized

Author's personal copy

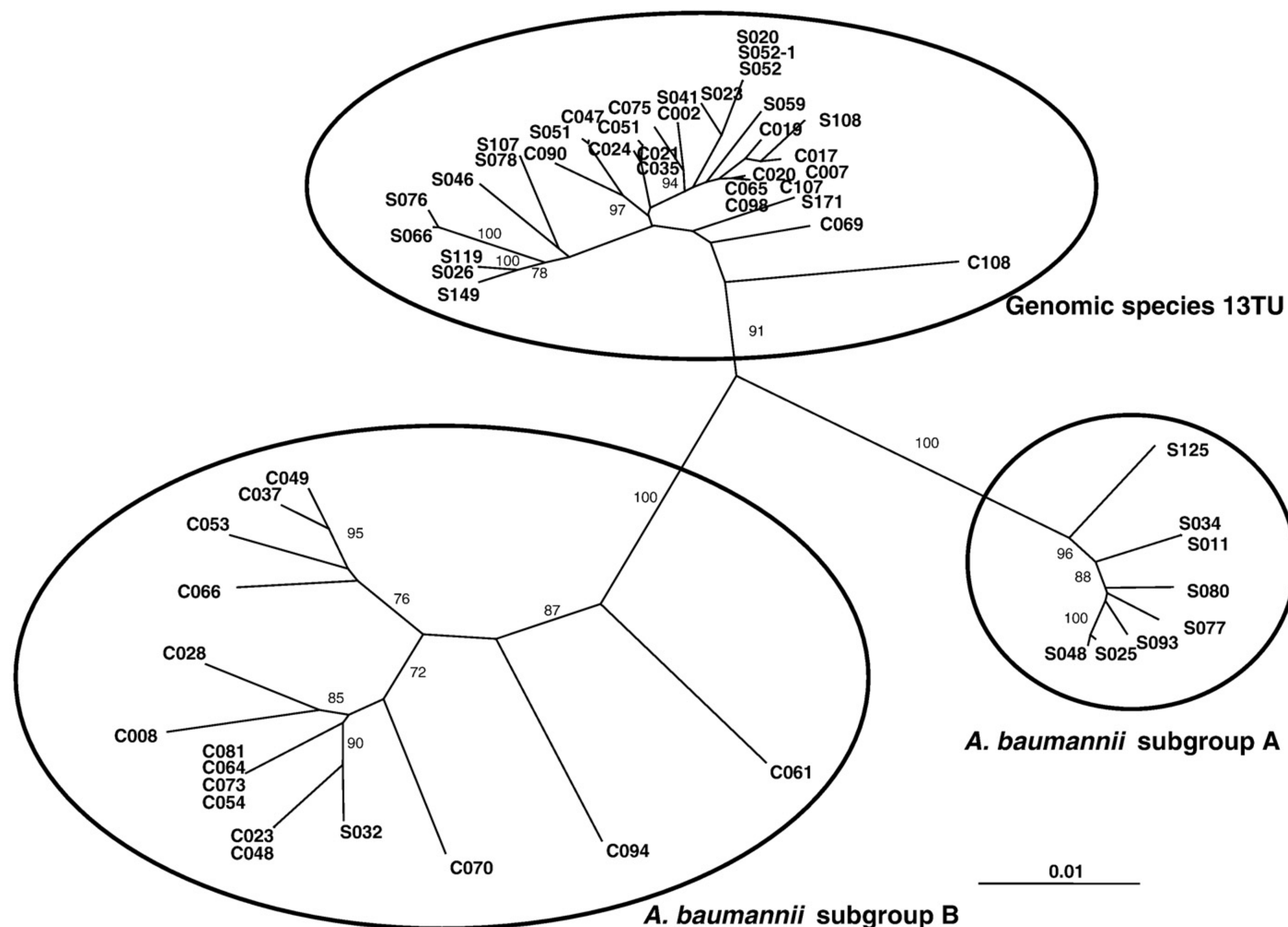


Fig. 1. Unrooted phylogenetic tree of 58 colistin-resistant *Acinetobacter* spp. isolates as derived from concatenated sequences of the 7 gene loci used in MLST. The tree was constructed using the neighbor-joining method. Three *Acinetobacter* groups are represented. Only bootstrap values higher than 70% in the bootstrap analysis are indicated at the corresponding branches. Scale bar indicates 1 substitution per 100 nucleotides.

carbapenem-resistant *A. baumannii* isolates. MLST analysis showed that only 2 major distinct clones were found among the carbapenem-resistant *A. baumannii* isolates acquired from Korean hospitals (Ko et al., unpublished).

