The core root microbiome of sugarcanes cultivated under varying nitrogen fertiliser application

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Summary

Diazotrophic bacteria potentially supply substantial amounts of biologically fixed nitrogen to crops, but their occurrence may be suppressed by high nitrogen fertiliser application. Here, we explored the impact of high nitrogen fertiliser rates on the presence of diazotrophs in field-grown sugarcane with industry standard or reduced nitrogen fertiliser application. Despite large differences in soil microbial communities between test sites, a core sugarcane root microbiome was identified. The sugarcane root enriched core taxa overlap with those of *Arabidopsis thaliana* raising the possibility that certain bacterial families have had long association with plants. Reduced nitrogen fertiliser application did not increase the relative abundance of root-associated diazotrophs or *nif* gene counts. Correspondingly, low nitrogen fertiliser crops had low biomass and nitrogen content reflecting a lack of major input of biologically fixed nitrogen, indicating that manipulating nitrogen fertiliser rates does not improve sugarcane yields by enriching diazotrophic populations under the test conditions. These findings advance our understanding of the relation between crop management practices and nitrogen metabolism in root associated microbiota, and should be considered when formulating guidelines for improved agricultural management procedures.

Introduction

The global sugarcane industry is driven to increased production by a strong demand for sugar and biofuel. To ensure high yields, it is common practice in Australia to supply as much as 200 kg ha^{-1} yr^{-1} synthetic nitrogen (N) fertiliser (Wood *et al.*, 2010) and up to 5-times higher rates in some sugarcane systems in China and India (Robinson *et al.*, 2011). Despite considerable N fertiliser input and intensive agronomy, the Australian sugarcane industry is experiencing

declining yields (Garside *et al*., 2005). On average, only 50% of N fertiliser is used by sugarcane crops globally (Robinson *et al*., 2011), which is similarly inefficient as reported for other crop systems (Chen *et al*., 2008). Over half of N fertiliser losses from agricultural soils occur because N-metabolising microorganisms gain energy from N fertiliser via urea hydrolysis, nitrification and denitrification, and generate nitrate and gaseous forms of N (Webster & Hopkins, 1996; Wrage *et al*., 2001). In Australia, high N fertiliser rates drive emissions of the potent greenhouse gas nitrous oxide (Allen *et al*., 2010) and off-site losses that cause algal blooms which threaten the integrity of the World Heritage listed Great Barrier Reef (Brodie *et al*., 2012; Park *et al*., 2012). Other problems linked to the use of synthetic N fertiliser include soil acidification (Guo *et al*., 2010) and loss of soil organic matter (Mulvaney *et al*., 2009).

Brazilian studies suggest that sugarcane can obtain in excess of 100 kg N ha^{-1} yr^{-1}, or 60% of the crops' requirement, from biological nitrogen fixation (BNF). It is therefore considered that sugarcane does not require intensive N fertiliser application (Urquiaga *et al*., 1992; Boddey *et al*., 2001; Urquiaga *et al*., 2012), with N derived from diazotrophic bacterial endophytes such as *Beijerinckia* spp. (Döbereiner, 1961), *Acetobacter diazotrophicus* (Cavalcante & Döbereiner, 1988), *Herbaspirillum* spp. (Baldani *et al*., 1986) and *Burkholderia* spp. (Perin *et al*., 2006). However, diazotrophic bacteria are inhibited under high N concentrations (Fuentes-Ramírez *et al*., 1999; Robertsen, 2006) similar to those generated with industry-standard N fertiliser application in Australia (Robinson *et al*., 2011; Holst *et al*., 2012). In addition to direct suppression of diazotrophic bacteria, it is possible that diazotrophic associations are indirectly selected for in the Brazilian sugarcane breeding program due to low N fertiliser input (≈50 kg N ha^{-1} yr^{-1}, Baldani *et al*., 2002) while in Australia high N fertiliser rates are used (150–200 kg N ha^{-1} yr^{-1}, Robinson *et al*., 2011). Accounts of diazotrophic bacteria associated with Australian

sugarcanes are limited to culture-based studies that have identified *A. diazotrophicus* (Li & MacRae, 1992) and *Klebsiella* spp. (Chapman *et al*., 1992), and an isotope study that concluded that BNF was an unlikely source of N for Australian sugarcanes under standard fertiliser application (Biggs *et al*., 2002).

Here, we used culture-independent profiling and metagenomics of sugarcane soil and root-associated microbial communities to assess the types and relative abundance of N-metabolising bacteria under low (40 kg N ha^{-1} yr^{-1}) and standard (160–180 kg N ha^{-1} yr^{-1}) fertiliser application at two field sites. Based on 16S ribosomal RNA (rRNA) gene amplicon profiling, we show that previously reported sugarcane diazotrophs belonging to the genera *Beijerinckia, Acetobacter* and *Klebsiella* were below detection in all samples and that certain *Herbaspirillum* and *Burkholderia* species were enriched in root communities but their relative abundance was not correlated with N treatment. No consistent enrichment of inferred diazotrophic populations was observed, and this was consistent with reduced sugarcane biomass and N content associated with low N fertiliser input. We conclude that passive manipulation of native diazotrophic populations by reducing N fertiliser rates is ineffective and does not generate conditions conducive of biological N fixation under the test conditions.

Results

Sample collection, sequencing and phenotypic metadata

In total, 432 samples (216 root plus rhizosphere, 216 bulk soil) were collected for microbial profiling representing two soil types and two geographic locations (Ayr and Mackay), three time points across two growth stages in the sugarcane crop cycle, two N application rates and three sugarcane varieties (**Table 1**). Of the 432 samples, 389 (195 root, 194 soil) were successfully

extracted and the 16S rRNA genes PCR-amplified and sequenced to produce community profiles. Sugarcane plastid sequences were removed from the data before microbial community analysis, leaving 1,236,674 quality-filtered reads. Average contamination from plastid reads was 7.9% in root samples and 0.4% in soil samples. Reads were clustered into a total of 12,199 operational taxonomic units (OTUs) each comprising ≥2 reads, at a sequence threshold of 99% identity which roughly corresponds to species-level units (Stackebrandt & Goebel, 1994). An additional 12 bulk soil samples each pooled from nine soil cores were collected for chemical analysis. The largest differences in soil chemistry were seen between sampling locations, followed by differences between growth season and N fertiliser application within each sampling location, particularly in the Mackay samples (**Table S1**). Sugarcane crop biomass and N content was measured during harvest at the end of each growing season. The primary variable associated with differences in biomass was N fertiliser application with standard application rates leading to consistently higher biomass and N content than the reduced rates for all sugarcane varieties at both locations (**Fig. 1**). Secondarily, a drop in yield was noted between the 2012 and 2013 growth seasons at both locations and for all sugarcane varieties (**Fig. 1**).

Effects of test parameters on microbial community composition

To quantify the relative effects of the test parameters on community composition, we used PERMANOVA to relate metadata describing each sample to its community composition. We found that compositional differences in both root and soil microbial communities were significantly related to soil type, sampling season and N fertiliser application in descending size of effect. Sugarcane variety (plant genotype) had the most subtle effect on community composition which was significant only in a subset of samples (**Table S2**). This order of effect was also supported by a PCA of community composition showing primary segregation of

samples by sampling location (soil type) and sample fraction (**Fig. 2**). We repeated this analysis separately on samples by location (Ayr and Mackay) and sampling season (Feb12, Nov12 and Feb13) to exclude the effects of geography and seasonality. We found that N fertiliser application was significantly associated with changes in the composition of the microbial community of all samples except for those obtained in November 2012 (Nov12) from Mackay (**Table S3**).

