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Chemical Structures of Manure from Conventional and Phytase Transgenic Pigs Investigated by Advanced Solid-State NMR Spectroscopy

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Nonpoint phosphorus (P) pollution from animal manure is becoming a serious global problem. The current solution for the swine industry includes the enzyme phytase as a component in oil meal and cereal grain-based swine diets. A long-term approach is the production of transgenic phytase pigs that express phytase in the salivary glands and secrete it in the saliva. This study provides a detailed comparison of chemical structures of manure from conventional pigs and transgenic pigs that express phytase under growing and finishing phases using new solid-state NMR techniques. Spectral editing techniques and quantitative NMR techniques were used to identify and quantify specific functional groups. Two-dimensional $^{1}H-^{13}C$ heteronuclear correlation NMR was used to detect their connectivity. Manure from conventional and transgenic pigs had similar peptide, carbohydrate, and fatty acid components, while those from transgenic pigs contained more carbohydrates and fewer nonpolar alkyls. There was no consistent effect from diets with or without supplemental phosphate or growth stages.

KEYWORDS: NMR; transgenic; characterization; pig manure; spectral editing

INTRODUCTION

Nonpoint phosphorus (P) pollution arising from the application of animal manure is becoming a serious global problem (*1*), as runoff waters carry excess P from fields receiving manure into water bodies and cause eutrophication (*2*). This is especially important if the manure is from monogastric animals such as pigs, because those animals inherently lack the ability to digest plant phytate, the major form of organic P in cereal grains. Therefore, as phytate passes through the digestive tract of these animals, the P concentration excreted in the manure is often concentrated by 3−4 times as compared to that of the ingested feed. To compensate for the indigestible phytate, bioavailable mineral P is often added to swine diets to meet P nutritional requirements and to ensure that maximum growth rates are achieved. This practice is nutritionally successful but not environmentally beneficial.

In recognition of the negative environmental impact of excess P in animal manure, various strategies have been developed to minimize the need for mineral P in swine diets. First, the enzyme phytase can be included in the diet (*3*). This enzyme digests dietary phytate, making nonavailable phytate P available to the pigs in their intestinal tracts. However, the enzymes are an added cost and can decrease the already small profit margins of swine producers. A second approach is to genetically modify plants so that there is phytase in the seeds or simply lower levels of phytate P in the grain (*4*). Problems with this approach are that seed phytase may be unstable and lose its activity during pelleting and storage, and phytate-reduced cereal grains may have reduced yields and be less drought resistant. A third option is to use a new breed of transgenic Yorkshire pigs, trademarked Enviropig, that excrete phytase from their salivary glands (*5, 6*). These animals produce sufficient phytase to digest most of the phytate P in cereal grain diets. Initial studies showed that these pigs fed a diet without mineral P excreted feces with 75% less P than nontransgenic pigs (*6*).

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Transgenic pigs thus offer a unique biological approach for managing both P nutrition and potential pollution within the pork industry. The transgenic pigs producing phytase, Enviropig, are similar to conventional Yorkshire pigs in health status, growth rate, and reproductive characteristics but, at this time, do not have regulatory approval for use as food for human consumption (*7*).

In addition to issues regarding the safety of the meat, there are also questions regarding the safety of the manure from transgenic pigs. The manure from conventional pigs is a good fertilizer except for the high phosphorus content. The question with manure from transgenic pigs expressing phytase is whether the manure is modified in any other way than having a reduced phosphorus content, which may lead to unintended effects when spread on land. Toward this objective, a useful first step would be to understand how the chemical structures in manure from transgenic Enviropig pigs differ from those of conventional pigs. This would begin to provide the information needed (i) to determine any potentially negative impact of manure from transgenic pigs on the environment and human health and (ii) to gain insights into potential metabolic changes in the pigs arising from the gene modification.

Analysis of swine manure is complicated because a significant portion of the material is insoluble. Different spectroscopic methods such as FT-IR (Fourier transform infrared) and NMR (nuclear magnetic resonance) spectroscopies can be used to investigate the chemical structures of complex organic materials such as pig manure. Among them, solid-state NMR is arguably the most powerful one because it is nondestructive and can handle insoluble samples directly (*8*). Solid-state NMR has been extensively applied to the study of natural organic matter (*9–12*). However, because of technical and availability limitations, solid-state NMR spectroscopy has not been extensively used to study the chemical structures of animal manures. In a few studies of manure using solid-state NMR, the sole technique used has been routine CP/MAS (cross-polarization/magic angle spinning) (*13–15*).

In this study, we obtained detailed chemical structural information for eight manure samples from transgenic and nontransgenic pigs with different diet (conventional containing supplemental P vs unsupplemented) and growth stage (growing vs finishing) treatments. Advanced solid-state NMR techniques that we developed recently for characterizing complex organic matter in plants, soils, water, sediments, sludge (*8, 16–30*), and meteorites (*31*) were used. Traditional ^{13}C NMR spectra for complex organic matter can identify only about 10 types of chemical groups, because routine ^{13}C solid-state NMR spectra consist of broad and heavily overlapping bands in which functional groups cannot be clearly distinguished. With our new techniques, we have clearly identified more than 36 different moieties. Our spectral-editing techniques can selectively retain certain peaks and eliminate others, clearly revealing specific functional groups. In addition, we can use ^{1}H spin diffusion to detect domains or heterogeneities on a 1−50 nm scale (*22*). To the best of our knowledge, this is the first project to investigate the detailed chemical structures of pig manures and also the first effort to use advanced solid-state NMR spectroscopy to distinguish differences in the chemistry of manure from transgenic and nontransgenic pigs.