Although the mechanism of colistin resistance in *Acinetobacter* spp. has not been fully revealed, it is known that changes in the outer membrane causes colistin resistance in *Pseudomonas aeruginosa* and *Escherichia coli* (Bonomo and Szabo, 2006; Li et al., 2006b). Although resistance to colistin in *Acinetobacter* spp. is rare worldwide, heteroresistance to colistin associated with colistin therapy and a high colistin resistance rate in Korea have been reported (Hawley et al., 2008; Ko et al., 2007). In addition, Tan et al. (2007) revealed the extensive emergence of colistin-resistant *A. baumannii* subpopulations provoked by exposure to colistin. They suggested that the current recommended dosage regimens for colistin are inadequate to prevent the emergence of resistance in vitro (Tan et al., 2007). Thus, it can be speculated that the independent emergence of colistin-resistant *Acinetobacter* spp. isolates in this study may be related to such inadequate dosage regimens. In reality, Gilad et al. (2005) showed that emergence of colistin resistance in *A. baumannii* might be related to increased use of colistin. Hawley et al. (2008) also showed using PFGE that colistin-heteroresistant *Acinetobacter* spp. isolates associated with previous colistin therapy were not derived from a single clone. The frequent appearance of colistin resistance in *Acinetobacter* spp. isolates, as evidenced in this study, should be of concern. Although clonal spreading of colistin-resistant *Acinetobacter* spp. isolates was rarely found in this study, combination of frequent development of colistin-resistant isolates and their clonal dissemination in hospitals may result in a high prevalence of colistin resistance, which limits the therapeutic options for treating *Acinetobacter* infections. Thus, careful use of colistin for treatment of Gram-negative pathogens is needed, and appropriate dosage regimens for colistin so as to prevent colistin-resistant mutants should be defined (Hawley et al., 2008; Li et al., 2006a, 2006b; Tan et al., 2007).

Gene trees of the 58 colistin-resistant *Acinetobacter* spp. isolates from each gene sequence were shown in Fig. 2. Except the *gdhB* and *gpi* gene trees, the other gene trees

Author's personal copy

showed a clear distinction among the 3 *Acinetobacter* groups. Although the 3 clusters generally corresponding to *Aba* A, *Aba* B, and GS 13TU could also be identified in the *gdhB* gene tree, 7 GS 13TU isolates and 4 *Aba* B isolates were grouped with the *Aba* A isolates (Fig. 2C). In contrast, isolates of the 3 groups were more intermingled within the *gpi* gene tree (Fig. 2F). However, the 3 phylogenetic clusters were also found in the *gpi* gene tree. The first cluster (*) consisted of mostly GS 13TU isolates. The 2nd (#) included mostly *Aba* B isolates, some GS 13TU isolates, and all the *Aba* A isolates except S125. Some GS 13TU and 1 *Aba* B were grouped into the 3rd cluster (†) . Thus, it is considered that the colistin-resistant *Acinetobacter* spp. isolates in this study are separated into 3 distinct clusters, which are generally consistent with species and subgroups. Along with the tree from the concatenated sequences, the distinction within each gene tree may indicate the divergence of these 3 groups over an extended period.

However, the phylogenetic relationships among the 3 groups were inconsistent within the gene trees. In contrast to the general conservation of subgroupings, interrelationships among the subgroups were not concordant with each other. Gene trees derived from the *gltA*, *gyrB*, *gdhB*, and *rpoD* sequences showed close relationships between *Aba* A and GS 13TU, whereas *Aba* B and GS 13TU were closely related in those trees derived from *recA* and *cpn60* sequences. Interestingly, such closeness did not correlate with physical relatedness of genes within the genome. A genomic map of

Fig. 2. Phylogenetic trees of 58 colistin-resistant *Acinetobacter* spp. isolates from each gene sequence of the MLST loci: *gltA* (A), *gyrB* (B), *gdhB* (C), *recA* (D), *cpn60* (E), *gpi* (F), and *rpoD* (G). Each gene tree was constructed using the neighbor-joining method and rooted using 2 divergent strains (S117-1 and S133) as out-groups. Scale bars indicate 10 (F, *gpi*) or 1 (all others) substitution per 100 nucleotides. In panel F, the first cluster (*) consisted of mostly GS 13TU isolates. The 2nd (#) included mostly *Aba* B isolates, some GS 13TU isolates, and all the *Aba* A isolates except S125. Some GS 13TU and 1 *Aba* B were grouped into the 3rd cluster (†).