Microbial taxa enriched in Australian sugarcane root communities

Consistent with the rank order of influencing parameters, the composition of root microbial communities mirrored the composition of their corresponding soil communities, indicating that sugarcane recruits a subset of the native soil community to its root microbiome (**Fig. S1**). Despite the strong influence of soil type, a number of genera were enriched at least twofold in root communities irrespective of their relative abundance in the surrounding soil, with *Bradyrhizobium* having the highest relative abundance in the roots (4.92%) and *Erwinia* having the highest fold enrichment relative to soil (59 fold; **Table 2, Fig. S2**). The enrichment of these particular taxa suggests that sugarcane roots harbour a core microbiome that is dominated (in rank order) by Betaproteobacteria, Alphaproteobacteria, Bacteroidetes, Gammaproteobacteria, Actinobacteria, Chloroflexi and Firmicutes (**Table 2**).

Effects of fertiliser addition on diazotrophs

N fertiliser rate produced the largest difference in sugarcane yield and N content (**Fig. 1**), however, its effect on root and soil microbial community composition was smaller than soil type and sample fraction (root *versus* bulk soil). The significantly higher crop growth under standard fertiliser conditions indicates that native diazotrophs associated with sugarcane roots do not

increase sufficiently in number and/or activity to substitute for N fertiliser under the tested conditions. Of the classical sugarcane-associated diazotrophs, *Herbaspirillum* (0.59% average relative abundance in roots) and *Burkholderia* (2.35% average relative abundance in roots) were not enriched under low N conditions (**Table 2**). One other putative diazotrophic genus only recently reported to be associated with sugarcane, *Bradyrhizobium* (3.43% average relative abundance in roots) (Fischer *et al*., 2012; Rouws *et al*., 2014), was also not enriched under low N conditions. Instead, we noted nine OTUs representing a wide diversity of lineages that were significantly enriched in root communities under low N conditions, although the degree of enrichment was modest (1.2 to 3.7-fold; **Table 3**). Since most of these OTUs have unknown involvement in N cycling, we decided to directly assess changes in relative abundances of key N-metabolising genes by shotgun sequencing a subset of samples receiving standard or low N fertiliser application rates. Six root and eight soil samples from Ayr collected February 2012 were shotgun-sequenced for functional gene analysis. Using Hidden Markov Models (HMMs) to identify amino acid sequences in the shotgun data, we found no significant differences in the relative abundance of key N fixation genes (*nifDHK*) in root or soil samples receiving low N compared to standard N rates (**Fig. 3**). This is in agreement with the 16S rRNA-based community profiling indicating no consistent increase in the relative abundance of native diazotrophs under low N conditions.

Effects of fertiliser addition on nitrifying and denitrifying populations

Biotic conversion of fertiliser N through nitrification and denitrification is thought to be a major factor in observed loss of N (Azam *et al*., 2002); therefore we investigated the metagenomic datasets for key nitrifying and denitrifying genes, as well as urease, which converts urea fertiliser to ammonia. In soils receiving standard N fertiliser, relative abundances of *amoA*, *hao*, *narG*,

nirK and *norB* were elevated compared to soils under low N fertiliser application indicating increased nitrifying and denitrifying capacity for urea fertiliser and associated conversion to gaseous N forms, including nitrous oxide (**Fig. 3**). A slight increase in *nasB* (assimilatory nitrite reductase) abundance in standard compared to low N fertiliser soil indicates that there is likely increased assimilation of fertiliser N into microbial biomass. Root samples, however, showed no significant difference in gene abundances between low or standard N fertiliser (**Fig. 3**). Based on these results, we predict that certain soil populations opportunistically use N fertiliser as an additional energy source. These populations increase in abundance in the soil with increased fertiliser application but are unable to compete with the plant for this N source at the root. Analysis of microbial taxa and their function, including at high spatial and temporal resolution, will advance insight into the competitive ability of microbes and plants when sourcing N. There was no increase in urease genes (conversion of urea to ammonia), which could be due to the 4-month time lag between fertiliser application and sample collection and depletion of urea. The putative identities of these enriched soil populations were determined by matching the functional genes to the IMG reference genome database and to our 16S profile data. They include denitrifiers *Cryocola*, *Conexibacter*, *Mycobacterium*, *Bradyrhizobium*, *Rhizobium*, *Agrobacterium*, *Burkholderia*, *Ralstonia*, *Rubrivivax*, *Enterobacter* and *Dyella* and the nitrifier *Nitrosovibrio* (**Table 2**).

Discussion

The plant root microbiome is integral to host plant function (Weyens *et al*., 2009; Panke-Buisse *et al*., 2014). Microorganisms associated with the root system have been studied using culture-independent 16S rRNA-based methods such as denaturing gradient gel electrophoresis (DGGE),

terminal restriction fragment length polymorphism (TRFLP) and 16S rRNA clone libraries which provide a better overview of the *in situ* populations than culture-based studies (Amann *et al*., 1995). Using next generation sequencing platforms, culture-independent molecular methods have sufficiently improved to provide high resolution microbial community profiles (Lebeis *et al*., 2012). The first exemplar of a high-resolution analysis of the root microbiome is of the model plant *Arabidopsis thaliana* (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012; Bodenhausen *et al*., 2013; Schlaeppi *et al*., 2014). In these studies, soil type was the main determinant of the overall root community composition by acting as a 'seed bank' of microorganisms that can be recruited into the root-associated community. Despite major differences in the soil community composition, the *Arabidopsis* root microbiome is composed of a core set of microbial taxa. The present survey of root-associated communities of field-grown sugarcane is, to our knowledge, the first high resolution community profiling of a commercial crop investigating the effects of agricultural management practices including soil type, sampling season, fertiliser application and crop variety on root community composition. Similar to *Arabidopsis*, there was enrichment of a core set of microbial taxa in sugarcane root communities relative to bulk soil (27 bacterial families from a total of 397 detected) (**Table 2**) despite striking differences in the composition of the two soil microbial communities investigated, (**Fig. S1, Table S2**). The enriched core taxa in sugarcane root communities (**Table 2**) substantially overlap with the reported core set for *Arabidopsis* (**Fig. 4**; 18 out of a total of 41 families), raising the interesting possibility that a number of bacterial families have had long association with plants (Schlaeppi *et al*., 2014) at least since the divergence of monocots and dicots 150 million years ago (Chaw *et al*., 2004). Our data further show that many species-level OTUs (99% identity) are consistently enriched in the sugarcane rhizosphere if they were detected in both soil types (**Fig. S2**).

