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Isolation and Identification of Hyper-Ammonia Producing Bacteria from Swine Manure Storage Pits

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Abstract. Storage of swine manure is associated with the microbiological production of a variety of odorous compounds, including ammonia, organic acids, and alcohols, phenolics, and sulfides. Until recently, little was known about the microorganisms responsible for their production. Results from our laboratory have demonstrated that the predominant microbial populations of stored swine manure are anaerobic, low (G + C), Gram-positive bacteria. However, studies on pure cultures isolated from manure have found few microorganisms that produce appreciable ammonia concentrations. Therefore, selective and enrichment techniques were employed to isolate ammonia-producing bacteria from stored swine manure by using media containing peptone and amino acids as carbon and energy sources. We now report on the isolation of 40 bacterial cultures, a number of which are capable of producing at least 40 mM ammonia in peptone-amino acid medium, concentrations similar to those produced by hyper-ammonia producing (HAP) bacteria isolated from the rumen of cattle. The manure HAP isolates are phylogenetically distinct from the ruminal isolates and may prove to be intimately involved in the production of ammonia during storage of swine manure.

Modern concentrated animal confinement operations have resulted in the concentration of greater numbers of domesticated animals into fewer operations. This concentration has created the necessity to store larger amounts of manure for longer periods of time prior to their being applied as fertilizer. Lagoon treatment and under-barn pit storage are among the more popular methods used to handle liquid manure, as produced by large-scale swine facilities. However, storage of swine manure is associated with the production of a variety of odorous chemicals including ammonia, organic acids and alcohols, phenolics and sulfides. Production of ammonia, in particular, within a confined facility from urine and nitrogenous compounds found in stored manure can pose health problems to both the animals and human workers. Although production of these chemicals is the result of microbiological activity, until recently little was known about the types of microorganisms responsible for their production. Results from our laboratory with both culture-independent, direct 16S rDNA sequencing from total DNA and isolation of pure bacterial cultures have demonstrated that the predominant microflora of stored manure from under-barn pits are anaerobic, low (G + C), Gram-positive bacteria [6, 15]. However, biochemical evaluation of the pure cultures isolated in our laboratory indicated that relatively few of these isolates produce ammonia as an end-product of fermentation. These results suggest that the microorganisms responsible for ammonia production might be present in lower concentrations and thus not easily isolated by non-selective culture procedures.

Previous work with the ruminal ecosystem has demonstrated the presence of bacteria capable of producing large amounts of ammonia (>40 mM) and termed hyper-ammonia producing (HAP) bacteria [1, 4, 12, 14]. These bacteria were present in lower concentrations in the rumen, but could be isolated by using enrichment and selection media. Although present in lower numbers than

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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more commonly isolated species, their ammonia-producing activities were of sufficient magnitude for them to be quantitatively important in the production of ammonia observed in the rumen. In order to investigate the possibility of analogous HAP bacteria present in stored swine manure, selection and enrichment experiments were carried out to isolate peptide and amino acid-fermenting bacteria from manure samples. We now present the first report of ammonia-producing bacteria from stored swine manure, including isolates capable of producing concentrations of ammonia similar to those of HAP bacteria from the rumen.

Materials and Methods

Swine manure pit sampling. A swine facility near Peoria, IL, was used as the source of the manure pit sample. Samples (50–100 mL) from under-barn manure storage pits were collected by using a Tank Sampler (NASCO, Ft. Atkinson, WI) and kept on ice until returning to the laboratory.

