The Success of *Acinetobacter* Species; Genetic, Metabolic and Virulence Attributes

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Abstract

An understanding of why certain *Acinetobacter* species are more successful in causing nosocomial infections, transmission and epidemic spread in healthcare institutions compared with other species is lacking. We used genomic, phenotypic and virulence studies to identify differences between *Acinetobacter* species. Fourteen strains representing nine species were examined. Genomic analysis of six strains showed that the *A. baumannii* core genome contains many genes important for diverse metabolism and survival in the host. Most of the *A. baumannii* core genes were also present in one or more of the less clinically successful species. In contrast, when the accessory genome of an individual *A. baumannii* strain was compared to a strain of a less successful species (*A. calcoaceticus* RUH2202), many operons with putative virulence function were found to be present only in the *A. baumannii* strain, including the *csu* operon, the acinetobactin chromosomal cluster, and bacterial defence mechanisms. Phenotype microarray analysis showed that compared to *A. calcoaceticus* (RUH2202), *A. baumannii* ATCC 19606 was able to utilise nitrogen sources more effectively and was more tolerant to pH, osmotic and antimicrobial stress. Virulence differences were also observed, with *A. baumannii* ATCC 19606, *A. pittii* SH024, and *A. nosocomialis* RUH2624 persisting and forming larger biofilms on human skin than *A. calcoaceticus*. *A. baumannii* ATCC 19606 and *A. pittii* SH024 were also able to survive in a murine thigh infection model, whereas the other two species were eradicated. The current study provides important insights into the elucidation of differences in clinical relevance among *Acinetobacter* species.

Introduction

In contemporary medicine, certain *Acinetobacter* species have proven to be highly successful in their ability to cause outbreaks and develop antibiotic resistance [1,2]. However, great diversity exists in the clinical importance of the various species, with some being dominant as human pathogens and others merely acting as colonizing or environmental organisms [2]. To date, with the recent description of the novel species *Acinetobacter pittii* (former name *Acinetobacter* genomospecies [gen. sp.] 3) and *Acinetobacter nosocomialis* (former name *Acinetobacter* gen. sp. 13TU) [3], the genus *Acinetobacter* comprises 27 validly named species and 9 DNA–DNA hybridization groups (gen. sp.) with provisional designations. *A. baumannii* has long been considered the most clinically important species, with the greatest number of healthcare–related outbreaks and reports of multidrug resistance. More recently, and likely as a consequence of improved laboratory identification, *A. pittii* and *A. nosocomialis* have also surfaced as clinically significant, with increasing reports of outbreaks and antibiotic resistance [4,5,6,7,8,9]. Species that have less commonly been associated with human disease include *A. iwoffii*, *A. junii*, and *A. haemolyticus*, and some species have only been identified as colonizing human skin or very rarely described as causing human disease, such as *A. johnsonii* and *A. radioresistens* [2,6]. To our knowledge, *A. calcoaceticus* has never been implicated in serious human disease [2]. However, given the difficulty in phenotypically differentiating it from *A. baumannii*, *A. pittii* and *A. nosocomialis*, these species are often grouped together in diagnostic microbiology laboratories as the ‘*A. calcoaceticus – A. baumannii* complex’.

Thus far, the attributes that make one *Acinetobacter* species more adept at causing human outbreaks and disease than another are poorly understood. Previous studies have shown that *A. baumannii*
has the ability to survive in both wet and dry conditions in the hospital environment [10,11,12]. A recent clinical study showed that relative to *A. nosocomialis*, *A. baumannii* was an independent predictor of mortality [4]. A variety of virulence mechanisms have been identified in *A. baumannii*, including siderophore–mediated iron acquisition systems, biofilm formation, adherence and outer membrane protein function, the lipopolysaccharide (LPS), capsule formation, and quorum–sensing [13,14,15,16,17,18,19,20]. Significantly less is known about the non–*baumannii* species. In this study, we used genomics, phenotype microarray analyses and virulence studies, to identify species characteristics that may explain why some *Acinetobacter* species are successful as human pathogens and others are not.

