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Correspondence and requests for materials should be addressed to N.S.C. (chenn@sfu.ca) or B.-Y.X. (xiebingyan2003@yahoo.com.cn)

* These authors contributed equally to this work.

Metagenomic analysis of the pinewood nematode microbiome reveals a symbiotic relationship critical for xenobiotics degradation

Xin-Yue Cheng[1]*, Xue-Liang Tian[2,4]*, Yun-Sheng Wang[2,5], Ren-Miao Lin[2], Zhen-Chuan Mao[2], Nansheng Chen[3] & Bing-Yan Xie[2]

[1]College of Life Sciences, Beijing Normal University, Beijing, China, [2]Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China, [3]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada, [4]College of Henan Institute of Science and Technology, Xinxiang, China, [5]College of Plant Protection, Hunan Agricultural University, Changsha, China.

Our recent research revealed that pinewood nematode (PWN) possesses few genes encoding enzymes for degrading α-pinene, which is the main compound in pine resin. In this study, we examined the role of PWN microbiome in xenobiotics detoxification by metagenomic and bacteria culture analyses. Functional annotation of metagenomes illustrated that benzoate degradation and its related metabolisms may provide the main metabolic pathways for xenobiotics detoxification in the microbiome, which is obviously different from that in PWN that uses cytochrome P450 metabolism as the main pathway for detoxification. The metabolic pathway of degrading α-pinene is complete in microbiome, but incomplete in PWN genome. Experimental analysis demonstrated that most of tested cultivable bacteria can not only survive the stress of 0.4% α-pinene, but also utilize α-pinene as carbon source for their growth. Our results indicate that PWN and its microbiome have established a potentially mutualistic symbiotic relationship with complementary pathways in detoxification metabolism.

The pinewood nematode (PWN) *Bursaphlenchus xylophilus* is an important invasive plant parasitic nematode that has caused heavy mortality of pine trees, which is called pine wilt disease (PWD), in introduced regions including Japan, Korea, China, and Portugal[1,2]. Pines are extremely rich in resins containing resin acid, terpenoids (especially α-pinenes), benzoate, phenolic compounds, stilbenoids, etc., and those aromatic compounds play important roles in mediating various plant–herbivore interactions. Living in pine trees, PWNs have to survive the stresses of those compounds. Some of them such as terpenoids are membrane-destructive and could inhibit reproduction and development of the nematodes[3–5]. Nematode activities including feeding cause injuries to pine trees, which cause their parenchyma cells to synthesize more terpenoids, benzoic acid and ethylene thus further deteriorate the living condition of the PWNs[6–9]. Consequently, PWNs have to cope with a rather toxic condition within the pine trees. Our recent study of the PWN transcriptomes showed that the main metabolic pathway for xenobiotics detoxification in PWN is cytochrome P450 metabolism and PWN does not have a functional pathway that contains all enzymes needed for terpenoids degradation[10], suggesting that PWNs may utilize alternative mechanisms for terpenoids degradation.

In this study, we explored the role of microbiome of PWN in terpenoids degradation by metagenomics methods, trying to explore the mechanism of PWN detoxification from the relationship and interaction of microbe-eukaryotic organisms. Recently, by applying new technologies including metagenomics, most eukaryotic organisms have been found to be intimately associated with a complex community of beneficial microbes, called microbiomes, which can be critical for their development and survival[11–14]. Nematodes have developed a variety of association types with their microbiomes and the association between nematodes and their corresponding microbiomes have been extensively investigated[15]. In particular, entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) and their endosymbiotic bacteria (*Xenorhabdus* and *Photorhabdus*) has been studied as a model system for nematode-bacterium symbiosis[16,17]. Additionally, microbial-eukaryotic lateral gene transfer (LGT) has been documented through transcriptome and genome analysis of plant-parasitic nematodes,

