Antagonism and population dynamics of *Acinetobacter baumannii* from US military treatment centers

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Antibiotic-resistant bacteria complicate many infections and can be difficult to eradicate from hospitals. The population dynamics and ecology of these organisms in the hospital setting, however, is not well understood. Here, we report extensive strain-based antagonistic interactions occurring in military clinical isolates of *Acinetobacter baumannii*, a bacterial species that causes many drug-resistant hospital-associated infections. Sequence-based phylogenetic analysis of isolates allowed for differentiation to two major clades, with one of the clades representing two closely related genetic groups. Antagonistic activity was detected using a spot-plate assay to test pairwise interactions of all isolates. Isolates exhibited extensive and diverse patterns of antagonism against other isolates. One major clade of isolates had a distinct change in antagonism phenotype between isolates that differed by one base pair out of ~1500bp sequenced, with consistent antagonism of one group of isolates by the other. Both the antagonistic and the sensitive group exhibited extensive drug resistance. The first isolate of the antagonistic group was cultured in May 2010. The proportion of isolates from the antagonistic group collected before and after July 2010 increased from 2% to 76%. The results of this early study of the ecology of hospital-associated bacterial populations are discussed in the context of the species ecology of bacteria in natural environments. This work is a potential starting point for investigations into ecological interventions for infection control in hospitals.

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Abstract: Antibiotic-resistant bacteria complicate many infections and can be difficult to eradicate from hospitals. The population dynamics and ecology of these organisms in the hospital setting, however, is not well understood. Here, we report extensive strain-based antagonistic interactions occurring in military clinical isolates of *Acinetobacter baumannii*, a bacterial species that causes many drug-resistant hospital-associated infections. Sequence-based phylogenetic analysis of isolates allowed for differentiation to two major clades, with one of the clades representing two closely related genetic groups. Antagonistic activity was detected using a spot-plate assay to test pairwise interactions of all isolates. Isolates exhibited extensive and diverse patterns of antagonism against other isolates. One major clade of isolates had a distinct change in antagonism phenotype between isolates that differed by one base pair out of ~1500bp sequenced, with consistent antagonism of one group of isolates by the other. Both the antagonistic and the sensitive group exhibited extensive drug resistance. The first isolate of the antagonistic group was cultured in May 2010. The proportion of isolates from the antagonistic group collected before and after July 2010 increased from 2% to 76%. The results of this early study of the ecology of hospital-associated bacterial populations are discussed in the context of the species ecology of bacteria in natural environments. This work is a potential starting point for investigations into ecological interventions for infection control in hospitals.

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Introduction

Infections caused by antibiotic-resistant bacteria are an increasing threat to global health. With few new antibiotics in development, the medical community needs new strategies to combat this epidemic. *Acinetobacter* spp. are Gram-negative gammaproteobacteria with high rates of drug resistance. *Acinetobacter* causes a variety of infections in patients with critical illness or severe injury (Caricato et al., 2009; Dijkshoorn, Nemec, & Seifert, 2007; Petersen et al., 2011). This genus also occurs on surfaces in built environments including retail stores, homes, sewer systems and hospitals (Hoisington, Maestre, Kinney, & Siegel, 2016; Lax et al., 2014; Oberauner et al., 2013; Vandewalle et al., 2012). Clinical methods that determine bacterial species by phenotype do not typically differentiate among similar species of *Acinetobacter*. Instead, most isolates are identified as members of the *A. baumannii/calcoaceticus* (ABC) species complex. Of the species making up this complex, *A. baumannii* causes the majority of infections (Dijkshoorn et al., 2007).

Pathogens can often be cultured from the site of an infection, but molecular methods of bacterial detection have revealed that infections often contain more or different organisms than those identified by culture alone. Still, culture-based methods can be appropriate for some infectious disease applications. Many pathogens exhibit clonal evolution, and culture-based identification can help identify common pathogens in order to treat patients and prevent their spread (Achtman, 2012; Colpan et al., 2013; McDowell, Nagy, Magyari, Barnard, & Patrick, 2013; Spoor et al., 2013; Zimbler, Schroeder, Eddy, & Lathem, 2015). However, some types of infections are more difficult to attribute to a single, highly virulent organism. In these situations, molecular methods may be more appropriate for determining the composition and characteristics of bacteria in infections. Open wounds, for example, are in direct contact with the environment and thus bacteria-contaminated surfaces and particles. In chronic wound infections, polymicrobial communities appear to be the rule, not the exception (Price et al., 2009; Rhoads, Cox, Rees, Sun, & Wolcott, 2012; Wolcott et al., 2016). Synergistic pathogenic interactions have been reported in bacteria coexisting in wounds, even between strains of the same species (Keogh et al., 2016; Liao et al., 2014). The contribution of strain-based bacterial interactions to disease in the hospital environment is not yet well understood.

In this study, we aim to examine the population ecology of bacteria in the hospital environment by focusing on *Acinetobacter* clinical isolates through the lens of microbial ecology. Contrasted with the traditional clinical paradigm, environmental microbiology expects a wide variety of microbes to exist together in an open environment. Bacterial populations in soil, seawater and other natural environments often contain microdiverse strains coexisting (Acinas et al., 2004; Kashtan et al., 2014; Klepac-Ceraj et al., 2004; Nelson, Maezato, Wu, Romine, & Lindemann, 2016). These populations can persist for years (Szabo et al., 2013). Microheterogenous populations, rather than clones, have even been proposed to be the unit of pathogenesis for some infections in marine animals (Lemire et al., 2015). Given the tension between the medical expectation of pathogen clonality and the environmental expectation of diverse populations of strains, perhaps hospital-acquired infections that appear to be mono-microbial are actually polymicrobial below the species taxonomic level. Understanding the diversity and population structure of bacteria associated with nosocomial infections could provide insight on the way these populations could be controlled more effectively.

This examination of population ecology requires both genetic and trait-based differentiation of isolates. Genetic differentiation is relatively straightforward: portions of the genome of each isolate are sequenced and compared. For trait-based differentiation, this study selected isolate source metadata, drug resistance, and *in vitro* antagonism to investigate the community structure and ecology of clinical *Acinetobacter,* in particular *A. baumannii.* Bacterial antagonism

is the killing or growth inhibition of one bacterial organism by another. The antagonism phenotype is detected as a ring of clearing around a growth of the producer isolate upon a lawn of a sensitive isolate, similar to the zone of inhibition in a disk diffusion assay for testing antimicrobial susceptibility. Antagonism by isolates of *A. baumannii* against other isolates of the same species could illustrate kinship boundaries in strains of this species.

The objective of this study was to begin to understand the population dynamics of clinical isolates of *A. baumannii* from a military healthcare system. Many of the isolates belonged to two very closely related genetic groups, and there was extensive antagonism observed by isolates of one group against the other. In addition, there was a proportional increase in the antagonistic strain after July 2010. These findings reveal that bacteria in the hospital environment have population dynamics similar to those in natural environments characterized by periodic ecological disruption, with could provide a starting point for ecologically-oriented methods of infection control.

Methods

Bacterial Isolates

90 isolates were evaluated from a collection of drug-resistant clinical isolates held by the Wound Infections Department of Walter Reed Army Institute of Research (Silver Spring, MD, USA).

