Feng YZ, Chen RR, Hu JL, Zhao F, Wang JH, Chu HY,
Zhang JB, Dolfig J, Lin XG.

[Bacillus asahii comes to the fore in organic manure fertilized alkaline soils.](#)

Soil Biology and Biochemistry 2015, 81(2), 186-194.

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DOI: 10.1016/j.soilbio.2014.11.021

Link to published article:

<http://dx.doi.org/10.1016/j.soilbio.2014.11.021>

Date deposited:

27/01/2015

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1   **Title Page**

2   **Types of contribution:** Research Articles

3   **Title:** *Bacillus asahii* Comes to the Fore in Organic Manure Fertilized Alkaline Soils

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Abstract

Organic manure (OM) fertilization has a profound impact on agroecosystems. However, little is known about temporal responses and roles of the specific soil microbial guilds involved in the increases of soil fertility and crop yield triggered by OM fertilization. To unravel these interactions, a series of fresh and archived soil samples from a fertilization experiment started in 1989 in North China Plain (NCP) was systematically investigated. Molecular assays of contemporary fresh samples unravel that *Bacillus asahii* responded most distinctly to OM fertilization, while no shifts in microbial community structure were observed between chemical fertilizations and the control without fertilization; a series of archived soil samples from 1989-2009 reveal that the indigenous *B. asahii* took 2-4 years to become specifically dominant and its ratio fluctuated between 40% and 72% during 20 years. Culture-dependent assessments of isolated *B. asahii* strain further indicate that its rise subsequently played a key role in the increases of both crop yield and soil fertility, especially via accelerating carbon and phosphorus cycling. This insight deepens our understanding of how OM impacts agroecosystems through soil microbial processes, and highlights the possibility of using archived microbial information as a reference to develop an efficient and sustainable agricultural strategy.

Keywords: Organic manure fertilization, Soil fertility, Crop yield, *Bacillus*, Archived soils

1. Introduction

One of the biggest global challenges for this century is to resolve the incongruity between a continuously increasing human population and shrinking cropland area, while at the same time maintaining or improving soil fertility and protecting the environment. This issue is especially serious in China. Today, China has 9% of the world's arable land yet has to feed 21% of the world's population, while its soils are not particularly fertile. To resolve this contradiction, China has become the world's largest user of synthetic fertilizer, consuming 36% of the global total (Hvistendahl, 2010). Inevitably, the large amount of chemical fertilizer applied has adverse effects on soil fertility and environmental quality (Hvistendahl, 2010; Zhang et al., 2013). Given the need to boost crop yields and soil fertility while minimizing associated negative impacts on the environment (Zhang et al., 2013), it is imperative to understand the relationships between fertilization strategies and the agroecosystem. To unravel these relationships, a long-term field fertilizer experiment, including OM fertilization and various chemical fertilizations with NK (nitrogen-potassium), NP (nitrogen-phosphorus), and NPK (nitrogen-phosphorus-potassium) as well as a control without fertilization, was set up in 1989 at Fengqiu Agro-ecological Experimental Station, Chinese Academy of Sciences. The soil was a sandy loam (alluvial-aquic) soil with alkaline characteristics, which is the common type of soil in the agroecosystems in North China Plain (NCP). Data from this experiment after it had been running for 20 years show that compared to chemical fertilizations, in view of sustainability, OM fertilization is a promising agricultural practice to increase both crop yield (Table S1) and soil fertility (Table S2) (Chen et al., 2010), as it ameliorates soil properties abiotically (Gong et al., 2009) and biotically (Zheng et al., 2009) and confers greater benefit for the environment (Meng et al., 2005). However, in the first eight years OM

fertilization resulted in relatively low crop yields, compared to NPK fertilization (Table S1). Only after eight years of OM fertilization this yield difference disappeared. Similar observations have been made elsewhere (Mader et al., 2002; Seufert et al., 2012). These impediments could be due to the issue that, unlike chemical fertilizer, nutrients in organic manure are not directly available for crop uptake but await soil microbial help.