**Results**

**Genome Characteristics of the *Acinetobacter* Species**

As shown in Table 1, 14 genomes were included in this analysis, covering nine different *Acinetobacter* species (species names will be used for non–*baumannii* species throughout). Eight strains were sequenced as part of this study with mean coverage of 22–fold. Overall, the species that make up the *A. calcoaceticus – A. baumannii* complex had the largest genomes, with *A. radioresistens* having the smallest (3.16 Mb). Genome sizes of strains within the *A. baumannii* species varied by up to 289 Kb. The number of genes corresponded to genome size, ranging from 3,690 in *A. baumannii* to 2,874 in *A. radioresistens* (Table 1). Phylogenetic analysis showed that the species that make up the *A. calcoaceticus – A. baumannii* complex were most closely related (Figure S1). The other species formed distinct phylogenetic branches.

**Analysis of the *A. baumannii* Core Genome**

To understand the genetic core of *A. baumannii*, we first analysed the orthologous genes found in all six *A. baumannii* genomes. This analysis yielded 2,800 genes, indicating that the accessory genome, defined as the genes not found within the core *A. baumannii* genome, varied between 658–1,053 genes depending on the strain. A distribution of the *A. baumannii* core genome based on functional gene categories is shown in Figure 1. Apart from genes of general or unknown function, genes related to molecule transport and metabolism were most abundant (35%), including amino acid (11%), carbohydrate (5%), lipid (5%), nucleotide (3%), coenzyme (4%) and inorganic (7%) processing. Interestingly, despite *Acinetobacter* deriving its name from akineto meaning non–motile, *A. baumannii* has several core cell motility genes. These include a type IV pilus apparatus and pilus assembly genes (pilB, pilW, pilZ, pilJ, pilL, pilP, pilQ, pilO, pilN, pilM), fimbral biogenesis genes (fimT, pilZ), and twitching motility genes (pieU, pilT), which are important for pilus retraction. In fact, it has recently been shown that *A. baumannii* is motile under certain conditions [21,22], and this may play an important role in its ability to colonize and spread on surfaces, and to form biofilms [23].

**Comparison of A. baumannii Genome with Other *Acinetobacter* Species**

To begin to decipher the genetic attributes that may help explain why some *Acinetobacter* species are clinically more significant than others, we sought to identify genes only present in pathogenic species of *Acinetobacter* (the six *A. baumannii* genomes, and the *A. pittii* and *A. nosocomialis* genome) and not present in the other species. This analysis identified 51 genes, including 12 putative operons, shared among these eight genomes that were not present in the other species (Table S1). Importantly, one of these operons was the *csu* operon, which includes six genes, and codes for proteins involved in a chaperone – usher pilus assembly system [17]. This operon appears important for pilus assembly, adherence to abiotic surfaces and biofilm formation [17]. The finding that this operon is only present in pathogenic species of *Acinetobacter* further highlights its potential role in determining the clinical success of these species. The predominant functional categorization of the remaining genes was in molecule transport and metabolism, and transcription (Table S1).

**Comparison of Specific Strains of the *A. calcoaceticus – A. baumannii* Complex**

The data presented thus far provide some evidence that a small number of core genes may partly explain the clinical success of certain *Acinetobacter* species; however the number of genes that differed between pathogenic and less pathogenic species was few, suggesting that additional genetic characteristics that distinguish these strains may be found among the accessory genomes. To interrogate the accessory genome in more detail, we analysed representative strains from the four species that make up the *A. calcoaceticus – A. baumannii* complex (Table 1). A distribution of genes is shown in Figure 2A. A total of 2747 genes were common to all four species, with the greatest number of species–specific genes observed in *A. baumannii* ATCC 19606 (Figure 2A). Based on clusters of orthologous group (COG) functional classification, the distribution of genes unique to *A. baumannii* ATCC 19606 was compared to each one of the other three strains (individual pairwise comparisons) was similar, with the greatest number of ATCC 19606–specific genes having a presumptive role in amino acid, carbohydrate and lipid transport and metabolism, and transcription (Figure 2B).