All of the isolates were initially cultured from patient specimens at three military treatment facilities: Walter Reed Army Medical Center (n=82 isolates; WRAMC, Washington, DC, USA), Landstuhl Regional Medical Center (n=3; LRMC, Landstuhl, Germany), and National Naval Medical Center (n=5; NNMC, Bethesda, MD, USA). Isolates from NNMC and WRAMC were isolated between 2006 and 2010; date of isolation was not available for the isolates from LRMC (Table 1). All isolates were identified clinically as *A. baumannii/calcoaceticus* complex.

Isolates were stored frozen at -80°C in glycerol stocks. Isolation plates for the following assays were prepared by streaking frozen glycerol stocks of bacterial isolates on blood agar plates (R01198, Thermo Scientific, Waltham, MA, USA) and incubating 18-24h at 37°C. Liquid cultures were prepared by inoculating tryptic soy broth (211825, BD, Franklin Lakes, NJ, USA) with isolates and growing overnight with agitation at 37°C.

Isolates that have been published under other aliases or with full genome sequences available are listed in Table S1.

Genotyping

Phylomark software (Sahl, Matalka, & Rasko, 2012) was used to identify three genetic loci suitable for typing from a set of *A. baumannii* genomes. Primer pairs for each locus were designed using Lasergene (DNASTAR, Inc., Madison, WI, USA) and Primer-BLAST (NCBI, Bethesda, MD, USA) and synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA).

PCR with GoTaq Green Master Mix (M7122, Promega, Madison, WI, USA) was used to amplify the three loci from colonies of each isolate, and PCR products were sequenced by Macrogen (Rockville, MD, USA). Resulting sequences were trimmed for quality using Sequencher (Gene Codes, Ann Arbor, MI, USA).

Matching sequences from *A. baumannii* reference strains ATCC 19606 (NCBI: NZ_ACQB00000000), AYE (NCBI: NC_010410), and ACICU (NCBI: NC_010611) for the three

loci were identified and downloaded using BLAST (NCBI, Bethesda, MD, USA) from the RefSeq database (NCBI, Bethesda, MD, USA).

Sequences for each locus were aligned using MAFFT (Katoh & Standley, 2013), and gaps and highly divergent regions were removed using Gblocks (Talavera & Castresana, 2007). The aligned sequences for each locus were concatenated by isolate (Data S2). PHYLIP (version 3.695; J. Felsenstein, University of Washington [http://evolution.genetics.washington.edu/phylip.html]) DNAPARS was used with default settings plus a less thorough search to perform a parsimony tree search on the concatenated alignments, and one of the three most parsimonious trees was arbitrarily chosen as the observed tree.

PHYLIP BOOTSEQ was used with default settings on the concatenated alignments to create 100 pseudosequences. The pseudosequences were run through PHYLIP DNAPARS with default settings plus multiple (n=100) sets of data, one jumble and a less thorough search to generate a set of trees based on the bootstrap sample. Branch bootstrap support for the observed tree was calculated using the sumtrees utility of DendroPy (Sukumaran & Holder, 2010) on the set of trees generated from the bootstrapped sequences. The observed tree, with branch bootstrap support, was uploaded to the interactive Tree of Life (Letunic & Bork, 2007) for visualization.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed on the BD Phoenix system (BD, Franklin Lakes, NJ, USA) between August 2014 and July 2015. Test inocula of isolates were prepared from isolation plates. The specific antimicrobials tested included: cephalosporins (cefepime, cefotaxime, ceftazidime, ceftriaxone), aminoglycosides (amikacin, gentamicin, tobramycin), fluoroquinolones (ciprofloxacin, levofloxacin), trimethoprim-sulfamethoxazole, carbapenems (imipenem, meropenem), ticarcillin-clavulanate, and tetracycline. No susceptibility tests were run for any drug in the polymixin class. An isolate was considered sensitive to a drug class if it was scored as sensitive to all drugs tested from the class; otherwise, it was considered resistant.

Antagonism Testing

A pairwise reciprocal agar diffusion assay was used to investigate antagonistic interactions between the isolates. Each clinical isolate was tested both for production of antagonism and sensitivity to antagonism when grown on agar plates in contact with each of the other isolates.

To test for antagonism production, a test plate was prepared by swab-inoculating the surface of a tryptic soy agar (236950, BD, Franklin Lakes, NJ, USA) plate with an overnight liquid culture of an isolate (test indicator). Approximately 2ul of an overnight liquid culture of another isolate (test producer) was spot-inoculated on the surface of the test plate. The test plate was allowed to dry after spot-inoculation, and then incubated 18-24h at 37°C. Antagonism was scored if test indicator growth was inhibited at the site of test producer inoculation. Each pair of isolates was tested for antagonism six times (Data S3). A schematic of the experimental protocol and a photo of an example plate are available in Supplemental Information (Figure S4).

Results

Sequence-based analysis was able to identify genetic clusters of clinical isolates. The three loci (L1-3) sequenced for each isolate were 600 base pair sections of the genome overlapping partially with one or two genes each. L1 overlaps part of a permease gene (NCBI: WP_001196403). L2 consists mostly of sequence encoding a tRNA glutamyl-Q (34) synthetase

GluQRS (NCBI: WP_000216736), but also overlaps a small segment of the cell division protein FtsW gene (NCBI: WP_000907680) and some non-coding DNA. L3 overlaps portions of a hemolysin D gene (NCBI: WP_000128703) and an MFS transporter gene (NCBI: WP_000857095). Internal primers amplified around 450-550 base pairs of DNA in all isolates for L1 and L2, but could only amplify DNA for some of the isolates for L3. For those isolates that did not amplify at L3 using internal primers at L3, external primers were used to successfully amplify the locus (Table 2). All experimentally determined sequences in this study have been published by NCBI (Table 3).

The phylogenetic tree generated for the isolates reveals that there are a few large clusters of very closely related isolates, as well as many individual isolates on their own branches or in small clusters (Figure 1). The dendrogram designates an isolate in the assay belonging to species *A. calcoaceticus* (W0049) as the outgroup. The majority (n=61) of other isolates were *A. baumannii*. Many of the isolates (n=15 isolates, genetic group A1; Fig. 1, brown cluster) were indistinguishable from reference strain AYE by sequence, or differed by only one base pair in the L3 locus (n=25, genetic group A2; Fig. 1, green cluster). Another group of isolates (n=19) were indistinguishable from the reference strain ACICU (clade B; Fig. 1, purple cluster), with one isolate (W4052) differing from the other isolates in that clade by three base pairs in the L2 locus. The remaining 30 isolates belonged to other clades, and 14 of these isolates belonged to braches with no other isolates.

Antagonism was observed in 9,459 (19.5%) of the total 48,600 pairwise assays (Figure 2A). Of the 8,100 possible combinations of strains, 1,982 (24.5%) pairs of isolates had at least 1 replicate positive for antagonism and 1,168 (14.4%) combinations had 5 or 6 positives (Figure 2B). Antagonism was observed for each isolate against at least one other isolate in at least one of the six replicate tests. Also, all but three isolates (W0088, L0723, N0357A) were sensitive to antagonism by at least one other isolate (Figure 1, 2A).

