Selective advantages of two major clones of carbapenem-resistant Pseudomonas aeruginosa isolates (CC235 and CC641) from Korea: antimicrobial resistance, virulence and biofilm-forming activity

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The characteristics of carbapenem-resistant P. aeruginosa (CRPA) isolates from Korea were investigated. Two major clones, clonal complex (CC) 235 and CC641, were identified. CC235, an important international clone, might have been imported recently in Korea as this clone displayed a homogeneous genotype, oprD mutation and antimicrobial resistance profile. While 13 ST235 isolates harboured the blalmp-6 gene, which conferred high-level meropenem resistance, CC841 isolates showed high biofilm-forming activity. CC235 and CC641 isolates showed distinct distribution of ferripyoverdine receptor type and virulence markers. While all CC235 isolates were of the fpvAIIb type and exoS+/exoU+, CC641 isolates were exoS+/exoU, and all but one showed the fpvAIIB type. CC235 and CC641 isolates were also characterized by different extracellular protease activity: staphylolysin and elastase activities in CC235 and CC641, respectively. Two major CRPA clones in Korea seem to be predominant, reflecting their selective advantage by virtue of antimicrobial resistance, virulence and biofilm-forming activity.

INTRODUCTION

Pseudomonas aeruginosa is one of the major microorganisms responsible for nosocomial infections such as pneumonia, urinary tract infections, surgical site infections and bloodstream infections. The increasing prevalence of multidrug-resistant P. aeruginosa isolates is a major cause for concern, given the limited therapeutic options to treat severe infections caused by this pathogen (Poole, 2011). Although carbapenems such as imipenem and meropenem remain effective antibiotics for the therapy of serious infections by P. aeruginosa isolates, the development of high carbapenem resistance rates in P. aeruginosa isolates has been reported worldwide (Poole, 2011). Recently, an IMP-6-producing P. aeruginosa clone, ST235, was reported to have disseminated in Korea (Seok et al., 2011; Yoo et al., 2012). IMP-6, which emerged from IMP-1 by Ser196Gly substitution, displays increased hydrolytic activity for meropenem (Yano et al., 2001). ST235 has been also reported as an international clone, which has been associated with VIM-type metallo-β-lactamase (MBL) (Giske et al., 2006). Clonal spreading of ST235 isolates showing high resistance to meropenem due to IMP-6 would be a serious clinical concern. However, the characteristics contributing to its prevalence except antimicrobial resistance were not investigated.

The pathogenesis of P. aeruginosa infection depends on multiple cell-associated and extracellular virulence factors, including protease and toxins (Goodman & Lory, 2004). Proteases are important virulence factors (Tingpej et al., 2007; Zhu et al., 2002). Elastase (mainly LasB) acts alone or together with other P. aeruginosa proteases to degrade or inactivate several biologically important substrates, including connective tissues and immune system components (Zhu et al., 2002). Alkaline protease is able to degrade laminin and other substrates, suggesting a possible role for this enzyme in tissue invasion and dissemination (Twining et al., 1993). Additionally, P. aeruginosa uses a type III secretion system to inject toxic effector proteins into the cytoplasm of eukaryotic cells, which promotes severe illness. It has been shown that secretion of ExoU had the greatest impact on virulence while secretion of ExoS had an

Abbreviations: CC, clonal complex; CRPA, carbapenem-resistant P. aeruginosa; MBL, metallo-β-lactamase; MLST, multilocus sequence typing; ST, sequence type.
intermediate effect and ExoT had a minor effect (Shaver & Hauser, 2004).

In this study, we identified the molecular diversity of carbapenem-resistant P. aeruginosa (CRPA) isolates from Korea, and investigated the characteristics of major clones, such as ferricyanide receptor type, virulence marker, swimming motility and biofilm-forming activity.

METHODS

**Bacterial strains.** A total of 57 CRPA clinical isolates from Korea, which had been identified in a previous study (Lee & Ko, 2012), were included in this study. They were collected from November 2006 to August 2007 from seven Korean hospitals, and most of them were nosocomial.

**Antimicrobial susceptibility testing.** In vitro antimicrobial susceptibility testing was performed by a broth microdilution method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). Seven antimicrobial agents were tested: imipenem, meropenem, piperacillin/tazobactam, ceftazidime, tetracycline, ciprofloxacin and amikacin. Escherichia coli ATCC 25922 and P. aeruginosa ATCC 27853 were employed as the quality control strains. All tests were repeated with three independent cultures, each tested in duplicate.

