Association of the Genus Acinetobacter *with the Decomposition of a Swine Carcass and the Isolation and Characterization of a Novel Strain of* Acinetobacter *sp. P4*

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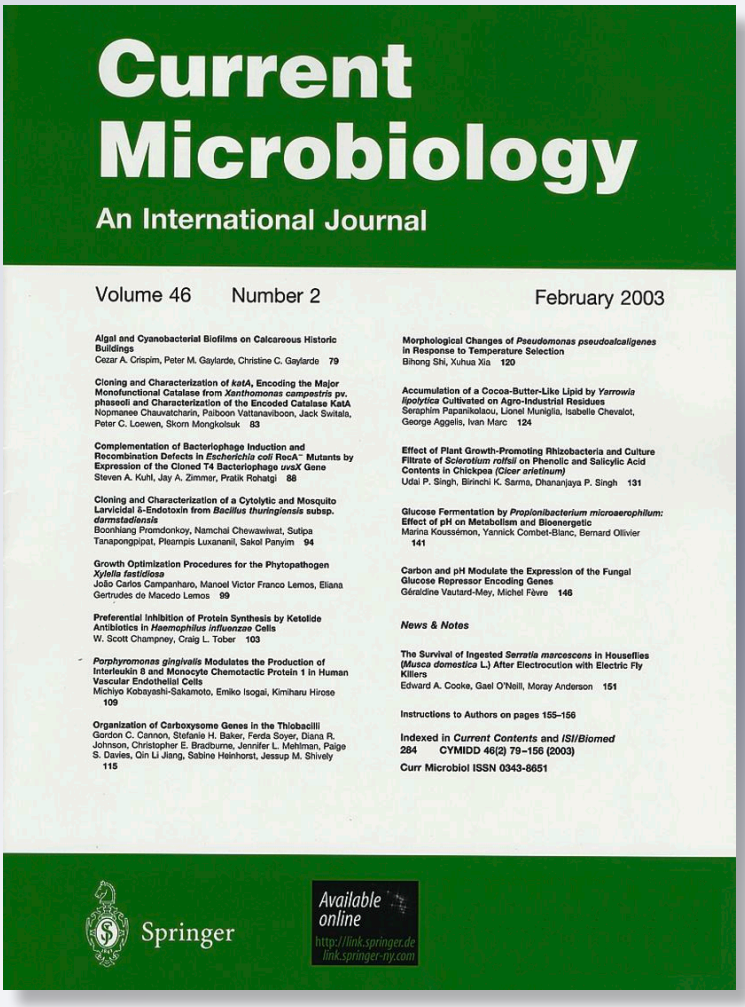

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Association of the Genus *Acinetobacter* with the Decomposition of a Swine Carcass and the Isolation and Characterization of a Novel Strain of *Acinetobacter* sp. P4

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Abstract Using fluorescence in situ hybridization (FISH) and a selective and differential medium, *Acinetobacter* numbers were enumerated over the time course of decomposition, from fresh to putrid/dry, of a swine carcass. In addition, *Acinetobacter* diversity and succession were also characterized. *Acinetobacter* bacterial counts were observed to be the lowest before exposure (undetectable) and increased to their highest during active decay then decreased and leveled during advanced decay through putrid/dry. FISH analysis revealed *Acinetobacter* cells were mostly clustered together, which is consistent with growth in a non-mixed environment, such as soil. The abundance of *Acinetobacter* cells decreased from active decomposition to putrid/dry. BLAST analysis using the 16S rRNA-gene sequence identified the isolates as one of the following *Acinetobacter* spp: *A. baumannii*, *A. haemolyticus*, *A. junii*, *A. johnsonii*, and *A. gerneri*. Phenotypic description of the identified isolates closely matched those of known genomic species. One isolate, P4, was observed to be unique in its phenotypic and phylogenetic characteristics and was more closely related to *A. sp* E10. The isolates from this study displayed multi-antibiotic resistance. The results from the study revealed the association of *Acinetobacter* spp. with that of carrion which adds to our knowledge of the ecology of this genus along with the potential implications of infection for this opportunistic pathogen.

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Introduction

In a previous study [13], the microbial diversity, succession, and lipolytic activity of the soil bacterial community associated with swine carcass throughout decomposition were elucidated. Given that the primary mediators of carbon and cycling of decaying remains are presumably microbial, an analysis of the microbial communities of both the carcass and site is essential to fully understand the carrion habitat. The major carbon sources available for bacterial degradation by a swine carcass are protein and lipid. Results from the previous study indicated that lipolytic bacterial counts were initially the lowest at day 0 (before exposure) and increased to their highest between days 9 and 12 (active decomposition) then decreased and leveled thereafter. Quantitative-PCR (qPCR) results using Group I lipase specific primers followed a similar pattern as the lipolytic CFU observed. Also, the lipid content of the carcass was observed to be contributing more organic carbon to the soil community than the available protein and the lipolytic bacteria were likely responding to an increase in lipid.