and some transferring genes are ancient acquisition and prevalent among plant-parasitic nematodes, which play a role in evolution of parasitism[15,18,19]. A variety of bacteria associated with plant-parasitic nematodes, such as cyst nematodes *Heterodera*[20,21], burrowing nematodes *Radophoblus*[22], Dagger nematodes *Xiphinema*[23], have been reported. Also, previous studies have shown that a number of bacteria were associated with pinewood nematodes, *Bursaphelenchus xylophilus*[24–30] and *B. mucronatus*[31]. Bacteria were obviously observed on the surface of *B. xylophilus* using a light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM)[32]. Some bacterial strains have been isolated and identified by culture-dependent method[24–26]. Recently, more bacteria were identified through sequencing the 16S rRNA gene sequences[27,28]. Bacterial diversity associated with the Chinese PWN was measured by construction of a fosmid library[29] and a 16S rRNA gene library[30]. Moreover, some bacterial species association with PWN were found even after nematodes were continuously reared in laboratory, indicating a strong specific bacteria–nematode relationship between *B. xylophilus* and its associated bacteria[28]. We also found that some bacteria were difficult to be removed from the nematode by surface sterilization (unpublished observation). A mutualistic symbiosis between *B. xylophilus* and bacteria strains are a reciprocal exchange of nutrients, in which bacteria could help the growth and reproduction of the nematode[33,34]. Furthermore, it has been hypothesized that the symbiotic bacteria associated with PWN could play a crucial role on pine wilt by producing a phytotoxin called phenylacetic acid (PA)[24–26,35–38]. However, negative experimental evidence has been presented[39]. Similar as above, some terpenoids of pine host are also harmful to bacteria associated with the nematode. It was reported that the Douglas fir α-pinene could inhibit the growth of a variety of bacteria, and all terpenoids could inhibit bacteria in guts of Douglas fir tussock moth larvae at concentrations normally present in the fir needle diet (0.27 mg/g)[40]. Therefore, metabolism of xenobiotics detoxification is important to both the phytophagous animals and their symbiotic bacteria.

To examine the role of microbiome in xenobiotics detoxification, which is essential for both bacteria and nematodes survival in their pine host, we have carried out metagenomic analysis of the microbiomes of PWNs. Metagenomics has been a major approach to study the composition, function, evolution of various microbiota, and interactions between microbiomes and their hosts[11,41,42]. We aim to examine the metabolic pathways in microbiome metagenomes and compare to that in the PWN genome for understanding the mechanisms underlying detoxification metabolism. More importantly, we have examined the potential capacity for utilization of α-pinene and benzoate as carbon sources in the cultivable symbionts.

Results

Metagenomic analysis and functional annotation. We obtained 38,750,000 reads (2.91 Gb in total) from genomic DNA harvested from enriched bacteria. After filtering out adapters, low quality reads and PCR duplication, 77.4% reads were available for analysis. These high quality reads were assembled into 99,049 contigs (> 200 bp), totalling 78 Mb. The mean coverage of bases in all contigs is 29 bp (range from 1 – 474 bp). The largest contig is 62,542 bp, and the average length is 786 bp (Table 1). A total of 155,722 open reading frames (ORFs) were predicted from assembled contigs, including 24% complete and 76% partial ORFs. The coding percentage is 87.8% of the total contig lengths (Table 1). After group analysis by CD-HIT-EST, we obtained 131,100 non-redundant metagenes. We annotated protein function encoded by these metagenes through BLAST searches. In total, 76,467 metagenes (58.3%) were assigned to 4104 KEGG orthologs (KOs). Among them, metabolisms of amino acid, carbohydrate, xenobiotics biodegradation and lipid were the four top abundant categories (supplemental Table S1). Glycine, serine and threonine metabolism (map00260), butanoate metabolism (map00650), benzoate degradation (map00632) and fatty acid metabolism (map00071) were the top pathway in each category, respectively. The top 24 pathways are shown in Figure 1.

Table 1 | Overview of two bacterial metagenomes symbiosis with *B. xylophilus*

Parameters	Solex/Illumina sequencing of a bacteria enriched library	454/Roche sequencing of a mixed DNA library
Total reads	38,785,880	423,675
Data size	2.91 Gb	25.81 Mb
Contig number (> 200 bp)	99,049	53,482
Total length	78 Mb	23 Mb
Reads mapped to the assembled contigs	27,398,730	374,385
Mapped reads percentage	70.64%	88.76%
Maximum length of contig	62,542 bp	7,144 bp
Average length	786 bp	428 bp
N50	2,194 bp	499 bp
GC content	53.53%	55.5%
ORF number	155,722	22,100
Total length of ORFs	68 Mb	5.1 Mb
Coding percentage	87.75%	22.17%
Maximum ORF length	16,173 bp	2,448 bp
Average ORF length	438 qbp	230 bp
Complete ORF	37,242 (~ 24%)	7,363(33.3%)
Incomplete ORF	118,549 (76%)	14,737(66.7%)
Contig length with a taxonomic assignment	44.9 Mb	4.55 Mb

