The fungus-growing termite *Macrotermes natalensis* harbors bacillaene-producing *Bacillus* sp. that inhibit potentially antagonistic fungi

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The ancient fungus-growing termite (Mactrotermitinae) symbiosis involves the obligate association between a lineage of higher termites and basidiomycete *Termitomyces* cultivar fungi. Our investigation of the fungus-growing termite *Macrotermes natalensis* shows that *Bacillus* strains from *M. natalensis* colonies produce a single major antibiotic, bacillaene A (1), which selectively inhibits known and putatively antagonistic fungi of *Termitomyces*. Comparative analyses of the genomes of symbiotic *Bacillus* strains revealed that they are phylogenetically closely related to *Bacillus subtilis*, their genomes have high homology with more than 90% of ORFs being 100% identical, and the sequence identities across the biosynthetic gene cluster for bacillaene are higher between termite-associated strains than to the cluster previously reported in *B. subtilis*. Our findings suggest that this lineage of antibiotic-producing *Bacillus* may be a defensive symbiont involved in the protection of the fungus-growing termite cultivar.

Beneficial symbiotic associations between prokaryotes and eukaryotes are widespread in nature. Such mutualistic relationships include defensive symbioses, which often involve selective antibiotics produced by prokaryotes against host antagonists. Recent studies exploring chemical mediators of symbiotic interactions in beetles, fungus-growing ants, marine sponges, and solitary wasps have provided insights into the fundamental functions of natural antibiotics, as well as the discovery of novel bioactive small molecules genetically coded by insect-associated microorganisms.

Fungus-growing termites (Blattodea, Mactrotermitinae) are major decomposers in the Old World tropics, where they form some of the most complex colony and mound structures known (Fig. 1a). The success of the Mactrotermitinae is undoubtedly attributed to their engagement in a mutualistic symbiosis with *Termitomyces* fungi (Basidiomycota: Agaricales: Lyophyllaceae), which aid in the degradation of plant material. The fungus is housed on a special substrate (fungus comb) in the nest, which is maintained by the termites through the continuous addition of partially digested plant material that has passed through the termite gut along with asexual *Termitomyces* spores (Fig. 1b). In return for continuous provisioning of a substrate for growth, *Termitomyces* serves as a nitrogen-rich food source for the termites. The association originated more than 35 million years ago and none of the more than 350 species of fungus-growing termites, or the fungus symbionts they maintain, have abandoned this long-term association.

The success of termite fungiculture is expected to rely on the termites effectively defending both themselves and their cultivar fungus from invading competitors and diseases. The maintenance of the cultivar fungus in monoculture within individual nests is predicted to make the fungus prone to exploitation in the absence of the termites, but only few candidate antagonists of the symbiosis have been identified. Ascomycete fungi in the subgenus *Pseudoxylaria* (Fig. 1d) are prevalent in fungus-growing termite nests and appear to compete with *Termitomyces* for the substrate provided by the termites, and *Trichoderma* will rapidly overgrow the termite fungus when termite workers are absent.

Whether or not *Pseudoxylaria* and *Trichoderma* act as specialized disease-causing microbes in the fungus-growing termite symbiosis is not clear; however, their competitive and/or antagonistic potential against *Termitomyces* supports that it would be beneficial for the termite-fungus association to assure that they are...
Isolates were obtained by the application of termite colony material from the same termite species in addition to the beneficial Termitomyces fungus: Trichoderma sp. (Fig. 1e), Coriolopsis sp. (Fig. 1f), Umbelopsis sp. and Fusarium sp. Each bacterial strain was cultivated with each of the fungi on YEME agar plates. Seven days after inoculation, all three Bacillus strains inhibited the growth of all fungi except Termitomyces, suggesting the presence of a selective antifungal compound, which prompted us to make further efforts to identify the responsible compound.