Soil microorganisms are responsible for degradation and transformation of soil organic matter and concomitant release of nutrients. Meanwhile the soil microbial community is also sensitive to OM amendment. Shen et al. (2010) found that 20-year OM fertilizations bring shifts in soil bacterial community composition in acid soil in Northeast China. Ling et al. (2014) revealed that long-term OM application can increase soil bacterial diversity and their enzyme activities. Thus eight years of OM fertilization apparently successfully reshapes soil microbial community structure to efficiently degrade organic manure, which in turn releases sufficient nutrients to meet the needs of crop growth. Insight in the succession and role of this microbial community therefore can contribute to a comprehensive understanding of how OM fertilization influences agroecosystems and helps to establish a point of reference which hopefully can guide us towards more effective OM fertilization strategies, first and foremost by shortening the lag time of the increase in crop yield. To achieve this goal, five Ws should be answered first. Which microbial species respond most distinctly to OM fertilization? Where are they from? When and why do they become dominant and what are they doing in the OM fertilized soil? Unfortunately, due to the limitation of techniques used, Shen et al. (2010) and Ling et al. (2014) only detect the difference in the relative abundances of DGGE band-like bacterial species between OM and other treatments and can't give the exact answers. To answer these questions,

a series of archived soil samples from 1989-2009 and current soil samples from a long term field experiment at the Fengqiu Agro-ecological Experimental Station were systematically investigated by culture-dependent and -independent approaches.

2. Materials and Methods

2.1. Description of the Long-term Experiment

The long-term field fertilizer experiment was conducted at Fengqiu Agro-ecological Experimental Station, Chinese Academy of Sciences, Fengqiu County, Henan Province, China (35°00'N, 114°24'E). The soil, a typical soil in the North China Plain (NCP) with a profile of sandy loam (about 9% clay, 21.8% silt) in the plough layer and loam in the subsoil, was derived from alluvial sediments of the Yellow River and is classified as Aquic Inceptisol (a calcareous fluvo-aquic soil). The crop succession was winter wheat (*Triticum aestivum* L.) and summer maize (*Zea mays* L.), and there was no substantial change in agronomic practice for more than 20 years. The soil contained 5.83 g kg⁻¹ of organic matter, 0.45 g total N kg⁻¹, 0.50 g total P kg⁻¹, and 18.6 g total K kg⁻¹ and had a pH of 8.65 at the beginning of the experiment in 1989. The detailed experimental design and fertilization regimes have been documented by Meng et al. (2005) and Gong et al. (2009). Briefly, seven treatments with four replicates in completely randomized blocks were established. The treatments were: (1) OM: application of organic manure (supplemented with P and K as chemical fertilizers for the same amount of nutrients as other treatments); (2) 1/2OMN: application of half organic manure plus chemical fertilizer NPK (3) NPK: balanced application of chemical fertilizer NPK; (4) NP: application of chemical fertilizer NP (5) PK: application of chemical fertilizer PK; (6) NK: application of chemical fertilizer NK; and (7) Control: no fertilization.

The organic manure applied was also described in detail by Meng et al. (2005) and Gong et al. (2009). The composition of organic manure was constant during all years. It contained wheat straw, soyabean oil cake and cotton cake in a ratio of 100:40:45. The chemical properties of the organic manures after composting are 422 g total C kg⁻¹ dried weight (DW), 54.4 g total N kg⁻¹ DW, 18.5 g P₂O₅ kg⁻¹ DW and 23.5 g K₂O kg⁻¹ DW.

2.2. Sample Collection and Determination

A series of soil samples from 1989 to 2009 under different fertilizations were collected. On 18 September 2011 (at maize harvesting stage), three plots for each of NK, PK, NPK, OM, and Control treatments (total of 15 out of 28 plots) were chosen for soil sampling. Soil samples were collected from 16 points at the depth of 0-15 cm from each plot, and then mixed and homogenized by passing through <2 mm sieve to remove aboveground plant materials, roots, and stones. Sub-samples for the strain isolation were pooled and kept at 4°C. Samples for molecular studies were kept at -80°C until further use.

2.3. Soil DNA Extraction

For each soil, genomic DNA was extracted from the same amount of moist soil (0.5 g) on the day after sampling using a FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. The extracted soil DNA was dissolved in 50 µl of TE buffer, quantified by a spectrophotometer and stored at -20°C until further use. A total of 12 DNA samples were used for qPCR and bar-coded pyrosequencing analyses.