MATERIALS AND METHODS

Pig Manure Samples. Manure samples representing eight different treatments associated with genetics, diet, and animal growth stage were collected from each of three transgenic JA line Yorkshire pigs consisting of two males and one female and the same number of age- and sex-matched conventional Yorkshire pigs. The treatments included (i) conventional Yorkshire pigs fed a conventional diet during the "late growing stage" (Yorkshire-conven-grower); (ii) Yorkshire pigs fed a diet without supplemental P during the growing stage (Yorkshire-lowP-grower); (iii) JA line of transgenic Yorkshire (Enviropig) pigs fed a conventional diet during the growing stage (Tg JA- conven-grower); (iv) JA line of transgenic Yorkshire pigs fed unsupplemented diet during the growing stage (Tg JA-lowP-grower); (v) conventional Yorkshire pigs fed a conventional diet containing supplemental P during the "finishing stage" (Yorkshire-conven-finisher); (vi) conventional Yorkshire pigs fed a low-P diet during the finishing stage (Yorkshire-lowP-finisher); (vii) JA line of finisher transgenic pigs fed a conventional diet during the finishing stage (Tg JA-conven-finisher); and (viii) JA line of transgenic Yorkshire pigs fed finisher diet lacking supplemental P (Tg JA-lowP-finisher). In these experiments, the "growing stage" refers to the pigs that received the grower diet beginning at 52−67 kg (123−147 pounds), while the "finishing phase" began at 74−88 kg (163−194 pounds). At the termination of the trial, the finishing pigs weighed between 97 and 112 kg (213 and 246.4 pounds). Manures from both periods were collected to determine whether animal growth stage affected their chemical structures. The conventional and low-P diets were fed during both growth stages for 10 day cycles according to a 2 × 2 Latin square design. After a 5-day adaptation period, all feces and urine were collected during days 6–10 to create composite samples for each treatment. The samples were thoroughly mixed with a stirring rod to homogenize the solid and liquid materials. The manure samples were freeze-dried and directly packed into NMR rotors for analysis.

Table 1. Formulation of Experimental Diets for the Transgenic and Conventional Pigs

ingredients (%, w/w)	grower diet		finisher diet	
	(A) grower, added P	(B) grower, no added P	(C) finisher, added P	(D) finisher, no added P
soybean meal, 47–48%CP	24.00	24.00	16.80	16.80
corn	72.92	72.60	80.48	80.18
corn starch		1.42		1.06
canola oil	0.25		0.16	
iodized salt	0.50	0.50	0.50	0.50
limestone ($CaCO_3$)	0.87	0.78	0.72	0.76
dicalcium phosphate[a]	0.76	0.00	0.64	0.00
vitamin premix	0.50	0.50	0.50	0.50
mineral premix	0.10	0.10	0.10	0.10
lysine-HCl (79%)	0.10	0.10	0.10	0.10
total (100 kg)	100.00	100.00	100.00	100.00
	calculated nutritive values			
DE (MJ/kg)	14.28	14.38	14.30	14.38
CP (%)	16.61	16.59	14.07	14.05
Ca (%)	0.60	0.40	0.50	0.37
total P (%)	0.50	0.36	0.45	0.33
Ca/total P ratio	1.20	1.11	1.09	1.11

[a] The key difference between control and low-P diets.

Diet Samples and 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Formulations for the conventional and low-P diets fed during the growing and finishing stages are presented in **Table 1**. The major ingredients were corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] meal. Dicalcium phosphate was added to the conventional diets (control diets) but not to the low-phosphorus diets. The diets for each growth stage were adjusted to provide the same levels of energy and crude protein and to maintain a constant calcium to phosphorus ratio. The other components including the iodized salt, vitamin premix, mineral premix, and crystalline lysine were constant for both growth stages. A model compound, POPC, was purchased from Avanti, Polar Lipids, Inc., and used to determine the existence of lipids in pig manure (see below).

NMR Spectroscopy. All experiments were performed in a Bruker Avance 300 spectrometer at 75 MHz for ^{13}C in a double-resonance probe, except for two-dimensional (2D) HETCOR experiments, which were performed in a Bruker Avance 400 spectrometer at 100 MHz for ^{13}C. About 90 mg of freeze-dried pig manure was placed in a 4 mm rotor.

High-Speed Quantitative ^{13}C DP/MAS NMR. Quantitative structural information was obtained using high-speed ^{13}C DP/MAS NMR. Characterizations were performed at a spinning speed of 13 kHz with a 90° ^{13}C pulse length of 4 μs. Recycle delays were tested by the CP/T_1-TOSS (cross polarization/spin–lattice relaxation time-total sideband suppression) technique to ensure that all carbon sites were fully (>95%) relaxed (*16*). On the basis of the CP/T_1-TOSS results, recycle delays of 50 s were used for these samples. To highlight mobile aliphatic components, a ^{13}C DP/MAS spectrum with a short recycle delay of 1.5 s was also recorded. The numbers of scans and recycle delays are listed in the figure captions.

^{13}C CP/TOSS and ^{13}C CP/TOSS Plus Dipolar Dephasing. Qualitative compositional information was obtained with good sensitivity by ^{13}C CP/TOSS (cross-polarization/total sideband suppression) NMR experiments. These experiments were run at a spinning speed of 5 kHz and a CP (cross-polarization) time of 1 ms, with a ^1H 90° pulse length of 4 μs. ^{13}C CP/TOSS combined with a 40 μs dipolar dephasing was employed to generate a subspectrum with signals of nonprotonated carbons and mobile groups such as CH_3. The recycle delay was 1 s.