Chemical identification of the antifungal compound. To find the compounds from the Bacillus strains responsible for the growth inhibition of the five fungi, we cultivated Bacillus strains in YEME liquid culture medium. An initial LC/MS (liquid chromatography and mass spectrometry) analysis of the ethyl acetate (EtOAc) extract of the cultures revealed a common major secondary metabolite in all three strains, and this compound displayed the typical polynye UV spectral feature (λmax at 346, 364, and 384 nm) and the low-resolution molecular ion [M + H]+ at m/z 581. Scaling up the culture conditions to 24 L of each of the Bacillus strains allowed for bioassay-guided fractionation to narrow down the active antifungal component in the extracts. The dried extract was fractionated under step gradient conditions using aqueous methanol (20, 40, 60, 80 and 100%) by open column reversed-phase chromatography on C18 resin. Each fraction was tested against Pseudoxylaria, Trichoderma, Coriolopsis, Umbelopsis and Fusarium using paper disk diffusion assays to trace active fractions.

The antifungal assays demonstrated that the 80% aqueous MeOH fraction of each Bacillus culture was the most active, and LC/MS analysis of the fraction revealed the common major compound initially detected in the crude extract. Purification of the compound by preparative reversed-phase high performance liquid chromatography (HPLC) yielded the pure compound (1), which possessed the molecular formula C34H48N2O6 based on electrospray high-resolution mass spectrum ([M + H]+ at m/z 581.3585). Subsequently, we analyzed 1H and two-dimensional NMR spectra of compound 1. Specifically, 1H-1H correlation (COSY; Fig. S3), heteronuclear single quantum coherence (HSQC; Fig. S4), and heteronuclear multiple bond correlation (HMBC; Fig. S5). The spectroscopic analysis and literature search identified compound 1 as bacillaene A, a polyene polyketide secondary metabolite29 (Fig. 2a).
We confirmed that bacillaeone A is responsible for the antifungal activity observed using an antifungal assay (Fig. 2b). Petri dishes were observed daily for 30 days (Fig. 2c; Fig. S1). Bacillaeone A (1) inhibited the growth of the fungi in a dose-dependent manner (Fig. 2c).

**Genomic identification of the bacillaeone biosynthetic gene cluster.** We obtained whole-genome data for two of the three Bacillus strains using mate-paired Illumina HiSeq sequencing, and genomes were assembled using Velvet32 and OSLay33 and annotated using BASYS34. The 16S rRNA genes were extracted from the genomes to obtain a phylogeny placement using the Ribosomal Database Project35 (RDP), which identified both strains as being indistinguishable from Bacillus subtilis (Fig. 3). Draft genome comparisons revealed that the two strains are almost identical with 4548 shared ORFs being 100% identical at the nucleotide level, 393 shared ORFs being less than 97% identical, despite the genomes being only at the draft level (Table 1; Table S1). However, when compared to B. subtilis 168, only 402 of the ORFs identified in Bacillus #9 are 100% identical to B. subtilis and only 455 of the ORFs identified in Bacillus #11 are 100% identical to B. subtilis (Table S1). We confirmed the presence of the genes necessary for bacillaeone production in the two draft Bacillus genomes of strains #9 and #11, and compared the 16 bac genes coding for the pks complex coding for bacillaeone A (1) to the published sequences obtained for Bacillus subtilis strain 16836–39. The ca. 80 kb gene cluster for the synthesis of bacillaeone in both Bacillus #9 and #11 also has 16 genes, which are organized identically to those of B. subtilis (Fig. 4). Pairwise comparisons of individual pks genes between genomes indicated comparable percentage identities between the termite-associated strains and B. subtilis. However, the two termite-bacilli strains are more similar to each other across the entire gene cluster (Fig. 4; Table S3). In fact, Bacillus #9 and #11 are nearly 100% identical both at the nucleotide and amino acid level in 15 of the 16 genes, with the only exception (98% identical) being the pksH gene, which functions to install a methyl group, not part of the backbone of bacillaeone (Fig. 4). This implies that termite-Bacillus produce the same chemical compound and are more genomically similar to each other, including with regard to the bacillaeone gene cluster, than to bacillaeone-producing species not associated with fungus-growing termites.