2.4. Preparation of the Amplicon Libraries for 454 Pyrosequencing

For each soil sample, the following primer set was used to amplify approximately

400 bp of bacterial 16S rRNA gene fragments for sequencing on the 454 GS-FLX pyrosequencing platform: 519F (CAGCMGCCGCGGTAATWC) and 907R (CCGTCAATTCMTTTRAGTTT) (Xia et al., 2011). The oligonucleotide sequences included the 454 Life Science A or B sequencing adapters (19 bp) fused to the 7-bp bar-coded primer set as follows: Primer B (GCCTTGCCAGCCCGCTCAG) + barcode + forward primer; and Primer A (GCCTCCCTCGCGCCATCAG) + reversed primer. PCR was carried out in 50- μ l reaction mixtures with the following components: each deoxynucleoside triphosphate at a concentration of 1.25 mM; 2 μ l (15 μ M each) of forward and reverse primers; 2 U of *Taq* DNA polymerase (TaKaRa, Japan); and 50 ng of DNA. Each reaction mix received 1 μ l of genomic community DNA as a template. Thirty-five cycles (95°C for 45 s, 56°C for 45 s, and 72°C for 60 s) were performed with a final extension at 72°C for 7 min. Triplicate reaction mixtures per sample were pooled, purified using the QIAquick PCR Purification kit (QIAGEN), and quantified using a NanoDrop ND-1000 (Thermo Scientific, USA). The bar-coded PCR products from all samples were normalized in equimolar amounts before pyrosequencing by means of a Genome Sequencer FLX System platform (454 Life Science Branford, CT, USA).

2.5. Processing of Pyrosequencing Data

The bacterial 16S rRNA gene data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) 1.4.0-dev pipeline ((Caporaso et al., 2010); <http://www.qiime.org>) using default parameters unless otherwise noted. In brief, sequences were quality trimmed (> 25 quality score and 200 bp in length), and assigned to soil samples based on unique 7-bp barcodes. Sequences were denoised (Reeder and Knight, 2010) and then binned into operational taxonomic units (OTUs) using a 97% identity threshold, and the most abundant sequence from each OTU was

selected as a representative sequence for that OTU. Taxonomy was assigned to bacterial OTUs against a subset of the Silva 104 database (<http://www.arb-silva.de/download/archive/qiime/>). OTU representative sequences were aligned using PyNAST, and chimera sequences were removed through QIIME. A phylogenetic tree was then constructed using FastTree2 (Price et al., 2009) to support phylogenetic diversity calculations.

Richness of phylotypes was calculated to compare community-level bacterial diversity at a single level of taxonomic resolution. We also estimated phylogenetic diversity using Faith's index (Faith, 1992), which provides an integrated index of the phylogenetic breadth across taxonomic levels. In this diversity analysis, 106,842 bacterial sequences that passed QIIME's quality filtering were included. We obtained between 3,030 and 11,068 sequences per sample for all soil samples (mean=7,123 and median=5,655). Because an even depth of sampling is required for beta diversity calculations, we reduced the datasets to the lowest number available to correct for differences in survey effort between samples. Namely, we calculated both diversity metrics using a randomly selected subset of 3,000 sequences per soil sample. This approach allows us to compare general diversity patterns among sites even though it is highly unlikely that we surveyed the full extent of diversity in each community (Shaw et al., 2008). The weighted pairwise UniFrac distances (Lozupone and Knight, 2005) were calculated for community comparisons using QIIME and were visualized using non-metric multidimensional scaling plots as implemented in PRIMER v6 (Clarke and Warwick, 2001).

2.6. PCR Amplification for Clone Library and DGGE

Primer set pB/pH was used to amplify the ca. 1300-bp fragment of *Bacilli* 16S rRNA gene fragment (De Clerck et al., 2004). PCR was carried out in 50- μ l reaction

mixtures with the following components: each deoxynucleoside triphosphate at a concentration of 1.25 mM; 2 μ l (15 μ M each) of forward and reverse primers; 2 U of *Taq* DNA polymerase (TaKaRa, Japan); and 50 ng of DNA. The PCR profile consisted of an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 68°C (for DGGE analysis) or 58°C (for clone library analysis) for 90 s and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. For DGGE analysis, the product of this selective PCR was further diluted 1:100 and used as template for a second PCR, using primers Ec1055 and Ec1392. The PCR profile consisted of an initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 45 s and extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

2.7. Clone Library Analysis

Three independent 1300-bp PCR products for each sample, amplified with primer set pB/pH, were mixed and purified using QIAquick PCR Purification kit (QIAGEN). Ligation into pMD18-T vector (TaKaRa) and transformation into *Escherichia coli* DH5a were performed following standard procedures. The ampicillin-resistant clones containing correct gene size were randomly picked out and were sequenced by Invitrogen Sequencing Department in Shanghai. Totally, nineteen clone libraries were constructed and 981 recombinant DNA clones (average 53 clones for each replicate of Control, NPK and OM samples on 2011; each 50 clones for the OM libraries on 1989, 1990, 1991, 1992, 1993, 1994, 1995, 2003 and 2005) were screened and analyzed by software MOTHUR v.1.33.3. Further, one representative clone sequence with 97% of similarity after sequence comparisons was used for phylogenetic identification.

