Development of a Quantitative Real-Time Polymerase Chain Reaction Assay to Target a Novel Group of Ammonia-Producing Bacteria Found in Poultry Litter

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ABSTRACT Ammonia production in poultry houses has serious implications for flock health and performance, nutrient value of poultry litter, and energy costs for running poultry operations. In poultry litter, the conversion of organic N (uric acid and urea) to NH_4-N is a microbially mediated process. The urease enzyme is responsible for the final step in the conversion of urea to NH_4-N. Cloning and analysis of 168 urease sequences from extracted genomic DNA from poultry litter samples revealed the presence of a novel, dominant group of ureolytic microbes (representing 90% of the urease clone library). Specific primers and a probe were designed to target this novel poultry litter urease producer (PLUP) group, and a new quantitative real-time PCR assay was developed. The assay allowed for the detection of 10^2 copies of target urease sequences per PCR reaction (approximately 1×10^4 cells per gram of poultry litter), and the reaction was linear over 8 orders of magnitude. Our PLUP group was present only in poultry litter and was not present in environmental samples from diverse agriculutural settings. This novel PLUP group represented between 0.1 to 3.1% of the total microbial populations (6.0×10^6 to 2.4×10^8 PLUP cells per gram of litter) from diverse poultry litter types. The PLUP cell concentrations were directly correlated to the total cell concentrations in the poultry litter and were found to be influenced by the physical parameters of the litters (bedding material, moisture content, pH), as well as the NH_4-N content of the litters, based on principal component analysis. Chemical parameters (organic N, total N, total C) were not found to be influential in the concentrations of our PLUP group in the diverse poultry litters Future applications of this assay could include determining the efficacy of current NH_4-N-reducing litter amendments or in designing more efficient treatment protocols.

Key words: ammonia, poultry litter, quantitative real-time polymerase chain reaction, urease

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INTRODUCTION

The physical and chemical properties of poultry litter, a combination of poultry excreta and bedding material, have been studied extensively (Barker, 1996; Martin et al., 1998; Williams et al., 1999; Lu et al., 2003; Fries et al., 2005). Poultry litter has been used as a dietary food source or supplement for a variety of livestock (Tanabe et al., 1985; Ilian et al., 1988; Arave et al., 1990) and is used as a land-applied fertilizer for a variety of crops (Liebhardt, 1976; Huneycutt et al., 1988; Ritter, 1990). Due to conditions of the handling, transportation, and application of the litter, its value as a fertilizer is reduced over time due to the significant losses of N (10 to 80%) attributed to the volatilization of NH_4-N (Pain et al., 1987; Hartung and Phillips, 1994). Ammonia emission and subsequent conversion to nitrates can be a major source of pollution (Koerkamp, 1994; Williams, 1995), causing eutrophication of surface waters (Edwards and Daniel, 1992; Paerl and Fogel, 1994), acidification of soils (Williams et al., 1999), and odor formation (Nahm, 2003; Wheeler et al., 2006). In the poultry house, NH_4-N emissions adversely affect the health, performance, and welfare of both animals (Wathes et al., 2002; Dawkins et al., 2004) and human operators (Kirychuk et al., 2006; Rylander and Carvalheiro, 2006).

Uric acid and urea constitute approximately 70% of the total N found in poultry litter (Shuler et al., 1979; Koerkamp, 1994). Microbial decomposition of these organic N sources leads to the production and subsequent volatilization of NH_4-N. One of the major limiting factors in the final stages of this decomposition reaction is the activity of the bacterial urease enzyme (Nahm, 2003), a nickel-containing trimeric enzyme that catalyzes the hydrolysis of urea to NH_4-N and CO_2. Most research dealing with bacterial ureases has focused on its role in the pathogenesis of urogential and gastroduodenal disease, including those caused by *Helicobacter pylori, Ureaplasma urealyticum,* and *Proteus mirabilis* (Mobley and Hausinger, 1989; Mobley et

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Table 1. Primers and probes used in this study

Oligo name	Target[1]	Sequence (5′-3′)[2]	Insert size (bp)	Reference
ureC1F	General *ureC*	AAG MTS CAC GAG GAC TGG GG	340	Koper et al. (2004)
ureC2R		AGR TGG TGG CAS ACC ATS AGC AT		
ureC-QRT-F	PLUP *ureC*	TTC ACA CCT TCC ACA CCG AA	103	This study
ureC-QRT-R		AAC GTC GGG TTG GTC GAG		
ureC-QRT-R		CGG TGG ACA CGC ACC AGA TAT CT		
1055F	All bacteria	ATG GCT GTC GTC AGC T	337	Harms et al. (2003)
1055F		ACG GGC GGT GTG TAC		
16STaq1115-BHQ		CAA CGA GCG CAA CCC		

[1]PLUP = poultry litter urease producer.
[2]Degeneracy codes: M = A or T, S = G or C, R = A or G.

al., 1995; Burne and Chen, 2000). Microbial populations from poultry litter have been characterized by both culture-dependent (Nodar et al., 1990; Martin et al., 1998) and independent means (Lu et al., 2003; Enticknap et al., 2006). Although urease activity has been measured in poultry litter (Tejada et al., 2006, 2007), no data are available on the organisms responsible for urease production in poultry litter.