Of most interest was the comparison between *A. baumannii* ATCC 19606 and *A. calcoaceticus*. This comparison identified 759 genes present in *A. baumannii* ATCC 19606 and not in *A. calcoaceticus* (Table S2). Of these, only 169 were found in the other five *A. baumannii* genomes analysed in this study, indicating that the majority (78%) were part of the accessory genome of *A. baumannii* ATCC 19606. Of the 759 genes, 333 had a COG classification, and they were significantly overrepresented in several functions necessary for basic bacterial growth and survival, including transcription (56 genes), DNA replication, recombination, and repair (33 genes), amino acid, inorganic ion and carbohydrate transport and metabolism (66 genes), and cell envelope biogenesis and outer membrane function (19 genes). Among the 333 genes with a COG classification, there were 69 putative operons (Table S2) that were enriched in virulence–related genes, including those involved in siderophore transport and biosynthesis, LPS biosynthesis, pilus and biofilm formation, Curli fimbriae assembly, and bacterial phage resistance mechanisms (Table 2). Several operons responsible for iron handling were identified, including the acinetobactin chromosomal locus (operons 36–39, Table 2), encoding a key *Acinetobacter* siderophore [24,25]. The genetic organisation of this locus and homologues in *A. pittii*, *A. nosocomialis* and *A. calcoaceticus* are shown in Figure 3A. *A. calcoaceticus* and *A. nosocomialis* lacked the full complement of genes that make this locus (Table 2 and Figure 3A). We also identified a more recently described siderophore operon (operon 17, Table 2 and Figure 3B) [21], made up of eight genes, with *A. baumannii* ATCC 19606 being the only strain with the full complement of genes, and *A. nosocomialis* and *A. calcoaceticus* being deficient in most of them.

Several genes related to bacterial defence mechanisms were also observed only in *A. baumannii* ATCC 19606 and not in *A. calcoaceticus*, including those coding for ABC transporters, and CRISPR – (Cas) and phage–resistance proteins (Table S2). Clustered regularly interspaced short palindromic repeats
**Table 1. Characteristics of the bacterial strains used in this study.**

| Bacterial species | Strain name | Origin (Place, year) | Source | Genome Size (Mb) | No. of Genes | Reference for Genome Sequence | Genbank Accession No. |
|-------------------|-------------|----------------------|--------|------------------|-------------|-----------------------------|----------------------|
| *A. baumannii*²   | ATCC 19606° | Unknown, before 1949 | Urine  | 3.97             | 3,766       | This study                  | ACQB00000000         |
| *A. baumannii*    | ATCC 17978 | Unknown, –1951       | Unknown| 3.98             | 3,791       | [50]                        | CP005621             |
| *A. baumannii*    | AB0057      | Washington, D.C., USA, 2003–2005 | Blood | 4.05             | 3,853       | [51]                        | CP001182             |
| *A. baumannii*    | AB307–0294  | Buffalo, NY, USA, 1994 | Blood | 3.76             | 3,458       | [51] | CP001172 |
| *A. baumannii*    | AYE         | Le Cremlin–Bicêtre, FR, 2001 | Urine | 3.94             | 3,607       | [52] | CU459141 |
| *A. baumannii*    | ACICU       | Rome, IT, 2005       | CSF    | 3.90             | 3,667       | [53] | CP00863  |
| *A. calcoaceticus*² | RUH2202 | Malmö, SE, 1980–82 | Wound  | 3.88             | 3,566       | This study                  | ACPK00000000         |
| *A. pittii*²      | SH024       | Cologne, DE, 1993    | Skin (axilla) | 3.97 | 3,689       | This study                  | ADCH00000000         |
| *A. nosocomialis*² | RUH2624 | Rotterdam, NL, 1987 | Skin (forehead) | 3.87 | 3,631       | This study                  | ACQF00000000         |
| *A. lwoffii*      | SH145       | Cologne, DE, 1994    | Skin (hand) | 3.48 | 3,134       | This study                  | ACPM00000000         |
| *A. junii*        | SH205       | Cologne, DE, 1994    | Skin (perineum) | 3.46 | 3,186       | This study                  | ACPO00000000         |
| *A. radioresistens* | SH164 | Cologne, DE, 1994 | Skin (forehead) | 3.16 | 2,874       | This study                  | ACPO00000000         |
| *A. johnsonii*    | SH046       | Cologne, DE, 1994    | Skin (perineum) | 3.69 | 3,363       | This study                  | ACPL00000000         |
| *A. baylyi*       | ADP1        | Atlanta, GA, USA, before 1958 | Soil  | 3.60             | 3,325       | [54] | CR543861 |

DE, Germany; FR, France; IT, Italy; NL, The Netherlands; SE, Sweden; US, United States.

²*A. baumannii SDF* was not included in this study due to its significantly reduced genome size and gene number compared to the *A. baumannii* isolates derived from human sources.