Enrichment and isolation of ammonia-producing bacteria. Media used for isolation of bacteria were based on routine growth medium [RGM, 8]. Selection/Enrichment (SE) medium contained buffer, salts, yeast extract (0.3%), and 1% Bacto-Tryptone (Becton, Dickinson and Co., Sparks, MD)/1% Casamino Acids (vitamin-free, salt-free casein hydrolysate, ICN Biomedicals, Inc., Aurora, OH) as carbon and energy sources and was prepared anaerobically with the technique of Hungate [9] as modified by Bryant [2]. SE-agar plates were prepared in an anaerobic chamber (Coy Laboratory, Ann Arbor, MI) in an atmosphere of 96% carbon dioxide-4% hydrogen. Serial dilutions of manure samples were prepared with the SE medium in the chamber. Aliquots (0.1 mL) of dilutions were plated directly onto SE-agar plates and allowed to incubate at room temperature (approx. 24°C), to simulate the pit environment, or at 37°C, until colonies were observed. The remaining dilutions were allowed to grow at either 24°C or 37°C for enrichment of peptide/amino acid fermenting bacteria. The enrichment cultures were then streaked out on SE-agar plates and incubated as above. All isolates were streaked several times for purity on SE-agar medium. Isolates were also tested on SE with 1% casein and RGM with and without 0.4% glucose as a carbon source. Ruminal HAP strains *Clostridium aminophilum* F, *Peptostreptococcus anaerobius* C, and *Clostridium sticklandii* SR were obtained from James Russell (Cornell University). Strains isolated at room temperature were designated with "RT," and those at 37°C with "37."

Identification of isolates by 16S rDNA sequence analyses. Aliquots of overnight cultures were centrifuged (23°C, 14,500 *g*, 2 min), the bacterial cell pellet was washed with Tris-EDTA (TE, pH 8.0) buffer, and the cells were suspended in TE. The suspensions were placed in a boiling water bath for 10 min to lyse the cells. 16S rDNA genes were isolated by PCR amplification from the boiled cells using the forward primer 5′-GAGAGTTTGAT (C/T)CTGGCTCAG-3′ (*E. coli* 8–28) and universal reverse primer 5′-GAAGGAGGTG(A/T)TCCA(A/G)CCGCA-3′ (*E. coli* 1522–1540) [13]. Conditions for PCR were 94°C, 45 s; 55°C, 30 s; 72°C, 2 min; 25 cycles. A final extension step of 72°C, 10 min completed the reactions. An aliquot of PCR products was analyzed by gel electrophoresis to confirm the presence of the correct size product. The PCR product was cloned into the plasmid pCR2.1 (Invitrogen, Carlsbad, CA) with the TA Cloning Kit according to the manufacturer's instruction. Randomly selected clones were analyzed for correct size inserts in the plasmids by PCR by using the m13 forward and reverse sequencing primers. Plasmid DNA was purified with the QIAGEN Wizard kits (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The 16S DNA inserts were sequenced at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois Biotechnology Center, Urbana, IL) by using the m13 forward and reverse primers and internal primers. Similarity analyses were carried out with the Advanced Blast Program of GenBank (NCBI, NIH, Washington, DC). Phylogenetic dendrograms were prepared by using the MegAlign programs of the Lasergene software (DNASTAR, Inc., Madison, WI). An Operational Taxonomic Unit (OTU) indicates those 16S rDNA sequences that have 99% or greater sequence similarity.

Bacteria growth and analyses. All bacterial isolates were grown at 37°C for 24 h for determination of growth by measuring the optical density at 660 nm by using a Spectronic 21 spectrophotometer (Milton Roy, Rochester, NY). For measurement of ammonia production, aliquots of cultures were removed, cells separated by centrifugation (23°C, 14,500 *g*, 2 min), and the supernatant fluid was used for assays. Ammonia concentrations were determined with the colorimetric method of Chaney and Marbach [3] as modified by Cotta and Russell [5]. Values reported are averages of at least duplicate experiments.

Nucleotide sequence accession numbers. The 16S rDNA gene sequences have been deposited in the GenBank database under accession numbers AY167932 through AY167971.

Results

Isolation of amino acid-fermenting bacteria. Swine manure pit samples were serially diluted into SE medium as described in Materials and Methods and either plated directly onto SE agar plates or allowed to incubate to enrich for bacteria capable of growth on peptides and/or amino acids. Both methods resulted in growth in dilutions as high as 10^{-8}. A variety of colony types was noted both on direct isolation plating and by streaking of enriched cultures, and different colony types were picked for further study. Following repeated streaking for purity, 40 isolated bacterial strains were recovered capable of growth on the SE medium. While strains indicated by RT were isolated at room temperature, all strains grew well at 37°C and were routinely grown at this temperature.