To examine whether the metagenome dataset obtained from the enriched bacteria is biased, we obtained another metagenome dataset by sequencing total genomic DNA of PWN, which was newly collected from a pine host in the same region (Zhejiang Province). Bacterial contigs were then isolated from the genome sequences of PWN. A total of 53,482 bacterial contigs (> 200 bp) with 23 Mb in length were obtained, and 22,100 ORFs were predicted from the dataset of directly sequencing metagenome (Table 1). As we expected, the result of KEGG functional annotation is similar to the result obtained from the bacteria enriched metagenome (Figure 1, supplemental table S1). We used the pared data, i.e. the numbers of metagenes involved in each KEGG pathways in the two metagomes (Xi, Yi), to calculate the sample Pearson correlation coefficient, and the result showed highly correlation between them ($r = 0.943$, $n = 130$). Therefore, the metagenome obtained from enriched bacterial is unbiased.

Taxonomic assignment of bacteria symbiosis with PWN. Taxonomic assignment was performed to determine composition of the bacterial consortia in the metagenome. We first matched the reads of the bacterial enriched metagenome to the bacterial genomes deposited in NCBI. To our surprise, only 9.4% reads could directly be matched (mismatches < or = 2 bp in the first 35-bp region, identity > 90%), and almost all of those matched reads were assigned to phylum Proteobacteria (99.8%). This low rate of matched reads is due largely to the stringent requirement by short reads alignment programs (in this case, SOAPaligner), which allow very low number of misalignment. Then, we used the assembled contigs to search against the NCBI bacterial database using BLAST. Altogether 44.9 Mb (57.7%) of assembled contigs with 21,891,219 reads (79.9% of the total) could be matched to the bacterial genomes and had a taxonomic assignment (cut-off 1×10^{-8}, identity > 90%). About 95% of mapped reads were assigned to the phylum Proteobacteria, including 46.6% to the *Alphaproteobacteria*, 38.3% to the *Gammaproteobacteria* and 10.1% to