²Representative strains of the *A. calcoaceticus – A. baumannii* complex that were analysed in detail.

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(CRISPRs) are recently described adaptive bacterial immune mechanisms that protect bacteria from invading foreign genetic elements such as bacteriophages [26,27]. Such systems, when combined with other phage resistance mechanisms, may provide a survival benefit to the bacterial host [26]. The A. baumannii ATCC 19606T CRISPR system includes cas1 and cas3; however we could not locate cas2, which is thought to be required with cas1 to form a functional CRISPR system [27]. The CRISPR operon was not found in the A. calcoaceticus, A. nosocomialis or A. pittii strains.

Comparison of the Metabolic Versatility of Specific Strains of the A. calcoaceticus – A. baumannii Complex

Given the predominance of metabolism genes differentiating pathogenic and less pathogenic strains (Table S1 and S2), we analyzed the metabolic profile of the four species of the A. calcoaceticus – A. baumannii complex using phenotype microarrays. Of the 1920 conditions tested, the four species shared 1356 metabolic responses (70.6%), of which 795 compounds or conditions could be utilized by all the species and 561 by none of them. A summary of the entire metabolic profile of the four species is shown in Figure 4A. A. baumannii ATCC 19606T appeared to utilize peptide nitrogen sources (PM 6-8) more effectively and to be more tolerant to pH stress (PM 10) than the other three species. A. baumannii ATCC 19606T and A. pittii had a reduced ability to utilise most of the phosphorus and sulfur sources (PM 4) (Figure 4A).

We then focused on the most clinically disparate of the species, and compared A. baumannii ATCC 19606T with A. calcoaceticus in more detail. In 195 conditions (Table S3), A. baumannii ATCC 19606T was significantly more metabolically active than A. calcoaceticus. These conditions comprised 10 carbon sources, 105 nitrogen sources (of which 98 were di- and tri-peptides) and 80 stress conditions, of which 26 related to osmotic and pH stress and 54 related to the presence of antimicrobials and other cytotoxic compounds (Table S3). Apart from the likely survival advantage inferred by the greater ability of A. baumannii ATCC 19606T to metabolise in the presence of osmotic, pH and antimicrobial exposure, one of the carbon sources utilized by this strain was D-glucarate. D-glucarate is found in the human body and has been shown to be a carbon source utilized by a range of gram–negative bacteria [28,29]. D-glucarate catabolism generates α-ketoglutarate, which enhances the citric acid cycle (Figure 4B). Recently, over–expression of the citric acid cycle was shown to occur in an A. baumannii strain with increased virulence in the presence of ethanol [30].

Virulence Differences between Strains of the A. calcoaceticus – A. baumannii Complex

Given the differences in the number of putative operons with virulence function that were observed between the four species of the A. calcoaceticus – A. baumannii complex, we performed a range of in vitro and in vivo virulence studies to characterise further the functional significance of their genetic differences. Given the predilection of A. baumannii to colonise or infect the respiratory tract, we first analysed the interaction of the four species with human bronchial epithelial cells. All strains could adhere to human bronchial epithelial cells and induce a pro-inflammatory cytokine response (IL-6 and IL-8). Adherence and IL-8 induction appeared to be most pronounced with A. pittii (Figure 5, A, B and C). Of note, cell monolayers remained intact and the morphology of the cells was not affected by any of the strains (data not shown).

Given the likely importance of biofilm formation to the success of Acinetobacter in hospitals, we next tested the four species in a unique biofilm assay. Thus far, the correlation between biofilm formation on abiotic surfaces and clinical significance has been poor [31]. Therefore, we used a novel assay that may predict the ability of Acinetobacter to colonise and form a biofilm on human skin [32]. Using a three-dimensional human skin construct, we observed that A. baumannii ATCC 19606T, A. pittii and A. nosocomialis were able to multiply rapidly and persist on human skin, whereas A. calcoaceticus grew to a significantly lower density (Figure 5D). In addition, biofilms of the former three species were visible on the stratum corneum after PAS-Alcian blue staining, whereas no such bacterial structures were seen for A. calcoaceticus (Figure 5, E and F).