**Discussion**

Our findings provide the first evidence of a specific role of bacillaeone A (1) in a biological system. We obtained this result through optimization of the production and isolation of bacillaeone A (1), combined with minimizing degradation of the compound by avoiding exposure to light. The compound was initially identified from Bacillus subtilis as an antibiotic agent inhibiting prokaryotic protein synthesis40. It was reported to display antibacterial activity against various gram-negative (Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, and Serratia marcescens) and gram-positive (Bacillus thuringiensis and Staphylococcus aureus) bacteria. However, it did not show antifungal activity against the yeasts Saccharomyces cerevisiae and Candida albicans40. Even though the gene cluster and the biosynthesis of bacillaeone have been relatively well studied31,41,42, its biological role in nature has been poorly understood, possibly because of its notorious instability41. Comprehensive analysis of Bacillus amyloliquifaciens, which is a prolific bioactive secondary-metabolite producer, including bacillaeone A (1), suggested a potential role of B. amyloliquifaciens as a defensive symbiont controlling plant pathogens43,44. Our findings suggest that bacillaeone A (1) produced by the Bacillus obtained from three different colonies of M. natalensis could...
aid in the suppression of antagonistic fungi of the cultivar fungus *Termitomyces*. If so, our findings suggest that i) bacillaene inhibits phylogenetically diverse filamentous fungi, ii) *Bacillus* strains and their secondary metabolites could play a symbiotic role in nature, and iii) *Bacillus* could play a symbiotic role in an ancient mutualism between social insects and fungi.

*Macrotermes natalensis* workers continuously bring in partly degraded plant material (mainly decaying wood) to their colony, and this substrate inevitably harbours microbes that have the potential to compete with or antagonize the termites’ mutualistic fungus. The substrate for *Termitomyces* is not directly incorporated into the fungus comb, but experiences obligate gut passage prior to incorporation17. The maintenance of defensive gut microbes that can aid in selective inhibition of antagonistic fungi consequently allows for the termites to control the characteristics of the comb substrate to avoid entry of harmful fungi. Our findings suggest that a lineage of *Bacillus* serves a defensive role through the production of a major compound, bacillaene A (1) that does not harm the termites’ mutualistic fungus, but suppresses the growth of known (*Pseudoxylaria* and *Trichoderma*) and putative antagonistic fungi of the symbiosis. The *Bacillus* lineage has so far been identified in *Odontotermes formosanus*30 and *M. natalensis* this study, two of the most ecologically important and phylogenetically diverse fungus-growing termite genera18. Phylogenetic comparison of the 16S rRNA gene between the *Bacillus*

Figure 3 | A 16S rRNA gene phylogeny placing *Bacillus* #9 and #11 (indicated with an arrow) in a global *Bacillus* phylogeny, showing that *Bacillus* sp. associated with fungus-growing termites are indistinguishable from *B. subtilis* based on the full-length 16S rRNA gene.
strains identified in this study with those identified by Mathew et al.60 (Fig. S6), showed that M. natalensis strains were 98.2 ± 0.03% (mean ± SE) similar across the 437 bp fragment to those obtained from O. formosanus guts, while they were more distant from isolates from fungus comb (94.1 ± 0.96%). This suggests that the same operational taxonomic unit likely is present in the two termite species and that the Bacillus strains we isolated in this study likely originated from the termite gut; however, whether a specific lineage of Bacillus associates with the entire Macrotermiteinae sub-family remains to be explored.

Bacillaene-producing Bacillus has so far been identified in both fungus-growing termite guts and within the fungus comb,9,10 this study. This suggests that Bacillus suppression can take place both during the passage of crude forage through the termite gut, which may allow for partial or complete suppression of incoming fungi, and also later within the fungus comb when Termitomyces hyphae decompose the comb substrate. This is possible because Termitomyces itself is not adversely affected by bacillaene, which provides an interesting contrast to the utilization of bacteria-derived antifungals in the other major fungus-farming symbiosis: the Neotropical fungus-growing termite. This suggests that the same operational taxonomic unit is present in the two termite species and that the Bacillus strains we isolated in this study likely originated from the termite gut; however, whether a specific lineage of Bacillus associates with the entire Macrotermiteinae sub-family remains to be explored.