Author's personal copy

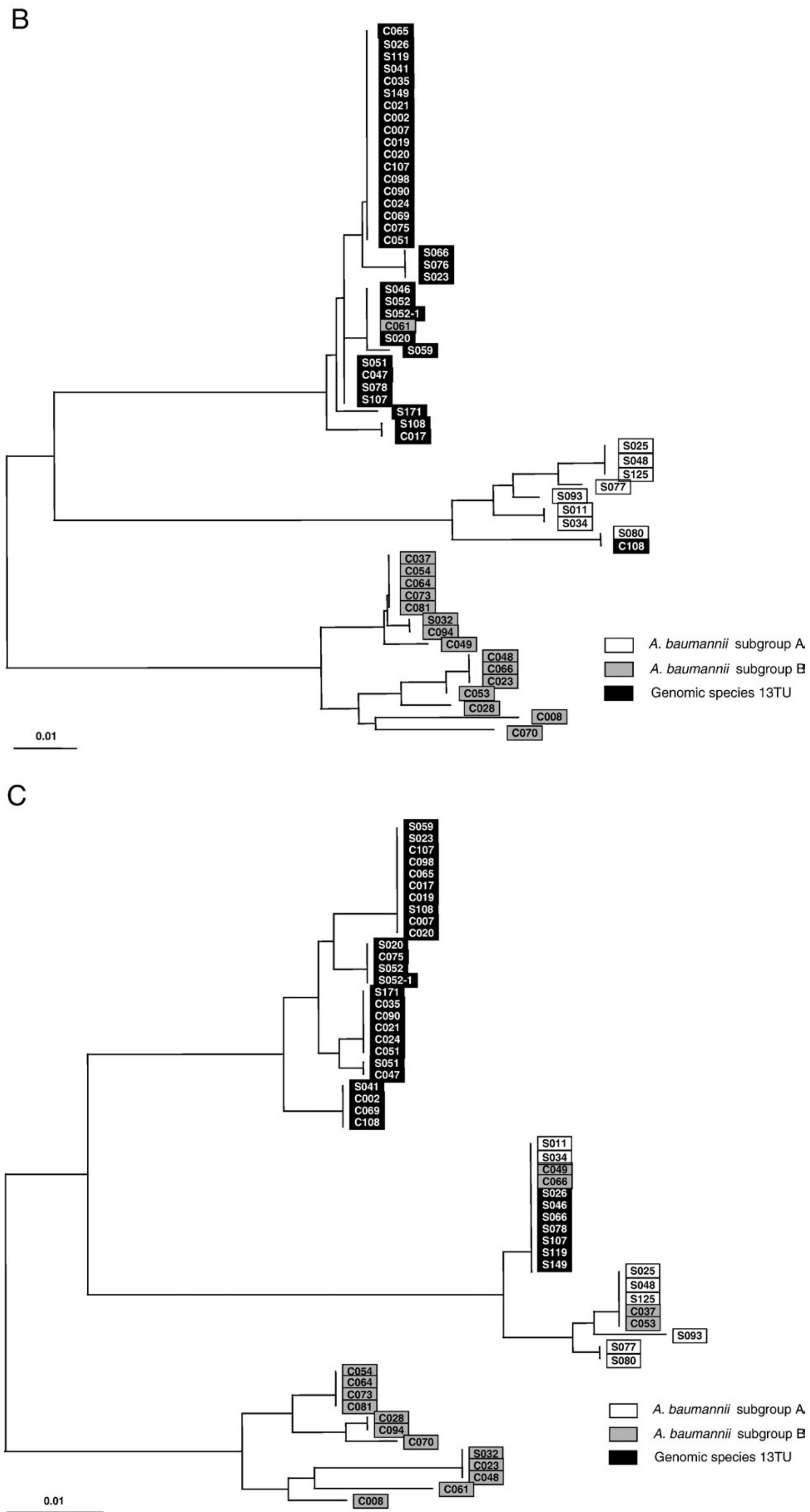


Fig. 2 (*continued*).