The goal of this study was to better characterize urease-producing microbes in poultry litter. The first objective was to identify the ureolytic microbial community in poultry litter by specifically targeting the urease gene using general *ureC* (largest structural subunit of the urease enzyme) PCR primers (Koper et al., 2004) and cloning and sequencing the resultant PCR products. A clone library was constructed to identify the dominant group(s) of urease producers in poultry litter. The second objective was to develop methods to specifically detect these dominant ureolytic group(s) from poultry litter using quantitative real-time PCR (**QRT-PCR**). Quantitative real-time PCR is a molecular microbiological technique that permits rapid, sensitive detection of target organisms in environmental samples. The abundance of a target sequence is determined by measuring PCR products as they are accumulating and quantifying the amount of target while the PCR reaction is still in the exponential range (Brunk et al., 2002; Ginzinger, 2002). Products are detected and quantified as they are generated during each PCR cycle through the use of fluorescently labeled probes and standards run along with samples. The advantage of the fluorogenic probes is that specific hybridization between probe and target is required to generate fluorescent signal. The polymerase will cleave the probe only while it remains hybridized to its complementary strand. It has been found that QRT-PCR has been used to target human pathogens in waste materials (Ibekwe et al., 2002, Inglis and Kalischuk, 2004) and to characterize bacteria in the rumen (Tajima et al., 2001) and in the human and swine intestinal tract (Hill et al., 2005). The final objective of this study was to use the developed assay to compare the concentrations of this novel group of ureolytic microbes from diverse poultry litter types, varying in location, bedding material, and physiochemical parameters, and determine which biological and physiochemical parameter directly correlated to or influenced the concentrations of this novel group in those poultry litters.

MATERIALS AND METHODS

Sampling

Poultry litter samples were obtained from poultry houses throughout Kentucky (n = 2), Mississippi (n = 1), and Oklahoma (n = 5). Samples were shipped on dry ice and remained frozen (≤–20°C) until sample processing. The Kentucky and Oklahoma poultry litter samples were bulk samples representative of their poultry houses, whereas the Mississippi litter samples were obtained as part of a spatial variability study within a single house (Lovanh et al., 2007). Ten subsamples from sampling sites throughout the Mississippi house were pooled to obtain a representative sample for the entire house.

Molecular Analysis of ureC Genes in Poultry Litter

Deoxyribonucleic acid was extracted from poultry litter samples (0.3 g) using the Q-Biogene FastDNA Spin Kit for soil (Q-Biogene, Irvine, CA) according to the specifications of the manufacturer. Urease genes (*ureC*) were specifically amplified from 2 μL of the 1:500 dilution of the community DNA extract using the ureC1F/ureC2R primer set (Table

Table 2. *ureC* quantitative real-time PCR (QRT-PCR) assay specificity using pure culture and environmental DNA templates

Sample	Urease[1]	QRT-PCR *ureC*[2,3]
Environmental samples		
Water	+	–
Soil	+	–
Cow manure	+	–
Pig lagoon slurry	+	–
Chicken litter	+	+
Pure cultures		
Bacillus subtilis (ATCC 6633)	+	–
Lactobacillus fermentum (ATCC 9338)	+	–
Pseudomonas aeruginosa (ATCC 9027)	+	–
Staphylococcus aureus (ATCC 25923)	+	–
Bacteroides thetaiotaomicron (ATCC 29148)	–	–

[1]Determined by ureC1F/*ure*C2R primer set (Koper et al., 2004).
[2]Determined by ureC-QRT-F/*ure*C-QRT-R primer set (this study).
[3]Any amplification before threshold cycle ≤33 was considered a positive result, based on threshold for 10^1 standard.