Culture-independent surveys of field-grown sugarcane in Brazil and China have previously been conducted using low-resolution 16S rRNA-based methods including DGGE (Dini-Andreote *et al*., 2010), TRFLP (Sheng *et al*., 2012) and 16S rRNA clone libraries (Pisa *et al*., 2011; Fischer *et al*., 2012; Magnani *et al*., 2013). 60 of 90 sugarcane root-associated genera reported in these previous studies were detected in the present study (**Table S4**), but only 18 of the subset of 45 core genera inferred to be enriched in sugarcane roots were previously reported (**Fig. S3; Table S4**). Of the enriched subset, the most frequently reported taxa are *Bradyrhizobium*, *Rhizobium*, *Burkholderia*, *Herbaspirillum*, *Bacillus* and *Streptomyces* (**Table 2**), suggesting that these genera commonly associate with sugarcane and likely constitute a major part of the core sugarcane root microbiome. Moreover, these six genera are associated with the roots of other plant species (Vessey, 2003; Seipke *et al*., 2012) and therefore likely constitute a generalist plant root core microbiome. Our survey substantially increases the number of bacterial taxa inferred to be specifically enriched in field-grown sugarcane roots including several uncharacterised lineages such as Ellin139 and HF_C_64, members of the Gammaproteobacteria and Bacteroidetes respectively (**Table 2**). These taxa were enriched to levels comparable to known important root-associated taxa such as *Bradyrhizobium*, therefore they may be potential candidates for the development of sugarcane 'probiotics' if proven to be beneficial.

One of the primary motivations for this study was to investigate the effect of N fertiliser application on the sugarcane root community and whether root-associated diazotrophs would become enriched in response to reduced N inputs and substitute for N fertiliser application. Nitrogen balance studies conducted on Brazilian-grown varieties showed that sugarcanes obtain

up to 60% of their N requirement through BNF (Boddey *et al*., 2001; Boddey *et al*., 2003; Urquiaga *et al*., 2012). In those studies, plant biomass was not significantly affected by N fertiliser application (Boddey *et al*., 2003; Taulé *et al*., 2012), suggesting that N fertiliser was not limiting crop productivity. Muthukumarasamy *et al*. (1999) and Reis-Junior *et al*. (2000) demonstrated that N fertiliser application significantly reduced counts of culturable diazotrophic bacteria in Indian and Brazilian sugarcanes, respectively. Similarly, the abundance of *Acetobacter diazotrophicus* was decreased in sugarcanes cultivated with high N supply (Fuentes-Ramírez *et al*., 1999; Medeiros *et al*., 2006). In contrast, our study shows that sugarcane biomass and N content was consistently higher in crops receiving the standard Australian fertiliser rate (**Fig. 1**) indicating that, in the tested conditions, lowered N fertiliser rates are insufficient for comparable crop productivity. This suggests that increased diazotrophic populations under low N conditions, if any, cannot substitute for synthetic N fertiliser under the test conditions. PERMANOVA and PCA analyses of microbial community composition (**Table S2**, **Fig. 2**) indicate that the effect of fertiliser application was subtle compared to other experimental variables such as soil type, microbial selection by roots and sampling season, further confirming that fertiliser application had only a small detectable effect on community composition as found in a long-term fertiliser input experiment (Ogilvie *et al*., 2008). Furthermore, no recognised diazotrophs were enriched in the roots under low N conditions relative to standard application rates (**Table 3**). Consistent with these observations, no nitrogen-fixing genes were significantly enriched under low N conditions (**Fig. 3**). Together, these findings further support our conclusion that passive manipulation of the Australian sugarcane root community through altered N fertiliser application rates does not substitute for fertiliser in achieving high crop productivity. The contrasting effects of lowered N fertiliser application on the productivity of sugarcane

varieties in Australia and Brazil and associated diazotrophic populations could be due to differences in sugarcane varieties, soils and/or N application rates and be the result of the overall agronomic history of sugarcane production. Accurate quantification of crop N sources remains a challenge due to the large endogenous soil N pool, that depending on soil type, consists of 1,000 to 10,000 kg N ha^{-1} (mostly as proteinaceous N), and N inputs from decaying plant matter, biomass burning and aerial deposition. Surrogate techniques for quantifying BNF, including acetylene reduction, ^{15}N-tracer dilution and ^{15}N natural abundance techniques have been criticised for their inaccuracy (Baldani *et al*., 2002). Another consideration are different growth potentials of sugarcane in the context of agronomic settings that may result in different N needs and associated crop yields, and indirectly affecting the contribution of BNF to the crops' N budget.

A key issue of high-production fertiliser intensive agriculture is associated nutrient loss that leads to environmental degradation (Beman *et al*., 2005). Run-off from Australian sugarcane agriculture is a significant source of N pollution in coastal waters (Paton-Walsh *et al*., 2011; Thorburn *et al*., 2011; Brodie *et al*., 2012). Soluble fertiliser breakdown products, predominantly nitrate, were elevated in Australian sugarcane soils following standard fertiliser application (Robinson *et al*., 2011) which, due to the high mobility of nitrate, is likely to contribute to N loss from soils. In the present study, significantly higher counts of nitrification- and denitrification-related genes were detected in soils receiving standard fertiliser application relative to the lower N rate (**Fig. 3**), suggesting that soil microorganisms use the additional fertiliser N for energy production through nitrification and denitrification. Our findings support current inferences that agricultural nutrient runoff and nitrous oxide emissions are exacerbated by biological processes, namely through microbial-driven nitrification and denitrification (Bremner, 1997; Azam *et al*.,

2002). These results call for improved fertiliser management to reduce N loss through biotic processes. Strategies could include addition of chemical or plant-generated nitrification inhibitors (Kleineidam *et al*., 2011; Subbarao *et al*., 2012), and/or use of enhanced-efficiency fertilisers that do not provide a readily usable substrate for microbial-driven N conversion (Chen *et al*., 2008).

Although passive manipulation of the sugarcane root microbiome to substitute for lowered fertiliser application was unsuccessful under our test conditions, active manipulation of the root microbiome by probiotic treatment may be a viable option to achieve equivalent crop yields under reduced fertiliser conditions. Greenhouse and field-grown sugarcanes inoculated with enriched cultures of sugarcane-associated microorganisms including *A. diazotrophicus*, *Herbaspirillum seropedicae* and *Burkholderia* species produced biomass comparable to non-inoculated controls supplied with fertiliser (Oliveira *et al*., 2002; Govindarajan *et al*., 2006; Muthukumarasamy *et al*., 2006; Oliveira *et al*., 2006). These promising results underpin the need for research and development of microbial inocula that provide tangible benefits to crop health including resistance against disease (Viswanathan & Samiyappan, 2000) and productivity (Oliveira *et al*., 2002; Oliveira *et al*., 2006; Paungfoo-Lonhienne *et al*., 2014). Our study provides a list of potential candidate genera naturally enriched in sugarcane roots that could contribute to this goal.

Experimental Procedures

Study site and sample collection

Samples were collected from two sugarcane (*Saccharum officinarum* x *spontaneum* L.) field trials situated approximately 300 km apart, near Ayr (Burdekin region; 19.733 S, 147.178 E) and

Mackay (21.164 S, 149.119 E) in north Queensland, Australia. Field experiments were established in 2010 (Ayr) and 2011 (Mackay) following cultivation of a sorghum (Ayr) or maize crop (Mackay) without N fertiliser addition to reduce the amount of available N in the soil. The set-up of each of plantings was replicated at each location. Each field trial consisted of 64 sugarcane varieties and unselected clones of diverse genetic background that were planted in a randomised design with six replicate blocks. Three blocks received the standard N fertiliser rate of 160 or 180 kg N ha^{-1} yr^{-1} at Mackay and Ayr, respectively, while the other three received a low N fertiliser rate of 40 kg ha^{-1} yr^{-1}. Each sugarcane type was grown in plots 10 m (Ayr) or 12 m (Mackay) long and consisting of four sugarcane rows with 1.8 m (Ayr) or 1.6 m (Mackay) spacing between rows. The two outer rows were considered guard rows protecting the middle two sampling rows from the influence of neighbouring sugarcane types (**Fig. S4**).