As a human corpse or animal carcass decomposes, the soft tissues of the body (particularly the dependant side) will become coated with a grayish-white waxy substance called adipocere (or "grave wax"). Adipocere consists of long-chain hydroxyl fatty acids and is the end product from the saponification of lipids (adipose tissue) in the presence of water and bacteria [12]. According to previous studies [14, 20], the composition of adipocere and the bacteria isolated from adipocere samples (*Pseudomonas, Serratia*, and *Bacillus*) have shown that lipolysis is involved in its decomposition. In

another study [17], frozen-thawed animal's decomposition proceeded from the outside to inside of the carcass through aerobic decomposition, whereas fresh-killed animal's decomposition proceeded from the inside to outside through anaerobic putrefaction. The microorganisms identified in these processes included enteric, Staphylococci, Streptococci, *Bacillus*, and *Clostridium*.

This study is a continuation from a previous study [13] to further characterize the *Acinetobacter* diversity and succession as well as to determine enumeration through isolation and identification of cultivable microbes of the soil bacterial community associated with the swine carcass throughout decomposition.

Materials and Methods

Sampling

The description of the study plot and sampling protocol for the adult swine carcass (47 kg) were previously described [13]. The carcass was monitored throughout decomposition corresponding to the different stages of decay [25] (fresh, bloat, active decay, advanced decay, and purtrid/dry remain stages). Soil cores (2-cm diameter × 2-cm depth, 12.6 cc each) were collected beneath the carcass at the torso every 3 days until day 15 of decomposition, and then sampled every 2 weeks until day 71. Soil cores (2-cm depth) composed of predominantly aerobic microorganisms associated with decaying detritus and organic-rich topsoil were mixed to form a composite sample for further analysis. Archive samples were used in this study that had been preserved at −70°C. To minimize vertebrate scavenging, the carcass was surrounded by a 1-m high 0.64-cm^2 hardware screen enclosure secured to wooden stakes.

Media and Culture Conditions

The number of cultivable *Acinetobacter* in each collected sample was determined by the pour plate method. For this method, 1 g of soil from each homogenized sample was diluted in 99 ml of sterile saline solution (0.9% NaCl, w v^{-1}) and mixed thoroughly. Standard serial dilutions followed, and a 1-ml aliquot of each dilution was used for enumeration.

A selective and differential medium were used for the enumeration of *Acinetobacter* [26]. The *Acinetobacter* Agar Medium (pH 7.4) contains in grams per liter: thiotone, 10; yeast extract, 3; NaC1, 5; sucrose, 10; mannitol, 10; sodium citrate, 0.5; sodium desoxycholate, 0.1; crystal violet, 0.00025; phenol red, 0.04 and agar–agar 15. This medium has the advantage of inhibiting the growth of cocci and Gram-positive bacilli, by the use of sodium citrate and sodium desoxycholate associated with the crystal violet; and of differentiating the Gram-negative bacilli from the Enterobacteriaceae, through the fermentative activity on the sucrose and/or mannitol, contrasting with the complete inactivity of the *Acinetobacter* genus bacteria over those substances.

Phenotypic Characterization

Identification consisted of Gram staining, catalase test, and oxidase test and then 16S rRNA-gene sequencing. The Biolog system was used for biochemical analysis (Hayward, CA).

Fluorescence in situ Hybridizations (FISH)

Samples from soil cores (1 g) were fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS) (composed of 0.13-M NaCl, 7-mM Na_2HPO_4, and 3-mM NaH_2PO_4 [pH 7.2 in water]) for 2–4 h on ice. The fixed samples were then washed in PBS and stored in ethanol–PBS (1:1) at −20°C. A fluorescently labeled oligonucleotide hybridization probe, Acin0659, targeting the 16S rRNA for members of the genus *Acinetobacter* was synthesized (5′ CTGG AATTCTACCATCCTCTCCCA 3′) and conjugated with the cyanine dye, Cy3, before purification with oligonucleotide probe purification cartridges [18]. Fluorescently labeled probes were diluted to 50 ng μl^{-1} with RNase-free water and stored at −20°C in the dark. Fixed samples were applied to a sample well on a ten-well Heavy Teflon Coated microscope slide (Cel-Line Associates, New Field, NJ) and air-dried. After dehydration with an increasing ethanol series (50, 80, 95% [v v^{-1}] ethanol, 1 min each), each sample well was covered with a mixture of 9.9 µl of hybridization buffer (20% [v v^{-1}] formamide, 0.9-M NaCl, 100-mM Tris HCl [pH 7.0], 0.1% (w v^{-1}) SDS) [9] and 0.1 µl of the stock fluorescently labeled oligonucleotide probe. The hybridizations were conducted in a moisture chamber containing excess hybridization buffer (to prevent dehydration of buffer on sample wells) for 1.5 h, in the dark, at 46°C. The slides were washed for 30 min at 48°C with 50 ml of pre-warmed washing buffer solution (215-mM NaCl, 20-mM Tris HCl [pH 7.0], 0.1% (w v^{-1}) SDS, and 5-mM EDTA) [8]. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) at a concentration of 1 µg ml^{-1} for 1 min and rinsed with DI water. Fixed hybridized cells were mounted with type FF immersion oil (Cargille, Cedar Grove, NJ) and a cover slip.

Image Capture

Whole cell fluorescence was visualized with an upright epiflourescence microscope (Leitz DiaPlan, Heerbrugg, Switzerland), and 8-bit digital images were captured using

a Spot-FLEX charge-coupled device (CCD) camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Images were collected using a ×40 and ×100 oil objectives and constant exposure time (1.0 s) and gain of 2.