Growth and ammonia production by isolated strains. Previous examinations in our laboratory of predominant isolated bacteria from stored swine manure demonstrated that almost all were saccharolytic and produced only low amounts of ammonia [6]. Therefore, we were interested in determining whether the isolates selected on SE medium could utilize glucose as a carbon and energy source. The 40 purified strains were inoculated into RGM medium containing 0.2% Trypticase with and without glucose (0.4%), and growth and ammonia production were monitored. The ruminal HAP strains were also tested as a comparison. The results are shown in Table 1. Twenty-four of the 40 strains, or 60%, demon-

strated improved growth when grown on RGM medium with 0.4% glucose versus RGM with no glucose. The ruminal HAP strains did not use glucose for growth. While inclusion of glucose in the medium did not affect production of ammonia by some isolates, others were profoundly affected. Strains such as RT-2D, RT-4B, RT-5A, and RT-13B demonstrated increased growth on glucose accompanied by a marked decrease in ammonia production (Table 1), suggesting a shift in biochemical pathways during growth on glucose.

When the swine manure isolates and ruminal HAP bacteria were grown on medium with different amino acid sources, growth and ammonia production varied markedly. As previously described [1, 4, 14], the ruminal HAP strains grew well on tryptone, casamino acids (CAA), and a mixture of both and produced high concentrations of ammonia (Table 2). The swine manure isolates were then ranked by production of ammonia following growth on the substrates, and the results are shown in Table 2. Fourteen of the forty strains produced ammonia concentrations comparable to those of the ruminal HAP isolates (40 mM ammonia or greater) from 1% tryptone/1% CAA medium. The remaining 27 strains produced between 11 mM and 37 mM ammonia on the same medium.

Production of ammonia by the isolates varied depending on the source of amino acids. The greatest ammonia production occurred with the combination of tryptone and casamino acids (Table 2). For the majority of the strains, ammonia production appeared to be additive for the combined tryptone/CAA medium. However, a number of strains (6 out of 40) did not produce ammonia from growth on tryptone, whereas all of the strains (including the ruminal HAP strains) were capable of producing ammonia from casamino acids. Interestingly, 19 of the 40 strains grew better and produced more ammonia from CAA than tryptone. In addition, 11 of 40 strains grew and produced ammonia from casein, demonstrating the ability to grow on intact protein.

Phylogenetic analyses of swine manure isolates. The 16S rDNA gene from the bacterial isolates were subjected to DNA sequencing and phylogenetic analyses. The isolates contain both Gram-positive (Fig. 1) and Gram-negative (Fig. 2) bacteria. Sequence similarity searches indicated that the swine manure isolates were phylogenetically distinct from other HAP bacteria. The Gram-positive isolates were primarily low (G + C) bacteria. Several, including strains 37-2 and 37-5, may represent new genera. Strains RT-8A, RT-13B, RT-18B, RT-10B, RT-23A, and 37-1 formed a cluster most closely related to *Peptostreptococcus anaerobius* and were among the most proficient hyperammonia

Table 1. Growth and ammonia production by swine manure isolates and ruminal HAP bacteria on routine growth medium (RGM) with and without 0.4% glucose