2.8. DGGE Fingerprinting Analysis

A DCode Universal Mutation Detection System (Bio-Rad, Hercules, Calif.) was used for DGGE analysis. Approximate 150-250 ng PCR amplicons of 350-bp *Bacilli* ribosomal genes from each soil sample were electrophoresed on a 10% acrylamide-bisacrylamide gel, with 30% to 70% denaturant at 130V for 8 h in 1×TAE running buffer at 60°C. The gels were visualized and digitalized by using a Gel Doc^{TM} EQ imager combined with Quantity one 4.4.0 (Bio-Rad). The representative bands were excised, left overnight in 25 µl Milli-Q water, reamplified and run again on the DGGE system to ensure purity and correct mobility of the excised DGGE bands. Correct PCR products were purified using the QIAquick PCR Purification kit (QIAGEN) before cloning.

The purified PCR amplicons of the excised DGGE bands were cloned into a pMD18-T vector (TaKaRa) and transformed into *Escherichia coli* DH5α competent cell. Six random clones containing correct gene size for each DGGE band were sequenced by Invitrogen Sequencing Department in Shanghai. DNASTAR software package was used to manually check and compare the clone sequences. One representative clone sequence with high quality after sequence comparison from each band was used for phylogenetic analysis.

2.9. Phylogenetic Identification

Together with the top three BLAST hits of homologous gene sequences and the *Bacilli* ribosomal gene sequences from cultured and well characterized species in Genbank, the representative clone sequences or DGGE band sequences were used to build a basic phylogenetic tree by the neighbor-joining method using the software package of MEGA 4.0 version (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2007). The tree topology was further evaluated by different methods including Minimum Evolution and Maximum Parsimony. The phylogenetic relationships of

Bacilli ribosomal gene sequences to the closest homolog in the GenBank were then inferred.

2.10. Isolation Methods and Phenotypic Characterization

One gram of archived soil sample of 1995 was added to 9 ml of sterile distilled water and heated in a hot water bath at 80°C for 20 min. Then the samples were diluted and appropriate dilutions were spread on sporulation medium with low amounts of nutrients (1.0 g glucose, 1.0 g peptone, 0.7 g yeast extract, 0.2 g MgSO₄ 7H₂O, 0.2 g (NH₄)₂SO₄ 1.0 g K₂HPO₄ and 20 g agar, dissolved in 1 L of distilled water). About 200 colonies were isolated from the sporulation medium and screened on the malt extract agar medium in comparison with the reference *Bacillus asahii* strain JCM12112. The colonies whose growth rate and morphology were similar to those of reference *B. asahii* strain JCM12112 were picked out and grown on the specific medium of *B. asahii* (10 g polypeptone, 2.0 g yeast extract, 1.0 g MgSO₄ 7H₂O and 20 g agar, dissolved in 1 L of distilled water). Finally one colony was phylogenetic identified by 16S rRNA gene.

Light microscopes were used to observe the shapes of the isolated strains. For light microscopes, the spores were stained by malachite green and *Bacillus* cells were stained by safranine. Metabolic properties and enzyme activities were determined using GP2 MicroPlate™ (BIOLOG), API 20 NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The activities of soil polyphenol oxidase and lipase were measured following the protocols of Keilin and Mann (1938) and Margesin et al. (2002) respectively. Bacterial ability to produce biosurfactant was assessed through the drop collapse test (Bodour and Miller-Maier, 1998).

2.11. Statistical Analyses

Statistical procedures were performed with the SPSS 13.0 software package for Windows. Data were expressed as the means with standard deviation (SD), and the letters indicate significant differences between the results of the different samples. Mean separation was assessed by Tukey's multiple range test. Differences at $p < 0.05$ were considered statistically significant. DNA fingerprints obtained from the *Bacilli* 16S rRNA gene banding patterns on the DGGE gels were photographed and digitized using Bio-Rad's Quantity One software. Using the digital matrix obtained from DGGE, the similarities (or dissimilarities) among genotypes from the different soils could be quantified using cluster analysis. Euclidean distances were calculated from relative positions and intensities of bands, and the samples were clustered using Pearson's product-moment coefficient and a UPGMA algorithm.