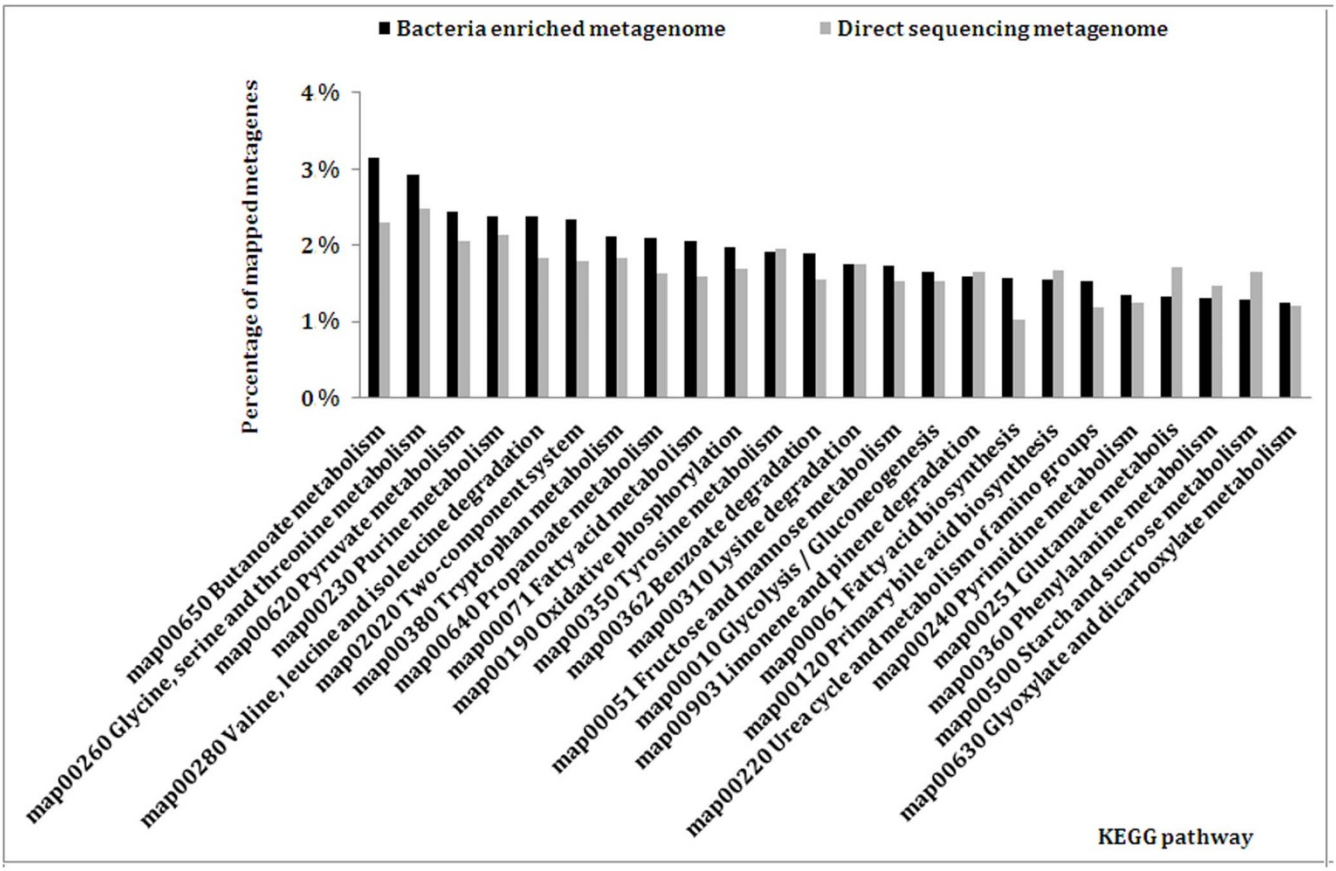

Figure 1 | The top 24 pathways in the two metagenomes of bacteria symbiosis with PWN.

Betaproteobacteria. The remaining was assigned to the phylum Bacteroidetes (4.4%) and other groups (0.6%). Among them, more than 93% of mapped reads were assigned to nine bacterial families (with a coverage $>$ 1%), in which two families Rhizobiaceae and Xanthomonadaceae occupied 60% of mapped reads (Figure 2). At the genus level, the most common genera of bacteria symbiosis with PWN were *Stenotrophomonas*, *Agrobacterium*, *Rhizobium*, *Pseudomonas*, *Herbaspirillum*, *Caulobacter*, *Pedobacter*, *Novospingobium* and *Sphingomonas*, for each with coverage larger than 1% (supplemental Figure S1A).

The bacterial diversity associated with the PWN strain was also measured by construction of a 16S rRNA gene library[30]. 26 operational taxonomic units (OTUs) were obtained from 73 clones (with 97% identity cutoff). Of them, four OTUs belonged to Alphaproteobacteria, eight to Betaproteobacteria, ten to Gammaproteobacteria, and four to Bacteoidetes. Among them, *Stenotrophomonas* had the most abundant clones, and then followed by *Achromobacter, Agrobacterium*, *Herbaspirillum, Variovorax, Dyella, Pedobacter, Sphingobacterium, Pseudomonas, Novosphingobium, Sphingomonas,* etc. (supplemental Figure S1C). Comparison of bacterial compositions obtained through 16S rRNA sequencing from metagenome sequencing showed these two methods revealed similar composition, although richness shows some difference (Figure 2).

Taxonomic assignment of the directly sequencing metagenome showed that less than 20% contigs have a definite taxonomic affiliation. Of them, with more than 70% mapped reads were assigned to *Stenotrophomonas* genus. So *Stenotrophomonas* is the most prevalent bacterial strain. *Agrobacterium* was next, and then followed by *Achromobacter*, *Variovorax*, *Rhizobium* and *Bordetella*. Genus *Pseudomonas*, *Sphingomonas* and *Pedobacter* were also richer than others (supplemental Figure S1C).