	RGM − 0.4% Glucose		RGM + 0.4% Glucose	
	OD_{660}[a]	NH_3[b]	OD_{660}	NH_3
Ruminal strains:				
P. anaerobius C	0.23	20.1	0.23	19.3
C. sticklandii SR	0.47	32.7	0.62	36.6
C. aminophilum F	0.12	16.0	0.29	14.9
Swine manure strains:				
RT-1A	0.07	24.2	0.18	3.8
RT-2A	0.16	0.0	1.1	1.0
RT-2B	0.10	11.3	0.53	9.9
RT-2C	0.09	11.0	0.54	9.4
RT-2D	0.24	12.2	1.1	6.8
RT-3A	0.20	6.8	0.62	8.0
RT-3B	0.10	5.9	0.56	4.9
RT-3C	0.10	2.2	0.59	2.5
RT-4A	0.19	4.2	0.78	6.8
RT-4B	0.28	28.2	0.80	12.7
RT-4C	0.20	9.1	0.54	7.4
RT-4D	0.13	2.7	0.62	2.8
RT-5A	0.24	15.3	1.0	0.0
RT-5C	0.09	3.3	0.08	0.0
RT-5D	0.15	2.5	0.95	4.2
RT-6A	0.24	6.3	0.85	6.1
RT-6B	0.30	15.3	0.84	7.0
RT-8A	0.18	2.0	0.37	0.0
RT-8B	0.30	14.1	0.30	20.0
RT-10A	0.20	3.7	0.68	2.0
RT-10B	0.27	5.5	0.52	0.0
RT-13A	0.12	2.4	0.60	0.0
RT-13B	0.23	8.0	0.78	0.0
RT-18A	0.11	0.0	0.50	0.0
RT-18B	0.10	0.0	0.45	0.0
RT-19A	0.13	6.8	0.29	2.3
RT-19B	0.17	1.7	0.81	4.0
RT-20	0.19	3.7	0.98	1.7
RT-21	0.18	2.4	0.88	1.8
RT-22	0.13	0.0	0.95	2.0
RT-23A	0.26	9.2	0.43	1.3
RT-23B	0.19	0.0	0.41	1.3
37-1	0.26	11.4	0.54	7.9
37-2	0.02	3.6	0.03	0.0
37-3	0.22	2.9	0.61	4.4
37-4	0.14	5.6	1.1	0.0
37-5	0.14	5.8	0.10	5.5
37-6	0.12	7.2	0.12	4.9
37-8	0.21	13.7	0.39	3.1
37-11	0.22	13.2	0.51	3.1

[a] Duplicate cultures grown for 24 h at 37°C. OD_{660} = optical density at 660 nm.

[b] Ammonia concentration in mM. 0.0 indicates no production above medium background.

Table 2. Growth and ammonia production by swine manure isolates and ruminal HAP bacteria on various amino acid sources