Finally, we assessed the survival of the four strains in a neutropenic mouse thigh muscle infection model, which is a soft-tissue model previously used for Acinetobacter infection [33]. A.
Calcoaceticus and A. nosocomialis were eradicated from the mouse thigh muscles, whereas bacteria of A. baumannii ATCC 19606T and A. pittii could be detected in the muscles after 48 hours (Figure 5G).

Discussion

This study provides a combined genomic, phenotypic and virulence assessment of a range of Acinetobacter species that have been variably associated with humans. From a genomic analysis of nine different Acinetobacter species, we identified a small number of genes unique to pathogenic species. The majority of these genes are predicted to be important for molecule transport and metabolism but also included the putative virulence csu operon. Investigating the accessory genome of individual strains of the four species of the A. calcoaceticus – A. baumannii complex, we found a range of putative operons with predicted functions related to host survival and virulence in A. baumannii ATCC 19606T but not in A. calcoaceticus. A. pittii appeared most similar to A. baumannii ATCC 19606T, whereas A. nosocomialis lacked several of these important operons, particularly the full repertoire of genes of the acinetobactin chromosomal locus. Phenotype microarray studies supported the genomic analysis in that A. baumannii ATCC 19606T was able to utilise more carbon and nitrogen sources, and was more tolerant to a range of cellular stresses than A. calcoaceticus. Moreover, the pathogenic species were able to multiply and form biofilms on human skin significantly more than A. calcoaceticus. Only A. baumannii and A. pittii were able to survive in a mammalian thigh infection model.

As a consequence of improved laboratory speciation, it is becoming apparent that non–baumannii species, particularly A. nosocomialis and A. pittii, are clinically significant human pathogens. For example, in a recent study from Norway, A. nosocomialis was the most common species (47%) isolated from blood cultures over a three–year period, followed by A. pittii (20%) [34]. With regard to their clinical impact, a more contemporary study has shown that relative to A. nosocomialis, bacteremia with A. baumannii was an
Table 2. Select operons with putative virulence function found in *A. baumannii* ATCC 19606\(^\text{\textregistered}\) and not in *A. calcoaceticus*.

| Operon ID | Function | Genes | *A. baumannii ATCC 19606\(^\text{\textregistered}\) ORFs\(^1\) | Homologues average similarity (ID% ± SD)\(^2\) |
|-----------|----------|-------|-------------------------------------------------------------|-------------------------------------------------|
| 2         | Pili assembly and biofilm form. | *csuA/B/ABCDE* | ACIB1v1_50001–6 | 91.8±7.1 91.8±7.1 |
| 17        | Siderophore transp. bios. | *cirA, menG, iucA/C, araA1, rhbE/C* | ACIB1v1_160094–101\(^3\) | 46.2±17.9 31.1±3.7 |
| 29        | Phage resistance | *cas1, cys1, cys2, cys3, cys4* | ACIB1v1_260071–75 | – – |
| 36        | Siderophore transp. bios. | *bauD, bauC, bauE, bauB and bauA* | ACIB1v1_480066–70 | 97.8±1.0 – |
| 37        | Siderophore transp. bios. | *basC and basD* | ACIB1v1_480071–72 | 97.4±1.0 – |
| 38        | Siderophore transp. bios. | *basE, basf, basG* | ACIB1v1_480073–75 | 97.6±0.3 47.8±0.4 |
| 39        | Siderophore transp. bios. | *barA, barB, basH, basI, basJ* | ACIB1v1_480076–80 | 95.1±6.3 32.8±0.8 |
| 40        | Siderophore transp. bios. | *tonB, PEPN* | ACIB1v1_490004–5 | 97.0±0.3 97.5±0.3 |
| 46        | Cell motility and secretion | *pilA* | ACIB1v1_560044–45 | 60.2±14.5 73.3±0.3 |
| 47        | LPS biosynthesis | *ipsC and lpsE* | ACIB1v1_600015–16\(^4\) | 33.8\(^5\) 68.2±29.8 |
| 50        | Curli fimbriae assembly | *casG* | ACIB1v1_700078–80 | 94.8±1.1 – |
| 56        | LPS biosynthesis | *wzx, degT, wbbi, mvIM and vipA* | ACIB1v1_740018–22\(^6\) | 42.6±23.8 48.3±17.2 |