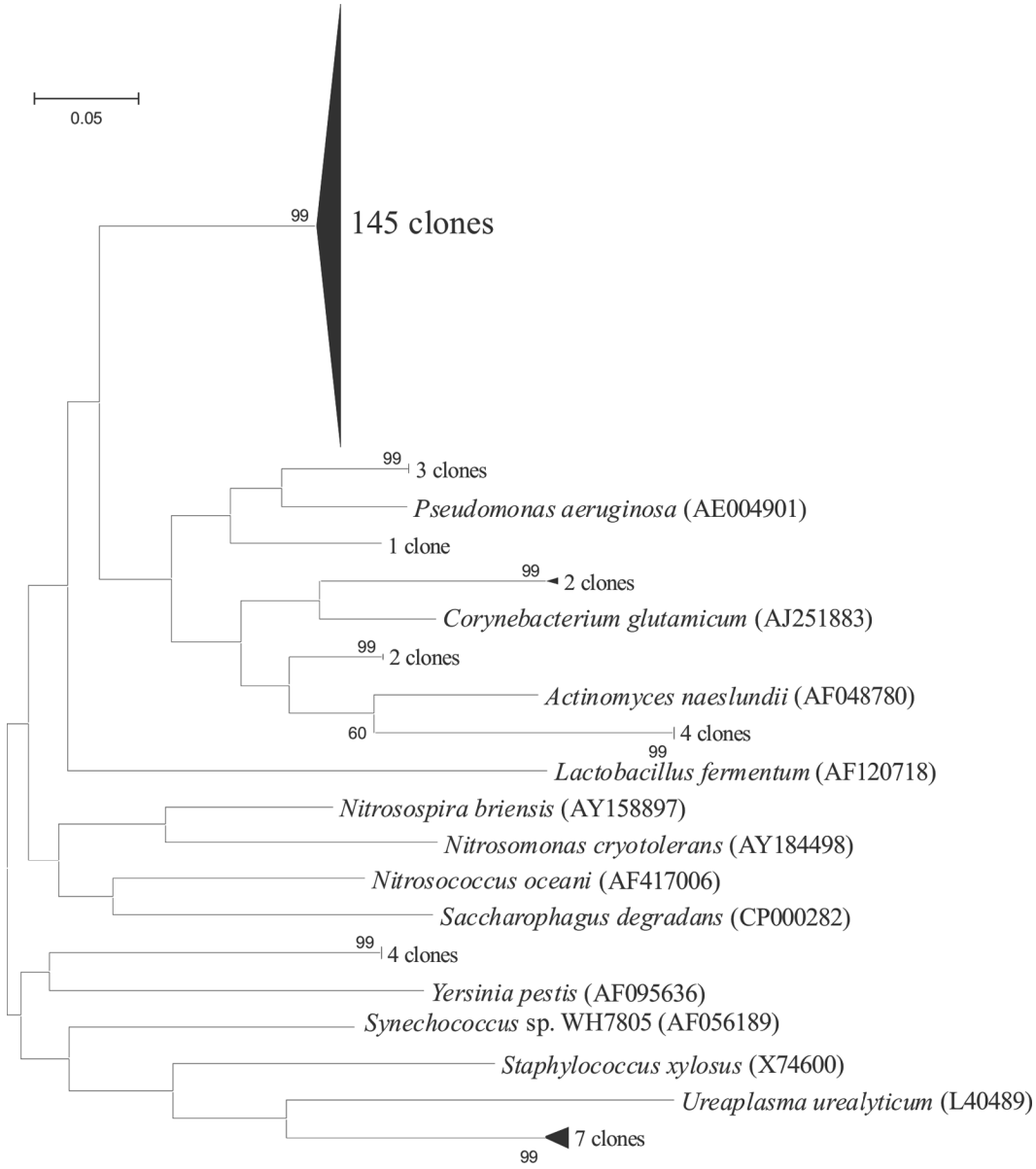

Figure 1. Neighbor-joining tree showing the phylogenetic relationship between community *ureC* clones from bulk community DNA extract from poultry litter and *ureC* gene sequences retrieved from the GenBank database (accession numbers in parentheses). The tree represents the alignment of a 338-bp region present in all sequences. Bootstrap values per 100 analyses were shown at the appropriate nodes for values greater than 50.

1) using thermocycling conditions as previously described (Koper et al., 2004) using a PTC-200 DNA thermal cycler (MJ Research, Las Vegas, NV). Sequences were amplified using Qiagen HotStarTaq Master Mix (Qiagen Inc., Valencia, CA), with 800 nM of each primer and 1 to 10 ng of template DNA from the genomic extracts of poultry litter. Annealing temperatures of 58 and 60°C were used in 2 separate cloning reactions to determine if annealing temperature had an effect on the resultant *ureC* clone libraries. The PCR products were cloned into the pCR2.1-TOPO plasmid using a TA TOPO Cloning Kit (Invitrogen, Carlsbad, CA) according to the specifications of the manufacturer and were sent to the USDA-ARS MSA Genomics Laboratory (Stoneville, MS) for sequencing. The new *ureC* sequences, combined with appropriate known *ureC* sequences from the GenBank database, were aligned using *MEGA* version 3.1 (Kumar et al., 2004). A 338-bp region of the alignment containing data from all sequences was selected for further phylogenetic studies. The alignment files were used to create bootstrapped (n = 1,000) neighbor-joining trees, using the Kimura 2-parameter model in the *MEGA* version 3.1 software package.

QRT-PCR Primer and Assay Design

The *ureC* sequences from 145 poultry litter clones were aligned using *MEGA* 3.1, and a 103-bp region was used as a target site for the development of QRT-PCR primers and a probe (Table 1). The primers were obtained from Sigma Genosys (St. Louis, MO), and the dual-labeled Black

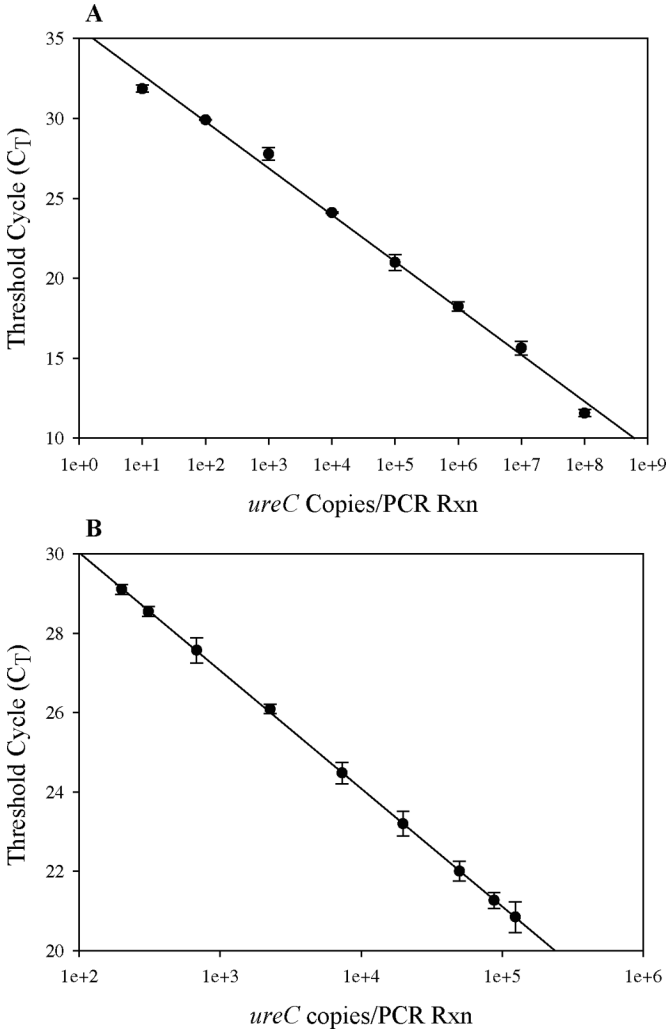

Figure 2. Relationship between the threshold cycle and the logarithm of the *ureC* copy number per quantitative real-time PCR (QRT-PCR) reaction using (A) standard *ureC* DNA, $y = 35.63 - 3.22x$, $r^2 = 0.994$ and (B) *ureC* DNA templates, $y = 35.98 - 3.27x$, $r^2 = 0.999$. Error bars indicate the deviation of triplicate QRT-PCR reactions.