	1%Tryptone/1% CAA[a]		1% Tryptone		1% CAA		1% Casein	
	OD_{660}[b]	NH_3[c]	OD_{660}	NH_3	OD_{660}	NH_3	OD_{660}	NH_3
Ruminal strains:								
P. anaerobius C	0.97	53.7	0.56	30.5	0.80	28.9	NG[d]	
C. sticklandii SR	0.56	64.9	0.75	29.5	0.47	29.4	NG	
C. aminophilum F	0.38	43.3	0.24	16.8	0.40	23.6	NG	
Swine manure strains:								
RT-5A	0.68	69.8	0.26	54.3	0.28	20.2	0.87	8.1
RT-4B	0.62	68.7	0.50	49.1	0.43	28.4	0.30	27.6
RT-6B	1.1	63.7	0.70	32.5	0.80	33.3	1.2	6.1
RT-1A	0.60	59.5	0.39	25.9	0.22	25.9	1.5	18.3
RT-10B	0.85	54.9	0.41	39.9	0.55	39.9	0.17	22.1
RT-23A	0.61	53.7	0.28	28.2	0.52	29.8	NG	
RT-19A	0.74	51.5	0.27	24.8	0.38	19.9	NG	
RT-8B	0.84	50.1	0.58	50.1	0.49	27.5	0.90	47.6
RT-13B	0.85	50.4	0.49	24.1	0.52	32.9	NG	
37-8	0.80	45.2	0.38	18.8	0.52	17.4	0.30	6.8
37-11	0.98	45.1	0.44	18.1	0.58	15.3	NG	
37-1	0.75	45.1	0.48	24.3	0.64	42.5	0.30	11.1
RT-8A	0.80	43.5	0.40	20.3	0.27	18.5	NG	
36-6	0.56	39.9	0.22	7.1	0.43	13.8	0.15	5.3
RT-4C	0.62	36.7	0.27	15.3	0.45	23.2	NG	
37-5	0.60	35.1	0.29	17.6	0.43	26.8	NG	
RT-6A	0.56	35.1	0.35	26.8	0.40	21.8	NG	
RT-10A	0.34	30.3	0.30	11.9	0.24	10.5	NG	
RT-20	0.35	25.7	0.24	9.5	0.27	12.4	NG	
RT-22	0.24	24.5	0.18	11.3	0.17	14.3	NG	
RT-2D	0.48	22.8	0.33	0.0	0.40	18.2	NG	
37-4	0.24	22.7	0.24	10.4	0.18	15.9	0.16	3.1
RT-21	0.21	22.1	0.18	8.1	0.14	7.7	NG	
RT-4A	0.33	21.5	0.24	7.3	0.29	9.1	NG	
37-3	0.42	21.1	0.30	10.3	0.23	10.9	0.2	2.1
RT-5C	0.31	20.3	0.18	12.9	0.19	7.8	NG	
RT-18B	0.22	19.6	0.13	9.2	0.07	2.4	NG	
RT-19B	0.33	18.9	0.21	8.9	0.23	7.8	NG	
RT-18A	0.22	18.5	0.14	8.3	0.06	3.5	NG	
RT-3A	0.30	18.4	0.25	0.0	0.22	13.8	NG	
RT-13A	0.90	16.3	0.17	11.1	0.18	5.9	NG	
RT-4D	0.24	14.2	0.17	8.2	0.21	9.9	NG	
RT-5D	0.35	13.8	0.20	7.1	0.25	3.2	NG	
RT-3B	0.21	13.7	0.14	0.0	0.14	9.1	NG	
RT-23B	0.26	13.2	0.22	7.2	0.16	5.2	NG	
RT-3C	0.18	12.9	0.13	3.5	0.12	5.3	NG	
RT-2C	0.20	12.8	0.14	0.0	0.16	13.8	NG	
37-2	0.27	11.9	0.03	6.6	0.14	9.3	NG	
RT-2B	0.19	11.8	0.14	0.0	0.15	9.1	NG	
RT-2A	0.43	11.1	0.26	0.0	0.34	9.7	NG	

[a] CAA = Casamino acids.
[b] Duplicate cultures grown for 24 h at 37°C. OD_{660} = optical density at 660 nm.
[c] Ammonia concentration in mM. 0.0 indicates no production above medium background.
[d] NG = No growth.

producers. Interestingly, strain 18B produced much lower amounts of ammonia when compared with the other strains (Table 2). A number of strains were found to cluster with *Streptococcus*, *Enterococcus*, and *Staphylococcus*. The remaining strains were primarily related to *Clostridium* species. Most of the Gram-negative strains were found to be most closely related to *Proteus* species, including nine strains that had 99% or greater 16S rDNA sequence similarity (RT-OTU1, Fig. 2). Two other HAP strains, 37-8 and