^{13}C Chemical Shift Anisotropy Filter. To separate the signals of anomeric carbons (O−C−O) from those of aromatic carbons, both of which may resonate between 120 and 90 ppm, a five-pulse ^{13}C chemical shift-anisotropy (CSA) filter [CP-t_{CSA}-180°-pulse-t_{CSA}-90°-pulse-t_z-90°-pulse (*26*)] was inserted into ^{13}C CP/TOSS to separate sp^3-hybridized anomeric carbons (O−C−O) from sp^2-hybridized aromatic carbons. This technique selectively suppressed sp^2-hybridized carbons and retained sp^3-hybridized carbons. The ^1H 90° pulse length was 4 μs, the CP contact time was 1 ms, and the CSA filter time was 47 μs. Four-pulse TOSS (*32*) was employed before detection, and TPPM (two-pulse phase-modulated) decoupling was applied for optimum resolution. The CSA filter scheme was combined with an increment of the z-period in four steps of tr/4, which provided a "γ-integral" that suppressed side bands up to the fourth order (*18*).

CH Spectral Editing. The CH spectral editing technique was employed to select CH-only information. The CH-only spectrum typically showed five or six bands: aromatic or olefinic CH near 130 ppm, acetal O−CH−O near 100 ppm, alkyl OCH near 70 ppm, alkyl NCH between 50 and 62 ppm, and nonpolar alkyl CH (bonded to three carbons) between 35 and 55 ppm. For this technique, the dipolar distortionless enhancement by polarization transfer (DEPT) method was used (*33*) at a spinning speed of 4 kHz. The first of a pair of recorded spectra contained signals of CH, as well as residual quaternary-carbon and CH_3 peaks. The latter two were removed and reduced, respectively, by taking the difference with a second spectrum acquired using the same pulse sequence except for an additional 40 μs dipolar dephasing before detection. The recycle delay was 0.5 s with a 0.5 s ^{13}C T_1 filter. The details of this technique have been described elsewhere (*33*).

CH_2 Spectral Editing. Spectral editing of CH_2 signals was achieved by selection of the three-spin coherence of CH_2 groups, using a ^{13}C 90° pulse and ^1H 0°/180° pulses applied after the first quarter of one rotation period with MREV-8 decoupling (*27*). Typically, two or three bands were observed as follows: nonpolar CH_2 (bonded to two carbons) resonated between 20 and 40 ppm; CH_2−OH resonated between 60 and 70 ppm, while CH_2−O−C ethers may contribute signals between 68 and 75 ppm. The spinning speed was 5.787 kHz.

^1H−^{13}C Heteronuclear Correlation NMR (HETCOR). The scale on which ^1H−^{13}C proximities are probed can be chosen by the cross-polarization method. Three or less bond ^1H−^{13}C connectivities were revealed by 0.5 ms of Lee−Goldburg cross-polarization (LG-CP), which suppressed ^1H−^1H spin diffusion during polarization transfer. The experiments were performed at a spinning speed of 6.5 kHz. Also, 40 μs dipolar dephasing was inserted in the LGCP HETCOR to reveal connectivities for unprotonated carbons and mobile groups such as CH_3. A total of 128 t_1 increments of 6.65 μs were used. A more detailed discussion of the 2D HETCOR experiments has been provided by Mao et al. (*19*).

RESULTS AND DISCUSSION

NMR Spectra. Figure 1 shows the ^{13}C DP/MAS spectra for the manure from all eight treatments. The similarity of the eight spectra is striking and indicates that all samples contain similar major components.

To identify the common major components of the manure samples, we chose the sample of grower stage manure from Yorkshire pigs fed the conventional P-supplmented ration for detailed characterization. **Figure 2** shows a series of ^{13}C CP/MAS NMR spectra acquired with suitably designed radiofrequency pulse sequences to select subspectra for specific chemical groups, such as nonprotonated carbons and mobile groups, sp^3-carbons, CH (methine), and CH_2. Initially, it should be noted that in this study two sets of data—quantitative and qualitative—were collected separately, using high-spinning speed DP/MAS and low-spinning speed experiments inserted with TOSS, respectively. Sidebands are removed or reduced in both sets of data. There are two common ways to remove or reduce side bands: fast spinning of samples and insertion of a sequence of total suppression of sidebands (TOSS) before detection. Fast magic angle spinning (MAS) of DP/MAS at 14 kHz (**Figure 1**) requires small 4 mm rotors and thus low sensitivity because of reduced sample amounts, but it provides quantitative structural information. When only qualitative structural information is needed, low-spinning speeds using large 7 mm rotors are used to increase sensitivity with larger sample sizes (**Figures 2**, **5**, and **6**). Under this circumstance, the insertion of four π-pulse TOSS is employed to remove sidebands. Another advantage of TOSS is that it can avoid baseline distortions due to the dead time problem.

Figure 2a is ^{13}C CP/TOSS spectrum that shows qualitative structural information and is used primarily as reference spectrum for the selective subspectra. The corresponding ^{13}C CP/TOSS spectrum after 40 μs of dipolar dephasing, **Figure 2b**, exhibits only signals of nonprotonated carbons and mobile segments, including rotating CH_3 groups, which have a reduced C−H dipolar coupling due to their fast motions. This spectrum shows (i) strong signals from highly mobile CH_3 and $-(CH_2)_n$ components between 0 and 48 ppm; (ii) a very small amount of carbons between 97 and 165 ppm arising from nonprotonated anomerics, mobile C═C, nonprotonated aromatics, and aromatic C−O; and (iii) significant COO/CON groups (between 165 and 190 ppm). We did not detect an OCH_3 peak around 56 ppm in **Figure 2b**, suggesting that the band around 48 and 63 ppm is primarily attributed to NCH, which might be peptides.