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**Methods**

**Collections.** Three isolates of Bacillus (#9, #11 and #13) were obtained from three different Macrotermes natalensis colonies collected in MooiKloof (previously Naboospruit, S24°40’30.5”E28°47’50.4”, elevation 1,045 m), South Africa on the 15th of January 2010. Isolates were obtained by crushing workers in PBS and plating on Chitin (per liter: 4 g chitin, 0.7 g KHPO4, 0.3 g KH2PO4, 0.5 g MgSO4·7H2O, 0.01 g FeSO4·7H2O, 0.001 g ZnSO4·0.001 g MnCl2 and 20 g agar) or microcrystalline (per liter: 5 g microcrystalline and 20 g of agar) medium. After ca. 14 days of growth on these low-nutrient media, Bacillus-like CFUs were transferred to Yeast Malt Extract Medium (per liter: 10 g malt extract, 4 g yeast extract, 4 g glucose, 15 g agar).

**Chemical analyses.** Bacillus strains #9, #11 and #13 were cultivated in 25 mL YEME liquid medium (per liter: 10 g malt extract, 4 g yeast extract, 4 g glucose) of a 100 mL Erlenmeyer flask with shaking at 200 rpm at 30°C for 2 days. Then 10 mL of culture was inoculated to 1 L of YEME medium in 2.8 L Fernbach flask and cultured at 180 rpm at 30°C for 2 days. 24 L of each (total 72 L for three strains) were prepared and cultured. The liquid cultures were extracted with a total of 72 L of EtOAc. The EtOAc layer was concentrated with a rotary evaporator to yield 3 g of dry extract. Because the 80% fraction was the most active in the antifungal assay, the 80% material. The dry crude extract was re-suspended in MeOH and dried with celite. The EtOAc layer was concentrated with a rotary evaporator to yield 3 g of dry extract material. The dry crude extract was re-supended in MeOH and dried with celite. The cell-bound material was fractionated by column chromatography on C18 resin with combinations of MeOH and water (2:8, 4:6, 6:4, 8:2, and 10:0 MeOH to water). Because the 80% fraction was the most active in the antifungal assay, the 80% fraction was further purified through preparative reversed-phase HPLC (Phenomenex Luna column C18, 250 × 21.2 mm, UV detection 360 nm, flow rate 10 mL/min). Bacillaene (1) eluted at 25 min using isocratic 70% aqueous MeOH with 0.1 formic acid and overall 9 mg of pure bacillaene (1) were obtained.

**Table 1 | Genome characteristics of Bacillus associated with Macrotermes natalensis**

|               | Bacillus #9 | Bacillus #11 |
|---------------|-------------|--------------|
| Median coverage depth | 157.1       | 155.9        |
| Number of nodes | 733         | 1051         |
| n50/max/total   | 23077/114783/3958212 | 11318/62079/3932419 |
| Number of reads used | 11407671/11598752 | 11207443/11250000 |
| Number of supercontigs | 22          | 26           |
| Number of gaps in supercontigs | 265          | 509          |
| Percentage covered | 93%         | 89%          |
| ORFs identified and annotated | 4590        | 4879         |
| Genome draft length (bp) | 4101765     | 4244028      |