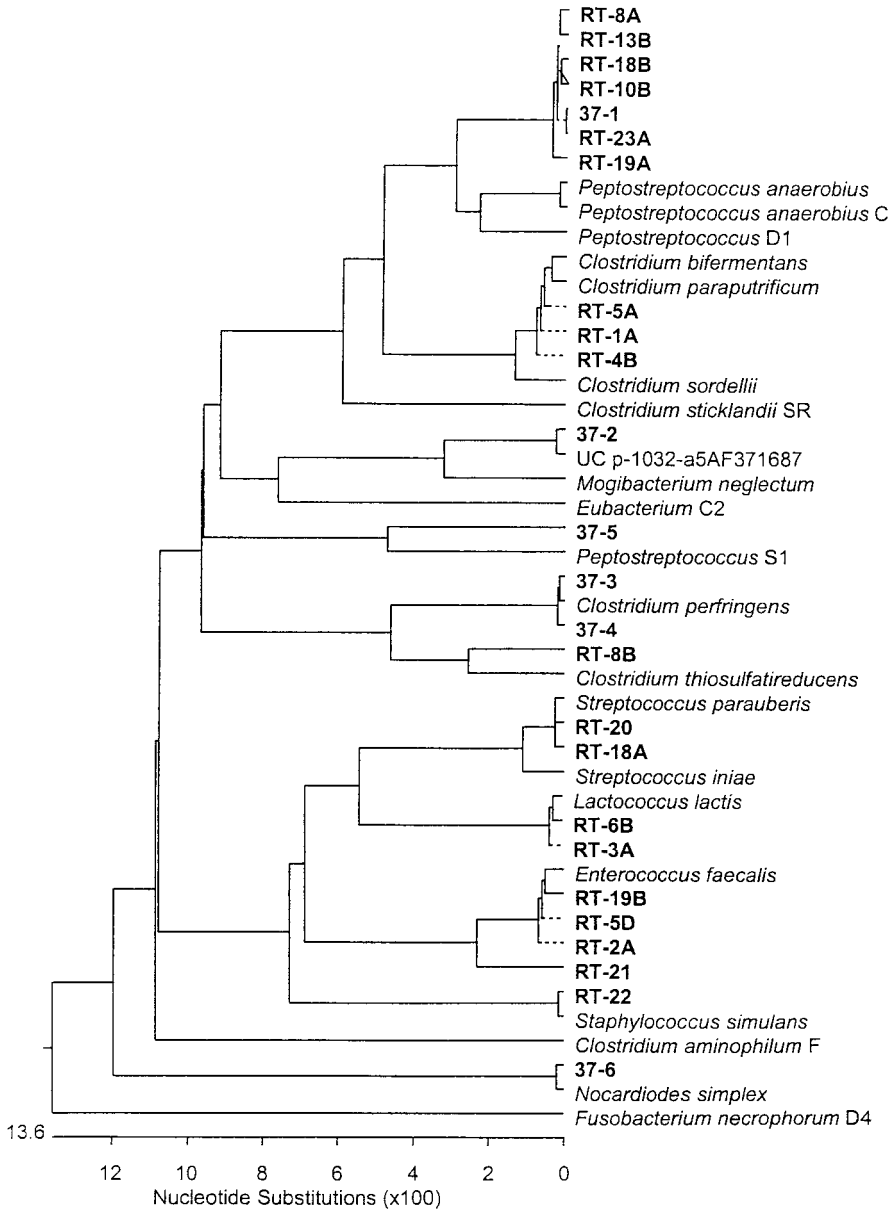

Fig. 1. Phylogenetic analysis of 16S rDNA gene sequences from Gram-positive swine manure isolates. Bacterial names indicate sequences obtained from GenBank. Bold letters indicate sequences from isolates from this study.

37-11, also fell into the Gram-negative classification. Strain 37-8 was most similar to *Pseudomonas migulae*, while strain 37-1 was most similar to *Klebsiella pneumoniae* with about 97% 16S rDNA sequence similarity.

Discussion

The results presented in this report confirm the presence of an ammonia-producing metabolic group of bacteria. Although lower in total numbers, activity of such magnitude undoubtedly contributes significantly to the production of ammonia in stored manure. On the basis of the plating and enrichment data, these bacteria are present at concentrations of at least 10^8/mL.

Previous studies from our laboratory with both culture-independent and pure culture isolations showed that the primary bacterial compositions of stored manure and swine feces are low (G + C), Gram-positive bacteria [6, 10, 15]. However, studies on the predominant pure cultures from the manure found very few bacterial strains that produced appreciable amounts of ammonia (M.A. Cotta, R.L. Zeltwanger, and T.R. Whitehead, submitted for publication). These results prompted us to determine