Detoxification metabolism of bacteria symbiosis with PWN by metagenome analysis. We are particularly interested in metabolism pathways of xenobiotics biodegradation, because xenobiotics detoxification systems are able to remove compounds from the complex mixture produced by metabolic process or from environment. We found that enzymes involved in metabolism of xenobiotic biodegradation were very abundant in the bacterial metagenome. A total of 12,601 metagenes (16.5%) were involved in this category in the bacteria enriched metagenome. Among them, metagenes encoding enzymes involved in the pathway of benzoate degradation were more abundant than in other pathways (Figure 3), with a complete 3-oxoadipate pathway for benzoate degradation through catechol- the hydroxylation pathway (supplemental Figure S2). Benzoate is a common intermediate in the anaerobic metabolism of aromatic compounds. Toxic compounds, such as phenolic compounds, polycyclic aromatic hydrocarbons, could firstly be degraded to benzoate through some other detoxification pathways, and then enter into the pathway of benzoate degradation to be further degraded. The terminal product acetyl-CoA could be utilized in the pathway of citrate cycle. So that benzoate degradation and its related pathways are very important for xenobiotics detoxification in the bacterial consortia symbiosis with PWNs.

Pinenes are the major components of turpentine which are produced in significant quantities by pine trees. KEGG function annotation of the bacterial metagenome showed that enzymes encoded by 1212 metagenes have activities in metabolism of limonene and pinene degradation (Figure 3). Except for the alpha-pinene oxide lyase (EC 5.5.1.10), which was not found because of no reference (i.e., nucleotide) sequence kept in GenBank, all enzymes involved in the pathway of α-pinene degradation are found in the dataset of bacteria enriched metagenome (Figure 4). The same results were also