Bios., Biosynthesis; Form., Formation; ID, Identity; SD, Standard Deviation; Transp., Transport.  
\(^1\)Expressed as the average identity at the nucleotide level ± standard deviation.  
\(^2\)Only three, two and two genes (out of eight) are found in *A. pittii*, *A. nosocomialis* and *A. calcoaceticus*, respectively. The homologues identified exhibited low similarity.  
\(^3\)Both genes belong to an LPS operon that spans from ORF ACIB1v1_600009 to 16, and which is only partially present within *A. calcoaceticus* (three of eight genes are absent).  
\(^4\)PilC is absent from *A. pittii* genome.  
\(^5\)Only *vipA* is present in *A. calcoaceticus* and exhibited moderate similarity. The operon is poorly conserved and partially present also in *A. pittii* and *A. nosocomialis*.  
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Figure 3. Genetic organisation and conservation of the siderophore clusters found in *A. baumannii* ATCC 19606\(^\text{\textregistered}\) and not in *A. calcoaceticus*.  
(A) Siderophore cluster 1 (operons 36–39) is known as the acinetobactin chromosomal cluster, and (B) siderophore cluster 2 (operon 17) (See Table 2 for details about the operons). The presence of homologues for each gene in *A. pittii*, *A. nosocomialis*, and *A. calcoaceticus* is shown.  
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independent predictor of mortality [4]. Interestingly, and consistent with our study findings, there was no significant difference between *A. baumannii* and *A. pittii*, however the number of patients in the *A. pittii* group was small [4]. Genetically and metabolically, we showed that *A. pittii* appeared similar to *A. baumannii*, and they also behaved similarly in the mammalian infection model. Despite its description of causing bloodstream infection, *A. nosocomialis* lacked several of the putative virulence related operons, particularly the acinetobactin siderophore cluster, and its phenotype was more closely aligned to *A. calcoaceticus*, both of which may explain its failure to survive in the murine model and its association with a lower mortality in clinical studies [4].

Within mammalian hosts, free iron is often a scarce resource and for pathogenic organisms to survive *in vivo* they often utilize a range of iron scavenging systems. Such systems have been analysed across different *A. baumannii* strains [21,35] however this is the first analysis, to our knowledge, of such genes in non-*baumannii* species. In addition to the acinetobactin chromosomal locus, we observed another siderophore cluster and a putative iron uptake receptor in *A. baumannii* ATCC 19606\(^\text{\textregistered}\) that was not present in *A. calcoaceticus*. This second cluster (operon 17 in Table 2) is a recently described siderophore cluster made up of eight genes that is well conserved across *A. baumannii* species. In addition to the acinetobactin siderophile cluster and a putative iron uptake receptor in *A. baumannii* ATCC 19606\(^\text{\textregistered}\) that was not present in *A. calcoaceticus*. This second cluster (operon 17 in Table 2) is a recently described siderophore cluster made up of eight genes that is well conserved across *A. baumannii* strains [21]. The full repertoire of genes from this cluster was not found in *A. pittii*, *A. nosocomialis*, and *A. calcoaceticus*, and the few homologues identified exhibited low similarity (Table 2). Such genetic differences between *Acinetobacter* species in key virulence attributes may help explain why some species have greater clinical impact.

Apart from genes involved in metabolism and transcription, we identified the *csu* operon as an operon found in pathogenic species of *Acinetobacter* (six *A. baumannii* strains, *A. pittii* and *A. nosocomialis*) but not in non-pathogenic species. Loss of function of this operon leads to a lack of pili-like structures on the surface of *A. baumannii* and to loss of cell attachment and biofilms on abiotic surfaces [17].
Interestingly, this operon was not shown to be important for attachment to and cytokine production by human bronchial epithelial cells [36]. We hypothesize that this operon may aid in Acinetobacter attachment and colonization of plastic medical devices such as ventilator tubing and catheters, with a subsequent increased risk of invasive infection. The definitive role of this operon in mammalian virulence requires further evaluation.