Antagonism occurred significantly more frequently between genetically distinct isolates than between isolates from the same genetic cluster (Figure 3A, 3B). The phenotype was particularly consistent in pairs where one isolate came from group A1 and the other from group A2 (Figure 3A). Antagonism between those pairs was generally unilateral: 82% of interactions testing A2 isolates against A1 isolates were antagonistic, while only 3% of interactions testing A1 isolates against A2 isolates were antagonistic. Antagonistic interactions also occurred between A2 isolates and clade B isolates consistently, albeit at a lower rate (52%; Figure 3B). These interactions, unlike the antagonistic activity between A1 and A2, were characterized by antagonism directed in both directions: antagonism of A2 by B and of B by A2 (9%). Unlike group A1 and A2 isolates, which were not internally antagonistic, clade B isolates antagonized other isolates from clade B (13%; Figure 3B). One isolate in clade B (W4052) had an antagonism phenotype more similar to the A2 isolates than the B isolates (Figure 1, 2).

Antagonistic activity was more common in tests of isolates collected during or after July 2010 against those collected prior to July 2010 than the reciprocal test (44% vs 11%; Figure 3C). Before that month, clade B (33%, n=19/58) and group A1 (22%, n=13/58) together represented the majority of isolates (Figure 1). Only one isolate (W0051) from group A2 (2%, n=1/58) was collected prior to July 2010; that isolate was collected in May 2010. The aberrant isolate in clade B (W4052) was cultured in September 2007 (Table 1). Between July and August of 2010, group A2 isolates represent 76% (n=22/29) of isolates collected during that period. Of the seven remaining isolates collected after July 2010, one is from clade B (3%, n=1/29) and two are from group A1 (7%, n=2/29).

Isolates were collected from a variety of patient specimens: wounds (n=23, 26%), blood (n=20, 22%), respiratory (n=17, 19%), surveillance (n=17, 19%), tissue (n=10, 11%), and urine (n=3, 3%) (Table 1). Overall, the isolates in this study exhibited high levels of antibiotic resistance to all drug classes tested. Susceptibility scores (susceptible, intermediate, resistant) to individual drugs were condensed by class (Magiorakos et al., 2012). Rates of resistance by drug class are as follows: 99% were resistant to cephalosporins (n=89 isolates/90 tested), 97% to aminoglycosides (n=87/90), 92% to fluoroquinolones (n= 83), 92% to ticarcillin-clavulanate (n=56/61), 88% to trimethoprim-sulfamethoxazole (n=79/90), 87% to tetracycline (n=53/61), 83% to carbapenems (n=75/90) (Table 4). Two isolates (N0326, W0967) were resistant to only one class of antibiotic, cephalosporins. Sixty-seven isolates (74%) were resistant to all classes of antibiotic tested (Figure 1). No isolate was susceptible to all antibiotics tested.

No pattern of the antagonism data can be observed based on isolate specimen source (not shown) or drug class resistance (Figure 3D, 3E).

Discussion

This study is the first to describe strain-based patterns of pairwise antagonistic interactions in a large set of drug-resistant clinical isolates of *Acinetobacter baumannii*. Antagonism has been reported in clinical isolates of *Acinetobacter* previously on a small scale without genetic differentiation of isolates (Andrews, 1986). In the present study, we found antagonistic activity to be widespread among the isolates screened and most consistent between two closely related groups of isolates. Isolates from genetic clusters with high sensitivity to antagonism were generally isolated prior to July 2010, and antagonistic isolates were typically isolated after that time.

The goal of this study was to investigate the population dynamics and bacterial interactions of a species associated with hospital acquired infection, *A. baumannii*, in order to understand more about the ecology of pathogens in nosocomial environments. *A. baumannii* belongs to the gammaproteobacteria class, which contains many of the most common Gram-negative nosocomial pathogens, including *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Escherichia coli* (Dijkshoorn et al., 2007; Gaynes, Edwards, & National Nosocomial Infections Surveillance, 2005). High rates of drug resistance alone can make patient infections with hospital-acquired bacteria difficult to treat (Boucher et al., 2009; Dijkshoorn et al., 2007). In addition, some species have a remarkable ability to withstand desiccation and ethanol exposure, allowing them to survive on hospital surfaces as well (Fiester & Actis, 2013; Tashiro et al., 2014). The problems caused by nosocomial transmission of pathogens are not expected to ease.

Hospitals house a remarkable diversity of bacteria, especially considering the frequency of sterilization and disinfection of the hospital environment (Lax & Gilbert, 2015). If sterilization of surfaces and eradication of microbes from the hospital is not a reasonable goal, it becomes increasingly important to understand the ecology of drug-resistant pathogens that benefit from the current paradigm of disinfection, and to identify new strategies for mitigating the spread of infection.

Antagonism is a widespread bacterial trait resulting from the production of diffusible inhibitory or antibiotic biochemicals (Abrudan et al., 2015; Al-Saedi, Stones, Vaz, & Krachler, 2016; Buffie & Pamer, 2013; Christensen et al., 2016; Cordero et al., 2012; Grossart, Schlingloff, Bernhard, Simon, & Brinkhoff, 2004; Hawlena, Bashey, & Lively, 2012; Zapien-Campos, Olmedo-Alvarez, & Santillan, 2015). The functional impact of antagonism on community ecology is debated, particularly with regard to whether antagonism is primarily a cooperative trait promoting

population cohesion and synergy (Cordero et al., 2012), or a competitive trait used as a weapon against other microbes (Abrudan et al., 2015). Another open question is the impact of environmental factors (community diversity, nutrient availability) on the expression of the antagonism phenotype (Abrudan et al., 2015; Rivett et al., 2016).

The isolates surveyed for antagonism in this study are similar to *Acinetobacter* isolates identified in other studies. Our finding of extensive genetic diversity among clinical isolates, with a few dominant genetic clusters, is consistent with previous studies of hospital-associated *A. baumannii* (Wallace et al., 2016; Wright et al., 2014). By including the reference strains 19606, AYE and ACICU in this study, as well as previously published isolates (Table S1), we may infer that the major clades identified in this study correspond to Pasteur multi-locus sequence types (MLST) ST1 and ST2 (Wallace et al., 2016). Isolates with known ST in group A1 belong to ST1, and clade B appears to be equivalent to ST2. No isolate in A2 has been previously published. The isolates in this set have a higher incidence of resistance to many antibiotics than reported elsewhere (Lockhart et al., 2007), likely because these isolates come from a military repository of multi-drug resistant organisms.

The extent of antagonistic activity associated with the clinical isolates in this study emphasized that, to microbes, the hospital environment is similar to a natural environment and fosters similar microbial interactions. Studies of antagonistic interactions and population structure in bacteria from natural environments provide context and contrast with the results of this study. The short genetic distance between the antagonistic A2 isolates and sensitive A1 isolates is surprising, considering other publications that find that bacterial populations (i.e. operational taxonomic units, OTUs) can be defined as clusters of individuals with 70-99% sequence similarity, rather than being purely clonal organisms (Acinas et al., 2004; Cordero et al., 2012; Kashtan et al., 2014; Klepac-Ceraj et al., 2004; Lemire et al., 2015). The methods used in this study detected a genetic difference of <0.001% between A1 and A2 groups, which would seem to be too little distance to describe separate populations, although the strong antagonistic interactions between the groups indicate that the two groups are indeed separate populations. The genotyping method used in this study is designed to allow for minimal sequencing effort to identify core genome relationships. Thus, the genetic distance estimated excludes the more divergent accessory genome; the phylogenetic distance reported here is likely an underestimate of the whole genome sequence distance of individual isolates.