Image Analysis

Images were analyzed using the daime software package [7]. Ten images were analyzed for each sample and manual thresholding was used to remove background (2D segmentation mode) and only whole cell fluorescence was retained for analysis. Each sample (series of ten images) was processed with the same thresholding parameters and the "Biovolume fraction" feature was used to calculate the amount of cells present.

Transmission Electron Microscopy

A suspension of bacteria was placed in a 3-ml Eppendorf microcentrifuge tube and centrifuged at 12,000 × *g* for 5 min to form a pellet. The pellet was resuspended in a 0.1-M sodium cacodylate-buffered solution (pH 7.4) for 5 min and then centrifuged at 12,000 × *g* for 8 min. The supernatant was discarded and the pellet was placed in a 0.1-M sodium cacodylate buffered (pH 7.4) solution of 4% glutaraldehyde. After the cells were fixed at 4°C for 2 h, the pellet was rinsed three times with fresh buffer for 10 min each. The bacteria were post-fixed for 1 h at room temperature in a 0.1-M sodium cacodylate-buffered (pH 7.4) solution of 1% osmium tetroxide/2.5% potassium ferrocyanide. The pellet was rinsed three times with distilled water for 10 min each, dehydrated in a graded series of ethanol, and embedded in POLY/BED 812 resin. Thin sections of bacteria were collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 100S transmission electron microscope at 80 kV.

Scanning Electron Microscopy

One drop of a bacterial suspension was placed on a glass coverslip that had been coated with a 0.1% solution of poly-L-lysine. After a 10-min incubation period, the cover slip was rinsed in 0.1-M sodium cacodylate buffer (pH 7.4) and then immersed in a similarly buffered solution of 4% glutaraldehyde. Subsequent to their fixation at 4°C for 1 h, the bacteria were rinsed three times with fresh buffer for 10 min each. The bacteria were post-fixed for 1 h at room temperature by immersion of the cover slip in a 0.1-M sodium cacodylate-buffered solution (pH 7.4) of 1% osmium tetroxide. The cells were rinsed three times with distilled water, dehydrated in a graded series of ethanol, critical-point dried with liquid carbon dioxide, and sputtered coated with gold. The cells were examined with an FEI 20XL scanning electron microscope at 15 kV.

DNA Sequencing

Nucleotide sequences were determined by the Pennington Biomedical Genomic Center (Baton Rouge, LA) and analyzed using BLAST program in GenBank [1]. DNA sequence of 16S rRNA-gene was deposited in GenBank under the accession number FJ851148.

Phylogenetic Analysis

DNA sequences were aligned using the Greengenes program [9]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [24]. *Acinetobacter* sequences available from the GenBank database [1] were used. *Pseudomonas immobilis* was used as out-group for the rooted tree. Phylogenetically distinct clusters were assigned based on a bootstrap value >90%.

Antibiotic Sensitivity Analysis

To further characterize and differentiate isolates, susceptibility tests were performed using prepared 96-well microtiter plates from MicroScan® by Siemens Healthcare Diagnostics (West Sacramento, CA) and Sensititre® by TREK Diagnostic Systems (Cleveland, OH) that is an automatic system. Both methods used plates with a panel of several antimicrobials that are precision dosed at appropriate dilutions and equates to the classical microbroth dilution method. The MicroScan® plates were read manually, whereas the Sensititre® plates were read by the TREK instrument that detects growth as a fluorescent substrate is used by bacterial surface enzymes. The amount of detected fluorescence is proportional to bacterial growth. The TREK data system interprets the MIC (minimal inhibitory concentrations) values following CLSI (Clinical and Laboratory Standards Institute) recommendations although manual interpretations can be performed with novel antimicrobials. Fail-safe features built into the database preclude interpreting tests read at inappropriate times, correctly interpret manually read tests, and automatically flag unusual results. All results were compared and interpreted following CLSI recommendations.

Before testing, the isolates were taken out of the −80°C freezer and subcultured twice on trypticase soy agar plates containing 5% sheep blood (BA) (BD BioSciences, Sparks, Md.). Incubation temperature was 35°C. Overnight growth (4–5 colonies) is taken from BA and resuspended in demineralized water and adjusted to a 0.5 MacFarland. Inoculation of plates followed the manufacturers' directions to give about 1×10^5 cells/ml per well and plates

were sealed with non-removable plastic seals and incubated in ambient air at 35 ± 2°C for 24 h. An inoculum count plate was set up for each test isolate. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were the control organisms for the tests.

Results

Enumeration of *Acinetobacter* spp.

This study was conducted between 24 September and 3 December 2008. Soil from the field plot was determined to have a bulk density of 1.51 g cc^{-1} and a soil texture was sandy loam as described by [23]. Ambient temperatures for the first 15 days of decomposition were observed to have maximum temperatures averaging 30°C and minimum temperature of 23°C. Whereas, by days 28 through day 59 of decomposition, the average maximum and minimum temperatures were 25 and 17°C, respectively. The lowest daily maximum (17°C) and minimum (10°C) ambient temperatures of the study was observed on Day 71.