3. Results

3.1. Shifts in Bacterial Community Composition in Response to Long-term OM Fertilization

In 2011 pyrosequencing of soil bacterial DNA was conducted. A total of 106,842 sequences of bacterial 16S rRNA gene fragments were obtained from soils that had been exposed to five different fertilization regimes (Fig. S1). The sequences were analyzed and clustered and a heat map of the relative abundance of dominant bacterial phylotypes (OTUs) was produced (Fig. 1A). Hierarchical clustering of OTUs revealed that OM fertilization had a greater influence on bacterial community composition than fertilization with NK, NP and NPK: the bacterial community compositions after 22 years of OM fertilization clustered together and were distinct from those of other fertilizations. In contrast, chemical fertilizations didn't influence the bacterial community structure as compared to the control. A non-metric multi-dimensional

scaling (NMDS) plot also indicated that OM fertilization resulted in a shift in the soil bacterial community (Fig. S2); Chao1 values indicating the maximal number of species were significantly higher in OM fertilization than in other fertilizations (Table S3). The heatmap diagram (Fig. 1A) and the 100% stacked column chart (Fig. S1) further showed that the relative abundance of Firmicutes was significantly increased under OM fertilization: from 5% in the control up to 14% ($p<0.05$) (Figs. 1A and S1).

3.2. Shifts in *Bacilli* Community Composition in Response to Long-term OM Fertilization

With a higher resolution, pyrosequencing data revealed that OM fertilization significantly increased the ratio of *Bacilli*-like sequences in Firmicutes to 92.1% in comparison to 84.0% in the control ($p<0.05$) (Fig. 1B and Table S4), while chemical fertilizations didn't have an effect. Meanwhile, the composition of the *Bacilli*-like OTU-ensemble under OM fertilization was different from those under other fertilizations (Figs. 1B and S4).

To accurately identify the specific *Bacilli* species that positively respond to OM fertilization, a clone library targeting *Bacilli* 16S rRNA gene fragments (length 1250-bp) was constructed (Fig. 2A). The clone library (84.6% coverage) with on average 53 amplicons per sample, with triplicates, revealed that long-term OM fertilization significantly influenced the composition and diversity of the *Bacilli* community (Figs. 2A and B, Table S5). The Shannon (S), Richness (H) and Evenness (Eh) indices all were significantly decreased by OM fertilization ($p<0.05$). In contrast, the ratio of OTU1 in OM fertilization was significantly increased to 23.5% ($p<0.05$), compared to 4.2% in the control. Meantime, the ratio of OTU2 (27.6%) was also significantly ($p<0.05$) higher in OM fertilization than that (2.3%) in the control, while the ratios of OTU1 and OTU2 were not different between NPK fertilization and the

control. Phylogenetic analysis indicated that both OTU1 and OTU2 were affiliated with *Bacillus asahii* (Fig. 2C). Collectively, the ratio of *Bacillus asahii* was 51.1% in the *Bacilli* community in fresh OM fertilized soils.

3.3. Succession of *Bacilli* Community Composition during 20 Years of OM

Fertilization

PCR-DGGE targeting *Bacilli* species in archived soil samples from 1989 to 2009 was then conducted to chart their community composition during 20 years of OM fertilization (Fig. 3). Cluster analysis implied that four groups of *Bacilli* community compositions were formed during 20 years of OM fertilization: 1989, 1990-1995, 1996-2003 and 2004-2009, that is the *Bacilli* community structure had gone through three phases. In contrast, no changes in *Bacilli* community composition were observed under NPK fertilization (Figs. S5-S8) and in the control (Figs. S9-S10). For validation of Fig. 3, triplicates of several representative OM fertilization samples of various years – indicated in red in Fig. 3 –, and of manure were chosen and re-analyzed by DGGE (Fig. S11). The results supported the abovementioned conclusion that OM fertilization greatly affected *Bacilli* community composition (Fig. S12), and revealed that the *Bacilli* community composition in manure was most distinct from the others (Figs. S11 and S12). With respect to specific phylotypes, DGGE band 4 appeared in the 1993 DGGE fingerprinting profile and became dominant after 1994; DGGE band 7 came to the fore in 1990; no other changes in the DGGE fingerprints bands were observed (Figs. 3 and S11). Both DGGE bands did not appear in the manure DGGE profile (Fig. S11). These observations indicate that these are indigenous *Bacilli* species that are stimulated by manure input. The phylogenetic analysis revealed that the majority of excised DGGE bands including DGGE band 7, were affiliated with *Virgibacillus*; DGGE band 4 was closely related to *Bacillus asahii* (Fig. S13).