**Genotyping.** Multilocus sequence typing (MLST) was performed as described previously (Curran et al., 2004). Allelic profiles and sequence types (STs) were assigned at the PubMLST database (http://pubmlst.org/paeruginosa/). New STs were submitted to the PubMLST website and approved. Pulsed-field gel electrophoresis (PFGE) was performed for 13 IMP-6-producing CRPA isolates. For PFGE, genomic DNA plugs prepared as described previously (Struelens et al., 1993) were digested with 10 U SpeI (New England Biolabs). The restricted fragments were separated using a temperature-controlled CHEF DR III system (Bio-Rad) at 6.0 V cm⁻¹, with pulse times ranging from 5 to 30 s, for 22 h (Yan et al., 2001).

**Detection and identification of MBLs.** All CRPA isolates were screened by PCR for blaIMP, blaVIM, blaSPM, blaGIM and blaSIM. Presence of MBL genes was confirmed by sequencing of PCR products.

**Multiplex PCR for the detection of ferricyanide receptor genes.** Ferricyanide (Fpv) receptor type was determined using a multiplex PCR method as described previously (Bodilis et al., 2009). The primers used for this study are listed in Table 1. Specific PCR conditions were adopted from Bodilis et al. (2009).

**Virulence marker assays.** The virulence markers exoA, exoS, exoT, exoY and exoU were also identified using PCR with specific primers listed in Table 1 as described previously (Kaszab et al., 2011).

**Zymography.** Gelatin zymography for extracellular protease activity was carried out using 10 % polyacrylamide gels containing 0.1 % gelatin (Sigma-Aldrich). Unconcentrated supernatants of exponential-phase cultures were subjected to SDS-PAGE. Following electrophoresis, gels were washed in 2.5 % Triton X-100 (Sigma-Aldrich) to renature proteins, incubated overnight at 37 °C in 20 mM Tris/HCl (pH 8.5) and 0.05 % sodium azide to induce protease activity, and then stained with 0.25 % Coomassie blue for 2 h with gentle shaking. Extracellular protease activity appeared as clear bands in blue-stained gels (Tingpej et al., 2007). Experiments were repeated at least three times.

**Biofilm assay.** To assess biofilm formation, 96-well microtitre plate assays were performed as previously described (Merritt et al., 2005) with minor modification for all CRPA isolates. Briefly, overnight

### Table 1. Primers used in this study

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<th>Primer</th>
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<td><strong>Primers for exotoxin detection</strong></td>
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cultures were normalized by measuring the optical density at 600 nm and diluted 1:100 in Luria–Bertani medium supplemented with 0.5% glucose. Aliquots (200 µl) of this suspension were inoculated into the wells of 96-well flat-bottom polystyrene plates and incubated for 18 h at 37 °C. The planktonic bacteria from each well were removed and the wells were gently washed and air-dried. A well to which sterile LB lacking bacteria was added served as a control. The biofilm bacteria were visualized by staining with 0.5% crystal violet for 15 min and resuspended by adding 200 µl 95% ethanol. The absorbance at 600 nm (A_{600}) of the extracted crystal violet was then measured by microplate reader, yielding a measure of biofilm formation (relative to the control). Experiments were repeated with five independent cultures, each tested in duplicate (n=10 tests). One-way analysis of variance was used to determine whether there were any significant differences in biofilm formation capacity between the three independent groups [colonal complex (CC) 235, CC641 and singleton group]. Data were analysed using spss version 11.5 software (SPSS Inc.). Differences were considered statistically significant at a P value of <0.05.

Swimming motility assay. The swimming assay was done by stabbing 0.3% LB agar plates with a single colony of bacteria as described previously (Murray & Kazmierczak, 2006). The swimming zone was measured after overnight incubation at 30 °C. All isolates were tested in duplicate on five independent cultures (n=10 tests), which showed identical results.