Acinetobacter bacterial counts were initially the lowest before exposure (undetectable) and increased to their highest during active decay ($146.7 \times 10^2 \pm 7.37$), then decreased and leveled during advanced decay through putrid/dry. Figure 1 shows the cultivable *Acinetobacter* counts as $\log_{10}$ colony forming units (CFU) per gram soil sample from each time interval during decomposition. In addition, FISH was performed using the Acin0659 probe, which targets most *Acinetobacter* spp. The *Acinetobacter* present in the samples were short rod-shaped cells ranging in size from 1.0 to 1.5 by 1.5 to 2.5 μm and were typically found in pairs or in clusters. *Acinetobacter* cells were not present in the day 43 (putrid/dry) sample. If present, the *Acinetobacter* cells were mostly clustered together, which is consistent with growth in a non-mixed environment, such as soil. Results from the daime analysis are shown in Table 1. The abundance of *Acinetobacter* cells decreased from 3.4 to 0% from day 12 (active decomposition) to 43 (putrid/dry). These results are in agreement with the plate count enumeration data.

Acinetobacter spp. Identification and Characterization

BLAST analysis using the 16S rRNA-gene sequence identified the isolates as one of the following *Acinetobacter* spp: *A. baumannii* (97–98% identity), *A. haemolyticus* (98–99% identity), *A. junii* (97–98% identity), *A. johnsonii* (98–99% identity), and *A. gerneri* (99% identity). One isolate, P4, was observed to be more closely related to *A. sp* E10 (99% identity).

Phenotypic description of the identified isolates closely matched those of known genomic species as noted in Tables 3 and 4 [5, 6, 16]. The exception was for isolate P4, which was unique in its biochemical properties. Growth for isolate P4 was noted at 30, 37, and no growth at 41°C. Isolate P4 hydrolyzed the following: Tween 40, Tween 80, Wesson oil, pyruvate, acetate, α-ketobutyric acid, α-ketoglutaric acid, D-L-lactate, propionate, succinate, and proline; however, it was the only isolate unable to use citrate. In addition, isolate P4 was unable to metabolize L-phenylalanine, L-histidine, and L-aspartate. Isolate P4 was observed to be a Gram-negative oxidase negative, catalase positive, coccobacillus. Almost the entire 16S rRNA gene of isolate P4 was sequenced (1394 nt, accession # FJ851148). The gene sequence has a 99% identity with that of *Acinetobacter* sp. E10 (accession # FJ392125) and clusters with *A. baumannii* (Fig. 2). Electron microscopy confirmed that P4 is a Gram-negative rod-shaped cell, ranging in size from 1.0 to 1.5 by 1.5 to 2.5 μm and are typically found in pairs or in clusters (Fig. 3).

An evaluation of antibiotic susceptibility was performed on each isolate. Minimal inhibitory concentration (MIC) for antibiotics tested (μg ml^{-1}) is denoted in Table 2. The isolates from this study displayed multi-antibiotic resistance in addition to that for cefazolin and cephalothin. Isolates identified as *A. baumannii* also conferred antibiotic resistance to ampicilin, aztreonam, cefuroxime cefoxitin, cefpodoxime, and tetracycline (Table 3). One isolate identified as *A. junii* displayed resistance to ertapenem and

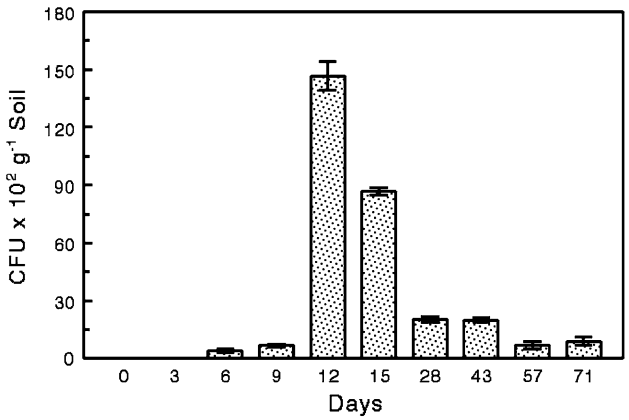

Fig. 1 Bacterial colony counts as determined by the pour plate method for each sample time interval taken during decomposition

Table 1 Relative abundance of *Acinetobacter* in samples based on daime analysis of digital images

Stage of decay (Day)	Abundance (%)	Standard deviation
Active (12)	3.4	5.67
Active (15)	2.51	2.86
Advanced (28)	0.23	0.452
Putrid/dry (43)	0	0