If each clade identified in this study then represents sufficient genetic distance to constitute a population, the consistency of phenotype displayed by A2 isolates is surprising, as is the frequency. In cooperating natural populations, it is typical for some individuals to produce an antibiotic as a public good while other members of the population retain resistance to the antibiotic but do not expend energy producing it (Allen, Gore, & Nowak, 2013; Cordero et al., 2012). However, all A2 isolates appear to produce whatever effector is responsible for the antagonism trait.

Environmental microbiology has frequently shown that the expression of antagonism varies with the microbial diversity of the testing paradigm as well as the richness of the substrate used during testing (Abrudan et al., 2015; Rivett et al., 2016). The screening paradigm used here is similar to the “social growth” condition used in the Abrudan *et al* study, because multiple isolates were tested for production of antagonism on the same lawn of test indicator isolate, at less than 1 cm distance (Abrudan et al., 2015). According to the cited study, social interactions of isolates in a nutrient-rich environment can suppress antagonism. It is possible that the high observed incidence of antagonism could be even higher *in situ*, on more nutrient-poor hospital surfaces and in patient infections.

The results of this study found compelling evidence that there was a mass turnover of strains in the US military hospital system around July 2010. The fact that it was the antagonists that overtook their sensitive cousins in group A1 begs the interpretation that antagonism was part of the mechanism enabling the epidemiological transition. This interpretation is further supported by the appearance of a lone isolate (W0051) belonging to A2 in May 2010. Additionally interesting, a phenotypic outlier of clade B (W4052) possesses a nearly identical antagonism phenotype to that of A2 isolates. The connections between these disparate yet compelling lines of evidence could be determined with genomic comparison and further *in vitro* testing.

This study did not investigate the mechanism of antagonism, but direct interbacterial toxin-mediated inhibition typically occurs via one of two mechanisms: two-partner contact dependent inhibition (CDI) or type V secretion systems (T6SS). Both systems require direct contact between the antagonist and target cell and effectors are delivered to the cytosol of the target cell. However, CDI requires binding with a receptor on the outer membrane of the target cell, while T6SS includes a mechanism for penetrating the target cell's outer membrane to deliver effectors (Carruthers, Nicholson, Tracy, & Munson, 2013; Harding et al., 2017; Ruhe, Low, & Hayes, 2013; Silverman, Brunet, Cascales, & Mougous, 2012). CDI systems have been recently identified in the *A. baumannii-calcoaceticus* complex species *A. nosocomialis* as well as in *A. baumannii* (Harding et al., 2017). Two different CDI loci were identified in *A. baumannii* 19606, which had differential inhibitory activities against immunity gene knockout mutants (Harding et al., 2017). T6SS has been implicated in interspecies inhibitory interactions between *A. baumannii* and *E. coli* (Carruthers et al., 2013).

In spite of the evidence that both CDI and T6SS play a role in *A. baumannii* cell-cell interaction, the antagonism reported in our study is probably not due to a contact-dependent system. Firstly, the zone of inhibition around antagonistic isolates implies that the effector is secreted into the environment, not delivered directly into the cytosol of the target cell. Secondly, preliminary experiments have indicated that the antagonism effector is present in agar where antagonistic strains are grown in the absence of a target strain, evidenced by chloroform killing of the antagonist grown alone and subsequent inhibition of the target after cross-streaking (data not shown). Future experiments should include comparative genomics and proteomics of producer (A2, W4052) and sensitive (A1) isolates, as well as new *in vitro* experiments to rule out the contribution of contact-dependence to the phenotype.

Investigating the interactions of natural populations of bacteria, including those in the hospital and other built environments, provides valuable information about the way those populations function. Only by understanding the whole can we identify elements that can be changed or manipulated to achieve desired outcomes. This type of manipulation of microbial systems is advancing in the area of biofuel production and agricultural science, but has not yet been attempted by the medical research community. In addition to its utility in tracking strain dynamics, antagonism may hold potential as a component of a system that uses ecological mechanics to perform infection control. However, this will only be possible by further investigation and understanding of the interactions of pathogenic organisms in the environments they inhabit.

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Figure 1

Phylogeny of isolates

One of three maximum-parsimony (MP) trees, showing bootstrap values greater than 25% for tidiness. Major clades and subclades labeled on tree branches. Date of culture labeled on blue to orange scale, first cultured to most recent cultured. MDR isolates are defined as those resistant to 3 or more classes of drugs tested. Average antagonistic activity against major clades was calculated by averaging observed antagonism of an isolate against all isolates in a group (A1, A2, B). 50% activity or greater labeled with darker color.

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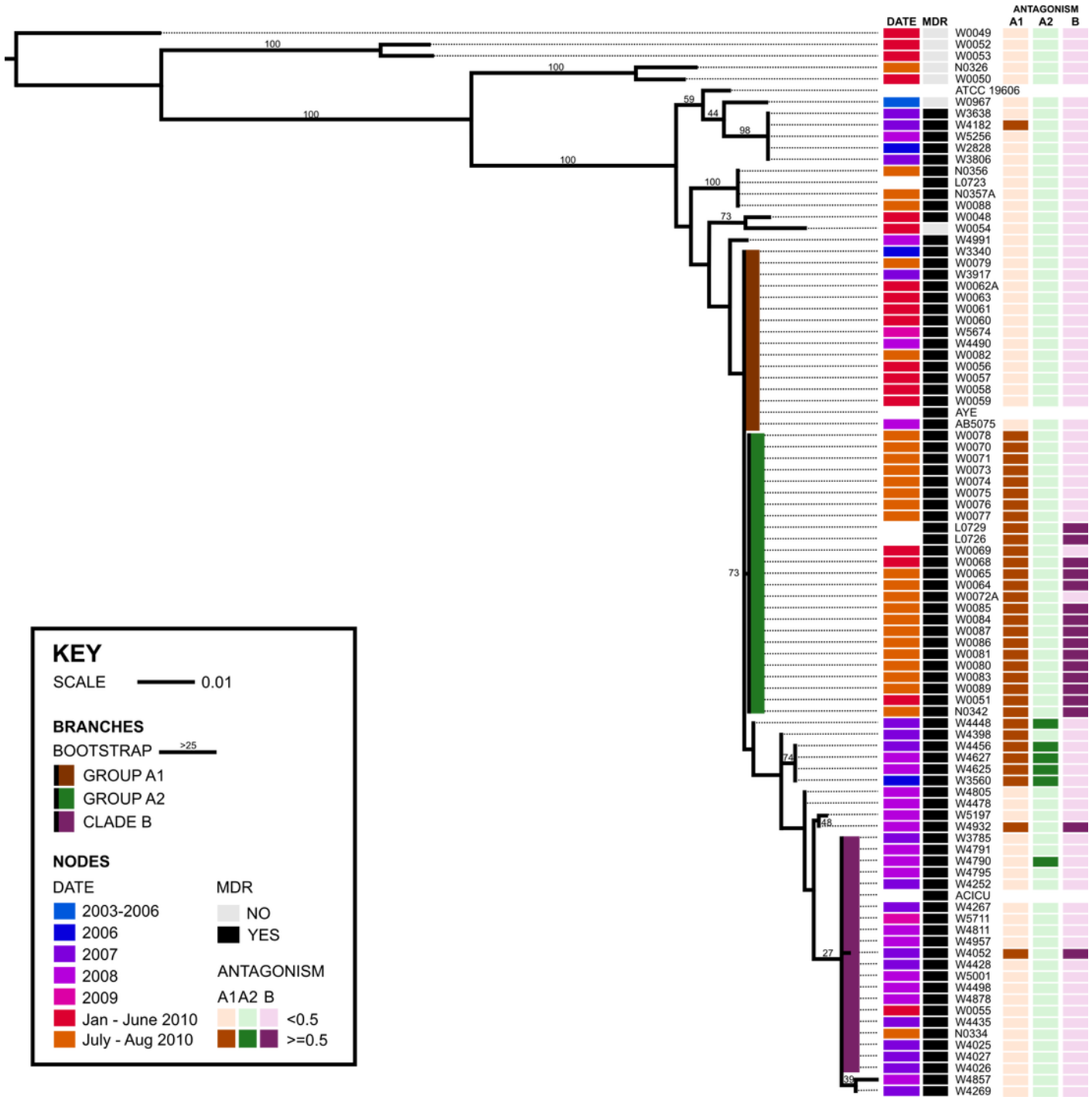