Root- and bulk soil-associated samples were collected from three of the 64 sugarcane types tested: Australian sugarcanes varieties Q186 and Q208 and Brazilian variety SP 79-2313. These varieties were chosen because both Australian varieties are widely planted in the region and initially showed contrasting responses to N treatment in the plant crop at Ayr, with Q186 performing much better with low N than Q208, but these differences were not pronounced at Mackay and in the ratoon crops at either location. The Brazilian variety was chosen to test if a low N environment stimulates BNF as has been stated for Brazilian varieties. Two replicate root and two corresponding soil samples were collected in each of three replicate blocks of both N fertiliser treatments, for a total of six biological replicates per variety. Roots of young (three months) and mature (eight months) sugarcanes with white root tips indicative of active growth were partially excavated in the row shoulder in the upper 15-20 cm of the soil. Root samples were standardised by taking the first 10 cm from the root tip. The soil immediately surrounding

the plant that was loosened by the root excavation was sieved through a 2 mm steel mesh laboratory sieve and collected as corresponding bulk soil samples. Samples were collected across one 12-month growth season from established first and second ratoon crops (Ayr) and plant and first ratoon crops (Mackay). Root and soil samples were stored in sterile containers and kept cool during field collection and transport, then frozen at -20°C until further processing.

Soil nutrient analyses

Soil types at Ayr and Mackay were podzol and chromosol, respectively. Nine soil cores from a randomly selected 25 x 25 m^2 area of the low and standard fertiliser treated plots were obtained for each location and each time point. Soils from the top 10 cm of each set of nine cores were pooled and thoroughly mixed to represent an average soil profile for the different fertiliser treatments. A portion of soils from each pool were weighed before and after drying at 105°C for one week to determine their moisture contents. The rest of the pooled soils were dried in paper bags and approximately 300 g of each pool was sent to the Soil and Plant Analysis Laboratory (CSBP Limited, Western Australia) for nutrient and elemental analysis. Measured properties and nutrients were soil conductivity, pH, ammonium (mg kg^{-1}), nitrate (mg kg^{-1}), phosphorus (mg kg^{-1}), potassium (mg kg^{-1}), sulphur (mg kg^{-1}), boron (mg kg^{-1}), organic carbon (%), copper (mg kg^{-1}), iron (mg kg^{-1}), manganese (mg kg^{-1}), zinc (mg kg^{-1}), aluminium (meq 100 g^{-1} soil), calcium (meq 100 g^{-1} soil), magnesium (meq 100 g^{-1} soil) and sodium (meq 100 g^{-1} soil).

DNA extraction and 16S rRNA gene sequencing

To facilitate the isolation of DNA from the sugarcane root system, root samples were frozen in liquid nitrogen and then ground using a sterile mortar and pestle. DNA was extracted according to manufacturer's instructions from 400–500 mg of ground root with an MP Biomedicals

FastDNA SPIN Kit (MP Biomedicals, LLC. Santa Ana, California). Similarly, 400–500 mg of soil was weighed and DNA extracted using a MO BIO PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, California) also according to manufacturer's instructions. DNA concentration was measured using a Qubit® fluorometer with Quant-it dsDNA BR assays (Invitrogen™, Thermo Fischer Scientific, Inc.) and then normalised with sterile water to 4 ng DNA μl^{-1}. 16S rRNA genes were amplified by PCR in 50 µl volumes containing 20 ng DNA, 1X PCR buffer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$, 0.3 mg bovine serum albumin, 0.02 U *Taq* DNA polymerase and 0.2 µM each of primers 803F (TTAGATACCCTGGTAGTC) and 1392R (ACGGGCGGTGWGTRC) modified to contain the 454 FLX Titanium Lib L adapters B and A, respectively. The 1392R primer contained a barcode sequence between the primer sequence and adapter. A unique barcode was used to amplify DNA from each sample to facilitate sample identification and demultiplexing after sequencing. Thermocycling conditions were: 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 90 s; and finally 72°C for 10 min.

Microbial community profile data processing and statistical analyses

Raw sequence reads were trimmed to 300 base pairs (bp) and quality-filtered with the Quantitative Insights Into Microbial Ecology (QIIME; Caporaso *et al*., 2010) script split_libraries.py and then checked for chimeras using UCHIME. Sequence homopolymer errors were checked and corrected using Acacia (Bragg *et al*., 2012). Using CD-HIT-OTU (Fu *et al*., 2012), sequences were clustered into operational taxonomic units (OTUs) of 99% similarity and assigned taxonomy with reference to the Greengenes 16S rRNA gene sequence database (May 2013 release) using BLAST (DeSantis *et al*., 2006). An OTU table containing OTU read counts and taxonomic assignments for all samples was generated using the QIIME script

384 make_otu_table.py. Plastid and mitochondrial OTUs were removed, and the number of reads per
385 sample was rarefied to 900 reads to normalise for sampling depth. OTU relative abundances of
386 the resulting rarefaction-normalised OTU table were then adjusted taking into account lineage-
387 specific 16S rRNA gene copy numbers using CopyRighter (Angly *et al*., 2014) to improve
388 microbial diversity estimates (Kembel *et al.*, 2012). Observed OTU richness and Simpson's
389 diversity index were calculated for each sample using QIIME. Differences in the composition of
390 root- and soil-associated microbial communities were assessed using permutational multivariate
391 analysis of variance (PERMANOVA) and principal component analysis (PCA). OTUs that
392 discriminated between treatments were identified using PCA. The significance of the differences
393 in these OTUs between treatments was then assessed using separate linear regression models. All
394 analyses were implemented using R version 3.0.1 (R Core Team, 2013) with the vegan package
395 (Oksanen *et al*., 2013).
396
397 *Root and soil metagenome sequencing and analysis*
398 Metagenomes were generated from bulk DNA of a subset of six rhizosphere and eight bulk soil
399 samples collected in Ayr February 2012. For root samples, DNA was processed with an
400 NEBNext® Microbiome DNA Enrichment kit to remove contaminating plant DNA and then
401 purified using Agencourt AMPure XP (Beckman Coulter, Inc) magnetic beads. Soil DNA was
402 directly purified using Agencourt AMPure XP magnetic beads. Concentration of the purified
403 DNA was adjusted to library preparation requirements and then submitted to the Ramaciotti
404 Centre for Genomics (University of New South Wales, NSW Australia) for sequencing. Root
405 DNA sequencing libraries were prepared using the Illumina® Nextera DNA Sample Preparation
406 Kit and soil DNA sequencing libraries were prepared using the Illumina® TruSeq DNA Sample
407 Preparation Kit. All DNA libraries were paired-end sequenced on an Illumina HiSeq 2500