Hole Quencher probe was prepared by Biosearch Technologies Inc. (Novato, CA). The annealing temperature (58 to 70°C), primer (300, 600, 900 n*M*) and probe (50, 100, 200 n*M*) concentrations, and $MgCl_2$ (3 to 5 μ*M*) concentrations were optimized for this assay. The QRT-PCR assays were run on the DNA Engine Opticon 2 (MJ Research Inc., Waltham, MA) and were carried out using the Qiagen HotStarTaq Master Mix (Qiagen) in a total volume of 25 μL. The amplification mixture contained 3.0 m*M* of $MgCl_2$, 300 n*M* of each primer and 200 n*M* of probe, and sample DNA or standard (from 10^1 to 10^8 copies). The QRT-PCR program was 15 min at 95°C followed by 35 cycles at 95°C for 20 s and 63°C for 20 s. Baseline values were set as the lowest fluorescence signal measured in the well over all cycles. The baseline was subtracted from all values, and the cycle threshold (**C_T**) was set to 1 standard deviation of the mean. A positive result was determined to be amplification of product at a C_T value ≤33, which represented 1 cycle beyond the average C_T (31.99) for the 10^1 *ureC* standard. Template DNA consisted of DNA extract (5 μL of a 1:500 dilution) run in triplicate, with all PCR runs including duplicates of standards and control reactions without template (repeatability). All reactions were run on at least 2 different plates (reproducibility). Standard DNA consisted of plasmid PCR 2.1 vector (Invitrogen) carrying a *ureC* insert from a larger fragment of a representative *ureC* poultry litter clone, contained within our novel group (338 bp), and amplified using the ureC1F/ureC2R primer set (Koper et al., 2004).

QRT-PCR Efficiency and Sensitivity

The DNA concentration of a representative poultry litter extraction was determined using the Hoechst 33258 nucleic acid stain (Invitrogen) and measured with a Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences, San Francisco, CA) according to the instructions of the manufacturer. A series of dilutions of the poultry litter extract (1,000 ng to 0.1 ng of template DNA per PCR reaction) were amplified using the designed assay, as were plasmid standards containing 10^8 to 10^1 copies of the *ureC* insert. No inhibition was observed using DNA template concentrations between 0.1 and 100 ng per 25 μL of PCR reaction. To determine the amplification efficiency of this assay, the original number of *ureC* copies per PCR reaction was compared with the C_T value for that sample, and a regression analysis was performed. Amplification efficiency was calculated using the following formula: $E = 10(-1/x) - 1 \times 100$, where x = the slope of the regression line. Assay efficiency of the poultry litter extracts and the plasmid standards were compared.

To determine the specificity of this assay for *ureC* sequences found in poultry litter, DNA was extracted (using the above procedure) from environmental samples, including cow manure, soil, swine lagoon slurry, and water samples. Fifty to 150 pg of genomic DNA extract was analyzed using the *ureC* QRT-PCR assay described above. Five pure cultures were analyzed to further determine the specificity of the assay. *Bacillus subtilis* ATCC 6633, *Lactobacillus fermentum* ATCC 9338, and *Staphylococcus aureus* ATCC 25923 were obtained by growing cultures from Kwik-Stik (Microbiologics Inc., St. Cloud, MN) on Bacto-Tryptic Soy Broth (**TSB**) agar (BD, Franklin Lakes, NJ) with 5% sheep's blood (Hemostat Laboratories, Dixon, CA). *Pseudomonas aeruginosa* ATCC 9027 from a BioBall (TCS Water Sciences, Buckingham, UK) was rehydrated in 100 μL of 1× phosphate-buffered solution and grown on TSB-Blood agar. Pure cultures of *Bacteroides thetaiotaomicron* ATCC 29148 were obtained from ATCC and grown on anaerobic brain heart infusion agar (BD). Isolated colonies from each organism were grown up in 5 mL of TSB or anaerobic brain heart infusion broth (*B. thetaiotaomicron*) overnight at 37°C, spun down (10,000 × *g* for 10 min), and genomic DNA was extracted using the FastDNA Kit (Q-Biogene) according to the specifications of the manufacturer, and 1:10 extract dilutions were analyzed using the *ureC* QRT-PCR assay.

Evaluation of QRT-PCR Assay

The concentration of poultry litter urease producers (**PLUP**) cells per gram of poultry litter was calculated by

dividing the copy number per gram of litter (calculated from the regression analysis of the plasmid standard DNA) by average copy number of *ureC* genes per cell (1.5; Koper et al., 2004). To compare our PLUP cell concentrations to total cell numbers in the poultry litter, QRT-PCR analysis of 16S rDNA was performed. The QRT-PCR analysis of 16S rDNA copies was carried out as previously described (Harms et al., 2003) using the 1055f and 1392r primers at 600 nM each and the 16STaq1115-BHQ at a concentration of 200 nM (Table 1). The amplification mixture contained 3.0 m*M* of $MgCl_2$, 600 n*M* of each primer, 200 n*M* of probe, and sample DNA (1:500 dilution) or standard (from 10^2 to 10^8 copies). The QRT-PCR program was 15 min at 95°C, 39 cycles at 95°C for 15 s, and 58°C for 45 s. Standard DNA consisted of plasmid PCR 2.1 vector (Invitrogen) carrying a 16S rDNA insert. All PCR runs included duplicates of standards and control reactions without template, and the amplification efficiency was calculated as shown above. Total concentration was calculated by dividing the copy number per gram of litter (calculated from the regression analysis of the plasmid standard DNA) by average copy number of 16S genes per cell (4.0; Klappenbach et al., 2001). The percentage of the novel PLUP cells in each poultry litter sample was determined as follows: (PLUP cell concentration/16S cell concentration) × 100.