The ^{13}C CP/TOSS spectrum after a ^{13}C CSA filter of 47 μs, which exhibits primarily sp^3-carbon signals, is displayed in **Figure 2c**. In particular, this technique separates overlapping anomerics (O–C–O) from aromatics between 90 and 120 ppm. A clear O–C–O band is displayed in this region. A small residual peak of COO/N–C═O exists due to their relatively smaller chemical shift anisotropies as compared with those of aromatics or C═C olefinics. **Figure 2d** depicts the spectrum with only methine (CH) signals, acquired using the dipolar DEPT technique (*33*). It selects NCH and OCH bands, around 56 and 76 ppm, respectively, as well as the expected aromatic CH or olefinic –CH═CH– resonances around 130 ppm. NCH groups can be found in all common peptides while OCH groups are typical of carbohydrates. The band centered at 105 ppm is mostly due to O–CH–O. Most of the anomerics are protonated, because there are only very small signals barely above the baseline around this region in the dipolar-dephased spectrum (**Figure 2b**). There are no nonpolar CCH groups in this pig manure sample; otherwise, their signals below 50 ppm would be detected. **Figure 2e** is the spectrum showing only CH_2 signals. It clearly displays a dominant C–CH_2 band at 33 ppm.

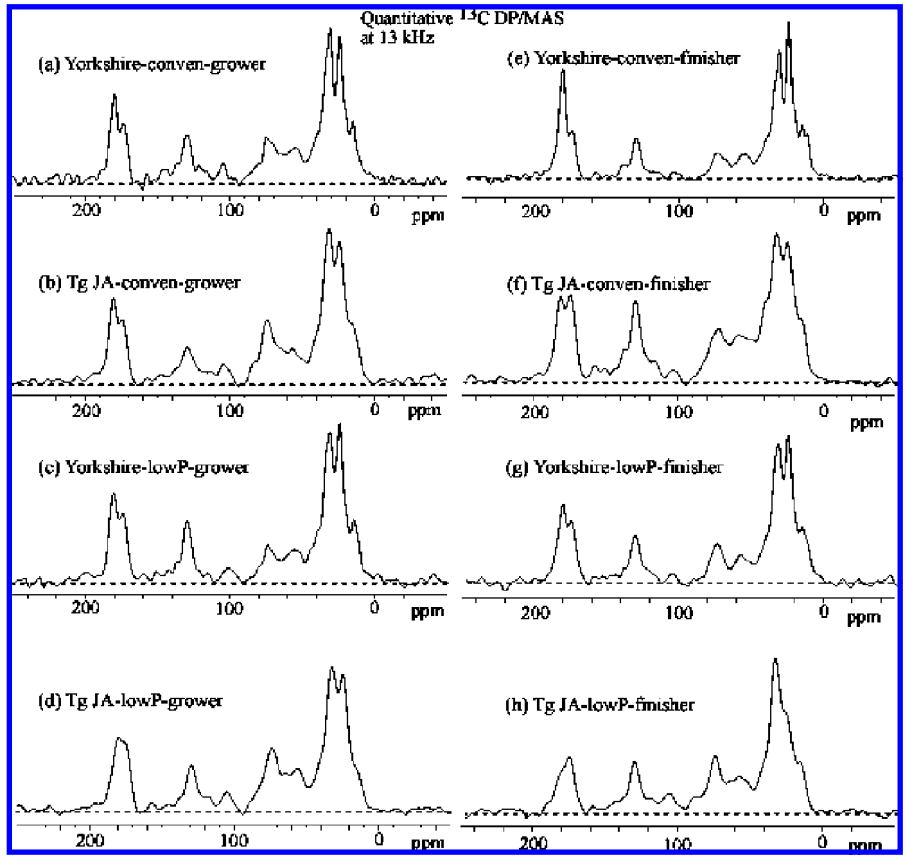

Figure 1. Quantitative ^{13}C DP/MAS NMR, at a spinning speed of 13 kHz, of manure from (**a**) nontransgenic Yorkshire pig fed conventional diet at the growing stage; (**b**) transgenic JA pig fed conventional diet at the growing stage; (**c**) nontransgenic Yorkshire pig fed low-P diet at the growing stage; (**d**) transgenic JA pig fed low-P diet at the growing stage; (**e**) nontransgenic Yorkshire pig fed conventional diet at the finishing stage; (**f**) transgenic JA pig fed conventional diet at the finishing stage; (**g**) nontransgenic Yorkshire pig fed low-P diet at the finishing stage; and (**h**) transgenic JA pig fed low-P diet at the finishing stage. Each with 1536 scans and a 50 s recycle delay.

To analyze the mobile aliphatic segments, we used ^{13}C DP/MAS with a short recycle delay of 1.5 s because the ^{13}C T_1's (spin–lattice relaxation times) of mobile groups are shorter than those of rigid groups. With a short recycle, rigid groups would be suppressed because they are far from fully relaxed. To determine the existence of lipids in manure, POPC was selected as a model compound because the acyl chain lengths of fatty acids, which likely exist in the swine diets, are similar to those of POPC. **Figure 3b** is the CP/TOSS spectrum of POPC, whose molecular structure is shown in **Figure 3a**. **Figure 3c** is the fully relaxed ^{13}C DP/MAS spectrum showing quantitative information. **Figure 3d** displays the ^{13}C DP/MAS spectrum with a short recycle delay of 1.5 s. It shows predominantly the mobile nonpolar alkyl region (0–48 ppm), a COO/NC═O band around 181 ppm, plus a small peak around 130 ppm within the olefinic C═C/aromatic region, assigned to olefinic CH═CH groups in mobile acyl chains of lipids, which is also observed in the dipolar-dephased spectra. Line broadening of the POPC spectrum would cause it to closely resemble large portions of the pig manure spectrum (**Figure 3d**: C1, C═C, long aliphatic $-(CH_2)_n-$ chains). Surprisingly, the COO/N−C═O peak at 181 ppm relaxes quickly whereas the peak at 174.6 ppm relaxes slowly. We are not clear why the COO/N−C═O peak at 181 ppm relaxes fast. On the basis of **Figure 3b**, a peak at 14 ppm in the nonpolar alkyl region (0–48 ppm) of manure is assigned to the methyl end chain (ω), a peak at 24 ppm is due to $\omega - 1$, and a peak at 32 ppm arises from $\omega - 2$. We also observe C═C and C1 from lipids in **Figure 3**.