Author's personal copy

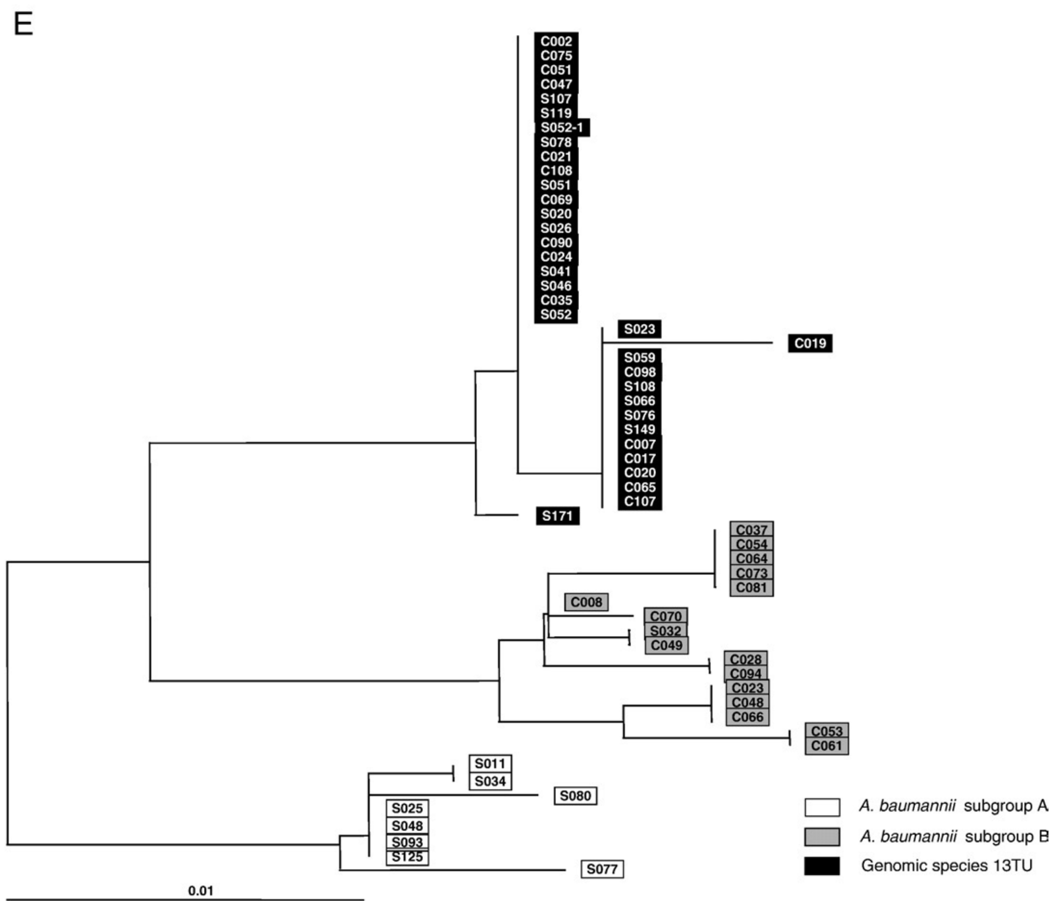
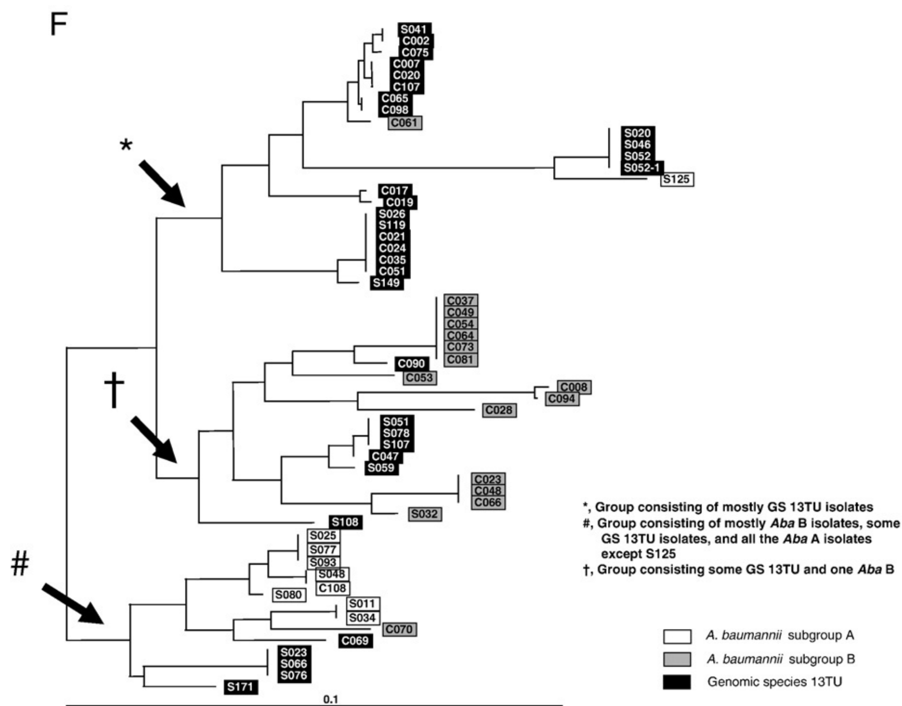


