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Acinetobacter baumannii is a nosocomial species frequently isolated from the traumatic wounds of injured military personnel and increasingly detected in civilian healthcare facilities. Many clinical isolates of *A. baumannii* are drug resistant, so new treatments are needed for infections. Recently, the ability of strains of conspecific bacteria to inhibit the growth of other strains has been observed in increasing numbers of species. We previously reported on intraspecific semisolid-phase growth inhibition (antagonism) among 94 clinical isolates of *A. baumannii*. These antagonistic interactions may be the result of genetically-encoded molecules, so more closely related isolates would be expected to produce similar patterns of interactions caused by identically active gene products. However, the phylogeny of clinical *A. baumannii* below the species level has not been established for this set of isolates.In this study, we used Phylomark to identify three genetic loci that recapitulated a whole-genome phylogeny of published *A. baumannii* genomes and we created a parsimony-based phylogeny from the 1.2 kilobase concatenated sequences. One clade appeared to exhibit the highest incidence of antagonistic interactions against all other isolates screened, except for itself and one relatively distant clade. This clade's nearest neighbor was susceptible to the most consistent antagonistic activity by the first clade; both of these clades appear to belong to MLST ST1 with reference strain AYE. Other isolates with high rates of antagonistic activity fall outside of ST1. Future studies aim to elucidate the genetic basis of the antagonism phenotype in clinical *Acinetobacter*, particularly in the most antagonistic isolates. The next step will be to mine these interactions to identify expressed antimicrobial molecules with potential for drug therapy.

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.436v1 | CC-BY 4.0 Open Access | received: 11 Jul 2014, published: 11 Jul

Resolving antagonistic interactions among clinical isolates of *Acinetobacter baumannii* with phylogenetic analysis

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Abstract

Acinetobacter baumannii is a nosocomial species frequently isolated from the traumatic wounds of injured military personnel and increasingly detected in civilian healthcare facilities. Many clinical isolates of *A. baumannii* are drug resistant, so new treatments are needed for infections. Recently, the ability of strains of conspecific bacteria to inhibit the growth of other strains has been observed in increasing numbers of species. We previously reported on intraspecific semisolid-phase growth inhibition (antagonism) among 94 clinical isolates of *A. baumannii*. These antagonistic interactions may be the result of genetically-encoded molecules, so more closely related isolates would be expected to produce similar patterns of interactions caused by identically active gene products. However, the phylogeny of clinical *A. baumannii* below the species level has not been established for this set of isolates.

In this study, we used Phylomark to identify three genetic loci that recapitulated a whole-genome phylogeny of published *A. baumannii* genomes and we created a parsimony-based phylogeny from the 1.2 kilobase concatenated sequences. One clade appeared to exhibit the highest incidence of antagonistic interactions against all other isolates screened, except for itself and one relatively distant clade. This clade's nearest neighbor was susceptible to the most consistent antagonistic activity by the first clade; both of these clades appear to belong to MLST ST1 with reference strain AYE. Other isolates with high rates of antagonistic activity fall outside of ST1. Future studies aim to elucidate the genetic basis of the antagonism phenotype in clinical *Acinetobacter*, particularly in the most antagonistic isolates. The next step will be to mine these interactions to identify expressed antimicrobial molecules with potential for drug therapy.

Background

Acinetobacter baumannii is a gram-negative bacterium associated with hospital settings, pneumonia and wound infections. It was distinguished from the soil bacterium *A. calcoaceticus* in 1986 by its ability to grow at higher temperatures (41C & 44C), to produce β-xylosidase from D-glucose and to utilize D-malate as a sole source of carbon (Bouvet & Grimont 1986). Clinical labs usually identify isolates to the species complex *A. calcoaceticus/baumannii*, which also includes two closely related species, *A. pittii and A. nosocomialis* (Dijkshoorn et al 2007; Nemec et al 2011). *A. baumannii* is predominatly isolated in hospitals, with low carriage rates in the community and environment. Strains are frequently multi-drug resistant (MDR) and may spread clonally among hospitals. *A. baumannii* infections are associated with natural and human-caused disasters, and the US military's challenge with infections of this species led in part to the establishment of the MDR-organism Repository and Surveillance Network (MRSN) at WRAIR. The data collected on these strains by the MRSN includes their drug resistance profile. High rates of drug resistance in the military hospital system and worldwide mean new therapies are desperately needed to treat *A. baumannii* infections (Dijkshoorn et al 2007).

Prior to the discovery of antibiotics, attempts to solve the problem of bacterial infections proceeded along a variety of lines of inquiry. The other two agents being investigated besides small molecules were bacteriophage and proteinaceous bacteriocidal molecules called bacteriocins (Kirkup 2006). Any of these agents may cause the phenomenon of antagonism: the killing or prevention of growth of a microorganism. *In vitro* manifestations of antagonism can be detected as a ring of clearing around a microbial plaque spotted and grown on another microbial lawn. When antibiotics were discovered, with their broad spectrum killing activity, the search faded out for phage and bacteriocins, which have narrower spectrums of activity, frequently against closely related organisms.