Figure 4a is the 2D HETCOR spectrum of manure from the Yorkshire grower pigs fed the conventional P-supplemented diet with an LG-CP (Lee−Goldburg cross-polarization) of 0.5 ms, showing correlations of protons and carbons separated by three or less bonds. There are $^1H-^{13}C$ HETCOR experiments in both solution-state and solid-state NMR. In solution state, the $^1H-^{13}C$ correlation is based on J couplings, which are through bonds. Thus, it can only show signals for CH_n fragments with $n \geq 1$, that is, protonated carbons, and there is no information on quaternary carbons. However, $^1H-^{13}C$ HETCOR experiments in solid-state are based on dipolar couplings. Dipolar couplings are through space and are also called indirect coupling. 2D $^1H-^{13}C$ HETCOR NMR is a valuable tool for characterizing the environments of nonprotonated carbon sites (*19*). Going beyond the simple identification of a carbon site and its directly bonded protons, the identification of the nearest protons for nonprotonated carbons such as COO/CON provides particularly useful structural information. Via 2D $^1H-^{13}C$ HETCOR experiments, junctions between different groups (e.g., aromatic and aliphatic) and larger structural units can also be identified (*22*). The COO/N–C═O at 174.6 ppm, carbohydrate rings, and NCH groups share significant cross-peaks, confirming the assignment of peptides and suggesting the existence of uronic acids or amino sugars. A cross-peak between COO/N−C═O at 174.6 ppm and nonpolar alkyls is also observed. The ^1H chemical shift of this peak exceeds 10 ppm, indicating the existence of OH of COOH (*34*). The peak at 181 ppm is

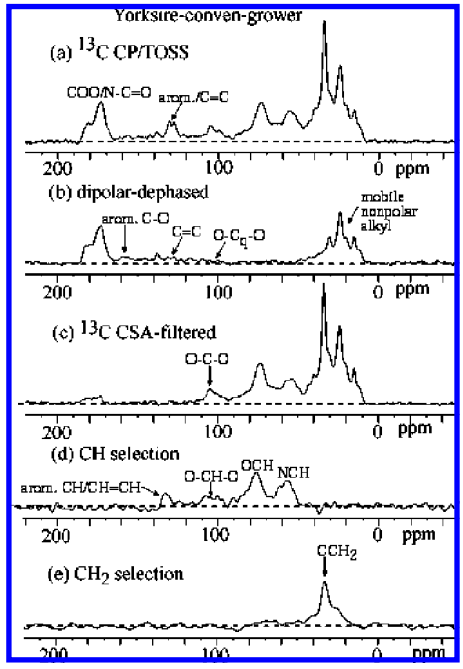

Figure 2. Spectral editing for identification of specific functional groups in manure from the nontransgenic Yorkshire pigs fed conventional diet at the growing stage, with a contact time of 1 ms at a spinning speed of 5 kHz. (**a**) Unselective ^{13}C CP/TOSS spectrum for reference with 8K scans and a recycle delay of 1 s; (**b**) corresponding dipolar-dephased ^{13}C CP/TOSS spectrum showing nonprotonated carbons and mobile segments such as CH_3, acquired after a 40 μs dipolar dephasng with 8K scans and a recycle delay of 1 s; (**c**) selection of sp^3-hybridized carbon signals by a chemical shift anisotropy filter, which in particular identifies OCO carbons, near 102 ppm, which are typical of carbohydrate rings (CSA filter time, 47 μs; other parameters are as in part **a**); (**d**) CH-only spectrum with 90K scans and a 1 s recycle delay; and (**e**) CH_2-only spectrum with 115140 scans and a 1 s recycle delay.

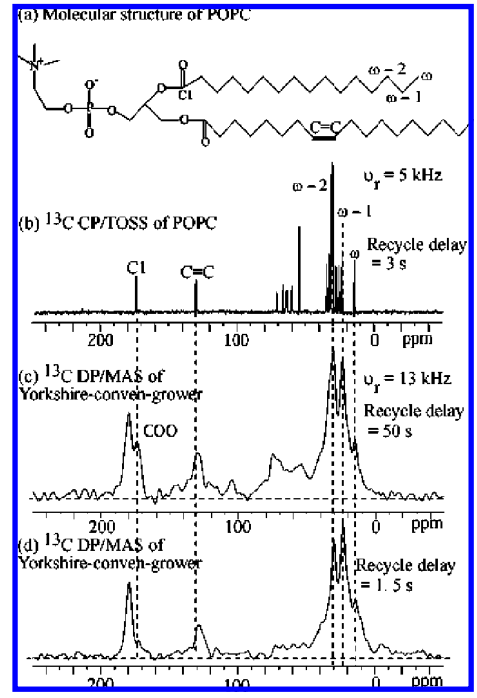

Figure 3. (**a**) Molecular structure of POPC; (**b**) ^{13}C CP/TOSS spectrum of POPC with 2K scans, 1 ms CP, and a recycle delay of 3 s; (**c**) DP/MAS with 1536 scans and a 50 s recycle delay and (**d**) with 4200 scans and a 1.5 s recycle delay of manure from nontransgenic Yorkshire pig fed the conventional diet at the growing stage.