Poultry Litter Analyses

The percentage of moisture of the poultry litter was determined by drying the litter at 65°C overnight and comparing the weight before and after drying the litter. Litter pH was determined using a combination electrode (Fisher Scientific, Hampton, NH) at a 5:1 deionized H_2O:litter ratio. Total N and C were determined by combustion of the litter, followed by gas chromatography using a Vario Max CN analyzer (Elementar Americas Inc., Mt. Laurel, NJ). The NH_4-N and NO_3-N content of the litter was determined by a 60:1 litter:2 *M* of KCl extraction followed by flow injection analysis using a Quickchem FIA+ (Lachat Instruments, Milwaukee, WI). Organic N was estimated by subtracting the NH_4-N and NO_3-N values from the total N value.

Principal Components Analysis

Factor analysis as a multivariate statistical method was used to find a small number of factors from a data set of many correlated variables. Factor analysis is a useful tool for extracting latent information or variables (principal component), such as underlying but not directly observable relationships between variables. The physiochemical parameters included litter composition, percentage of moisture, pH, NH_4-N, organic N, total N, and total C. The litter composition parameter was reduced to a single number to indicate bedding material (sawdust = 0, rice hulls = 1, no bedding = 2, and wood shavings = 3). STATISTICA 7.0 (Statsoft, Tulsa, OK) was used for the principal components analysis (**PCA**), which was performed using the Varimax raw method. The original data matrix was decomposed into the product of a matrix of factor loadings and a matrix of factor scores plus a residual matrix. The residual matrix identifies the part of variance of the data set that cannot be explained by common factors (e.g., analytical uncertainties or feature-own variances).

Nucleotide Sequence Accession Numbers

A total of 194 sequences were submitted to the GenBank database and were assigned the accession numbers of EF587504-EF587697.

RESULTS

Poultry Litter ureC Cloning and Sequence Analysis

One hundred sixty-eight sequences (338 bp in length) were obtained from 2 poultry litter *ureC* clone libraries (annealing temperature = 58 and 60°C). Phylogenic analysis showed that 89.5% (145/168) of the clone sequences formed a single unique cluster of *ureC* sequences whose closest match (73% similarity) in the GenBank database was to the *ureC* gene from *P. aeruginosa* strain PAO1 (AE004901; Figure 1). All of the 145 clone sequences within this novel cluster were ≥97% similar to each other. This novel PLUP group dominated both individual clone library groups equally, representing 87 and 85% of the 58 and 60°C libraries, respectively. The translated ureC protein sequences were only 76% similar to *Campylobacter lari* (BAD89502), which corroborated the novel nature of this group. The remaining 23 clones formed 6 smaller groups (n ≤ 7 clones), all of which ranged from 66 to 88% similar to their nearest known *ureC* sequences in the GenBank database.

Primer Design and Assay Sensitivity

The QRT-PCR primers and a probe were designed to amplify a 103-bp region of the *ureC* gene matching the PLUP group (Table 1). One of the *ureC* clones was selected as the standard and exhibited a strong linear fluorescent response over 8 orders of magnitude (10^1 to 10^8 *ureC* copies per QRT-PCR reaction; r^2 = 0.994). There was a strong linear relationship between the C_T and the logarithm of the number of *ureC* copies per PCR reaction using both poultry litter DNA template (Figure 2A; r^2 = 0.999, efficiency = 102%) and *ureC* standard DNA template (Figure 2B; r^2 = 0.994, efficiency = 104%). High amplification efficiencies, calculated from the slope of the regression analyses, using both poultry litter and standard *ureC* DNA templates, indicate that most of the target sequences were replicated at each amplification cycle. The similarity of these values also confirms the use of the standard *ureC* DNA as an accurate measure of the amplification and quantification of the target *ureC* sequence from poultry litter. Based on regression analysis, the lowest genomic poultry litter DNA concentration at which amplification was observed was 0.1 ng per PCR reaction, which is equiv-