Fig. 2 (*continued*).

Author's personal copy



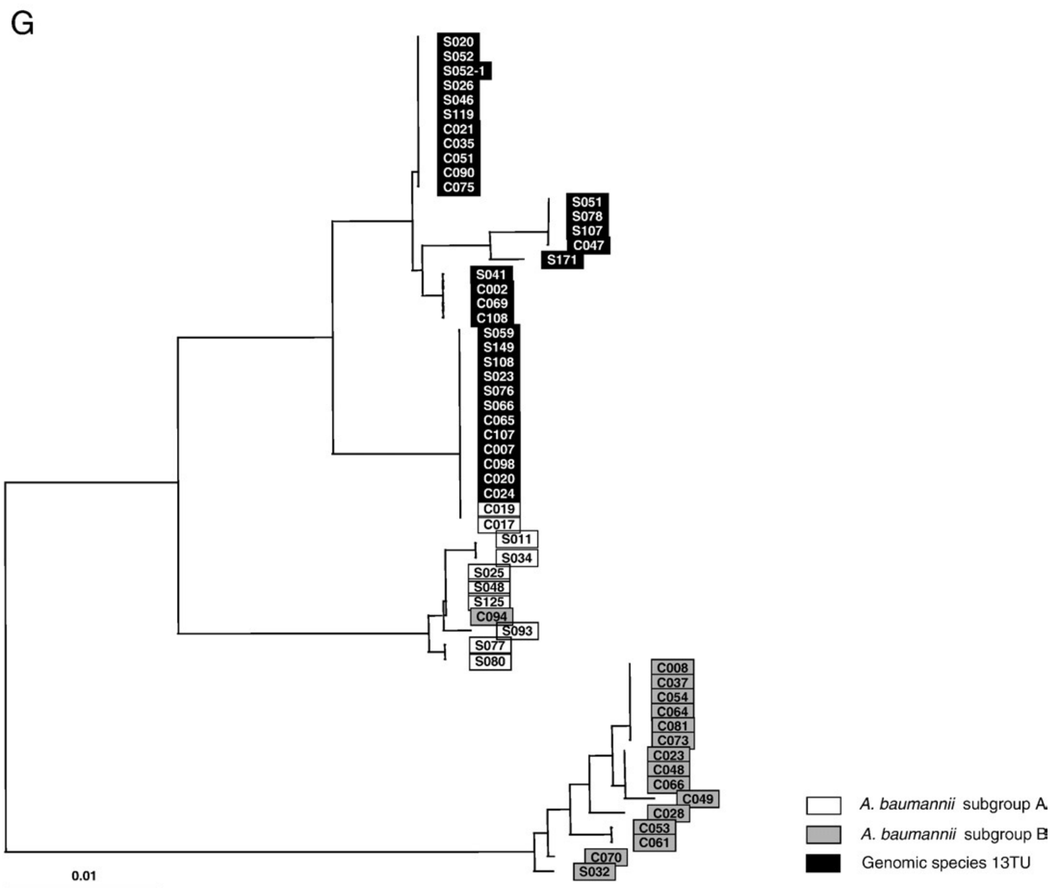


Fig. 2 (*continued*).