Figure 2

Pairwise antagonism of isolates

Heatmap of antagonistic interactions observed between isolates (A) and replicability of antagonism assays (B). Order of isolates in (A) as in Figure 1. Major clades and subclades labeled with colored bars vertically and horizontally as in Figure 1. Results of each pair of isolates (vertical-test isolate, horizontal-indicator isolate) indicated as greyscale squares (white: no antagonistic interactions observed, black: antagonism observed in all assays for that pair). The vast majority of isolate pairs yielded no antagonistic interactions, and for those pairs that did reveal antagonism, the trait was frequently observed in all six replicate tests (B).

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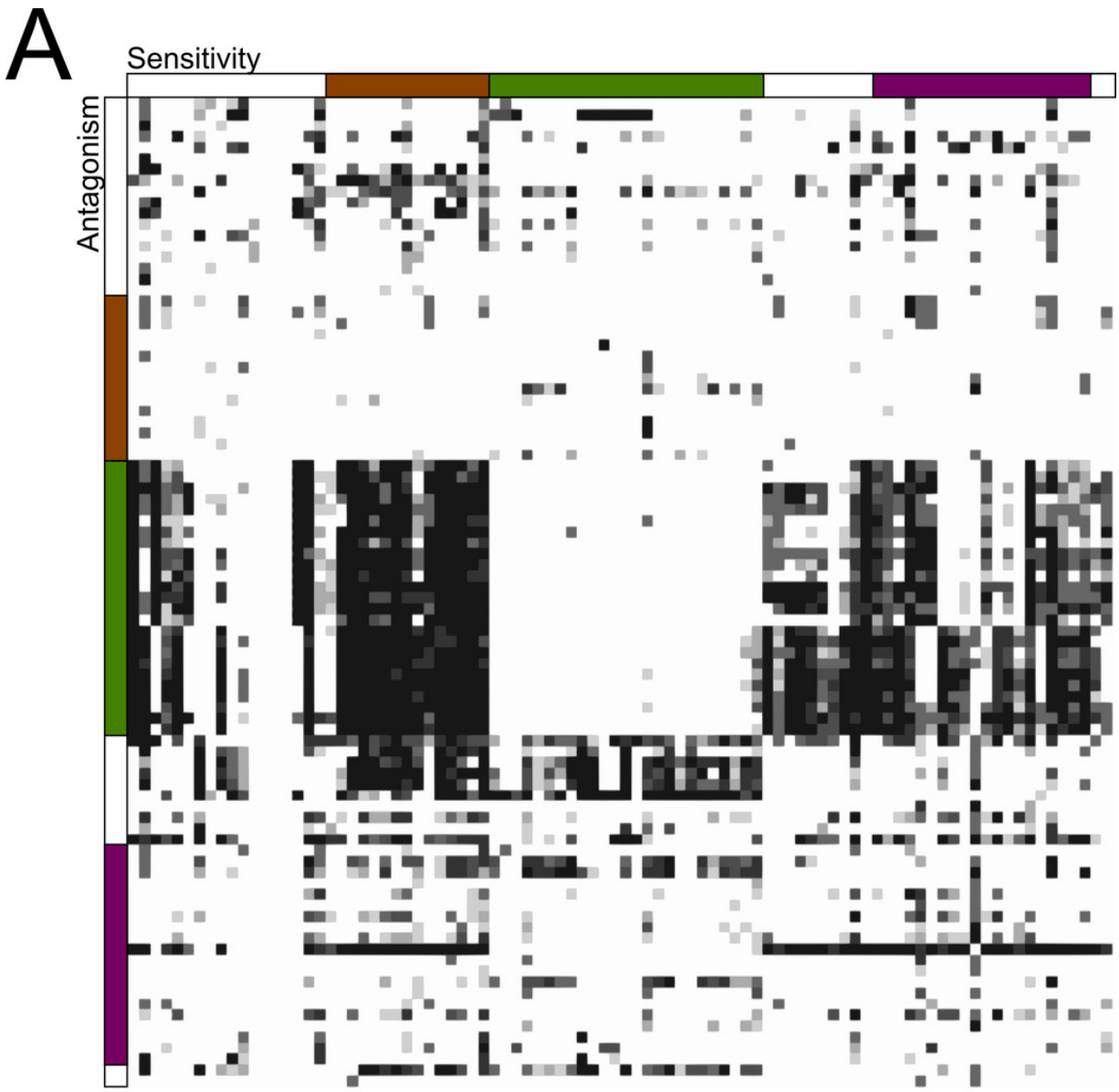
A
Sensitivity
Antagonism

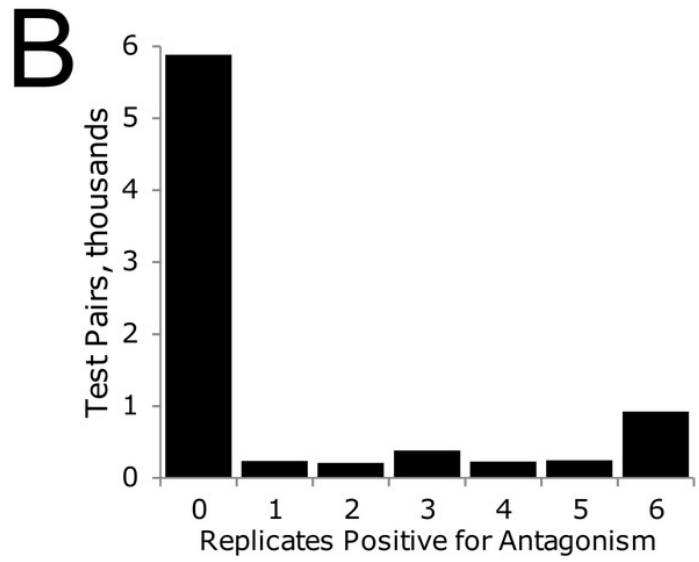
B
Test Pairs, thousands
0
1
2
3
4
5
6
Replicates Positive for Antagonism

Figure 3

Frequency of antagonism

Each isolate was screened against each other isolate for antagonistic activity. Frequency of antagonism occurrence (mean, 95% confidence interval, ***p<0.0001) was compared for isolates of different phylogenetic background (A, B), collected during different intervals (C), and with different resistance to some antibiotic classes (D, E). A1: genetic group A1; A2: genetic group A2; B: genetic clade B; Early: collected prior to July 2010; Late: collected during or after July 2010; CarbR: carbapenem resistant; CarbS: carbapenem sensitive; FluorR: fluoroquinolone resistant; FluorS: fluoroquinolone sensitive.