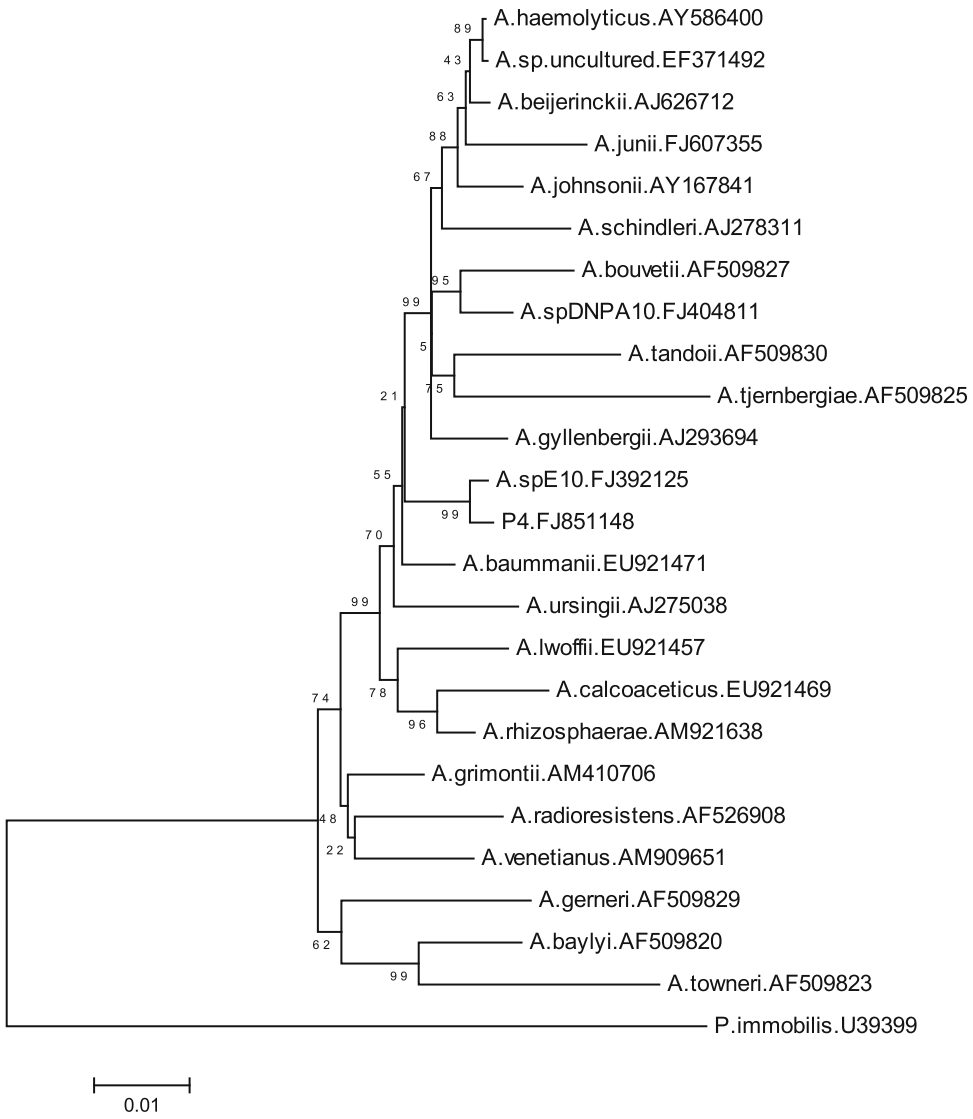

Fig. 2 Phylogenetic and molecular evolutionary analyses of 16S rRNA-gene sequences of *Acinetobacter* species. Analysis was conducted using MEGA version 4 (23). DNA sequences were aligned using the Greengenes program (9). *Acinetobacter* sequences available from the GenBank database (1) were used. *Pseudomonas immobilis* was used as out-group for the rooted tree. Phylogenetically distinct clusters were assigned based on a bootstrap value >90%

isolate P4 displayed resistance to cefoxitin and tetracycline. Isolates identified as *A. haemolyticus* displayed resistance to ampicilin, aztreonam, cefoxitin, cefpdoxime, and tetracycline (Table 4). Whereas, isolates identified as *A. johnsonii* displayed resistance to aztreonam and cefoxitin, and *A. gerneri* displayed resistance to ampicilin, aztreonam, cefoxitin, and cefpodoxime.

Discussion

Ambient temperature is one of the most important environmental factors affecting decomposition, and thus a pivotal parameter affecting both insect and bacterial activities. Precipitation, ambient temperatures, and insect species diversity observed during this study were typical for fall southeastern Louisiana [13].

In the previous study [13], it was observed that the overall bacterial counts for lipid-degrading microbes associated with the soil underneath a decomposing pig increased from day 0 ($5.5 \times 10^3 \pm 0.781$) to their highest between days 9 and 12 ($123.67 \times 10^3 \pm 3.06$ and $117 \times 10^3 \pm 4$, respectively), then decreased and leveled through the remaining time intervals. On the other hand, the overall bacterial counts for protein-degrading microbes slowly decreased from day 0 ($53 \times 10^5 \pm 5.8$) to day 9 ($11.33 \times 10^5 \pm 2.52$), followed by a second major decline at day 28 ($1.933 \times 10^5 \pm 0.153$), and then leveled throughout the remaining decay process. These results indicate that the lipid content of the carcass was contributing more organic carbon to the soil community rather than the protein content, and the lipolytic bacteria were likely responding to an increase in lipid in the soil. Another contribution to the decrease in bacterial numbers may be