We observed a diverse repertoire of core metabolic genes in A. baumannii, which is likely to be important for its ability to survive in vivo, as well as in unique ecological niches of healthcare institutions. To assess the global metabolic capabilities of the Acinetobacter species, we used phenotype microarrays, which enabled us to assess nearly 2000 metabolic and toxic compound conditions. Overall, A. baumannii ATCC 19606T was able to utilize nitrogen sources more effectively and was more tolerant to pH stress than A. nosocomialis, A. pittii and A. calcoaceticus. The differences were more marked when A. baumannii ATCC 19606T was compared to A. calcoaceticus. Interestingly, A. baumannii and A. pittii were unable to utilize most of the phosphorus sources despite both strains having the necessary genetic composition for phosphate metabolism. Several studies have highlighted the key role of the Pho regulon not only in phosphate management, but also in virulence and stress responses in many bacteria [37]. Whether the inability of A. baumannii and A. pittii to utilize phosphorus is linked to expression of the Pho regulon remains a question that needs further evaluation.

Taken together, these data are hypothesis generating and provide important insights into understanding the potential differences between species of the Acinetobacter genus. We describe genetic and metabolic characteristics that support why some species may be more clinically important than others, and also highlight the functional significance of these differences in various virulence models. Limitations of our study are that we only analysed one strain for each of the non-baumannii species, and our results need confirmation using a larger set of strains. However, to our knowledge this is the first genetic and metabolic description of such a diverse range of Acinetobacter species. Furthermore, confirmation of our findings using targeted gene deletion and complementation is required to define the significance and role of the species-specific operons found in pathogenic versus non-pathogenic species. Finally, our study has examined the presence or absence of genes between strains; however polymorphic differences in shared genes may also contribute to phenotypic differences. A full analysis of genotype-phenotype associations will

Figure 4. Metabolic diversity of specific strains of the A. calcoaceticus – A. baumannii complex. (A) Phenotype Microarray (PM) comparative results showing the number of compounds used (green) or not used (red) by A. baumannii ATCC 19606T [A], A. nosocomialis [B], A. pittii [C] and A. calcoaceticus [D]. The external circle and PM number represent the Biolog plate number. (B) A. baumannii ATCC 19606T is able to metabolise the carbon source, D-glucarate and produce α-Ketoglutarate through the functional enzymes, D-glucarate dehydrogenase and KDG dehydratase. α-Ketoglutarate is then utilized in the citrate cycle. These enzymes are not found in A. calcoaceticus.

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require data from many additional strains of each species. Overall, these data provide important insights into the potential differences in clinical relevance among Acinetobacter species.

Materials and Methods

Ethics Statement

The animal studies performed in this study were approved by the Leiden Experimental Animal Committee (permit number 10038) and were performed in compliance with The Experiments on Animals Act of 1996, which is the Dutch law related to the conduct of animal experiments. All efforts were undertaken to minimize suffering.

Bacterial Strains and Culture Conditions

The 14 strains included in this study are shown in Table 1. The genomes of eight strains were sequenced in the present investigation, while for six strains, the publicly available genomes were used (Table 1). Cultures were performed at 30°C or 37°C on sheep blood agar plates (bioMérieux, Boxtel, The Netherlands) or in Luria–Bertani (LB) broth.
Whole Genome Sequencing

Genomic DNA was extracted using the Invitrogen Easy-DNA kit (Invitrogen, CA, USA) or as described by Bomm et al. [38]. Genomes were sequenced using 454 FLX pyrosequencing (Roche) with DNA standard fragment and 3 kb jumping libraries according to the manufacturer’s recommendations [39]. Genomes were assembled using Newbler and annotation was performed using a combination of ab initio and evidence-based approaches (see Text S1).

Phylogenetic Analysis and Comparative Genomics

Predicted proteins from each Acinetobacter genome were compared using an all-against-all BLAST search and Pseudomonas aeruginosa PAO1 was used as the outgroup. Reciprocal best blast matches (RBM), regardless of percent identity, were stored in a custom MySQL relational database to facilitate identification of orthologous groups shared by selected phylogenetic and phenotypic groups of organisms. RBM matching proteins were clustered using the Markov clustering algorithm implemented in MCL [40], and clusters with one protein per genome were defined. These represent orthologous core genes that are present as a single copy in each genome. The protein sequences for each cluster were aligned using CLUSTALW [41] and the resulting multiple sequence alignments were concatenated for tree building. A neighbour joining (N) tree was made using MEGA4 [42] and evaluated using 100 bootstrap replicates. The criteria used to define a putative operon were (i) genes were consecutive, (ii) genes were transcribed in the same orientation, (iii) the intergenic distance between the genes was no longer than 150 bp, and (iv) gene length was at least 450 bp [43,44].