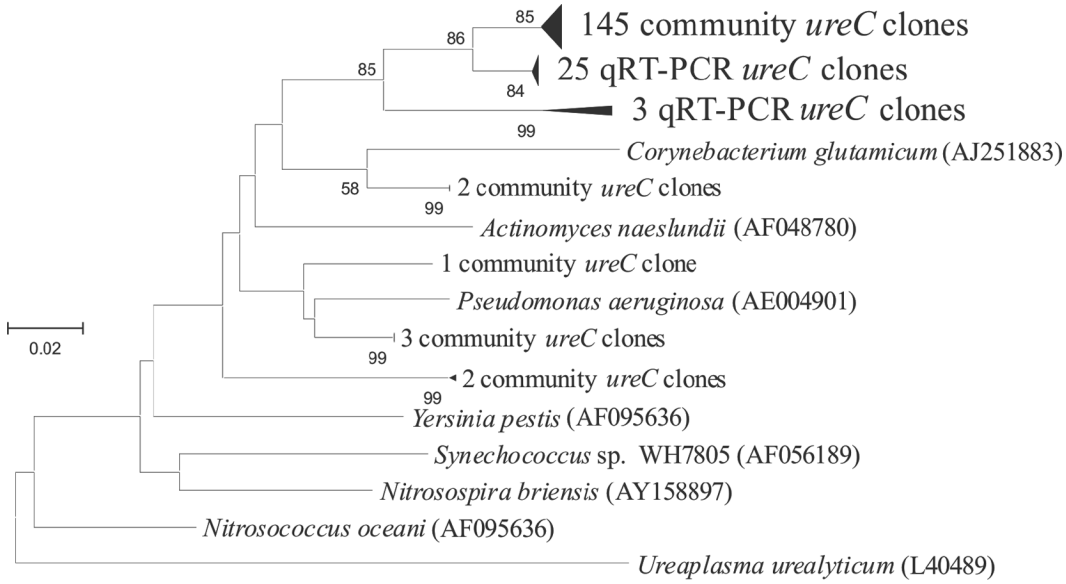

Figure 3. Neighbor-joining tree showing the phylogenetic relationship between clones from quantitative real-time PCR product (qRT *ureC* clones), community clones from bulk community DNA extract (community *ureC* clones), and *ureC* gene sequences retrieved from the GenBank database (accession numbers in parentheses). The tree represents the alignment of a 115-bp region present in all sequences. The portion of the tree that represents the novel group of *ureC* sequences targeted by the PCR primers/probe is denoted by the bold text. Bootstrap values per 100 analyses were shown at the appropriate nodes for values greater than 50.

alent to approximately 10^2 copies of the *ureC* gene matching the PLUP group per PCR reaction. This would be equivalent to approximately 1×10^4 PLUP cells per gram of poultry litter.

QRT-PCR Assay Specificity

To determine the specificity of this assay to poultry litter, we quantified *ureC* sequences from various environmental samples, including cow manure, soil, water, pig manure, and chicken litter, as well as pure cultures of known urease positive (*B. subtilis, L. fermentum, P. aeruginosa,* and *S. aureus*) and negative (*B. thetaiotaomicron*) organisms using the QRT-PCR primers and probe designed for our novel group. All samples were amplified first using the general *ureC* primer set (Koper et al., 2004) to determine if the samples contained the *ureC* gene (Table 2). All environmental and pure culture DNA extracts were positive for general *ureC* amplification except the *B. thetaiotaomicron.* The only sample that yielded a positive result when the new *ureC* QRT-PCR assay was performed was the poultry litter sample (Table 2), suggesting that the PLUP group sequences are unique to poultry litter. Attempts at cloning the QRT-PCR product failed to produce clones for any samples except for the poultry litter samples.

QRT-PCR ureC Sequence Analysis

To ensure that the assay was specifically targeting the PLUP group, QRT-PCR products from the amplification of poultry litter samples were cloned and sequenced. The phylogenetic analysis of sequences from cloned QRT-PCR products branched closely to the cluster of 145 *ureC* community clone sequences obtained using the general *ureC* primer set (Figure 3). All QRT-PCR *ureC* sequences (n = 28) were 94 to 97% similar to the cluster of *ureC* clone sequences used to design the QRT-PCR primers and probe. This indicates that the QRT-PCR *ureC* assay is amplifying the correct target sequence and supports the overall specificity of this assay to this novel PLUP group

Evaluation of NH_4-N Producers in Poultry Litter

Eight different poultry litter samples were analyzed for the presence of our PLUP group and on the basis of their biological (total cells per gram of litter) and physicochemical (litter composition, moisture content, pH, NH_4-N, organic N, total N, and total C) parameters (Table 3). Assuming an average *ureC* gene copy number of 1.5 per cell (Koper et al., 2004), the litters contained between 6.0×10^6 to 2.4×10^8 PLUP cells per gram of litter. Total cell numbers ranged from 1.3×10^{11} to 2.8×10^9 cells per gram of litter, which were based on 16S copy numbers assuming 4 copies of the 16S rDNA gene per cell (Klappenbach et al., 2001). Based on these data, the novel PLUP group represented between 0.1 to 3.1% of the total microbial populations in these poultry litters. Although all the litter parameters evaluated were positively correlated to PLUP cell concentrations, the only significant correlation was to total cell numbers (r = 0.76). None of the physiochemical parameters were found to be strongly correlated to PLUP cell concentrations, but the physical parameters (litter composition, moisture content, pH) were more positively correlated (r = 0.32 to 0.40) than were the chemical parameters (r = 0.05 to 0.13). Although none of the physiochemical parameters were directly correlated, it is possible that they may indirectly influence PLUP cell concentrations in the poultry litters. The PCA analysis of the PLUP cell concentrations

Table 3. Microbiological and physiochemical data for 8 different poultry litter types

Sample	Average # PLUP cells per gram of litter[1] × 10^7	Average # cells (16S) per gram of litter[2] × 10^9	Litter composition	Moisture (%)	pH	NH_4-N (mg/kg) × 10^3	Organic N (mg/kg)[3] × 10^4	Total N (% dry weight)	Total C (% dry weight)
CLE-MS	5.4 (4.8)	25.0 (13.0)	Sawdust	40	8.2	3.98	1.90	2.3	21
JW-KY	24.0 (4.3)	130.0 (53.0)	Sawdust	18	7.9	3.49	3.74	4.1	41
MH2-KY	17.0 (1.0)	5.4 (3.1)	Rice hulls	50	7.8	7.44	3.39	4.1	43
LX-OK	1.8 (1.1)	3.6 (1.3)	Rice hulls	37	8.8	1.11	3.74	3.9	38
H1-OK	0.6 (0.2)	5.4 (1.9)	No bedding	9	7.2	1.41	2.80	2.9	39
H2-OK	0.7 (0.1)	3.7 (1.5)	No bedding	11	7.0	1.71	4.51	4.7	39
UB-OK	1.3 (0.5)	2.8 (0.3)	Wood shavings	25	8.6	3.55	2.81	3.2	40
UL-OK	14.0 (8.1)	21.0 (7.9)	Wood shavings	12	8.5	1.33	4.31	4.4	40