producing 150 bp read lengths. Raw sequence data were processed by first removing sequencing adapters and then quality trimming using Nesoni clip (https://github.com/Victorian-Bioinformatics-Consortium/nesoni). Sequences were then translated in all six frames into amino acid sequences using EMBOSS Transeq (Goujon *et al*., 2010). Key functional genes for nitrogen fixation (*nifD*, *nifH* and *nifK* encoding the nitrogenase complex), urea hydrolysis (*ureC*, urease subunit α), nitrification (*amoA*, ammonia monooxygenase; *hao*, hydroxylamine oxidoreductase), denitrification (*narG*, nitrate reductase α subunit; *nirS*, nitrite reductase; *nirK*, copper-containing nitrite reductase; *norB*, nitric oxide reductase subunit B; *nosZ*, nitrous oxide reductase) and assimilatory/dissimilatory nitrate reduction (*nasA*, assimilatory nitrate reductase; *nasB*, assimilatory nitrite reductase; *narB*, nitrate reductase; *nirA* and *nirB*, nitrite reductases) were identified in the translated metagenomic datasets using using HMMER3 (Eddy, 2011) and Hidden Markov Model (HMM) profiles obtained from Pfam (Finn *et al*., 2014), TIGRFAM (http://www.jcvi.org/cgi-bin/tigrfams/index.cgi), FunGenes (http://fungene.cme.msu.edu) and the Functional Ontology Assignments for Metagenomes database (Prestat *et al*., 2014). Positive matches (E-value < 0.001) were counted individually for each HMM and abundance calculated as number of matches per million sequencing reads for comparison between samples. To determine which OTUs contributed to the key functional genes, DNA sequences from which translated amino acid sequences matched a target HMM profile were aligned to finished reference microbial genomes in the Integrated Microbial Genomes (IMG) database (Markowitz *et al*., 2012). 16S rRNA gene sequences from the matching IMG reference genomes were then aligned to the 16S rRNA sequences obtained from the community profile to identify probable OTUs harbouring these genes.

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Table and Figure legends

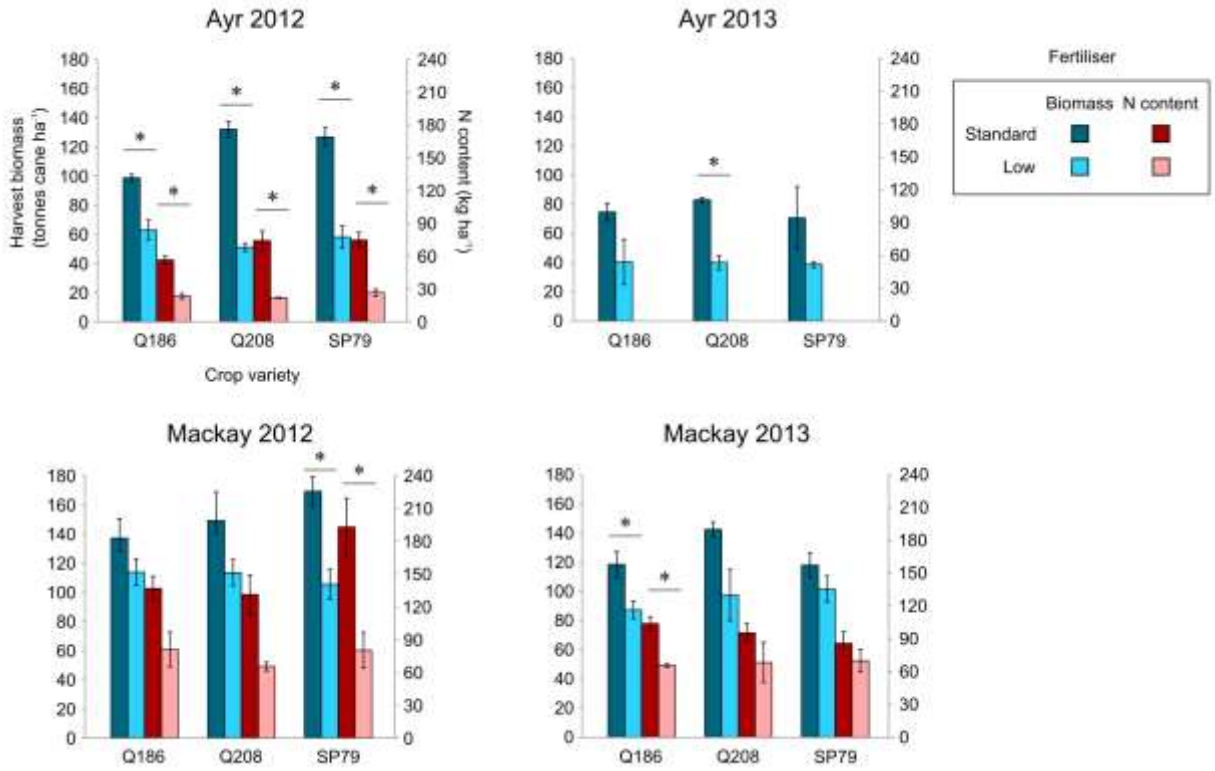
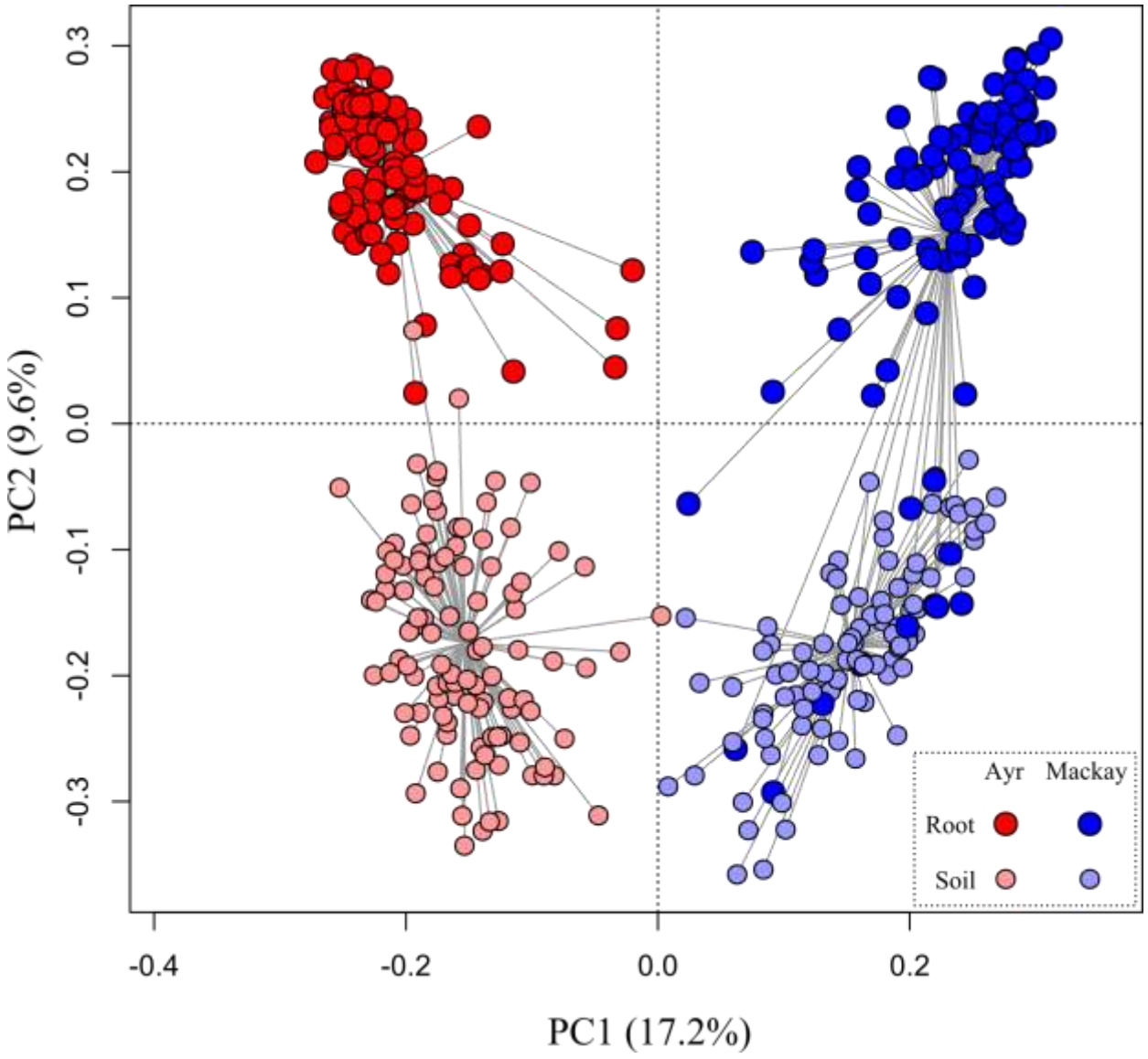