strongly correlated with nonpolar alkyls, but the ^{1}H ppm does not reach 5 ppm, suggesting little OH of COOH groups (*34*). The clear cross-peak between C−O (72 ppm) and O−C−O (105 ppm) indicates the existence of carbohydrate rings. We also see clear correlation peaks between aromatics or C=C olefinics and COO/N−C=O. On the basis of this evidence, we conclude that the peak at 174.6 ppm arises from N−C=O, COOH, and COO while the peak at 181 ppm is primarily due to COO^- associated with nonpolar alkyls. The HETCOR spectrum with dipolar dephasing, **Figure 4b**, shows the nontrivial two-bond correlation peaks of unprotonated carbons with the surrounding protons. This spectrum clearly indicates that the COO peak at 181 ppm is correlated with mobile nonpolar alkyls, which partially explains the fast relaxation of this peak, since high mobility can lead to its short ^{13}C T_1 (spin–lattice relaxation time) and thus fast relaxation.

To further demonstrate that all pig manure samples contain similar major components after the detailed analysis of the nongene-conven-growing sample, we present ^{13}C CP/TOSS, dipolar-dephased, and ^{13}C CSA-filtered spectra of all eight samples in **Figure 5**. The dipolar-dephased spectra are similar for all samples, and the same is true for the ^{13}C CSA-filtered, ^{13}C CP/TOSS, and DP/MAS spectra (**Figure 1**), confirming that all pig manure samples contain similar major components.

Figure 6 shows the ^{13}C CP/TOSS spectra of diets A (**Figure 6a**), B (**Figure 6d**), C (**Figure 6e**), and D (**Figure 6f**) and also the dipolar-dephased (**Figure 6b**) and ^{13}C CSA-filtered (**Figure 6c**) spectra of diet A. The spectra of all diet samples are the same, which is surprising, since their ingredients are not identical. One explanation may be that the proportions of the major components (i.e., carbohydrates, lipids, and proteins) of these diets are similar. The band from 0 to 45 ppm arises from nonpolar alkyls such as $C-C_{quat}$, C−CH, CCH_2, and CCH_3, most of which are mobile, as demonstrated in the dipolardephased spectrum in **Figure 6b**. The shoulder around 55 ppm is from NCH of proteins and completely dephased in the dipolar-dephased spectrum. The peaks at 62, 72, and 102 ppm are due to OCH_2, OCH, and OCHO (anomerics) of carbohydrates and are also completely dephased by dipolar dephasing. On the basis of the ^{13}C-CSA-filtered spectrum, the peak at 102 ppm is solely from sp^3-hybridized OCO, and its dipolar-dephased spectrum indicates that this peak is completely protonated. The peak at 174.6 ppm is attributed to COO or N−C=O and cannot be dephased by dipolar dephasing. In summary, the NMR spectra indicate that the dominant component in all diets is carbohydrates. Signals from lipids and proteins are small as compared with those of carbohydrates.

Peak Integrals from Quantitative NMR Spectra. On the basis of the detailed ^{13}C NMR peak assignments described in the previous section, we can evaluate the quantitative spectra of **Figure 1**. The information on chemical shift ranges and corresponding peak areas is listed in **Table 2**. Roughly, the assignments are as follows: 0–48 ppm, nonpolar alkyls; 48–63 ppm, NCH; 63–112 ppm, carbohydrate; 112–164 ppm, aromatics or olefinics; and 164–210 ppm, COO and N–C=O.

When diet and growth stage are held constant, the manure from the transgenic pigs contains more carbohydrates (63–112 ppm) than that from corresponding conventional pigs, whereas the reverse is true for the nonpolar alkyl region (0–48 ppm). We also found that manure from transgenic pigs contain less COO/N−C=O than those from conventional pigs except the samples of grower stage Yorkshire pigs receiving the conventional ration vs the JA line receiving the same ration. These

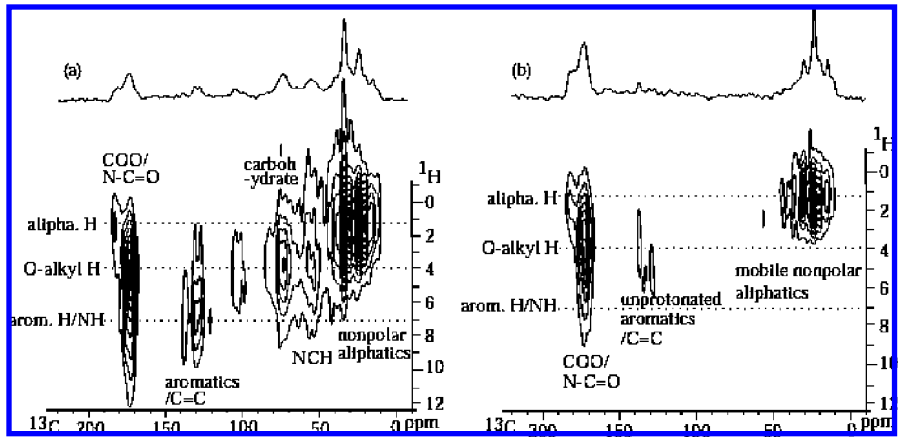

Figure 4. Two-dimensional $^{1}H-^{13}C$ heteronuclear correlation NMR spectra of manure from the nontransgenic Yorkshrie pigs fed conventional diet at the growing stage, with an LG-CP of 0.5 ms, showing correlations of protons and carbons separated by three or less bonds: (**a**) whole spectrum and (**b**) spectrum with a 40 μs dipolar dephasing. Each with 2048 scans and a 1 s recycle delay. The spectra above the 2D HETCOR spectra are the corresponding 1D ^{13}C spectra of this sample.