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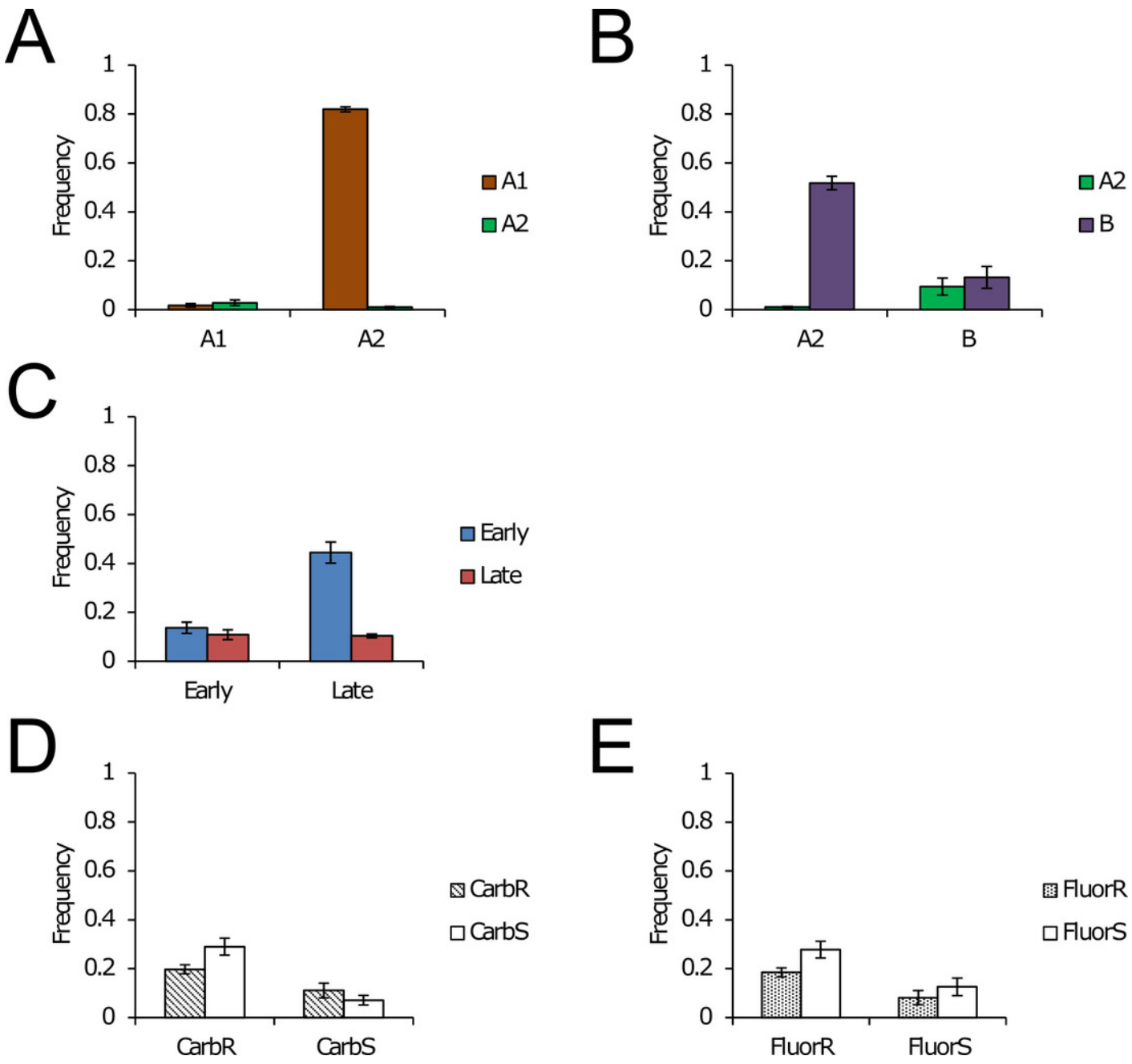

Table 1(on next page)

Metadata

Data regarding isolate collection time and location.

Table 1. Metadata.

Isolate	Culture Date	Specimen Type	Hospital	Location
L0723	ND	Respiratory	LRMC	ND
L0726	ND	Blood	LRMC	ND
L0729	ND	Surveillance	LRMC	ND
N0326	7/20/2010	Wound	NNMC	Ward
N0334	7/25/2010	Respiratory	NNMC	ICU
N0342	7/31/2010	Respiratory	NNMC	ICU
N0356	7/10/2010	Wound	NNMC	ICU
N0357A	7/10/2010	Wound	NNMC	ICU
W0048	1/2/2010	Respiratory	WRAMC	ICU
W0049	2/19/2010	Urine	WRAMC	Clinic
W0050	3/26/2010	Blood	WRAMC	ICU
W0051	5/25/2010	Surveillance	WRAMC	Ward
W0052	5/29/2010	Surveillance	WRAMC	Ward
W0053	6/8/2010	Urine	WRAMC	ICU
W0054	6/9/2010	Surveillance	WRAMC	Ward
W0055	6/16/2010	Respiratory	WRAMC	ICU
W0056	6/20/2010	Wound	WRAMC	ICU
W0057	6/21/2010	Wound	WRAMC	ICU
W0058	6/22/2010	Wound	WRAMC	ICU
W0059	6/23/2010	Respiratory	WRAMC	ICU
W0060	6/23/2010	Sterile site	WRAMC	ICU
W0061	6/25/2010	Respiratory	WRAMC	ICU

W0062A	6/26/2010	Respiratory	WRAMC	ICU
W0063	6/27/2010	Wound	WRAMC	ICU
W0064	7/2/2010	Wound	WRAMC	Ward
W0065	7/2/2010	Wound	WRAMC	Ward
W0068	7/7/2010	Blood	WRAMC	ICU
W0069	7/20/2010	Blood	WRAMC	ICU
W0070	7/20/2010	Surveillance	WRAMC	ICU
W0071	7/20/2010	Wound	WRAMC	ICU
W0072A	7/21/2010	Wound	WRAMC	ICU
W0073	7/21/2010	Wound	WRAMC	ICU
W0074	7/21/2010	Wound	WRAMC	ICU
W0075	7/21/2010	Respiratory	WRAMC	ICU
W0076	7/22/2010	Respiratory	WRAMC	ICU
W0077	7/24/2010	Respiratory	WRAMC	ICU
W0078	7/26/2010	Blood	WRAMC	ICU
W0079	7/30/2010	Wound	WRAMC	Ward
W0080	7/30/2010	Respiratory	WRAMC	ICU
W0081	7/30/2010	Wound	WRAMC	ICU
W0082	8/2/2010	Wound	WRAMC	Ward
W0083	8/4/2010	Wound	WRAMC	ICU
W0084	8/5/2010	Wound	WRAMC	ICU
W0085	8/7/2010	Wound	WRAMC	Ward
W0086	8/3/2010	Blood	WRAMC	ICU
W0087	8/3/2010	Blood	WRAMC	ICU
W0088	8/3/2010	Surveillance	WRAMC	ICU