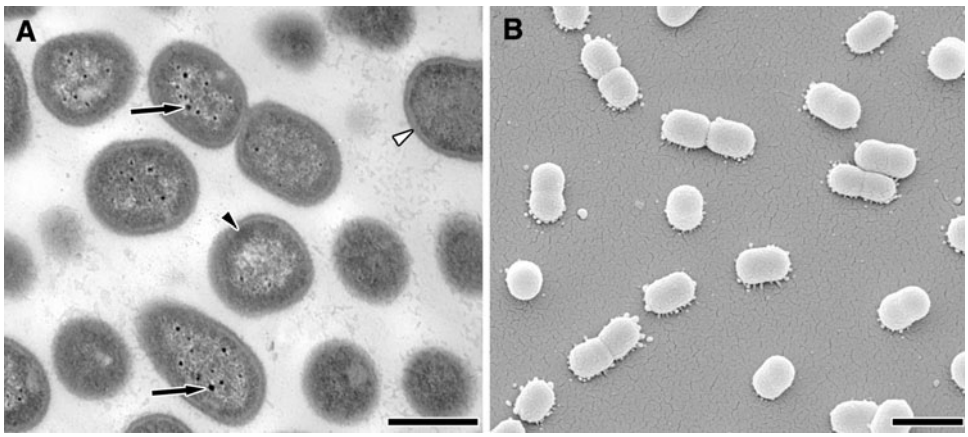

Fig. 3 Electron micrographs of *Acinetobacter* P4. **a** Transmission electron micrograph of bacterial cells illustrating Gram-negative rods. *Line scale* represents a length of 2 μ. **b** Scanning electron micrograph of P4 cells illustrating rod shape of 1.3 μ in length and which usually cluster in pairs. *Line scale* represents a length of 2 μ

Table 2 MIC interpretive standards (μg ml^{-1}) for *Acinetobacter* spp.

	Susceptible	Intermediate	Resistant
Amikacin	≤16	32	64
Ampicilin	≤8	16	≥32
Ampicillin/sulfabactam	≤8/4	16/8	≥32/16
Augmentin	≤8/4	16/8	≥32/16
Aztreonam	≤8	16	≥32
Cefazolin	≤8	16	≥32
Cefepime	≤8	16	≥32
Cephalothin	≤8	16	≥32
Meropenem	≤4	8	≥16
Ertapenem	≤2	4	≥8
Cefuroxime	≤8	16	≥32
Gentamicin	≤4	8	≥16
Ciprofloxacin	≤1	2	≥4
Levofloxacin	≤2	4	≥8
Piperacillin/Tazobactam	≤16/4	32/4–64/4	≥128/4
Cefoxitin	≤8	16	≥32
Trimethoprim/ sulfamethoxazole	≤2/38		≥4/76
Cefpodoxime	≤2	4	≥8
Ceftazidime	≤8	16	≥32
Tobramicin	≤4	8	≥16
Tigecycline	≤2	4	≥8
Ticarcillin/clavulanic acid	≤16/2	32/2–64/2	≥128/2
Ceftriaxone	≤8	16/32	≥64
Tetracycline	≤4	8	≥16
Minocycline	≤4	8	≥16

because of the decrease in average daily temperature after day 28.

The response of the soil *Acinetobacter* bacterial population because of the presence of the decomposing pig was similar to that observed for the lipolytic bacterial population [13]. The *Acinetobacter* bacterial counts were initially the lowest before exposure (undetectable) and increased to their highest during active decay ($146.7 \times 10^2 \pm 7.37$), then decreased and leveled during advanced decay through putrid/dry. FISH analysis was used in conjunction with plate counting. FISH analysis supported the observations obtained from the *Acinetobacter* bacterial counts. The abundance of *Acinetobacter* cells decreased from 3.4 to 0% from day 12 (active decomposition) to 43 (putrid/dry).

Members of the genus *Acinetobacter* are ubiquitously distributed in nature. Although the majority of strains of described species have been isolated from clinical sources, many of the described species also include environmental strains. Overall, the ecology of species belonging to the genus *Acinetobacter* is not well elucidated. The genus *Acinetobacter* was observed to be the more predominant group isolated from soil associated with the decomposition of a pig [13]. In addition, the abundance of *Acinetobacter* correlated with the abundance of lipolytic bacteria during the same time periods of decomposition. Several *Acinetobacter* species were described including the isolation and characterization of a unique isolate. The results observed in this study for the bacterial enumeration, identification of bacterial isolates, and qPCR indicate that lipid biodegradation is more prevalent than protein biodegradation by soil bacteria involved in the degradation of swine carrion. *Acinetobacter* resembles saprophytic pseudomonads in their ability to use a wide variety of organic compounds as carbon and energy sources including many aromatic compounds. Aromatic compounds are available through normal breakdown of dead plant and animal tissues, and it is not surprising that these microorganisms would be associated with their degradation process.

Phenotypic characterization and 16S rRNA-gene sequences analysis were used to identify the 19 *Acinetobacter* isolates. All isolates were observed to be oxidase negative, catalase positive, and Gram-negative rod-shaped cells ranging in size from 1.0 to 1.5 by 1.5 to 2.5 μm and were typically found in pairs or in clusters and did not display the presence of flagella. Phenotypic characteristics

Table 3 Phenotypic characteristics and antibiotic susceptibility of P-4, *Acinetobacter baumannii*, and *A. junii* isolates