Fig. 1 Effects of fertiliser rate on sugarcane harvest biomass (blue columns) and nitrogen content (red columns). Dark coloured columns denote standard fertiliser samples while light coloured columns denote low fertiliser samples. Harvest biomass values are on the left vertical axis while nitrogen content values are on the right vertical axis. During both 2012 and 2013 harvests, the standard fertiliser crops were consistently associated with higher biomass and nitrogen content compared to the low fertiliser crops. Error bars denote SEM. *, $P < 0.05$. N content data unavailable for Ayr 2013.



675

676 **Fig. 2** Principal Component analysis (PCA) of field-grown sugarcane root and soil microbial
677 community composition. Community composition was determined by sequencing 16S rRNA
678 gene amplicons. The colour of the symbols indicates sampling location and sample fraction: dark
679 red (Ayr root samples), pink (Ayr soil samples), dark blue (Mackay root samples) and light blue
680 (Mackay soil samples). Principal components 1 and 2 explained 17.2% and 9.6% of the variance,
681 respectively.

682

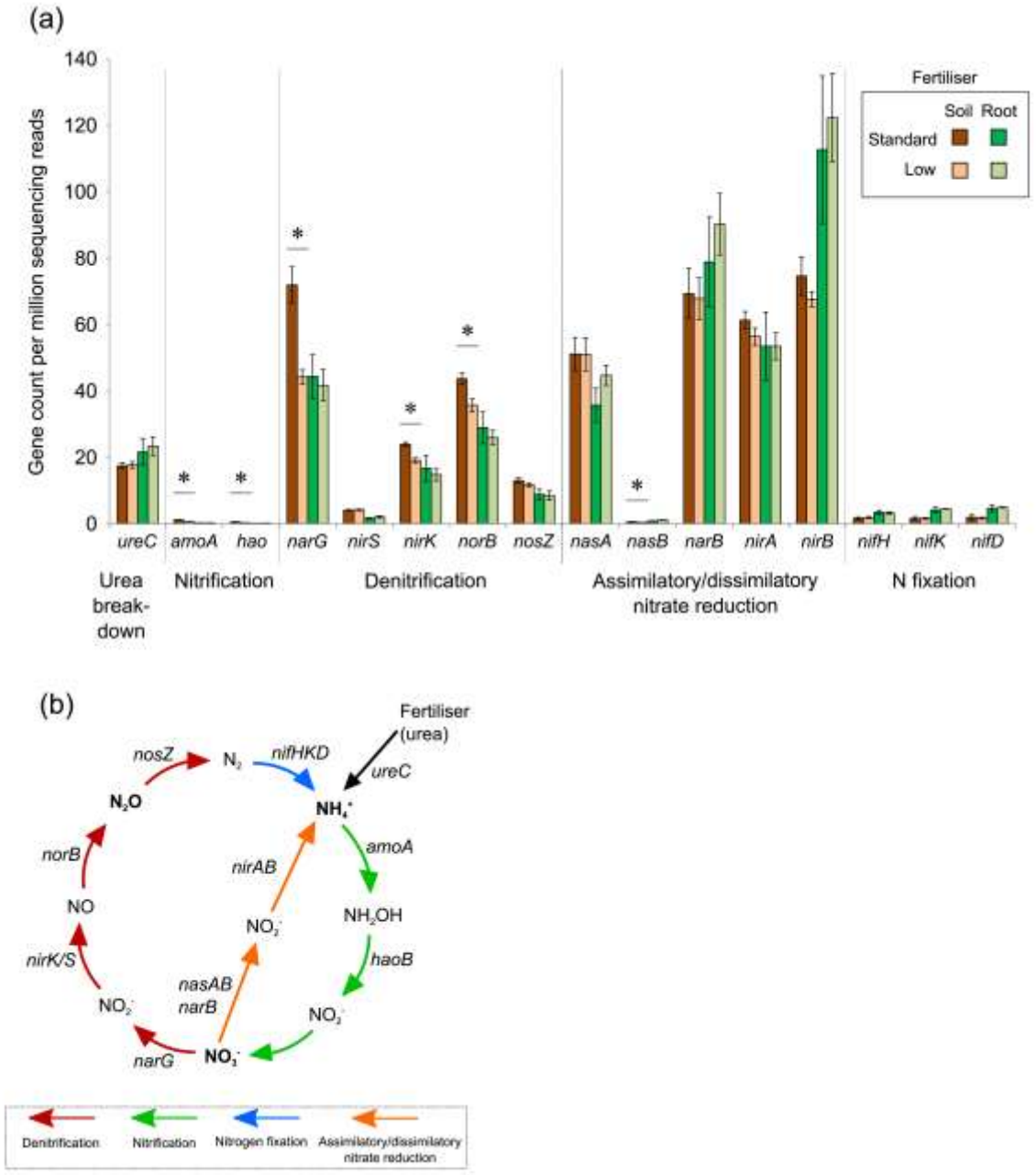


Fig. 3 Effects of fertiliser rate on the abundance of nitrogen cycling related genes. (a) A subset of six root (green columns) and eight soil samples (brown columns) were shotgun sequenced. Dark coloured columns denote standard fertiliser samples while light coloured columns denote low fertiliser samples. Gene counts were normalised by sample-specific number of sequencing reads to compare between samples. *ureC*, urease subunit A; *amoA*, ammonia monooxygenase; *hao*,

hydroxylamine oxidoreductase; *narG*, nitrate reductase α subunit; *nirS*, nitrite reductase; *nirK*, copper-containing nitrite reductase; *norB*, nitric oxide reductase subunit B; *nosZ*, nitrous oxide reductase; *nasA*, assimilatory nitrate reductase; *nasB*, assimilatory nitrite reductase; *narB*, nitrate reductase; *nirA* and *nirB*, nitrite reductases; *nifDHK*, nitrogenase complex. A diagram of the nitrogen cycle is provided to show the conversion of fertiliser nitrogen into various forms of nitrogenous compounds facilitated by microbial genes (b). Error bars denote SEM. *, $P < 0.05$.