W0089	7/28/2010	Wound	WRAMC	Ward
W0967	9/23/2003	Blood	WRAMC	NT
W2828	3/28/2006	Blood	WRAMC	ICU
W3340	10/22/2006	Blood	WRAMC	ICU
W3560	12/14/2006	Blood	WRAMC	ICU
W3638	1/10/2007	Sterile site	WRAMC	Ward
W3785	3/18/2007	Blood	WRAMC	ICU
W3806	3/19/2007	Sterile site	WRAMC	ICU
W3917	5/13/2007	Blood	WRAMC	ICU
W4025	6/24/2007	Wound	WRAMC	Clinic
W4026	6/26/2007	Wound	WRAMC	Clinic
W4027	6/26/2007	Wound	WRAMC	Clinic
W4052	9/14/2007	Surveillance	WRAMC	ICU
W4182	7/16/2007	Surveillance	WRAMC	Clinic
W4252	10/19/2007	Respiratory	WRAMC	ICU
W4267	10/14/2007	Surveillance	WRAMC	ICU
W4269	10/16/2007	Surveillance	WRAMC	ICU
W4398	12/11/2007	Surveillance	WRAMC	ICU
W4428	12/24/2007	Urine	WRAMC	ICU
W4435	12/21/2007	Sterile site	WRAMC	ICU
W4448	12/25/2007	Surveillance	WRAMC	ICU
W4456	12/30/2007	Respiratory	WRAMC	ICU
W4478	1/5/2008	Blood	WRAMC	ICU
W4490	1/12/2008	Surveillance	WRAMC	Ward
W4498	1/13/2008	Blood	WRAMC	ICU

W4625	3/9/2008	Blood	WRAMC	ICU
W4627	3/4/2008	Sterile site	WRAMC	Ward
W4790	5/4/2008	Surveillance	WRAMC	ICU
W4791	5/4/2008	Surveillance	WRAMC	ICU
W4795	5/5/2008	Sterile site	WRAMC	Ward
W4805	5/11/2008	Respiratory	WRAMC	Clinic
W4811	5/12/2008	Respiratory	WRAMC	ICU
W4857	5/28/2008	Sterile site	WRAMC	Ward
W4878	6/6/2008	Surveillance	WRAMC	ICU
W4932	7/4/2008	Respiratory	WRAMC	Ward
W4957	7/17/2008	Sterile site	WRAMC	Ward
W4991	8/3/2008	Surveillance	WRAMC	ICU
W5001	8/5/2008	Blood	WRAMC	ICU
AB5075	9/1/2008	Sterile site	WRAMC	ICU
W5197	10/15/2008	Blood	WRAMC	Ward
W5256	11/11/2008	Blood	WRAMC	Ward
W5674	5/22/2009	Blood	WRAMC	ICU
W5711	6/9/2009	Blood	WRAMC	ICU

ND: No data
LRMC: Landstuhl Regional Medical Center
NNMC: National Naval Medical Center
WRAMC: Walter Reed Army Medical Center
ICU: Intensive care unit

1

Table 2(on next page)

PCR and sequencing primers

Oligonucleotide sequences of primers used for polymerase chain reaction (PCR) and DNA sequencing.

Table 2. PCR and Sequencing Primers.

Locus Name	Direction	Sequence
L1	Forward	5'-AACATGGGATGGCTTGGTTTT-3'
L1	Reverse	5'-CAGATCTACCCGTGCCTTGATAA-3'
L2	Forward	5'-CCATTCGGGTAAAAGTTCAAGA- 3',
L2	Reverse	5'-CCTGAGAGGGAAGAATCAAACTT-3'
L3	Forward (internal)	5'-CAAACCGCATAGGAAAGAAAAGA-3'
L3	Reverse (internal)	5'-AAGTCCGCCCAGGTCAGC-3'
L3	Forward (external)	5'-GCGCATGTAGAACAAGCTCAG-3'
L3	Reverse (external)	5'-GGTCATTGCCCATAGGCCC-3'

1

Table 3(on next page)

Sequences submitted to NCBI GenBank

These sequences are available as a single set of PopSet sequences (identifier: 1018196084).

The sequences have been trimmed for quality from raw sequencing data.

Table 3. Sequences Submitted to NCBI GenBank

Isolate	L1	L2	L3
L0723	KR995876	KR996054	KR995965
L0726	KR995905	KR996083	KR995994
L0729	KR995904	KR996082	KR995993
N0326	KR995867	KR996045	KR995956
N0334	KR995947	KR996125	KR996036
N0342	KR995920	KR996098	KR996009
N0356	KR995875	KR996053	KR995964
N0357A	KR995877	KR996055	KR995966
W0048	KR995879	KR996057	KR995968
W0049	KR995864	KR996042	KR995953
W0050	KR995868	KR996046	KR995957
W0051	KR995919	KR996097	KR996008
W0052	KR995865	KR996043	KR995954
W0053	KR995866	KR996044	KR995955
W0054	KR995880	KR996058	KR995969
W0055	KR995945	KR996123	KR996034
W0056	KR995892	KR996070	KR995981
W0057	KR995893	KR996071	KR995982
W0058	KR995894	KR996072	KR995983
W0059	KR995895	KR996073	KR995984
W0060	KR995888	KR996066	KR995977
W0061	KR995887	KR996065	KR995976

W0062A	KR995885	KR996063	KR995974
W0063	KR995886	KR996064	KR995975
W0064	KR995909	KR996087	KR995998
W0065	KR995908	KR996086	KR995997
W0068	KR995907	KR996085	KR995996
W0069	KR995906	KR996084	KR995995
W0070	KR995897	KR996075	KR995986
W0071	KR995898	KR996076	KR995987
W0072A	KR995910	KR996088	KR995999
W0073	KR995899	KR996077	KR995988
W0074	KR995900	KR996078	KR995989
W0075	KR995901	KR996079	KR995990
W0076	KR995902	KR996080	KR995991
W0077	KR995903	KR996081	KR995992
W0078	KR995896	KR996074	KR995985
W0079	KR995883	KR996061	KR995972
W0080	KR995916	KR996094	KR996005
W0081	KR995915	KR996093	KR996004
W0082	KR995891	KR996069	KR995980
W0083	KR995917	KR996095	KR996006
W0084	KR995912	KR996090	KR996001
W0085	KR995911	KR996089	KR996000
W0086	KR995914	KR996092	KR996003
W0087	KR995913	KR996091	KR996002
W0088	KR995878	KR996056	KR995967