To verify and extend the results of DGGE analysis with respect to the succession of *Bacilli* community composition during 20 years of OM fertilization, nine representative clone libraries (82.4% coverage) of on average 50 *Bacilli* amplicons of 1250-bp length per sample were constructed (Fig. 4) plus one for manure (Fig. 4 and Table S6). Consistent with the above DGGE results, OM fertilization resulted in profound changes in the *Bacilli* community composition (Table S7). OTU1 and OTU2, which are closely related to *B. asahii* (Figs. 4 and 2C), first appeared in the *Bacilli* community in 1990 and 1991 respectively. OTU1 became dominant in 1991, the 2nd year of OM fertilization, while OTU2 followed in 1992 (Fig. 4). Subsequently, their ratios fluctuated between 22% and 42% for OTU1 and between 6.5% and 30% for OTU2 (Table S6). Namely, the ratio of *B. asahii* in the *Bacilli* community fluctuated between 40% and 72% during nearly 20 years. No OTU affiliated with *Bacillus asahii* was observed in manure.

3.4. The Assessment of the Isolated *B. asahii* Strain from OM Fertilized Soil

To study the potential roles of *B. asahii* in OM fertilized soil, we isolated an indigenous *Bacillus* strain (no. KJ528250) from an archived OM fertilized soil sample from 1995. The sequence similarity of the 16S rRNA gene fragment of this *Gram*-positive, spore-forming, rod-shaped aerobic bacterium (Fig. 5B), with *B. asahii* JCM 12112 (GenBank/EMBL/DDBJ no. NR_024817) was 100% (Fig. 5C). The isolate can use a wide variety of carbon sources (Fig. S14), including β -hydroxy butyric acid and several short-chain fatty acids (Fig. S14B). To evaluate its *in situ* effect on soil enzyme activities, we inoculated this strain to current NPK fertilized soil, with the control without inoculation. As shown in Fig. 5D, the inoculation significantly increased the activities of both soil polyphenol oxidase and lipase ($P < 0.05$). Furthermore, we detected that the new isolate could produce biosurfactant

(Fig. S15).

4. Discussion

The NCP covers an area of nearly 310,000 km² and is among the most important agricultural production bases in China. With a dominant winter wheat-summer maize double cropping system, it provides more than 50% of the nation's wheat and about 33% of maize production (Wang et al., 2012), yet its land has low indigenous soil fertility. Nearly 20-year data at Fengqiu Agro-ecological Experimental Station reveal that OM fertilization is the most promising practice, considering the sustainability of soil fertility and crop growth, though a lag in the response of crop yield is recorded (Tables S1 and S2). Due to the important roles in this network, the responses of soil microorganisms to long-term OM fertilization have been systematically investigated previously (Chu et al., 2007; Lin et al., 2012; Zheng et al., 2009). However, the information on the temporal responses of key soil microorganisms as well as their roles is still elusive.

4.1. B. asahii Responds Most Distinctly to Long-term OM Fertilization

To unravel this information, the first question raised is what changes had occurred in the soil microbial community. Pyrosequencing of contemporary soil bacterial DNA was conducted. It is found that OM fertilization had a greater influence on bacterial community composition than fertilization with NK, NP and NPK (Figs. 1A, S1 and S2 and Table S3), which resulted from the significant changes in soil chemical properties under long-term OM fertilization (Table S8). This finding is consistent with other reports on long-term OM fertilization experiments. Shen et al. (2010) found that 20-year OM fertilizations bring shifts in soil bacterial community composition in acid soil in Northeast China, dominated by *Acidobacteria*,

Bacteroidetes, Gemmatimonadetes, Alphaproteobacteria, Actinobacteria, Gammaproteobacteria and Firmicutes. Lentendu et al. (2014) reported that the community composition of eukaryotic microorganisms is influenced by OM fertilization to a greater extent than by mineral fertilizations. Both Mäder et al. (2002) and Ling et al. (2014) observed that OM fertilization enhances the biodiversity of soil microbial community dominated by Actinobacteria, γ -Proteobacteria, Acidobacteria and abundant unclassified species, as well as their enzyme activities. Ling et al. (2014) further speculated that these changes could result from the shift of specific functional bacterial guild. In this investigation, the relative abundance of Firmicutes was significantly increased under OM fertilization ($p<0.05$) (Figs. 1A and S1): OM fertilization resulted in the significantly increased *Bacilli* abundance ($p<0.05$) and changes in *Bacilli* composition (Fig. 1B and Table S4). These results validate our previous conclusion (Chu et al., 2007) that OM fertilization selectively stimulates some *Bacilli* species.