Author's personal copy

the *Acinetobacter baylyi* strain ADP1 indicates that the *gltA*, *cpn60*, and *rpoD* genes; the *gyrB* and *gpi* genes; and *gdhB* and *recA* genes are located proximally to each other (Bartual et al., 2005). In the *A. baumannii* strain AYE, of which the genome has been recently sequenced, the physical relatedness of genes are similar to that of the ADP1 strain (Vallenet et al., 2008). Inconsistent groupings may indicate mosaic genomes of *A. baumannii* and GS 13TU. It may also be evidence of gene fragment replacement after divergence of these 3 groups, probably by horizontal gene transfer (Feil and Spratt, 2001). However, an alternative explanation that some MLST loci are unsuitable for typing of *Acinetobacter* may be possible.

In addition to the complicated clustering within the *gdhB* and *gpi* gene trees, some isolates showed incongruent phylogenetic clustering in other gene trees: C075 in *gltA* (Fig. 2A), C061 and C108 in *gyrB* (Fig. 2B), and C094 in *rpoD* (Fig. 2G). This may indicate recent genetic recombination events. *A. baylyi* ADP1 is known to be naturally transformable and has a propensity for frequent recombination (Metzgar et al., 2004). Although natural transformability has not been reported in other *Acinetobacter* spp., it is suggested that frequent recombination in *Acinetobacter* spp. may contribute to high antimicrobial resistance. Furthermore, it has been suggested that colistin resistance may be associated with modification of lipid A with 4-aminoarabinose in *E. coli* (Gatzeva-Topalova et al., 2005), though it requires further investigation to determine if such mutated genes might have been transferred into colistin-susceptible isolates.

In this study, it was shown that the high resistance to colistin of *Acinetobacter* spp. isolates from Korea was not due to clonal dissemination of specific clones but that they likely arose independently. Three colistin-resistant *Acinetobacter* groups were conserved within the phylogenetic tree from concatenated sequences and in most of the individual gene trees. However, gene replacement and recombination might also be inferred in the phylogenetic analyses. Owing to the increasing use of colistin against Gram-negative pathogens and the high recombination rate of *Acinetobacter* spp., it is of concern that colistin resistance in *Acinetobacter* spp. isolates may increase rapidly.

Acknowledgments

The authors thank Ms Ji Young Choi for her technical assistance. This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry of Health, Welfare, and Family Affairs, Seoul, Republic of Korea (A080330-AA1723-08N1-00010A).

References

Bartual SG, Seifert H, Hippler C, Luzon MAD, Wisplinghoff H, Rodriguez-Valera F (2005) Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. *J Clin Microbiol* 43:4382–4390.

Bonomo RA, Szabo D (2006) Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. *Clin Infect Dis*) 43 (Suppl 2):S49–S56.

Clinical and Laboratory Standards Institute (CLSI) (2007) Performance standards for antimicrobial susceptibility testing; 16th informational supplement. Document M100-S17. Wayne, Pa: CLSI.

Falagas MW, Kasiakou SK (2005) Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis* 40:1333–1341.

Feil EJ, Spratt BG (2001) Recombination and the population structures of bacterial pathogens. *Annu Rev Microbiol* 55:561–590.

Gales AC, Jones RN, Sader HS (2006) Global assessment of the antimicrobial activity of polymyxin B against 54,731 clinical isolates of Gram-negative bacilli: report from the SENTRY antimicrobial surveillance programme (2001–2004). *Clin Microbiol Infect* 12:315–321.

Gardam MA, Burrows LL, Kus JV, Brunton J, Low DE, Conly JM, Humar A (2002) Is surveillance for multidrug-resistant Enterobacteriaceae an effective infection control strategy in the absence of an outbreak? *J Infect Dis* 186:1754–1760.