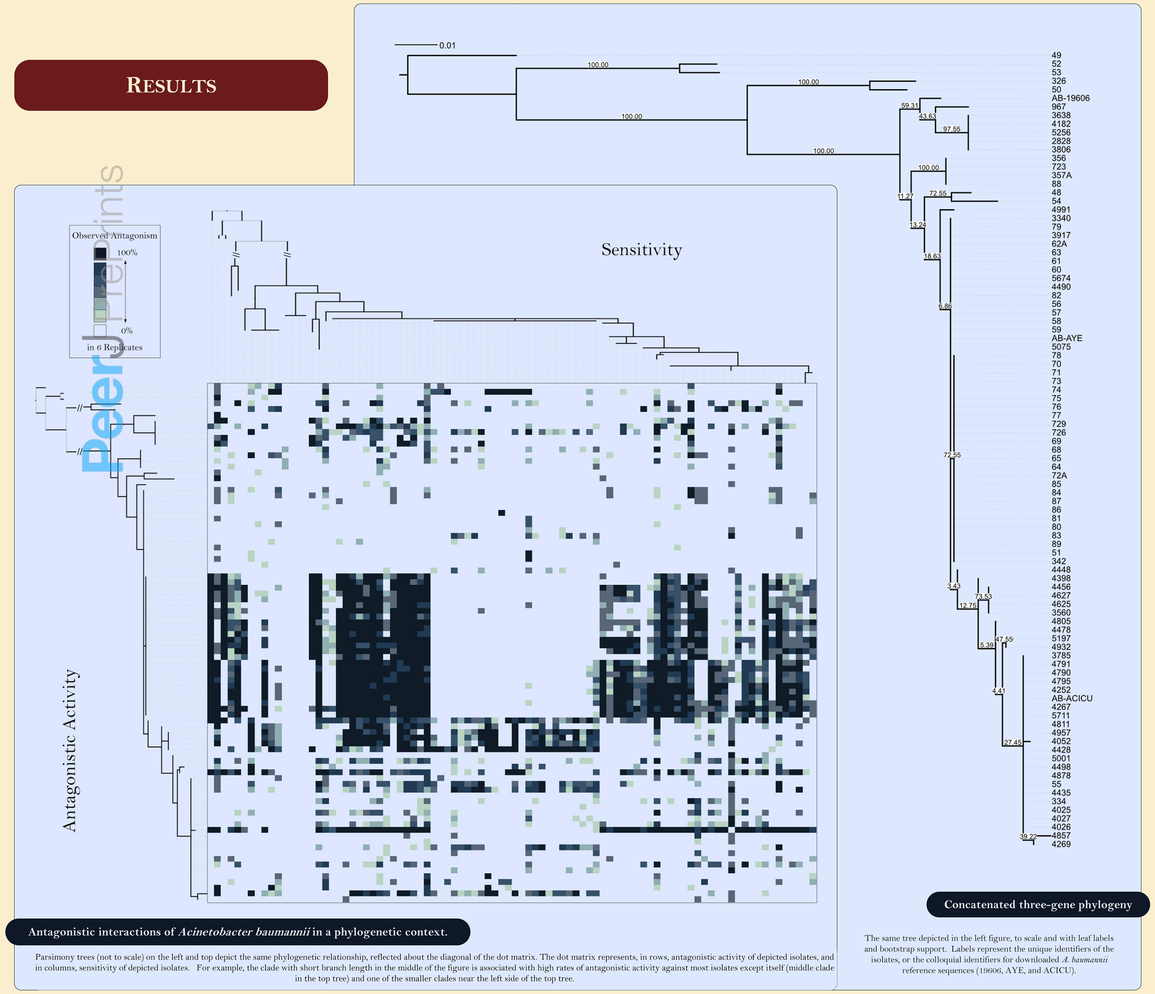
With the evolution of drug-resistant bacteria and the lack of new antibiotics in the development pipeline, a new approach to infection control may involve narrow-spectrum eradication of problematic strains and repopulation of the niche with innocuous or beneficial strains or species (Kirkup 2006).

We used a subset of *A. baumannii* collected by the MRSN and tested them pairwise for antagonism. In 2013 we presented the pattern of antagonism we observed in the data, and we reported high levels of antagonism in some isolates and very low levels in others (Summers et al 2013).