	A. baumannii						*A. junii*			New
	G[a,b]	L-3	L-13	L-15	L-17	P-1	G[a,b]	P-5	P-6	P-4
Growth at 41/37°C	+/+	+/+	+/+	+/+	+/+	+/+	90/+	+/+	+/+	±
Gelatin hydrolysis	−	−	−	−	−	−	−	−	−	−
Hemolysis of Sheep blood	−	−	−	−	−	−	−	−	−	−
Acid from glucose	+	+	+	+	+	+	−	−	−	−
Use of:										
L-Phenylalanine	+	+	+	+	+	+	−	−	−	−
L-Histidine	+	+	+	+	+	+	+	+	+	−
L-Aspartate	+	+	+	+	+	+	40	−	−	−
L-Leucine	38	+	+	+	+	+	11	−	−	−
β-Alanine	+	+	+	+	+	+	−	−	−	−
Phenylacetate	+	+	+	+	+	+	−	−	−	−
Citrate	+	+	+	+	+	+	82	+	+	−
Amikacin		≤0.5	ND	ND	ND	ND		ND	ND	ND
Ampicilin		8	**>32**	**32**	**32**	**16**		≤4	≤4	≤4
Ampicillin/sulfabactam		≤4	16	16	16	16		8	≤4	≤4
Augmentin		4	ND	ND	ND	ND		ND	ND	ND
Aztreonam		**>32**	**32**	**32**	8	16		16	≤4	≤4
Cefazolin		**>32**	**>32**	**>32**	**>32**	**>32**		16	**>32**	**>32**
Cefepime		2	ND	ND	ND	ND		ND	ND	ND
Cephalothin		**>16**	**>16**	**>16**	**>16**	**>16**		**>16**	**>16**	**>16**
Meropenem		0.25	ND	ND	ND	ND		ND	ND	ND
Ertapenem		4	≤2	≤2	≤2	4		4	**8**	4
Cefuroxime		16	**32**	≤4	≤4	≤4		≤4	≤4	≤4
Gentamicin		≤2	≤2	≤2	≤2	≤2		≤2	4	≤2
Ciprofloxacin		≤0.5	≤0.5	≤0.5	≤0.5	≤0.5		≤0.5	≤0.5	≤0.5
Levofloxacin		0.06	ND	ND	ND	ND		ND	ND	ND
Piperacillin/Tazobactam		1	ND	ND	ND	ND		ND	ND	ND
Cefoxitin		**>32**	**>32**	8	16	8		≤4	≤4	**32**
Trimethoprim/sulfamethoxazole		≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5		≤0.5/9.5	≤0.5/9.5	≤0.5/9.5
Cefpodoxime		**8**	4	≤2	≤2	4		≤2	≤2	≤2
Ceftazidime		8	4	≤1	≤1	2		ND	ND	≤1
Tobramicin		≤4	≤4	≤4	≤4	≤4		≤4	≤4	8
Tigecycline		0.25	ND	ND	ND	ND		ND	ND	ND
Ticarcillin/clavulanic acid		≤16	≤16	≤16	≤16	≤16		≤16	≤16	≤16
Ceftriaxone		8	4	≤1	≤1	2		≤1	≤1	4
Tetracycline		2	**>16**	**>16**	**>16**	**>16**		≤0.5	≤0.5	**>16**
Minocycline		≤0.5	ND	ND	ND	ND		ND	ND	ND

G corresponds to genomospecies. Numbers correspond to percent of strains exhibiting phenotypic characteristic. Boldfaced MIC denotes resistance

[a] Phenotypic description of genomic species [6]

[b] Phenotypic description of genomic species [16]

for each isolate were obtained and compared with known genomic species [5, 6, 16]. The phenotypic results for 18 of the isolates tested closely matched that of 4 known genomic species [5] and 1 type strain [6]. These phenotypic results were also in agreement with the 16S rRNA-gene analysis in identifying these isolates. According to the description assigned to each genomic species [5], Genospecies 2 was named *A. baumannii*, Genospecies 4 was named *A. haemolyticus*, Genospecies 5 was named *A. junii*, and Genospecies 7 was named *A. johnsonii*. The relationship among

Table 4 Phenotypic characteristics and antibiotic susceptibility of *Acinetobacter haemolyticus*, *A. johnsonii*, and *A. gerneri* isolates