RESULTS

Genotypes
MLST analysis revealed 27 different STs, including six new STs, among the 57 CRPA isolates (Table 2). The most prevalent clone was ST235 (21 isolates, 36.8%), followed by ST641 (six isolates, 10.5%). CC235 included ST1081 as well as ST235, and CC641 included 12 CRPA isolates of six STs (Table 2). Although three CCs were identified, very diverse genotypes (STs) were found among the CRPA isolates. MLST analysis revealed that the carbapenem resistances have emerged independently in diverse P. aeruginosa clones from Korea, with the exceptions of ST235 and ST641.

MBL prevalence
MBL genes were identified in 15 of the 57 carbapenem-resistant P. aeruginosa isolates (26.3%). Thirteen isolates were positive for bla_{IMP}-like genes, and two isolates were positive for bla_{VIM}-like genes. No bla_{SPM}+, bla_{GIM}+, or bla_{SIM}-like genes were identified in the isolates. The bla_{IMP}- and bla_{VIM}-like genes identified were bla_{IMP-6} and bla_{VIM-2}, respectively. All IMP-6-harbouring isolates belonged to ST235 (Table 2) and showed a nearly identical PFGE pattern (Fig. 1). Two VIM-2-harbouring isolates belonged to different STs, ST235 and ST298.

Antimicrobial resistance
Twenty-two CC235 (including ST235 and ST1081) isolates were from six different hospitals, and most (20 isolates) were isolated from patients with urinary tract infections. CC235 isolates showed very high MICs of meropenem, partly due to the presence of IMP-6. In addition, CC235 isolates showed very high antimicrobial resistance rates: all CC235 isolates were resistant to amikacin and ciprofloxacin, and resistance to ceftazidime and tetracycline was each detected in two isolates (Table 2). As a result, 15 CC235 isolates were resistant to all six antimicrobial classes tested in this study. On the other hand, the isolates of another major clone, CC641, displayed different antimicrobial resistance profiles from CC235 isolates. Most were not resistant to meropenem, ceftazidime, amikacin and ciprofloxacin. In addition, CC298 and singleton isolates also showed low resistance rates of ceftazidime, amikacin and ciprofloxacin.

Types of ferricytochrome receptors
Two major CRPA clones showed different distribution of ferricytochrome receptor type. While all CC235 isolates showed the fpvAllB type, all but one CC641 isolate showed the fpvAllI type (Table 2). One ST27 isolate of CC641 showed the fpvAll type. The fpvAllB and fpvAllI types were also found in another seven unrelated CRPA isolates. The fpvAll type was observed in another five CRPA isolates, and the fpvAllI type was found in four isolates. The fpvB type was identified in all CRPA isolates.

Exoenzyme production
The distribution of exoenzymes was also distinct among CC235 and CC641 isolates (Table 2). The exoA, exoT and exoY genes were identified in all CRPA isolates. However, the exoS and exoU genes were not both present in the same isolate. While all CC235 isolates possessed the exoU gene but not the exoS gene (exoS⁻/exoU⁺), all CC641 isolates were exoS⁺/exoU⁻.

Extracellular protease activity
Fifty-four of the 57 isolates (94.7%) produced positive results in SDS-PAGE zymography, which produced a protease profile of up to three bands for each sample (Fig. 2). Bands were detected at molecular masses of approximately 120, 98 and 51 kDa. Elastase (LasB) and staphylolysin (LasA) were represented by bands at 120 and 98 kDa, respectively, and alkaline protease (AprA) was represented by a band at 51 kDa (Zhu et al., 2001). Twenty-five of the 57 (43.8%) isolates produced alkaline protease, and 30 (51%) and 24 (42.1%) of 57 isolates produced staphylolysin and elastase, respectively (Table 2). The amount of protease secreted by individual isolates as indicated by band intensity was variable (Fig. 2). While all ST235 isolates exhibited staphylolysin activity, ST641 isolates exhibited high activity of elastase (Table 2, Fig. 2).

Biofilm-forming activity and swimming motility
Biofilm-forming activity was compared among three groups: CC235, CC641 and the other CRPA isolates
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<th>Source‡</th>
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</tr>
<tr>
<td>ST27</td>
<td>6-5-6-7-</td>
<td>4-6-7</td>
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Table 2. Genotype, antimicrobial resistance, Fpv receptor type and distribution of virulence markers in carbapenem-resistant *P. aeruginosa* isolates from South Korea.
Table 2. cont.