This leads up to the question exactly which microbial species responded most distinctly to OM fertilization. Based on the information derived from pyrosequencing, a clone library targeting *Bacilli* 16S rRNA gene fragments (length 1250-bp) was constructed (Fig. 2A). Consistently with the results presented above, it was found that long-term OM fertilization influenced the composition and diversity of the *Bacilli* community (Figs. 2A and B, Table S5). With respect to specific species, the ratios of OTU1 and OTU2 were significantly higher in OM fertilized soil than in the control ($p<0.05$), while their ratios were not different between NPK fertilized soil and the control. Phylogenetic analysis indicated that OTU1 and OTU2 were highly affiliated with *Bacillus asahii* (Fig. 2C). Thus, *B. asahii* most distinctly responded to long-term OM fertilization and currently, its ratio was 51.1% in the *Bacilli* community. The type

strain of *B. asahii* was originally isolated from soil (Yumoto et al., 2004). Until now, this microorganism has been exclusively detected in alkaline soils exposed to a large amount of organic matter input, such as an Indian soil that had been irrigated with pulp and paper mill effluent for 25 years (Yadav et al., 2011), and a Kuwaiti soil that had been polluted with oil (Sorkhoh et al., 2010). *B. asahii* has the ability to metabolize short-chain fatty acids and does not produce acid from carbohydrates, which is quite rare among members of the genus *Bacillus* (Yumoto et al., 2004). We speculate that it could be these specific physiological characteristics that make *B. asahii* become dominant in the abovementioned soils. Furthermore, the pervasiveness of alkaline soil with heavy organic matter input could stimulate extensive interest into this microorganism.

4.2. Indigenous B. asahii Takes 2-4 Years to Become Specifically Dominant in OM Fertilized Soils

The above results raise the further questions when *B. asahii* became dominant in response to OM fertilization and from where the organism originated. Previous work has revealed that microbial information is well preserved in and can be retrieved from archived soils (Dolfing et al., 2004). Clark and Hirsch (2008) subsequently demonstrated that air-dried soil can protect microbial DNA for more than 150 year and offer an invaluable resource for research, especially for the soil amended OM. They further revealed that the information of spore-forming Firmicutes is especially well preserved, probably due to their stress resistance. Also, *Bacilli* are capable of forming endospores with improved long-term viability under adverse conditions (Nicholson et al., 2000), and therefore should be relatively well conserved in dried soils. Thus we used the availability of archived soil samples from 1989 to 2009 under different fertilizations at Fengqiu Agro-ecological Experimental Station to unravel the

historical responses of *B. asahii* to OM fertilization.

PCR-DGGE fingerprinting (Figs. 3, S11 and S13) and clone library (Figs. 2C and 4) analyses targeting *Bacilli* species in archived soil samples were reciprocally conducted to chart their community composition during 20 years of OM fertilization. They consistently indicate that *B. asahii* is indigenous in NCP arable soil but not in manure, and becomes dominant in the soil *Bacilli* community after 2-4 years of OM fertilization. The high percentage of *B. asahii* - up to 72% in the *Bacilli* community - implies that this microorganism was even dominant in the bacterial community as a whole, which is corroborated by our previous observation of a dominant DGGE-band affiliated with *Bacillus* that only appeared in OM-fertilized soil samples in 2007 (10). Although the intricacies of the mechanism by which indigenous *B. asahii* became dominant are still elusive and need further investigation, the historical information unearthed here should be helpful in guiding us towards a more effective OM fertilization strategy, shortening the lag time of the increase in crop yield and further increasing that yield. Furthermore, the difference in times between the 2-4 years needed by *B. asahii* to come to the fore and the eight years needed by OM fertilization to catch up with the crop yield of NPK fertilization imply that *B. asahii* functions as a vanguard and plays a key role in soil microbial communities under OM fertilization.

4.3. The Increase of B. asahii Plays a Key Role in Promoting Crop Yield and Soil Fertility

To unlock the potential roles of *B. asahii* in OM fertilized soil, an indigenous *B. asahii* strain was isolated from an archived OM fertilized soil sample. This isolate can use a wide variety of carbon sources (Fig. S14), including β -hydroxy butyric acid and several short-chain fatty acids (Fig. S14B). Fermentation of the wheat straw based organic manure used in the Fengqiu experiment results in the formation of a variety of