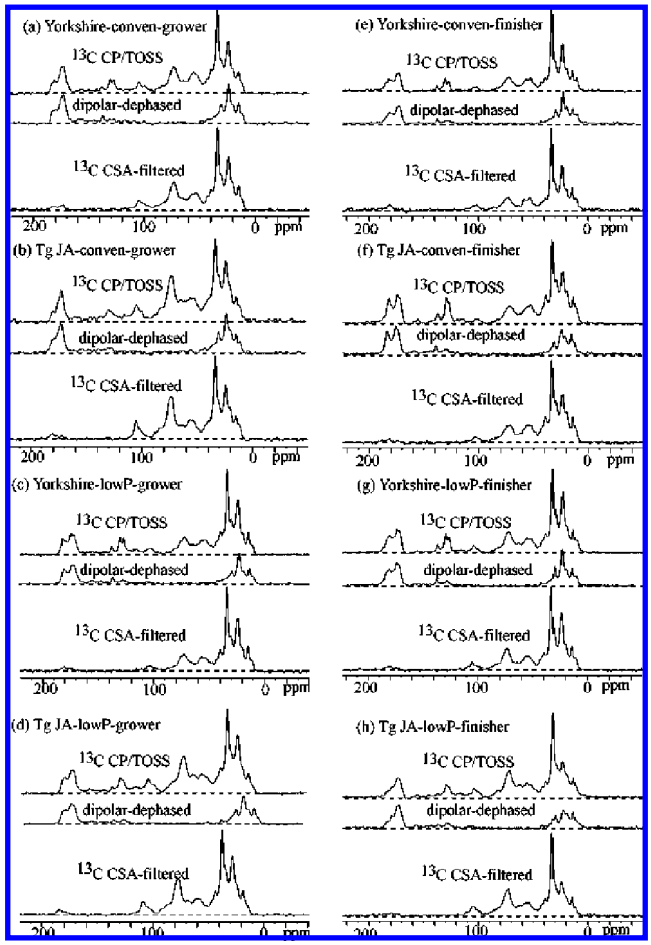

Figure 5. ^{13}C CP/TOSS, dipolar-dephased, and CSA-filtered spectra of manure from (**a**) Yorkshire-conven-grower; (**b**) Tg JA-conven-grower; (**c**) Yorkshire-lowP-grower; (**d**) Tg JA-lowP-grower; (**e**) Yorkshire-conven-finisher; (**f**) Tg JA-conven-finisher; (**g**) Yorkshire-lowP-finisher; and (**h**) Tg JA-lowP-finisher. Each with 8K scans and a 1 s recycle delay.

differences are confirmed in **Figure 1**. Finally, the peaks at 14, 24, and 32 ppm are broader in the spectra of samples from transgenic pigs as compared with those from the conventional pigs, suggesting that nonpolar alkyls in manure of transgenic pigs are of more diverse chemical environments than those in manure of conventional pigs.

Common Structures. The chemical structures of eight pig manure samples as revealed by advanced solid-state NMR methods are quite similar, and they contain three major components: lipids, peptides, and carbohydrates.

Lipids. Most of the nonpolar alkyls around the 0–48 ppm range in the pig manure are mobile (**Figure 3d**). On the basis of the previous discussion, nonpolar alkyls in pig manure could be mainly from lipids and side chains of peptides. In this study, the primary ingredients of diets A–D were corn and soybean meal (**Table 1**). The major fatty acids in corn and soybean are oleic acid (C18:1) and linoleic acid (C18:2) (http://www.nal.usda.gov/fnic/foodcomp/search/index/html). We chose POPC as a model compound because it contains C18 fatty acids whose acyl chain lengths are similar to those of fatty acids in the swine diets. We indeed note the partial matching between the NMR spectra of POPC and the pig manure (**Figure 3b**,**c**). In diets, lipids are not a major component (**Figure 6**), while in manure, they are.

Peptide. The ^{13}C NMR signals around 56 and 174.6 ppm originate from NCH and N−C═O, for instance, in peptides [(O═C)−N−(C−H)]. For many natural organic samples, OCH_3 signals overlap with NCH signals at around 56 ppm. The dipolar-dephased spectrum indicates that there are no OCH_3 groups in the pig manure (**Figures 2** and **5**). As shown in **Figure 6**, peptides are a small component in the ingested feed, but they are more prominent in pig manure.

Carbohydrates. Carbohydrates contribute to the signals around the 63–94 ppm range (OC) and those around the 94–112 ppm range (O−C−O). The area ratio of the O−C−O band (the anomeric carbon of carbohydrates) to the bands around the 63–94 ppm range (other carbons of carbohydrates) is ca. 4.5, suggesting the predominant presence of 5- or 6-membered ring carbohydrates in the samples. Anomerics and OC groups in the pig manure are protonated. The amount of carbohydrates in pig manure is significantly reduced as compared with that in the diets. Certainly, there are some other components in pig manure samples, but they are not as dominant as these three major components.