W0089	KR995918	KR996096	KR996007
W0967	KR995869	KR996047	KR995958
W2828	KR995873	KR996051	KR995962
W3340	KR995882	KR996060	KR995971
W3560	KR995926	KR996104	KR996015
W3638	KR995870	KR996048	KR995959
W3785	KR995931	KR996109	KR996020
W3806	KR995874	KR996052	KR995963
W3917	KR995884	KR996062	KR995973
W4025	KR995948	KR996126	KR996037
W4026	KR995950	KR996128	KR996039
W4027	KR995949	KR996127	KR996038
W4052	KR995940	KR996118	KR996029
W4182	KR995871	KR996049	KR995960
W4252	KR995935	KR996113	KR996024
W4267	KR995936	KR996114	KR996025
W4269	KR995952	KR996130	KR996041
W4398	KR995922	KR996100	KR996011
W4428	KR995941	KR996119	KR996030
W4435	KR995946	KR996124	KR996035
W4448	KR995921	KR996099	KR996010
W4456	KR995923	KR996101	KR996012
W4478	KR995928	KR996106	KR996017
W4490	KR995890	KR996068	KR995979
W4498	KR995943	KR996121	KR996032

W4625	KR995925	KR996103	KR996014
W4627	KR995924	KR996102	KR996013
W4790	KR995933	KR996111	KR996022
W4791	KR995932	KR996110	KR996021
W4795	KR995934	KR996112	KR996023
W4805	KR995927	KR996105	KR996016
W4811	KR995938	KR996116	KR996027
W4857	KR995951	KR996129	KR996040
W4878	KR995944	KR996122	KR996033
W4932	KR995930	KR996108	KR996019
W4957	KR995939	KR996117	KR996028
W4991	KR995881	KR996059	KR995970
W5001	KR995942	KR996120	KR996031
W5197	KR995929	KR996107	KR996018
W5256	KR995872	KR996050	KR995961
W5674	KR995889	KR996067	KR995978
W5711	KR995937	KR996115	KR996026

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Table 4(on next page)

Antimicrobial susceptibility

Results of antimicrobial susceptibility testing of isolates, condensed by class where multiple drugs from one class were tested.

Table 4. Antimicrobial susceptibility.

Isolate	Amino-glycoside	Carba-penem	Cephalo-sporin	Fluoro-quinolone	Folate Pathway Inhibitor	Penicillin with Beta-Lactamase Inhibitors	Tetracycline	MDR
L0723	R	R	R	R	S	NT	NT	Yes
L0726	R	R	R	R	R	NT	NT	Yes
L0729	R	R	R	R	R	R	R	Yes
N0326	S	S	R	S	S	NT	NT	No
N0334	R	R	R	R	R	R	R	Yes
N0342	R	R	R	R	R	R	R	Yes
N0356	R	R	R	R	S	NT	NT	Yes
N0357A	R	R	R	R	S	NT	NT	Yes
W0048	R	R	R	R	R	R	S	Yes
W0049	S	S	R	S	S	NT	NT	No
W0050	R	S	R	S	S	S	S	No
W0051	R	R	R	R	R	NT	NT	Yes
W0052	R	S	S	S	R	S	S	No
W0053	R	S	R	S	S	S	S	No
W0054	R	S	R	S	S	NT	NT	No

W0055	R	R	R	R	R	R	R	Yes
W0056	R	R	R	R	R	R	R	Yes
W0057	R	R	R	R	R	R	R	Yes
W0058	R	R	R	R	R	R	R	Yes
W0059	R	R	R	R	R	R	R	Yes
W0060	R	R	R	R	R	R	R	Yes
W0061	R	R	R	R	R	R	R	Yes
W0062A	R	R	R	R	R	R	R	Yes
W0063	R	R	R	R	R	R	R	Yes
W0064	R	R	R	R	R	R	R	Yes
W0065	R	R	R	R	R	R	R	Yes
W0068	R	R	R	R	R	R	R	Yes
W0069	R	R	R	R	R	R	R	Yes
W0070	R	R	R	R	R	R	R	Yes
W0071	R	R	R	R	R	R	R	Yes
W0072A	R	R	R	R	R	R	R	Yes
W0073	R	R	R	R	R	R	R	Yes
W0074	R	R	R	R	R	R	R	Yes

W0075	R	R	R	R	R	R	R	Yes
W0076	R	R	R	R	R	R	R	Yes
W0077	R	R	R	R	R	R	R	Yes
W0078	R	R	R	R	R	R	R	Yes
W0079	R	R	R	R	R	NT	NT	Yes
W0080	R	R	R	R	R	R	R	Yes
W0081	R	R	R	R	R	R	R	Yes
W0082	R	R	R	R	R	R	R	Yes
W0083	R	R	R	R	R	R	R	Yes
W0084	R	R	R	R	R	R	R	Yes
W0085	R	R	R	R	R	R	R	Yes
W0086	R	R	R	R	R	R	R	Yes
W0087	R	R	R	R	R	R	R	Yes
W0088	R	R	R	R	S	R	S	Yes
W0089	R	R	R	R	S	R	S	Yes
W0967	S	S	R	S	S	S	S	No
W2828	R	R	R	R	R	R	R	Yes
W3340	R	R	R	R	R	NT	NT	Yes

W3560	R	S	R	R	R	NT	NT	Yes
W3638	R	R	R	R	R	R	R	Yes
W3785	R	S	R	R	R	NT	NT	Yes
W3806	R	R	R	R	R	NT	NT	Yes
W3917	R	R	R	R	R	NT	NT	Yes
W4025	R	S	R	R	R	R	R	Yes
W4026	R	S	R	R	R	R	R	Yes
W4027	R	S	R	R	R	R	R	Yes
W4052	R	R	R	R	R	NT	NT	Yes
W4182	R	R	R	R	R	R	R	Yes
W4252	R	R	R	R	R	R	R	Yes
W4267	R	R	R	R	R	R	R	Yes
W4269	R	R	R	R	R	NT	NT	Yes
W4398	R	R	R	R	R	R	R	Yes
W4428	R	R	R	R	R	R	R	Yes
W4435	R	R	R	R	R	R	R	Yes
W4448	R	R	R	R	R	NT	NT	Yes
W4456	R	R	R	R	R	NT	NT	Yes

W4478	R	R	R	R	R	R	R	Yes
W4490	R	R	R	R	R	R	S	Yes
W4498	R	S	R	R	R	R	R	Yes
W4625	R	R	R	R	R	R	R	Yes
W4627	R	R	R	R	R	NT	NT	Yes
W4790	R	R	R	R	R	R	R	Yes
W4791	R	R	R	R	R	NT	NT	Yes
W4795	R	S	R	R	R	NT	NT	Yes
W4805	R	R	R	R	R	NT	NT	Yes
W4811	R	R	R	R	R	R	R	Yes
W4857	R	R	R	R	R	NT	NT	Yes
W4878	R	R	R	R	R	NT	NT	Yes
W4932	R	R	R	R	R	NT	NT	Yes
W4957	R	R	R	R	R	R	R	Yes
W4991	R	R	R	R	R	NT	NT	Yes
W5001	R	R	R	R	R	R	R	Yes
AB5075	R	R	R	R	R	NT	NT	Yes
W5197	R	R	R	R	R	NT	NT	Yes

W5256	R	S	R	R	R	NT	NT	Yes
W5674	R	R	R	R	R	R	R	Yes
W5711	R	R	R	R	R	S	R	Yes

R: Resistant to at least one drug in class
S: Sensitive to all drugs in class tested
NT: No drugs in class tested.
MDR: Multi-drug resistant; resistant to three or more classes of drugs.

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