However, we lacked an understanding of the genetic relationship of the isolates in the screen. In order to determine infraspecific phylogeny, we hoped to perform multi-locus sequence typing (MLST) to differentiate strains. However, MLST is occasionally plagued by a lack of resolution due to the selection of slow-evolving housekeeping genes as loci (Perez-Losada et al 2013); this lack of resolution can be further confounded in organisms with extensive lateral gene transfer and recombination (Wright et al 2014). Whole genome sequence typing (WGST) successfully demonstrated the lack of MLST resolution in *A. baumannii* isolates from a hospital in Cleveland, Ohio, USA, where almost all isolates were sequence type 2 (ST2; also known as global clone 2). Phylogeny based on single nucleotide variation in the WGST demonstrated that ST2 is populated by isolates of diverse genetic background, and that it is possible to define subclades of ST2 with similar genetic content.

Without the resources to do WGST on our 94 isolates, we mined alignments of published genomes using Phylomark, which selects loci that recapitulate the whole genome sequence tree. The sequences for each locus were assembled into a single partition and plotted on a parsimony tree. We used a non-parametric assembly algorithm because the concatenation of heterogeneously evolving loci violated the assumptions of parametric methods like maximum likelihood and Bayesian MCMC (Kolaczkowski & Thornton 2004). We display as a result of this study the matrix of antagonistic interactions and a phylogeny with bootstrap support and isolate names, including reference sequences.

Results



Antagonistic interactions of *Acinetobacter baumannii* in a phylogenetic context.

Parsimony trees (not to scale) on the left and top depict the same phylogenetic relationship, reflected about the diagonal of the dot matrix. The dot matrix represents, in rows, antagonistic activity of depicted isolates, and in columns, sensitivity of depicted isolates. For example, the clade with short branch length in the middle of the figure is associated with high rates of antagonistic activity against most isolates except itself (middle clade in the top tree) and one of the smaller clades near the left side of the top tree.

Concatenated three-gene phylogeny

The same tree depicted in the left figure, to scale and with leaf labels and bootstrap support. Labels represent the unique identifiers of the isolates, or the colloquial identifiers for downloaded *A. baumannii* reference sequences (19606, AYE, and ACICU).

Methods

Isolate Collection

All isolates tested came from a repository of isolates collected at Walter Reed Army Medical Center, Landstuhl Regional Medical Center or National Naval Medical Center between 2006 and 2010.

Pairwise Antagonism

The methods for testing competitive interactions among the clinical isolates were described in Summers et al 2013. A total of 6 replicate tests of each isolate against every other isolate were done.

Locus Determination

Phylomark was used to identify three 600 basepair regions corresponding to a putative permease, a glutamyl tRNA synthetase, and a region spanning multiple products. Primers were designed for these loci using DNASTAR Lasergene and ordered from IDT.

Antagonism was detected by stamping from 96 wells onto an innoculated agar plate. Zones of inhibition and clearing were scored as antagonistic interaction.

PCR and Sequencing

PCR products were sequenced by Macrogen USA. Chromatograms were trimmed using Gene Codes Sequencher with default settings. Isolates that failed to amplify or align at all three loci were removed from the analysis. Three reference *A. baumannii* sequences were downloaded from NCBI and included (19606, AYE, and ACICU).

Data Analysis

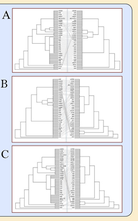
Loci were aligned using MAFFT and gaps and highly divergent regions were removed using gblocks. The reduced sequences were concatenated for each isolate. Phylip dnapars was used to perform a parsimony tree search. One of the most parsimonious trees was arbitrarily chosen and branches were annotated with boostrap support using the sumtrees utility of Dendropy. The tree was uploaded to the interactive Tree of Life (iTOL) for visualization. Parsimony trees of the 100 bootstrap replicates of each locus were consensed using Phylip, and loci were compared pairwise for agreement in Dendroscope.

Future Directions

- Confirm the species identity within *A. calcoaceticus-baumannii* complex of each clade in the three-gene phylogeny.
- Add data from outgroup gammaproteobacterial isolates to antagonism dataset.
- Add all available genomes from TGEN and NCBI to three-gene tree for completeness, context and comparison.
- Compare the topology of each of the three loci within the broader *Acinetobacter* population.
- Determine the antagonism patterns of *A. baumannii* isolates originating outside the Military Hospital System.
- Determine the genetic machinery responsible for the antagonistic phenotype.
- Determine the molecular agent responsible for the antagonistic phenotype.

Comparing three gene trees

Each tree was constructed from the consensus of 100 bootstrap parsimony trees of individual loci. Each panel is a tanglegram of two loci: (A) glutamyl tRNA synthetase [G] and multiple products [M], (B) M and permease [P], (C) G and P. The outgroup and each terminal leaf has bootstrap support of 100/100, and all other branches are scaled proportionally. The antagonistic clade emerges only in tree G. Grey lines connect isolates between the trees.



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Acknowlegements

Thanks to Mr. Mitchell Thompson for help designing some primers used in this study.

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