[1]Calculated assuming an average of 1.5 *ureC* genes per cell. Standard deviation shown in parentheses. PLUP = poultry litter urease producer.
[2]Calculated assuming an average of 4.0 16S rDNA genes per cell. Standard deviation shown in parentheses.
[3]Calculated by the following equations: (total N × 10^4) − (NH_4-N).

and both physical and chemical litter parameters showed that more than 75% of the total variance in the data set from the various poultry litter samples was accounted for by 2 factors, with factor 1 and 2 accounting for 42.3 and 33.2% of this variance, respectively (Figure 4). The log-transformed PLUP cell concentrations clustered near the physical (litter composition, pH, moisture content), as well as NH_4-N, litter parameters, whereas they were distinct from remaining chemical litter parameters (total N, total C, and organic N).

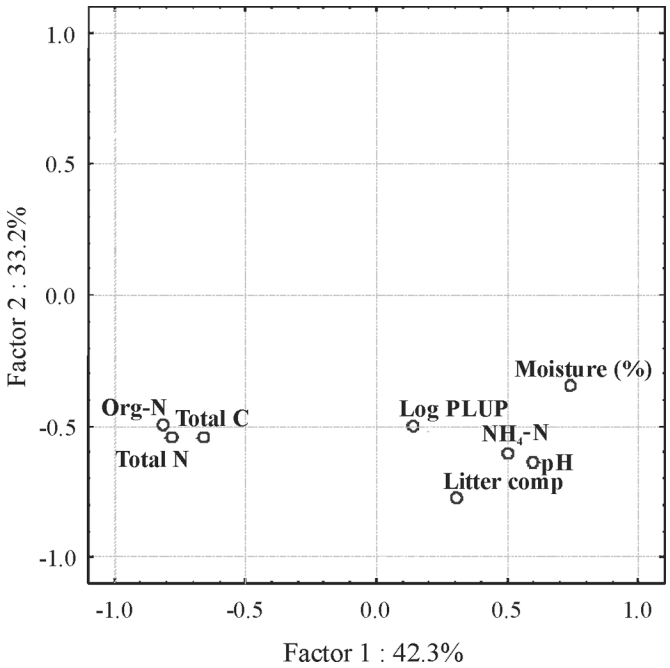

Figure 4. Principal component analysis of physiochemical poultry litter parameters, including log-transformed poultry litter urease producer (PLUP) cell concentrations, using the Varimax raw option in Statistica. Physiochemical parameters include organic N (Org-N), total N, total C, pH, moisture content (%), litter composition (Litter comp), and NH_4-N. The data set is shown in Table 3. Approximately 75% of the variance in the data set was explained by 2 factors (principal components), accounting for 42.3 and 33.2% of the variance, respectively. Parameters that are close to one another are affected similarly by the 2 factors.

DISCUSSION

Ammonia emissions from poultry houses affect overall air quality within the houses and in the surrounding environment. Reducing microbial populations responsible for this NH_4-N production is important, considering that the respiratory health of the flock is significantly affected when continuously exposed to NH_4-N concentrations as low as 10 ppm (Blake and Hess, 2001). Ammonia volatilization can be controlled by 2 methods in poultry houses, either by chemical or biological modification of the litter (Carlile, 1984). Chemical modification techniques are designed to attenuate or eliminate NH_4-N already present within the poultry litter. Commonly practiced methods include acidifying the litter using chemical amendments (i.e., alum) to transform NH_4-N to nonvolatile ammonium (Moore et al., 1995) or making the litter more alkaline to force all NH_4-N to be volatilized and then vent before the start of a new flock.

Although limited work has been performed to reduce or eliminate urease activity by adding chemical inhibitors (Singh et al., 2005), no work has concentrated on identifying the microbial communities responsible for production of the NH_4-N in the poultry litter. Poultry litter is an excellent environment for the survival and persistence of urease-producing microorganisms, given the abundance of urea and its precursors (uric acid, allatoin, allatoic acid; Nahm, 2003). Uric acid and urea represent 70% of the total N in poultry litter (Shuler et al., 1979), and the limiting step in the conversion of organic N to NH_4-N is the activity of microbial ureases. Although decomposition of organic N sources can be achieved abiotically, it is kinetically very slow, and therefore microbial ureases are essential to mediate this NH_4-N production (Nahm, 2003). In our study, the urease-producing community clone library from poultry litter was dominated by a single group of urease producers (145/168 clones, Figure 1), and this represented a novel group of urease producers (≤73% similarity to known urease producers in the GenBank database) found only in poultry litter. Given its dominance, the goal was to target our PLUP group from diverse poultry litters.