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<th>CC</th>
<th>ST</th>
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<th>MBL</th>
<th>Fpv type</th>
<th>Exotoxin genes</th>
<th>Extracellular protease§</th>
<th>MIC of antimicrobial agent (mg l⁻¹)¶</th>
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<td>AI</td>
<td>+  +  +  +  –  –  –  +</td>
<td>16  2  64  16  0.25  &gt;256/4</td>
<td>16</td>
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</table>

*aCSA-aroE-guaA-mull-nuoD-ppsA-trpE.
†SMC, Samsung Medical Center (Seoul); KSH, Kangbuk Samsung Hospital (Seoul); KNUH, Kyungpook National University Hospital (Daegu); DFH, Daegu Fatima Hospital (Daegu); CNUH-G, Chonnam National University Hospital (Gwangju); CNU-D, Chungnam National University Hospital (Daejeon), GNU, Gyeongsang National University Hospital (Jinju).
‡B, blood; U, urine.
§The degree of activity of the extracellular protease designated as – (negative) or + (positive).
||IMP, imipenem; MRP, meropenem; CAZ, ceftazidime; AMK, amikacin; CIP, ciprofloxacin; P/T, piperacillin/tazobactam; TET, tetracycline. Bold type indicates resistance.
The mean biofilm-forming activity was $4.08 \pm 1.61$, $5.69 \pm 2.29$ and $3.33 \pm 1.51$ in CC235, CC641 and other CRPA isolates, respectively, which was significantly different among the groups ($P = 0.001$). Biofilm-forming activity of CC641 was significantly higher than those of CC235 and the other isolates ($P = 0.028$ and $P = 0.001$, respectively). However, the difference in biofilm-forming activity was not significant between CC235 and the other CRPA isolates ($P = 0.192$). In assays of swimming motility, non-clonal CRPA isolates showed a larger swimming zone than ST235 and ST641 isolates (Fig. 4).

**DISCUSSION**

Previous studies established that CC235, including ST235, is a predominant clone in CRPA isolates from Korea (Seok et al., 2011; Yoo et al., 2012). It has also been found in Japan and European countries (Cholley et al., 2011; García-Castillo et al., 2011; Kitao et al., 2012; Maatallah et al., 2011); thus it should be considered as an internationally disseminated CRPA clone. One of the important features of the ST235 clone in Korea may be the presence of $\text{bla}_{\text{IMP-6}}$. ST235 isolates with $\text{bla}_{\text{IMP-6}}$ showed a nearly identical PFGE pattern, and $\text{oprD}$ gene sequences showed the identical mutation in all ST235 isolates carrying $\text{bla}_{\text{IMP-6}}$, that is, a 1 bp deletion at position 214 (Lee & Ko, 2012). These may indicate clonal dissemination of the clone. On the other hand, the other CC235 isolates without $\text{bla}_{\text{IMP-6}}$ showed diverse mutation patterns of the $\text{oprD}$ gene. Some postulates on the emergence of CC235 carrying $\text{bla}_{\text{IMP-6}}$ in Korea are possible: (i) the $\text{bla}_{\text{IMP-6}}$ gene might have been transferred in certain strains of ST235 in Korea from other species, (ii) the ST235 clone with the $\text{bla}_{\text{IMP-6}}$ gene might have been recently introduced into Korea from another country, or (iii) $\text{bla}_{\text{IMP-1}}$ in the ST235 clone may have changed into $\text{bla}_{\text{IMP-6}}$ by a single mutation at position 640. $\text{bla}_{\text{IMP-6}}$ has been identified in Klebsiella pneumoniae from Japan (Shigemoto et al., 2012), but it has not been found in other species or clones than $P. \text{aeruginosa}$ ST235 in Korea. Although $\text{bla}_{\text{IMP-6}}$ was first identified in Japan (Yano et al., 2001), it is unclear where it first emerged. Thus, the origin of ST235 carrying $\text{bla}_{\text{IMP-6}}$ is currently unknown, but low diversity of ST235 with $\text{bla}_{\text{IMP-6}}$ and its prevalence may indicate its recent emergence and advantage in survival and dissemination. For example, high-level resistance to meropenem would be an advantageous feature of ST235 isolates with $\text{bla}_{\text{IMP-6}}$ (Table 2) (Yano et al., 2001).