Metabolic Profiling

To assess for metabolic differences between Acinetobacter species, we used Phenotype Microarrays (PM) as described previously by Biolog Inc. (Hayward, CA, USA) [45]. This technology uses tetrazolium violet irreversible reduction to formazan as a reporter of active metabolism. Twenty 96–well microarray plates were used (PM 1–20) comprising 1920 different metabolic and toxic compound conditions. The analysis of PM data was carried out on the raw data-set provided by Biolog Inc., obtained by three replicates of each substrate. Binary coefficients (1/0) for positive metabolism (1) or no metabolic activity (0) were attributed to each PM well and a matrix of binary vectors, each representing a single Acinetobacter species, was prepared as previously described [16]. Binary data were then used to compute a similarity matrix by using Jaccard coefficient with the software PAST [47]. See text S1 for more details.

Growth on Human Skin Equivalents

Human keratinocytes were isolated from fresh mamma reduction surplus skin and human epidermal skin constructs were generated as previously described [32,48]. In brief, human epidermal skin constructs were incubated with 300 μl of a mid-logarithmic bacterial suspension (3×10⁷ colony forming units [CFU]/ml) at 37°C (7.3% CO₂). After 1 h, skin constructs were washed with phosphate buffered saline (PBS) to remove non-adherent bacteria and were incubated air-exposed for an additional 23 hr and 47 hr. Two circular biopsies (4 mm in diameter) were taken from the skin, homogenized in PBS and serially diluted to determine the number of CFU. A third biopsy of each skin construct was fixed in 4% formaldehyde, dehydrated and embedded in paraffin for subsequent staining with Alcian-blue PAS (Merck, Darmstadt, Germany) for morphological analysis. Three independent experiments were performed.

Bronchial Epithelial Cell Adhesion and Cytokine Production

Adherence of bacteria to human bronchial epithelial cells (HBE cells, ATCC CRL-1848, Manassas, VA, USA) and cytokine production by these cells was determined as described previously [36,49]. In brief, HBE cells were incubated for 1 hr at 37°C with 1×10⁶ CFU of an overnight bacterial culture on blood agar. Bacterial adherence to HBE cells was quantified by light microscopy and the average number of bacteria per 100 epithelial cells was recorded. Two independent experiments were performed in duplicate. For cytokine production, HBE cells were washed five times after 1 hr of bacterial infection (as described above) with prewarmed PBS, and fresh RPMI medium was added. After 24 hr incubation at 37°C, supernatants were collected and stored at −20°C until determination of cytokine levels. RPMI medium alone was used as a control. Interleukin (IL)–6 and IL–8 were determined by enzyme–linked immunosorbsent assays (ELISA, Biosource, CA, USA) according to the manufacturer’s instructions. The lower limit of detection was 15 pg/ml for IL–6 and 7 pg/ml for IL–8. Three independent experiments were performed.

Murine Thigh Infection Model

The survival of Acinetobacter strains in a mouse thigh muscle infection model was assessed as previously described [33], with modifications. Female Swiss mice (Charles River Nederland, Maastricht, The Netherlands) were made transiently neutropenic by intraperitoneal injection with cyclophosphamide (150 mg/kg body weight in 150 μl) on day 4 and 3 prior to infection. Approximately 1×10⁷ CFU (in 50 μl of saline) of a mid-logarithmic culture was injected in the right thigh muscle (three animals per strain). At 48 hr after infection, mice were sacrificed and infected thigh muscles were removed and homogenized in 1 ml PBS and viable counts were performed.

Statistical Analysis

All data were analysed for statistical significance using the Wilcoxon rank sum test. P values of ≤0.05 were considered statistically significant.

Supporting Information

Figure S1  Phylogenetic analysis of the eight sequenced strains of Acinetobacter species from this study.

Table S1 Unique genes found in pathogenic species of Acinetobacter (A. baumannii [six strains], A. pittii and A. nosocomialis) and not in other less or non-pathogenic species. Highlighted areas represent putative operons.

Table S2 Unique genes found in A. baumannii ATCC 19606T compared to A. calcoaceticus, and their functional characterisation. Highlighted areas represent putative operons.

Table S3 Metabolic gains found in A. baumannii ATCC 19606T but not in A. calcoaceticus using phenotypic microarrays (PM).

Text S1 Materials and Methods.
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