**Figure 4 | Comparative analyses of the bacillaene gene cluster between B. subtilis 168 (Bs), Bacillus #9 (B9) and Bacillus #11 (B11).** Sixteen genes code for the pks gene complex involved in bacillaene A (1) biosynthesis, and these are acpK, pksA, pksB, pksC, pksD, pksE, pksF, pksG, pksH, pksI, pksJ, pksL, pksM, pksN, pksR and pksS. The gene cluster was identified on a single contig in both #9 and #11. The figure shows a bp-scale bar of the ca. 80 kb gene cluster (top) together with a diagram of the orientation of the genes (bottom) in each of the three genomes. Pairwise comparisons (coloured bars) indicate comparable identities between the termite-associated strains and B. subtilis, showing that the two termite strains are more similar (almost identical) to each other across the entire gene cluster.
Antifungal biosynthesis: For the paper disk diffusion assay on agar plates against Corynebacterium (Fungus 48), Umbellularia (Fungus 14), Fusarium (Fungus 48), Trichoderma (Fungus 22) and Pseudoxylaria (Fungus 802-2), 9 cm diameter Petri dishes containing 20 mL of YEME agar medium were used. First, colonies of the fungal strains were inoculated in the center of the YEME agar plate and incubated at 30°C. After 3 days, four 6 mm diameter sterile paper disks were placed on the surface of each of the plates and then incubated. The diameters of the halo zones for each of the fungi were recorded for 18 h. The halo diameters were measured by the mean of 3–5 replicates: Bacillus, 9 cm diameter Petri dishes containing 20 mL of YEME agar medium were used. First, colonies of the fungal strains were inoculated in the center of the YEME agar plate and incubated at 30°C. After 3 days, four 6 mm diameter sterile paper disks were placed on the surface of each of the plates and then incubated. The diameters of the halo zones for each of the fungi were recorded for 18 h. The halo diameters were measured by the mean of 3–5 replicates: Bacillus

Genome sequencing and assembly. DNA was extracted from 50 mL LB (tryptone: 10.0 g, NaCl: 10.0 g) liquid broth culture of each Bacillus strain. 1 mL of culture was spun for 20 minutes at 13000 rpm, after which the supernatant was removed. 500 µL of CTAB buffer (10 mL 1 M Tris (pH 8.4), 5 mL 0.5 M EDTA (pH 8), 28 mL 5 M NaCl, 2 g cetyltrimethylammonium bromide, 57 mL ddH2O) was then added to each tube. Cells were subjected to two repeated cycles of freezing in 80°C and thawing at 65°C in a heat block. One volume of phenol–chloroform was added to samples, before vortexing and centrifugation for 10 min (13,000 rpm). Supernatants were transferred to clean 1.5 mL eppendorf tubes. 400 µL of cold 100% isopropanol were used for precipitation. After another round of centrifugation (20 min at 13,000 rpm), the pellet of DNA was washed with 70% ethanol and re-suspended in 50 µL ddH2O. Whole-genome sequences were achieved using mate-paired Illumina HiSeq at Beijing Genomics Institute (www.genomics.cn) and genomes of various fungi have been deposited to GenBank (Accession numbers KC832420-KC832451).

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Comparative bacilleaene gene cluster analyses. We confirmed the presence of the genes necessary for bacilleaene production in two Bacillus strains. The sixteen genes coding for the bae gene complex involved in bacilleaene biosynthesis were compared to published bae gene clusters. DNA was amplified using a combination of primers provided in Table S2. PCR was performed in a final volume of 20 µL using the VWR ready-to-use mix and 1 µL of each pair of primers: conditions: 94°C for 30 s, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, and final extension at 72°C for 5 min. 5 µL of each PCR product was run on a 1% agarose gel containing 1X GELRED for 30 min. PCR product (15 µL) was purified using the Invitrek kit, reeled in sterilized milliQ water and sent to MWG for sequencing. Comparisons of bacilleaene gene similarities between strains were performed using the OSLay software [33] with the B. subtilis genome sequence (ATCC 7003, AP012496) as a reference genome. Gene annotations and comparisons were performed using the BAYS software [4] (for full results, see Table S1). Contigs for draft genomes of Bacillus #9 and #11 are deposited in GenBank under the accession number.KC832420-KC832451.

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**Author contributions**

S.U., A.F., P.S., D.C.O. and M.P. designed the experiments; M.P. collected samples; S.U., A.F. and P.S. performed the experiments; D.C.O. and M.P. performed the general supervision of the project. D.C.O. and M.P. organized and drafted the paper with all authors contributing to the discussion of the data and to the writing.

**Additional information**

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