Gatzeva-Topalova P, May AP, Sousa MC (2005) Crystal structure and mechanism of the *Escherichia coli* ArnA (PmrI) transformylase domain. An enzyme for lipid A modification with 4-amino-4-deoxy-L-arabinose and polymyxin resistance. *Biochemistry* 44:5328–5338.

Gilad J, Eskira S, Riesenberg K, Schlaeff er F, Hyam E, Borer A (2005) Emergence of nosocomial colistin-resistant *Acinetobacter baumannii*. 45th Interscience Conference on Antimicrobial Agents and Chemotherapy; Washington, DC, USA; Dec 16–19.

Hawley JS, Murray CK, Jorgensen JH (2008) Colistin heteroresistance in *Acinetobacter* and its association with previous colistin therapy. *Antimicrob Agents Chemother* 52:351–352.

Higgins PG, Wisplinghoff H, Krut O, Seifert H (2007) A PCR-based method to differentiate between *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU. *Clin Microbiol Infect* 13:1199–1201.

Ko KS, Suh JY, Kwon KT, Jung SI, Park KH, Kang CI, Chung DR, Peck KR, Song JH (2007) High rates of resistance to colistin and polymyxin B in subgroups of *Acinetobacter baumannii* isolates from Korea. *J Antimicrob Chemother* 60:1163–1167.

La Scola B, Gundi VAKB, Khamis A, Raout D (2006) Sequencing of the *rpoB* gene and flanking spacers for molecular identification of *Acinetobacter* species. *J Clin Microbiol* 44:827–832.

Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L (2006a) Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 50:2946–2950.

Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, Paterson DL (2006b) Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 6:589–601.

Maiden MCJ (2006) Multilocus sequence tying of bacteria. *Annu Rev Microbiol* 60:561–588.

Metzgar D, Bacher JM, Pezo V, Reader J, Doring V, Schimmel P, Marliere P, de Crecy-Lagard V (2004) *Acinetobacter* sp. ADP1: an ideal model organism for genetic analysis and genome engineering. *Nucleic Acids Res* 32:5780–5790.

Nemec A, Krizova L, Maixnerova M, Diancourt L, van der Reijden TJ, Brisse S, van den Broek P, Dijkshoorn L (2008) Emergence of carbapenem resistance in *Acinetobacter baumannii* in the Czech Republic is associated with the spread of multidrug-resistant strains of European clone II. *J Antimicrob Chemother* 62:484–489.

Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA (2007) Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 51:3471–3484.

Shelburne SA, Singh KV, White AC, Byrne L, Carmer A, Austin C, Graviss E, Stager C, Murray BE, Atmar RL (2008) Sequential outbreaks of infections by distinct *Acinetobacter baumannii* strains in a public teaching hospital in Houston, Texas. *J Clin Microbiol* 46: 198–205.

Author's personal copy

Tan CH, Li J, Nation RL (2007) Activity of colistin against heteroresistant *Acinetobacter baumannii* and emergence of resistance in an in vitro pharmacokinetic/pharmacodynamic model. *Antimicrob Agents Chemother* 51:3413–3415.

Towner KJ, Levi K, Vlassiadi M, on behalf of the ARPAC Steering Group (2008) Genetic diversity of carbapenems-resistant isolates of *Acinetobacter baumannii* in Europe. *Clin Microbiol Infect* 14:161–167.

Vallenet D, Nordmann P, Barbe V, Poirel L, Mangenot S, Bataille E, Dossat C, Gas S, Kreimeyer A, Lenoble P, Oztas S, Poulain J, Segurens B, Robert C, Abergel C, Claverie JM, Raoult D, Medigue C, Weissenbach J, Cruveiller S (2008) Comparative analysis of Acinetobacters: three genomes for three lifestyles. *PLoS One* 3:e105.

van Dessel H, Dijkshoorn L, van der Reijden T, Bakker N, Paauw A, van den Broek P, Brisse S (2004) Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals. *Res Microbiol* 155:105–112.

Wisplinghoff H, Hippler C, Bartual SG, Haefs C, Stefanik D, Higgins PG, Seifert H (2008) Molecular epidemiology of clinical *Acinetobacter baumannii* and Acinetobacter genomic species 13TU isolates using a multilocus sequencing typing scheme. *Clin Microbiol Infect* 14:708–715.