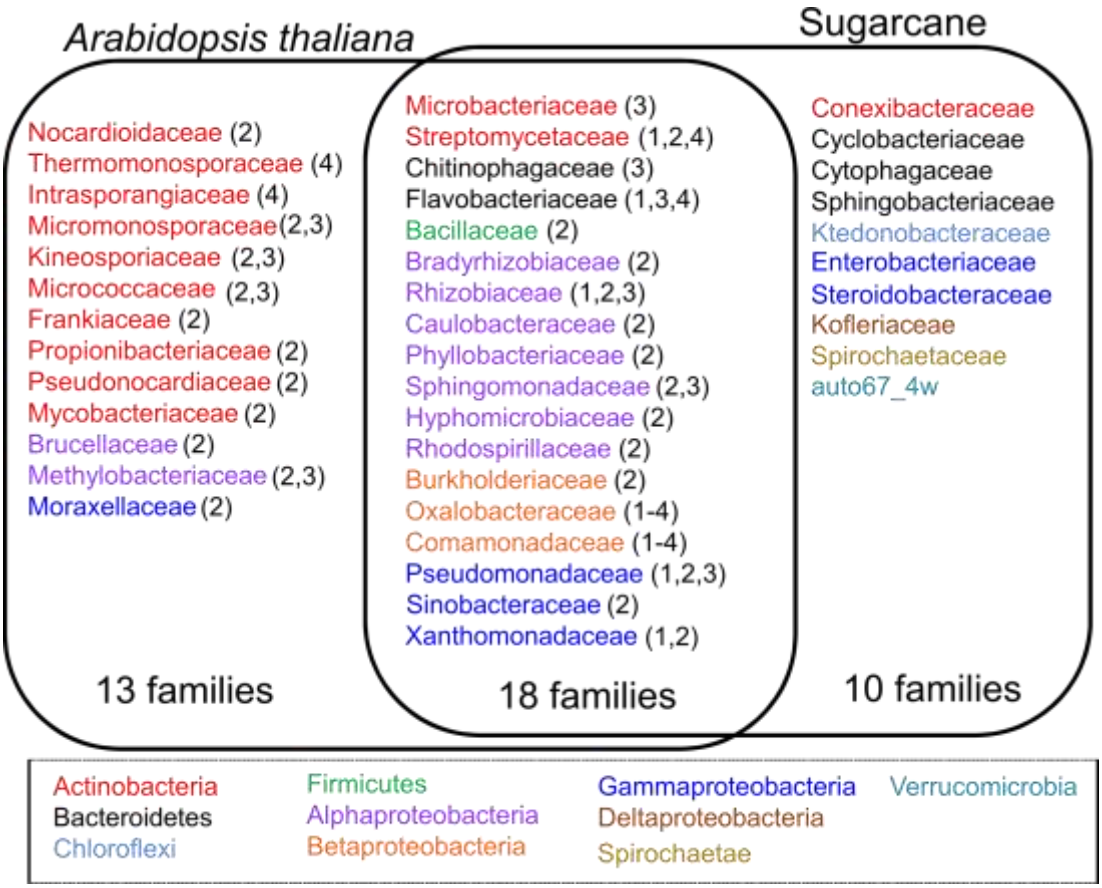


Fig. 4 Root-associated bacterial families shared between sugarcane and *Arabidopsis thaliana*. Families are colour-coded by phyla. *A. thaliana* bacterial families are based on (1) Bulgarelli *et al*., 2012; (2) Lundberg *et al*., 2012; (3) Bodenhausen *et al*., 2013 and (4) Schlaeppi *et al*., 2014 denoted by numbers in parentheses.

701 **Table 1** Experimental variables investigated in this study.

Variable		Levels
Sampling location	Ayr Mackay	2
Sample fraction	Root Soil	2
Sampling season	February 2012 November 2012 February 2013	3
Fertiliser application	Standard (160-180 kg N ha^{-1} yr^{-1}) Low (40 kg N ha^{-1} yr^{-1})	2
Crop variety	Q186 Q208 SP79-2313	3
Biological replicates	six	6
	Total number of samples	**432**

702

703

704 **Table 2** Microbial taxa enriched[1] in Australian field-grown sugarcane roots relative to

705 surrounding bulk soil.