	A. haemolyticus							*A. johnsonnii*					*A. gerneri*	
	G[a,b]	L-1	L-4	L-10	L-11	L-12	P-2	G[a,b]	L-2	L-5	L-6	L-9	T[c]	P-7
Growth at 41/37°C	∓	∓	∓	∓	∓	∓	∓	−/−	−/−	−/−	−/−	−/−	+/+	+/+
Gelatin hydrolysis	96	+	+	+	+	+	+	−	−	−	−	−	−	−
Hemolysis of sheep blood	+	+	+	+	+	+	+	−	−	−	−	−	ND	−
Acid from glucose	52	−	−	+	−	+	−	−	−	−	−	−	+	+
Use of:														
L-Phenylalanine	−	−	−	−	−	−	−	−	−	−	−	−	+	+
L-Histidine	96	+	+	+	+	+	+	−	−	−	−	−	−	−
L-Aspartate	64	+	+	+	+	+	+	61	+	+	+	+	−	−
L-Leucine	96	+	+	+	+	+	+	−	−	−	−	−	−	−
β-Alanine	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Phenylacetate	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Citrate	91	+	+	+	+	+	+	+	+	+	+	+	+	+
Amikacin		2	≤0.5	2	1	ND	ND		2	1	≤0.5	≤0.5		ND
Ampicilin		**32**	16	1	1	16	**32**		16	4	4	2		**>32**
Ampicillin/sulfabactam		≤4	≤4	≤4	≤4	16	16		≤4	≤4	≤4	≤4		≤4
Augmentin		8	4	0.5	0.5	ND	ND		2	1	1	1		ND
Aztreonam		**32**	**>32**	**32**	**32**	16	**>32**		**32**	**32**	16	≤4		**>32**
Cefazolin		**>32**	**>32**	**>32**	**>32**	**>32**	**>32**		**>32**	**>32**	4	≤4		**>32**
Cefepime		2	2	1	≤0.5	ND	ND		2	≤0.5	≤0.5	1		ND
Cephalothin		**>16**	**>16**	**>16**	**>16**	**>16**	**>16**		**>16**	**>16**	≤2	≤2		**>16**
Meropenem		0.25	0.25	0.25	0.12	ND	ND		0.25	≤0.06	0.25	0.25		ND
Ertapenem		4	≤2	4	4	4	4		4	≤2	≤2	≤2		≤2
Cefuroxime		16	16	≤4	≤4	≤4	16		16	16	≤4	≤4		16
Gentamicin		≤2	≤2	≤2	≤2	≤2	≤2		≤2	≤2	≤2	≤2		≤2
Ciprofloxacin		≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5		≤0.5	≤0.5	≤0.5	≤0.5		≤0.5
Levofloxacin		0.06	0.06	0.03	0.03	ND	ND		0.03	0.12	0.03	0.03		ND
Piperacillin/Tazobactam		≤0.06	1	0.25	0.25	ND	ND		≤0.06	0.5	0.25	≤0.06		ND
Cefoxitin		**>32**	**>32**	**32**	**32**	**32**	**>32**		**32**	**>32**	≤4	≤4		**>32**
Trimethoprim/sulfamethoxazole		≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	1/19		≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5		1/19
Cefpodoxime		**16**	**8**	≤2	≤2	≤2	**16**		4	≤2	≤2	≤2		**8**
Ceftazidime		4	8	2	4	2	8		4	4	≤1	≤1		ND
Tobramicin		≤4	≤4	≤4	≤4	≤4	≤4		≤4	≤4	≤4	≤4		≤4
Tigecycline		0.25	0.25	0.25	0.12	ND	ND		0.25	2	0.5	0.03		ND
Ticarcillin/clavulanic acid		≤16	≤16	≤16	≤16	≤16	32		≤16	≤16	≤16	≤16		≤16

Table 4 continued

	A. haemolyticus						*A. johnsonnii*					*A. gerneri*	
	G[a,b]	L-1	L-4	L-10	L-11	L-12	P-2	G[a,b]	L-2	L-5	L-6	L-9	T[c]	P-7
Ceftriaxone		32	8	4	4	16	8		16	4	≤0.06	2		4
Tetracycline		0.5	4	≤0.5	≤0.5	**>16**	**>16**		0.5	2	≤0.5	≤0.5		4
Minocycline		≤0.5	≤0.5	≤0.5	≤0.5	ND	ND		≤0.5	2	≤0.5	≤0.5		ND

G corresponds to genomospecies, *T* corresponds to type strain 9A01. Numbers correspond to percent of strains exhibiting phenotypic characteristic. Bold-faced MIC denotes resistance

[a] Phenotypic description of genomic species [5]

[b] Phenotypic description of genomic species [16]

[c] Phenotypic description of type species [6]

these groups based on 16S rRNA-gene sequence analysis revealed that genomic species 4 and 7 clustered together and genomic species 2 and 5 clustered together [15].

In addition to the 18 identified isolates, one isolate, P4, was observed to be unique from the others. Phylogenetic and molecular evolutionary analyses showed that isolate P4 was more closely related to the phenol-degrading *Acinetobacter* sp. E10 [21] and clustered with *A. baumanniii*. The P4 isolate shared similar phenotypes with those of genomic species 8 and 9 [5, 16]. However, isolate P4's phenotype was more closely related to *Acinetobacter towneri* and *Acinetobacter tandonii* [6]. The only dissimilar feature between P4 and *A. towneri* was P4 did not grow at 41°C; however, P4 had three features that distinguished it from that of *A. tandoni*: P4 is unable to metabolize L-phenylalanine, L-histidine, and L-aspartate.

Acinetobacter spp. has gained increased recognition in recent years as pathogens that have the potential to cause severe nosocomial infections in critically ill patients [2, 24]. Strains from genomic species 2 (*Acinetobacter baumannii*), 3, and 13 sensu Tjernberg and Ursing (13TU) [3] are frequently isolated from clinical specimens and are often associated with nosocomial outbreaks [2, 24]; they belong, together with genomic species 1 (*Acinetobacter calcoaceticus*), to the so-called *A. calcoaceticus–A. baumannii* complex [10, 11]. *A. calcoaceticus* strains are seldom isolated from patients or associated with infections [2]. Other *Acinetobacter* strains are also isolated infrequently from patients, although both *Acinetobacter junii* and *Acinetobacter johnsonii* have been reported to be involved in cases of septicemia [4, 22]. *Acinetobacter baumannii* has emerged as one of the most troublesome pathogens for health care institutions globally. Its clinical significance, especially during the last 15 years, has been propelled by its remarkable ability to up-regulate or acquire resistance determinants, making it one of the organisms threatening the current antibiotic era. *A. baumannii* strains resistant to all known antibiotics have now been reported, signifying a sentinel event that should be acted on promptly by the international health care community [19]. Interestingly, these *Acinetobacter* spp. were also isolated in our study and displayed multi-antibiotic resistance.

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