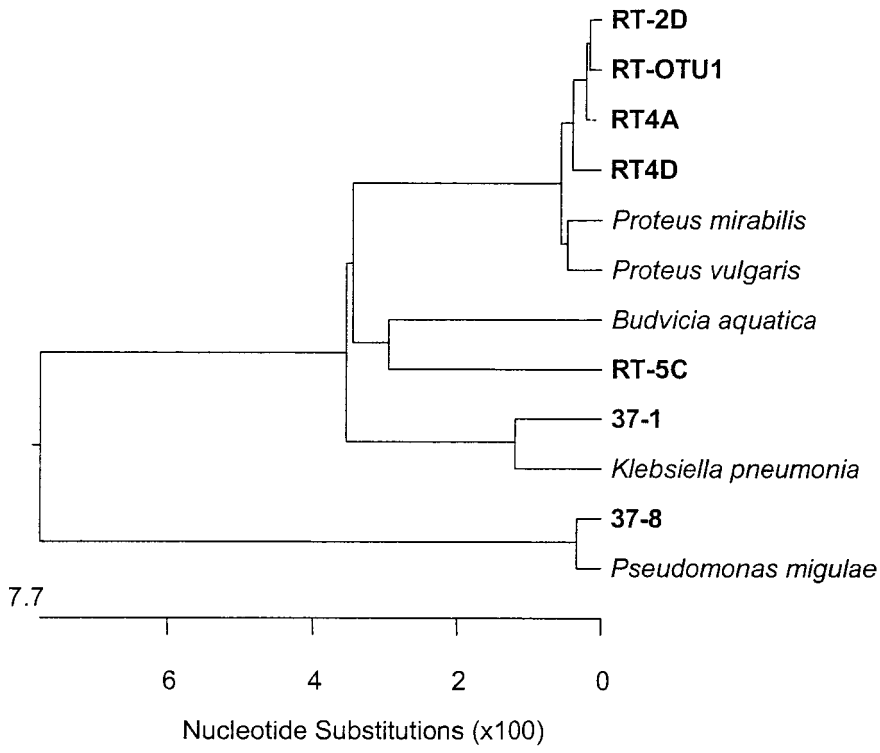

Fig. 2. Phylogenetic analysis of 16S rDNA gene sequences from Gram-negative swine manure isolates. Bacterial names indicate sequences obtained from GenBank. Bold letters indicate sequences from isolates from this study. OTU1 (>99% 16S rDNA sequence identity) contains strain RT-2B, RT-2C, RT-3B, RT-3C, RT-4C, RT-6A, RT-10A, RT-10B, and RT-13A.

whether protein-, peptide-, and/or amino acid-fermenting bacteria might be present in the manure at lower concentrations than those used for previous isolations. Such bacteria, capable of producing large amounts of ammonia and termed hyperammonia-producing (HAP) bacteria, have been isolated from the rumen of cattle in the United States [4, 14] and elsewhere around the world [1, 7, 11]. These bacteria are also found in lower concentrations in the rumen.

A large number of bacterial isolates from stored swine manure were recovered that could grow on a variety of amino acid sources, such as tryptone, casamino acids, and, in some cases, casein. These isolates varied in their ability to produce ammonia from the different amino acid sources, but 14 produced concentrations of ammonia comparable to those of HAP bacteria from the rumen. On the basis of direct plating and enrichment cultures, these bacteria are present at concentrations of at least 10^8/mL, or approximately 1%–0.1% of the culturable bacterial population. Twelve of the 14 HAP strains were found to be Gram-positive, low (G + C) bacteria, while the other two strains were Gram-negative and most closely related to *Klebsiella* and *Pseudomonas*. These results correlate well with previous work indicating the primary bacteria in the pits are Gram-positive, low (G + C) bacteria [6, 15]. The presence of the HAP and other similar bacterial strains in the stored manure may prove to be an important factor in the digestion and fermentation of proteinaceous material in the manure and production of ammonia and other compounds. Of particular interest would be the fermentation of amino acids such as tryptophan, phenylalanine, and tyrosine. Fermentation products from these amino acids can give rise to the production of indole and phenolic compounds (such as skatole), which can contribute greatly to the foul odors associated with swine facilities. We are also studying potential compounds that may interfere with the fermentation of these compounds and reduce the production of ammonia and other volatile organic compounds.

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