Structural Variations with the Phytase Gene Modification. The chemical structures in manure from the JA line of transgenic pigs are in different proportions from those of conventional pigs. More carbohydrates and less nonpolar alkyls or COO/N−C═O were found in manure from transgenic pigs. Also, the chemical environments of nonpolar alkyls were more diverse in the manure from transgenic pigs than those from conventional pigs, suggesting that nonpolar alkyls are more altered in the digestive tracts of transgenic pigs. Previous studies have demonstrated a

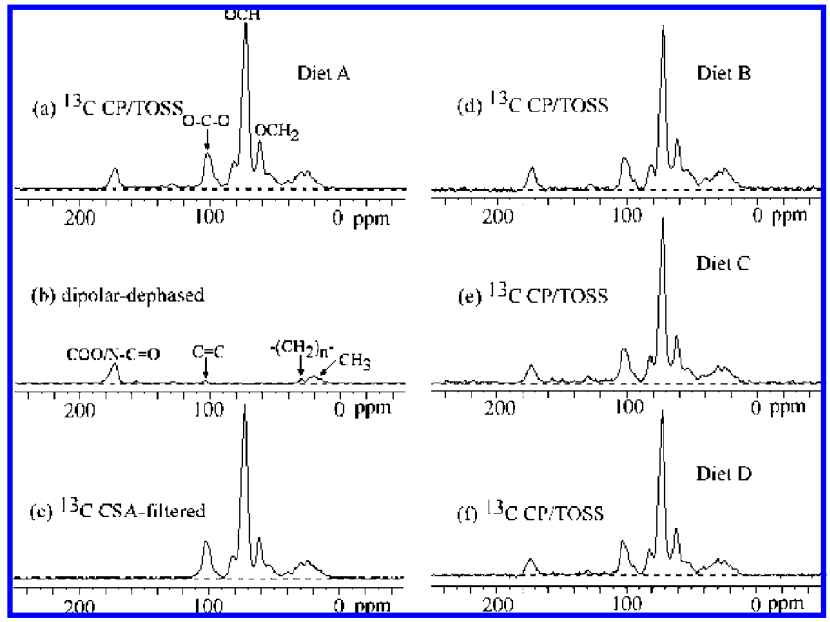

Figure 6. ^{13}C spectra of swine diets: (**a**) 13C CP/TOSS, (**b**) dipolar-dephased, and (**c**) ^{13}C CSA-filtered spectra of diet A; (**d**) ^{13}C CP/TOSS spectrum of diet B; (**e**) ^{13}C CP/TOSS spectrum of diet C; and (**f**) ^{13}C CP/TOSS spectrum of diet D. Each with 2K scans and a recycle delay of 3 s.

Table 2. Quantitative Information of Different Functional Groups of Manure from Pigs Fed with Different Diets and at Different Growth Stages[a]

chemical shift region	164–210 ppm	112–164 ppm	63–112 ppm	48–63 ppm	0–48 ppm
assignment	COO/N−C═O	arom./olefinic C═C	carbohydrate	NCH	nonpolar alkyls
Yorkshire-conven-grower	17.2%	11.4%	13.9%	7.3%	50.2%
Tg JA-conven-grower	17.8%	9.5%	**16.8%**[b]	6.7%	**49.1%**
Yorkshire-lowP-grower	19.5%	12.9%	10.3%	6.7%	50.4%
Tg JA-lowP-grower	**17.1%**	10.6%	**18.2%**	7.8%	**46.4%**
Yorkshire-conven-finisher	22.3%	11.1%	8.2%	6.1%	52.4%
Tg JA-conven-finisher	**18.4%**	16.6%	**11.6%**	8.5%	**45%**
Yorkshire-lowP-finisher	19.6%	12.5%	10.2%	6.7%	51%
Tg JA-lowP-finisher	**13.0%**	12.4%	**18.2%**	8.5%	**47.8%**

[a] Abbreviations are defined in the text. Information was derived from the quantitative DP/MAS spectra in **Figure 1**. [b] Some values are shown in bold to highlight the differences between transgenic and conventional treatments.

significant contribution of the gastrointestinal endogenous P in feces, and this contribution is affected by dietary P levels and sources and P availability (*35, 36*). Thus, this might have resulted from the fact that manure P from the transgenic pigs was more of gastrointestinal endogenous origins than the dietary origins, as a majority of the dietary P was digested and absorbed in the upper tract.

This research has demonstrated the effectiveness of NMR techniques for characterizing swine manure, and it has provided some of the first information available regarding properties of manure from transgenic pigs. The potential significance of our findings to environmental and human health issues is unknown, but it provides valuable leads for follow-up studies to explore the differences.

ABBREVIATIONS USED

CP/MAS, cross-polarization magic angle spinning; CP/T_1-TOSS, cross-polarization/spin–lattice relaxation time−total sideband suppression; CSA, chemical shift anisotropy; DEPT, distortionless enhancement by polarization transfer; DP/MAS, direct polarization magic angle spinning; FT-IR, Fourier transform infrared; Tg JA-lowP-finisher, JA line of Yorkshire transgenic (Enviropig) pigs fed a low-P diet during the finishing stage; Tg JA-conven-grower, JA line Yorkshire transgenic pigs fed a conventional diet during the growing stage; Tg JA-conven-finisher, JA line Yorkshire transgenic pigs fed a conventional diet during the finishing stage; Tg JA-lowP-grower, JA line of Yorkshire transgenic pigs fed a low-P diet during the growing stage; HETCOR, heteronuclear correlation; T_1, spin–lattice relaxation time; TPPM, two-pulse phase-modulated; LG-CP, Lee−Goldburg cross-polarization; NMR, nuclear magnetic resonance; Yorkshire-conven-grower, Yorkshire conventional pigs fed a conventional diet during the growing stage; Yorkshire-conven-finisher, Yorkshire conventional pigs fed a conventional diet during the finishing stage; Yorkshire-lowP-grower, Yorkshire conventional pigs fed a low-P diet during the growing stage; Yorkshire-lowP-finisher, Yorkshire conventional pigs fed a low-P diet during the finishing stage; P, nonpoint phosphorus; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

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