It is known that type III secretion products such as ExoS and ExoU are important virulence markers (Kaszab et al., 2011). Although $\text{exoA}$, $\text{exoT}$ and $\text{exoY}$ were shared in all CRPA isolates, $\text{exoS}$ and $\text{exoU}$ were never present together, which was shown previously (Maatallah et al., 2011; Wiehlmann et al., 2007). It has been suggested that $\text{exoU}$ is transferred horizontally, followed by a targeted deletion of $\text{exoS}$ (Kulasekara et al., 2006). $\text{exoU}$ is a major factor of potential virulence of $P. \text{aeruginosa}$ (Lin et al., 2006). Kaszab et al. (2011) reported that, while compost isolates had a higher frequency of $\text{exoS}$ but a lower frequency of $\text{exoU}$, $\text{exoU}$ was exclusively found in clinical isolates. In this
study, CC235 with *exoU* showed a high resistance rate to ciprofloxacin, but CC641 with *exoS* did not, which is in agreement with a previous finding (Agnello & Wong-Beringer, 2012). In clinical *P. aeruginosa* isolates from the USA, *exoU*-positive isolates showed a high proportion of fluoroquinolone resistance compared with *exoS*-positive isolates (Agnello & Wong-Beringer, 2012). Although these authors did not explore their genotypes, our and the previous finding that *exoU*-positive isolates represented a clonal population forming the major clade of *P. aeruginosa* may indicate that the presence of *exoU* in ST235 contributes to the spread of the clone in patients (Maatallah *et al.*, 2011; Wiehlmann *et al.*, 2007).

As virulence markers such as *exoS* and *exoU*, extracellular proteases were produced strongly in the main clones. Extracellular proteases are also known as important virulence factors in *P. aeruginosa* (Tingpej *et al.*, 2007; Zhu *et al.*, 2002). Thus, high extracellular protease production by the main clones, especially in ST641, may contribute to their prevalence. In addition, two clones showed different main extracellular proteases: elastase in

---

**Fig. 3.** Relative quantity of biofilm in carbapenem-resistant *P. aeruginosa* isolates. Data are expressed as $A_{600}$ relative to the well with sterile LB that served as a control. The dashed line indicates quantity of biofilm in the reference strain, *P. aeruginosa* PAO1. Experiments were repeated with five independent cultures, each tested in duplicate. Filled bars, CC235; dashed bars, CC641; open bars, singletons.

---

**Fig. 4.** Comparison of swimming motility among *P. aeruginosa* isolates included in this study. The dashed line represents the mean zone diameters of 57 CRPA isolates. Filled bars, CC235; dashed bars, CC641; open bars, singletons.
ST235 and staphylolysin in ST641. Another main carbapenem-resistant 
P. aeruginosa clone, ST641, also showed the same mutation pattern of 
oprD, a 1 bp deletion at position 281 (Lee & Ko, 2012). No ST641 isolates 
contained any MBL genes. This ST presented different 
antimicrobial susceptibility profiles from ST235. That is, 
CC641 including ST641 showed susceptibility or low-level 
resistance to meropenem, ceftazidime, ciprofloxacin, ami-
kacin and tetracycline. Of note was that CC641 isolates 
showed significantly higher biofilm-forming activity than 
CC235 and the other isolates. Although the CRPA isolates 
included in this study were not from indwelling devices 
such as catheters, it seems that high biofilm-forming 
activity of CC641 isolates confers an advantage in survival 
in hospital settings. This clone was not identified in 
another study from Korea (Seok et al., 2011). Thus, a 
susceptible strain belonging to ST641 might have become 
imipenem resistant by a single deletion in 
oprD recently and then have been disseminated into some hospitals in 
Korea. Because ST641 has rarely been identified in other 
countries (Nemec et al., 2010), it may be endemic to Korea. In addition, identification of several single- or double-locus 
variants of ST641 may support the idea that ST641 was 
established relatively long ago and has differentiated. Unlike 
their biofilm-forming activity, virulence markers and 
antimicrobial susceptibility, isolates of the main clones 
showed lower swimming motility than non-clonal isolates. 
Although it has been proposed that swimming motility may contribute to the success of some clones (Murray & 
Kazmierczak, 2006), it is postulated that motility did not 
contribute to the success of the clones in Korea in this study.