short-chain fatty acids, especially butyrates (Namihira et al., 2011), which are excellent substrates for *B. asahii* (Yumoto et al., 2004). Besides, the ameliorated soil physical properties, such as aggregate stability, due to long-term OM fertilization and further plant growth should also be considered (Zhou et al., 2013). Both the abovementioned could be parts of underlying mechanisms that selectively stimulated *B. asahii* in OM fertilized NCP soils. Importantly, inoculation with this strain significantly increased the activities of both polyphenol oxidase and lipase ($P<0.05$) (Fig. 5D) in NCP soil. Both soil enzymes promote the transformation of soil organic matter into humus (Gupta et al., 2004; Sinsabaugh, 2010), the main constituent of soil organic carbon and a key factor in soil fertility. Besides, we detected that the new isolate could produce biosurfactant (Fig. S15), which can lower surface tension and facilitates biodegradation of organic manure and release of encapsulated nutrients (Jahanshah et al., 2013). Phosphorus availability is the limiting factor for crop growth (Lin et al., 2012) and microbial metabolism (Zheng et al., 2009) in NCP arable soils. Both alkaline phosphatase and naphthol-AS-BI-phosphate hydrolase were detected (Fig. S14A), indicating that the increase of *B. asahii* in the soil microbial community can enhance soil available phosphorus content for the growth of both crop and other soil microorganisms, the latter of which in return are in favor of crop yield and soil fertility in the long term (Lin et al., 2012). Finally, the increase in *B. asahii* might be of significance in biocontrol against plant pathogens. Sun et al. (2013) found *B. asahii* to be an excellent biological agent for cucumber downy mildew control. Collectively, these characteristics suggest that *B. asahii* plays a key role in enhanced soil fertility and crop yield in organic manure fertilized agroecosystems in NCP.

5. Conclusions

In this investigation, we analyzed a series of soil samples collected from a long

term field experiment at the Fengqiu Agro-ecological Experimental Station in China, showing that organic manure fertilization pushes *Bacillus asahii* to the fore in alkaline soils. Specifically, OM fertilization selectively stimulated the growth of indigenous *B. asahii* in alkaline arable soils, and *B. asahii* took 2-4 years to become specifically dominant. Subsequently, *B. asahii* functioned as a vanguard and its rise subsequently played a key role in the increases of both crop yield and soil fertility, especially via accelerating carbon and phosphorus cycling.

ACKNOWLEDGEMENTS

We thank Shengwu Qin and the other staff working for long-term located fertilization experiment, for their excellent field management. We also thank Huayong Zhang, Rui Wang, Qianhui Zu and Min Chen for their technical assistance. This work was supported by grants from the National Natural Science Foundation of China (41271256) to Y.Z.F. and (41371253) to C.R.R; Y.Z.F. was supported by grants from National Basic Research Program (973 Program) (2014CB954500), Foundation of the State Key Laboratory of Soil and Sustainable Agriculture (212000009) and Knowledge Innovation Program of Chinese Academy of Sciences (KSCX2-EW-G-16). C.R.R. was supported by grants from Knowledge Innovation Program of Chinese Academy of Sciences (ISSASIP1118-3). J.D. acknowledges a SAgE Faculty Research Fellowship from Newcastle University.

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Figure Legends

Fig. 1. 454 pyrosequencing-derived heatmap of dominant phyla of soil bacteria and cluster analysis of bacterial community composition based on 3000 sequences per sample (**A**) and ratio of *Bacilli*-like sequences in Firmicutes and shift in *Bacilli* community composition (**B**) under five different long-term fertilization regimes. Control: no fertilization; NK: application of chemical fertilizer NK; PK: application of chemical fertilizer PK; NPK: application of chemical fertilizer NPK; OM: application of organic manure.

Fig. 2. Composition (**A**) and diversity indices (**B**) of the dominant *Bacilli*-like sequences in response to different fertilizations in 2011 revealed by clone library (~1250 bp in length, 97% similarity). (**C**) The phylogenetic analysis of the dominant *Bacillus* OTUs in the clone library. Note: *S*, *H* and *Eh* mean Richness, Shannon and Evenness indices respectively.

Fig. 3. Cluster analysis of DGGE profiles of *Bacilli* community composition in archived OM fertilized soil samples from 1989 to 2009. Samples indicated in red were chosen to reconduct PCR-DGGE fingerprinting analysis of *Bacilli* 16S rRNA genes in triplicate (Fig. S11).

Fig. 4. Nine representative clone libraries of *Bacilli* communities during 20 years of OM fertilization plus one for manure (~1250 bp in length, 97% similarity).

Fig. 5. Colony (**A**) and light micrograph (**B**) of a 1-day-old culture of the isolated *Bacillus* sp.. The phylogenetic identification of isolated *Bacillus* strain (**C**), using the bacterial prime set 27F/1492R (~1500 bp in length). The effect of inoculation of the *Bacillus* strain (no. KJ528250) on the activities of soil polyphenol oxidase and lipase (**D**). The spores and cells were stained by malachite green and safranine respectively. Asterisks indicate significant differences between treatments.

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Figure 4

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