Quantitative real-time PCR assays have been developed targeting the *ureC* gene of specific urease producers such as *Ureaplasma urealyticum* and *Ureaplasma parvum* (Mallard et al., 2005) and *Helicobacter pylori* (He et al., 2002), but no assays were available for the amplification of *ureC* genes from an environmental microbial community. In this study, QRT-PCR primers and a probe were designed to target on a dominant group of ureolytic microbes in poultry litter. The designed assay was found to be highly efficient at amplifying both standard and environmental templates (Figure 2) and was able to amplify the target *ureC* DNA from 0.1 ng of genomic poultry litter DNA per PCR reaction. Considering that the PLUP cells represented approximately 1% of the total cells in the poultry litter, it is possible that as little as 10 pg of the target *ureC* DNA per PCR reaction could be detected. This assay was specific for this novel PLUP group, with no amplification found for DNA templates from other environmental samples or pure cultures shown to contain the urease enzyme (Table 2). The results from the pure cultures were not surprising, given the fact that the target sequences in the *ureC* gene for each of the known bacteria were only approximately 75% similar to the sequences of the designed QRT-PCR primers and probe (data not shown).

Because the copy number of the *ureC* gene is low (1 to 2 copies per genome; Koper et al., 2004), the concentration of *ureC* genes per gram of litter is a faithful estimate of the concentration of PLUP cells in the litter (Bach et al., 2002). Initially, the designed primers and probe were developed from a single litter type, with this novel group found in relatively high quantities (5.4×10^7 PLUP cells per gram of litter), but similar concentrations were found in poultry litter samples from various locations in other states (ranging from 6.0×10^6 to 2.4×10^8 PLUP cells per gram of litter) and from litters with different physiochemical properties (Table 3). The prevalence of dominant but specific microbial groups (i.e., Lactobacillales, Actinomycetes, Clostridia) in diverse poultry litters (varying locations, bedding materials) has been previously reported (Martin et al., 1998; Lu et al., 2003; Fries et al., 2005), so it was not unexpected that this group of ureolytic microbes was present in similar concentrations in our various poultry litter types. This novel group represented anywhere from 0.1 to 3.1% of the total number of cells (based on 16S QRT-PCR data) in the litter. These ratios are consistent with the other microbial enzymes vital for N cycling (*narG, nirK, nosZ*) that were shown to represent similar portions of the total bacterial populations from a variety of soils, including agricultural soils (Henry et al., 2004, 2006; Lopez-Gutierrez et al., 2004). These results indicate that this novel PLUP group is a significant component of the total bacterial population of poultry litter and could account for a significant amount of the NH_4-N produced from those houses.

In this study, we found the concentration of our PLUP group to be between 6.0×10^6 and 2.4×10^8 PLUP cells per gram of litter. Given published data on average flock concentration and litter production (Perkins et al., 1964) and the specific activity of the urease enzyme for a mixed microbial community from animal agriculture under optimal conditions (Mahadevan et al., 1977), we have determined that between 1.5×10^6 and 5.9×10^7 mg of NH_3 could be produced per house per day given our data. Assuming an average of 2×10^4 birds per house, NH_4-N production from larger poultry production facilities in the United States can range from 9.4×10^6 to 2.4×10^7 mg of NH_3 per house per day (Groot Koerkamp et al., 1998; Burns et al., 2003; Lacey et al., 2003; Wheeler et al., 2006), which indicated that our novel PLUP group could be responsible for more than 10% of the NH_4-N produced from a poultry house.

To assess the applicability of this assay, diverse litter types were tested microbiologically, physically, and chemically, and the variance in the data from the different litters was compared using PCA (Figure 4). Although only total cell concentrations were directly correlated to PLUP cell concentrations, other physiochemical parameters were found to influence their concentrations in the different litters. Log-transformed PLUP cells clustered most closely to physical litter parameters (pH, moisture content, bedding materials) and NH_4-N (Figure 4). This result was not surprising, considering that parameters such as pH (Elliot and Collins, 1982; Derikx et al., 1994) and moisture content (Nahm, 2003; Lovanh et al., 2007) have been previously reported to be the dominant environmental factors in NH_4-N volatilization within poultry houses. Because our PLUP group could potentially produce greater than 10% of that NH_4-N, it is logical that these physical parameters also affect the concentration of these PLUP cells. Chemical litter parameters (organic N, total N, and total C) did not cluster near the physical parameters or PLUP cell concentrations, while also exhibiting the lowest direct correlation to PLUP cell concentrations ($r \leq 0.13$). Previous studies have shown that there is either a very weak (Barbarika. et al., 1985; Serna and Fomares, 1991) or no correlation (Castellanos and Pratt, 1981) between total C/N ratio and N mineralization. Because the microbial urease activity is a major limiting step in the mineralization of organic (urea) to inorganic (NH_4-N) N, the total C and N numbers for each litter type were not expected to influence the concentrations of the PLUP cells.

In conclusion, primers and a probe specific to the *ureC* gene from a novel group of dominant ureolytic microbes found in poultry litter were designed and used to develop a new QRT-PCR assay. This assay was highly specific to poultry litter samples, and calculated concentrations of cells containing this target *ureC* gene were found in high numbers (~10^7 to 10^8 PLUP cells per gram of litter) in diverse litter types from 3 different states and using 4 different bedding materials, and these concentrations were found to be affected by the physiochemical parameters of the litter. Initial attempts at isolating the PLUP bacteria have been unsuccessful, although additional isolation experiments are currently ongoing. The characterization of our PLUP group will allow for a greater understanding of how they function within poultry litter and potentially lead to the development of remediation strategies to eliminate this group from the litter, which would aid in reducing NH_4-N volatilization. The use of appropriate NH_4-N-re-

ducing strategies will lead to increased bird performance and health, a reduction in energy costs associated with ventilation, and an increase in the fertilizer value of the poultry litter due to higher organic N content.

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