Three structurally different pyoverdines with different peptide 
chains have been identified from 
P. aeruginosa strains 
(Cornelis et al., 1989; Meyer et al., 1997; De Vos et al., 2001), each recognized at the level of the outer membrane by 
a specific receptor (Cornelis et al., 1989). Pyoverdine is 
essential for the virulence of 
P. aeruginosa in mouse models 
(Meyer et al., 1996; Handfield et al., 2000). Two predominant 
CRPA clones, CC235 and CC641, also showed distinct 
characteristics of FpvA type. The production of the iron-
scavenging, peptidic siderophore pyoverdine is important for 
the colonization of the host by 
P. aeruginosa, and 
P. aeruginosa can be classified into three ferrivopyoverdine 
receptor (FpvA) types (Bodilis et al., 2009). Although the 
different capacities to take up pyoverdine or virulence to the 
host according to the FpvA type have not been elucidated yet 
(Wiehlmann et al., 2007), it has been proposed that 
FpvAII was transferred more recently or from a more distantly related 
orGANism than the other two types (Bodilis et al., 2009). In a 
study comparing the 
P. aeruginosa isolates from bovine, human and environmental sources, 
FpvAI was prevalent in isolates from humans, but 
FpvAII was rare in such isolates (Szmolka et al., 2012). Thus, it is currently unknown which 
type contributes to the virulence of 
P. aeruginosa.

Our study demonstrates the diversity of CRPA isolates 
from Korea. However, two major clones, ST235 and 
CC641, were identified. They seemed to have selective 
advantage due to antimicrobial resistance (ST235) and 
biomembrane activity and extracellular proteases 
(CC641). Further investigations on the emergence of 
resistant clones and their dissemination are warranted.

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Korea). This study was supported by a grant from the Korea 
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REFERENCES

Agnello, M. & Wong-Beringer, A. (2012). Differentiation in quinolone 
resistance by virulence genotype in 
Pseudomonas aeruginosa. 
PLoS ONE 7, e42973.

Bodilis, J., Ghysels, B., Osayande, J., Matthijis, S., Pirnay, J. P., 
Denayer, S., De Vos, D. & Cornelis, P. (2009). Distribution and 
evolution of ferrivopyoverdine receptors in 
Pseudomonas aeruginosa. 
Environ Microbiol 11, 2123–2135.

Cholley, P., Thouverez, M., Hocquet, D., van der Mee-Marquet, N., 
Talon, D. & Bertrand, X. (2011). Most multidrug-resistant 
Pseudomonas aeruginosa isolates from hospitals in eastern France 
belong to a few clonal types. 
J Clin Microbiol 49, 2578–2583.

Testing. 21st Informational Supplement M100–S21. 
Wayne, PA: Clinical and Laboratory Standards Institute.

pyoverdine-mediated iron uptake systems among 
Pseudomonas aeruginosa strains. 
Infect Immun 57, 3491–3497.

Development of a multilocus sequence typing scheme for the 
opportunistic pathogen Pseudomonas aeruginosa. 
J Clin Microbiol 42, 5644–5649.

De Vos, D., De Chial, M., Cochez, C., Jansen, S., Tümmler, B., Meyer, 
by Pseudomonas aeruginosa isolated from cystic fibrosis patients: 
prevalence of type II pyoverdine isolates and accumulation of 
pyoverdine-negative mutations. 
Arch Microbiol 175, 384–388.

García-Castillo, M., Del Campo, R., Morosini, M. I., Riera, E., Cabot, 
dispersion of ST175 clone despite high genetic diversity of 
carbapenem-non-susceptible Pseudomonas aeruginosa 
clinical strains in 16 Spanish hospitals. 

Giske, C. G., Libisch, B., Colinson, C., Scoulia, E., Pagani, L., Füzi, M., 
between VIM-1-like metallo-β-lactamase-producing 
Pseudomonas aeruginosa strains from four European countries by multilocus 
sequence typing. 
J Clin Microbiol 44, 4309–4315.

Pseudomonas aeruginosa by genomewide transcriptional profiling. 
Curr Opin Microbiol 7, 39–44.

Handfield, M., Lehoux, D. E., Sanschagrin, F., Mahan, M.J., Woods, 
Pseudomonas aeruginosa. 