		Ayr		Mackay		Previously reported		Metagenome	
Phylum/Class Family	Genus[2]	Average relative abundance in roots (%)	Fold enrich-ment	Average relative abundance in roots (%)	Fold enrich-ment	Culture-based studies	Culture-independent studies	Diazotroph	Denitrifier
Actinobacteria		**1.42**	**13.5**	**2.24**	**7.3**				
Streptomycetaceae	*Streptomyces*	0.91	19.3	0.74	14.9	16	7, 10, 14		Yes
Microbacteriaceae	*Cryocola*	0.40	2.9	0.40	3.5				
Conexibacteraceae	*Conexibacter*	0.01	2.4	0.77	2.9				Yes
Streptomycetaceae	*Kitasatospora*	0.10	4.1	0.33	5.4				
Bacteroidetes		**8.47**	**15.0**	**3.48**	**6.6**				
Chitinophagaceae	*Niastella*	2.37	6.2	0.72	7.0		10		
Chitinophagaceae	*Chitinophaga*	1.66	13.7	0.46	9.6		10		
Chitinophagaceae	HF_C_64	0.79	8.6	1.22	4.5				
Cyclobacteriaceae	*Ohtaekwangia*	1.18	44.3	0.06	16.2				
Flavobacteriaceae	*Chryseobacterium*	0.82	11.9	0.21	16.1	3	10		
Sphingobacteriaceae	*Mucilaginibacter*	0.36	12.3	0.63	4.6		10		
Cyclobacteriaceae	*Chryseolinea*	0.51	11.5	0.11	3.2				
Chitinophagaceae	*Filimonas*	0.39	13.9	0.04	9.8				
Cytophagaceae	*Dyadobacter*	0.39	12.3	0.03	4.0	16			
Chloroflexi									
Ktedonobacteraceae	P8-90	0.01	4.6	2.38	3.1				
Firmicutes									
Bacillaceae	*Bacillus*	1.15	2.3	0.11	0.9	3, 13, 16	7, 10, 14		
Alphaproteobacteria		**8.30**	**9.0**	**10.60**	**4.3**				
Bradyrhizobiaceae	*Bradyrhizobium*	1.94	2.7	4.92	2.5	18	7, 10, 12	Yes	Yes
Rhizobiaceae	*Rhizobium*	2.61	16.7	1.49	6.5	8, 15	10, 12	Yes	Yes
Caulobacteraceae	*Asticcacaulis*	0.67	10.5	0.96	11.4				
Sphingomonadaceae	*Sphingomonas*	0.89	6.6	0.55	5.0	3, 16			
Phyllobacteriaceae	*Mesorhizobium*	0.94	5.2	0.38	4.7		10		
Sphingomonadaceae	*Kaistobacter*	0.16	2.6	1.16	2.2		7		
Hyphomicrobiaceae	*Devosia*	0.45	2.4	0.43	3.5				
Rhizobiaceae	*Agrobacterium*	0.6	10.1	0.27	6.7	3, 16			Yes
Rhodospirillaceae	*Telmatospirillum*	0.04	9.4	0.44	6.2				
Betaproteobacteria		**12.64**	**11.8**	**11.37**	**8.7**				
Oxalobacteraceae	*Undibacterium*	2.46	5.2	2.91	11.6				
Burkholderiaceae	*Burkholderia*	2.21	16.1	2.49	4.0	5, 6, 16	10, 12	Yes	Yes
Comamonadaceae	*Roseateles*	2.95	24.8	0.76	8.4				
Comamonadaceae	*Rubrivivax*	1.39	3.3	2.09	10.3			Yes	Yes
Oxalobacteraceae	*Massilia*	1.62	9.5	1.57	8.6		10		
Comamonadaceae	*Methylibium*	1.02	2.5	0.11	2.2		10		
Comamonadaceae	*Variovorax*	0.51	4.6	0.21	6.4	3	10		
Oxalobacteraceae	*Herbaspirillum*	0.33	3.0	0.84	8.7	1, 2	7, 12		
Oxalobacteraceae	*Ralstonia*	0.15	11.7	0.39	12.0		10		Yes
Gammaproteobacteria		**4.48**	**16.7**	**8.03**	**5.2**				
Steroidobacteraceae	Ellin139	1.72	3.0	3.35	2.7				
Xanthomonadaceae	*Dyella*	0.43	13.4	1.66	3.2				Yes
Xanthomonadaceae	*Frateuria*	0.22	23.0	1.08	4.7				
Xanthomonadaceae	*Stenotrophomonas*	0.73	26.7	0.40	5.7	11, 15, 16		Yes	
Enterobacteriaceae	*Erwinia*	0.35	59.3	0.28	39.9	8			
Pseudomonadaceae	*Pseudomonas*	0.43	5.4	0.16	5.0	3, 6, 8, 16	17		
Enterobacteriaceae	*Enterobacter*	0.28	30.6	0.39	9.7	4	17		Yes
Enterobacteriaceae	Unclassified genus	0.21	32.9	0.37	10.5				
Xanthomonadaceae	*Gynumella*	0.11	7.5	0.34	2.4				
Deltaproteobacteria									
Kofleriaceae	FFCH3218	0.30	5.9	0.53	9.5				
Spirochaetae									
Spirochaetaceae	*Spirochaeta*	0.17	6.2	0.40	7.5		14		
Verrucomicrobia									
auto67_4W	VC12	0.08	2.0	0.59	3.9				

1. Threshold to qualify as enrichment: > 0.3% relative abundance in root samples from either site and > 2x fold increase in root relative abundance compared to soil. Fold enrichment is weighted by OTU relative abundance in root fraction.
2. OTUs are collapsed at genus level classification based on Greengenes taxonomy
3. 1 Baldani *et al*., 1986 (Brazil); 2 Olivares *et al*., 1996 (Brazil); 3 Pankhurst *et al*., 2000 (Australia); 4 Boddey *et al*., 2003 (Brazil); 5 Perin et al., 2006 (Brazil, Mexico); 6 Mendes *et al*., 2007 (Brazil); 7 Dini-Andreote *et al*., 2010 (Brazil); 8 Magnani *et al*., 2010 (Brazil); 9 Mehnaz et al., 2010 (India); 10 Pisa *et al*., 2011 (Brazil); 11 Ramos *et al*., 2011 (Brazil); 12 Fischer *et al*., 2012 (Brazil); 13 Ratón *et al*., 2012 (Brazil); 14 Sheng et al., 2012 (China); 15 Taulé *et al*., 2012 (Uruguay); 16 Beneduzi *et al*., 2013 (Brazil); 17 Magnani *et al*., 2013 (Brazil); 18 Rouws *et al*., 2014 (Brazil)

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Table 3 Operational taxonomic units enriched in sugarcane roots receiving low fertiliser application relative to standard fertiliser.

	Average relative abundance in low N fertiliser roots (%)	Fold-enrichment relative to standard N fertiliser roots
Bacteroidetes		
Ohtaekwangia	2.22	3.19
Niastella	0.59	1.74
JJ008	0.47	1.44
Actinobacteria		
Streptomyces	0.44	1.73
Solirubrobacter	0.13	1.20
Alphaproteobacteria		
Kaistobacter	0.27	1.86
Acidobacteria		
Acidobacteria-5	0.14	1.89
Koribacteraceae	0.11	1.99
Candidatus Koribacter	0.10	3.72

OTUs significantly enriched under low fertiliser application were determined using linear regression models.

Standard N fertiliser rate: 160 (Mackay) or 180 kg N ha^{-1} yr^{-1} (Ayr)
Low N fertiliser rate: 40 kg N ha^{-1} yr^{-1}

Supporting Information

Fig. S1 Heat map showing operational taxonomic units (OTUs) of more than 1% relative abundance in sugarcane root or soil samples. Rows representing OTUs are grouped by phylum or class. Each column represents averaged replicate relative abundances of the listed taxa and are organised by experimental factors. '++' denotes standard fertiliser while '+' denotes low fertiliser samples. Only Actinobacteria, Bacteroidetes and Proteobacteria lineages are included to emphasise root-enriched taxa.

Fig. S2 Ranked abundance graph of top 150 sugarcane soil OTUs ranked by relative abundance (blue columns) overlaid with its corresponding root sample relative abundance (red columns) in Ayr (a) and Mackay (b). Taxonomic labels are followed by a unique OTU identifier number and average fold enrichment in sugarcane root relative to soil in parentheses. Red taxonomic labels denote OTUs commonly enriched in both Ayr and Mackay sugarcane root microbial communities.

Fig. S3 Number of bacterial genera detected in present study compared to previously reported culture independent-based surveys of the sugarcane root (References 25, 26, 29). The core root OTUs are listed in Table 2.

Fig. S4 Sampling site set-up. Samples for this study were collected from two duplicate field sites located in Ayr and Mackay in Queensland, Australia. Each of the 64 sugarcane genotypes was planted in blocks consisting of four rows. The outer two rows served as guard rows to minimise influence of neighbouring sugarcane varieties on the two middle sampling rows. Three replicate blocks received standard Australian fertiliser rate (160–180 kg nitrogen ha^{-1} yr^{-1}) while another

three received a lowered rate (40 kg nitrogen ha^{-1} yr^{-1}). For each of the three commercial sugarcane varieties Q186, Q208 and SP79-2313 included in this study, two duplicate root and soil samples were collected from the middle sampling rows of every replicate block for a total of six biological replicates.

Table S1 Soil chemistry measurements from pooled soil samples.

Table S2 Factors influencing microbial community composition ranked by size of effect (R^2).

Table S3 Association between fertiliser application, crop variety and microbial community composition in samples analysed separately by location, sample fraction and sampling season.

Table S4 Number of bacterial genera detected in roots of Brazilian-grown sugarcane compared to present study.