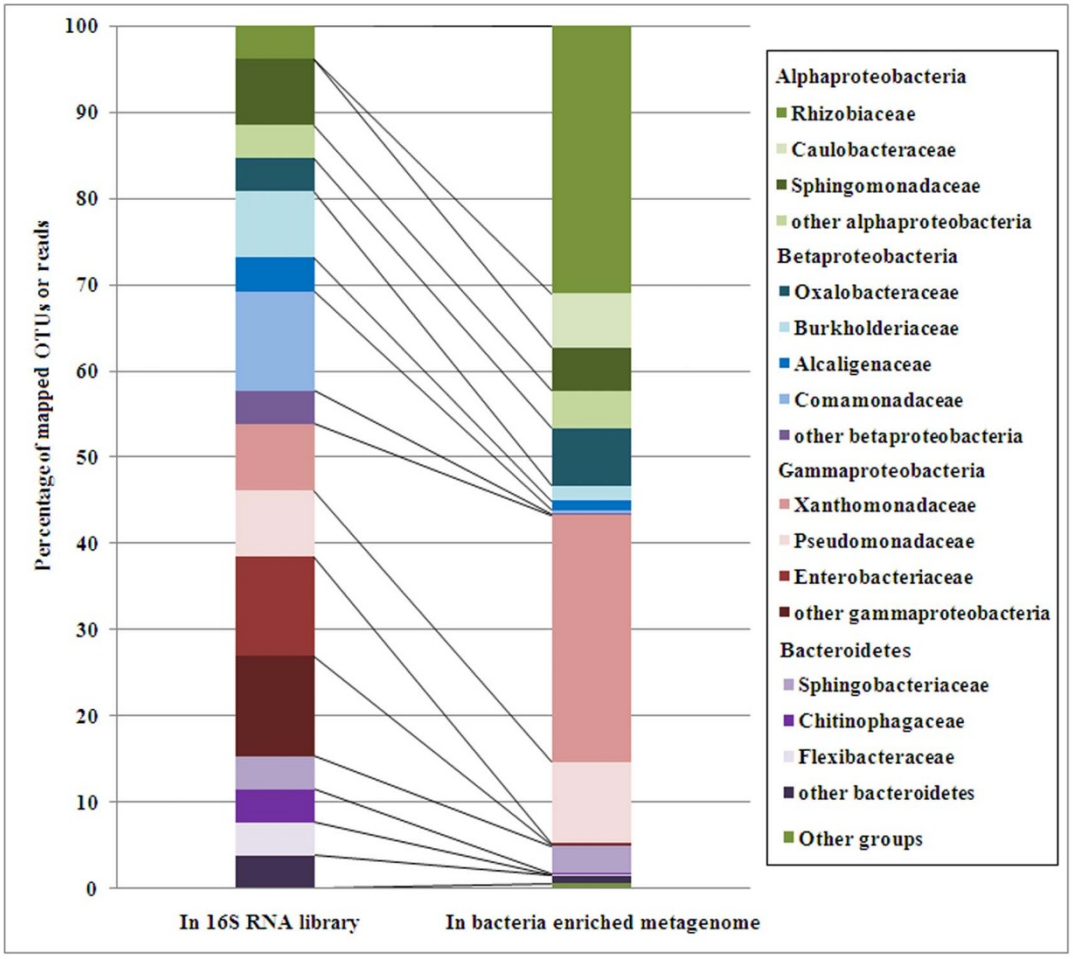

Figure 2 | Composition of family members in the bacterial consortia symbiosis with PWN.

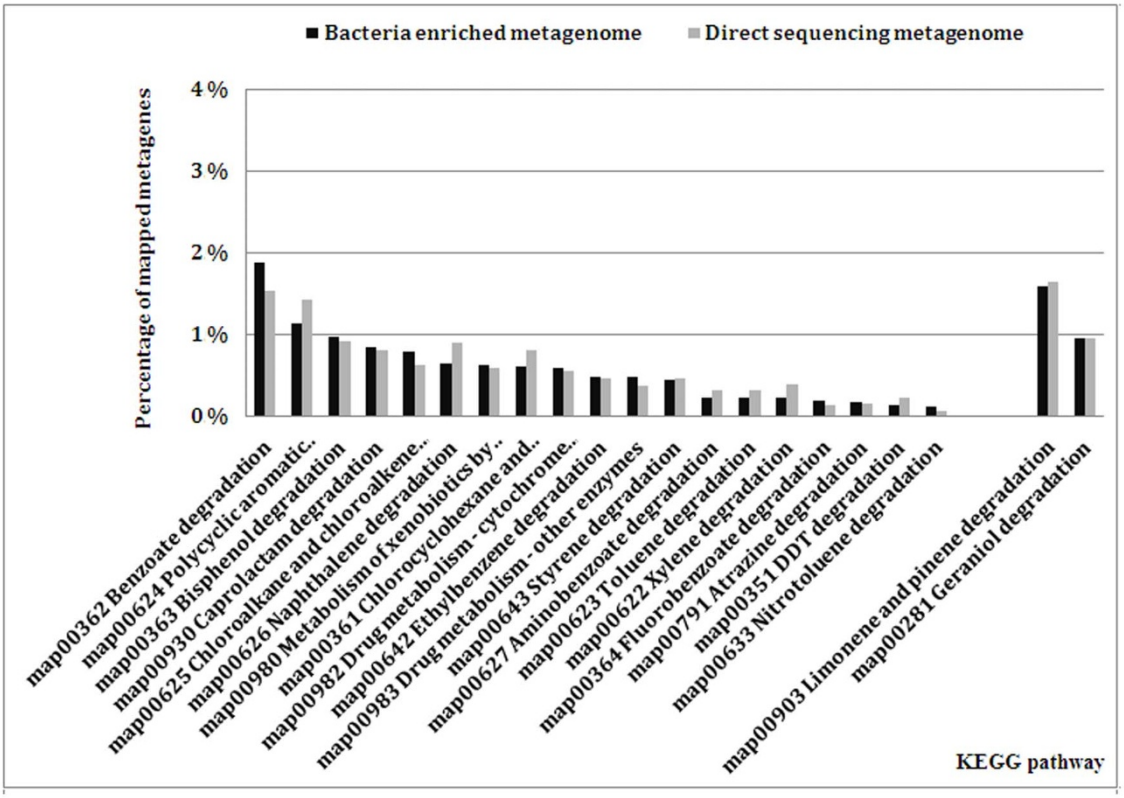

Figure 3 | KEGG pathways of xenobiotics biodegradation and metabolism in the bacterial metagenomes.

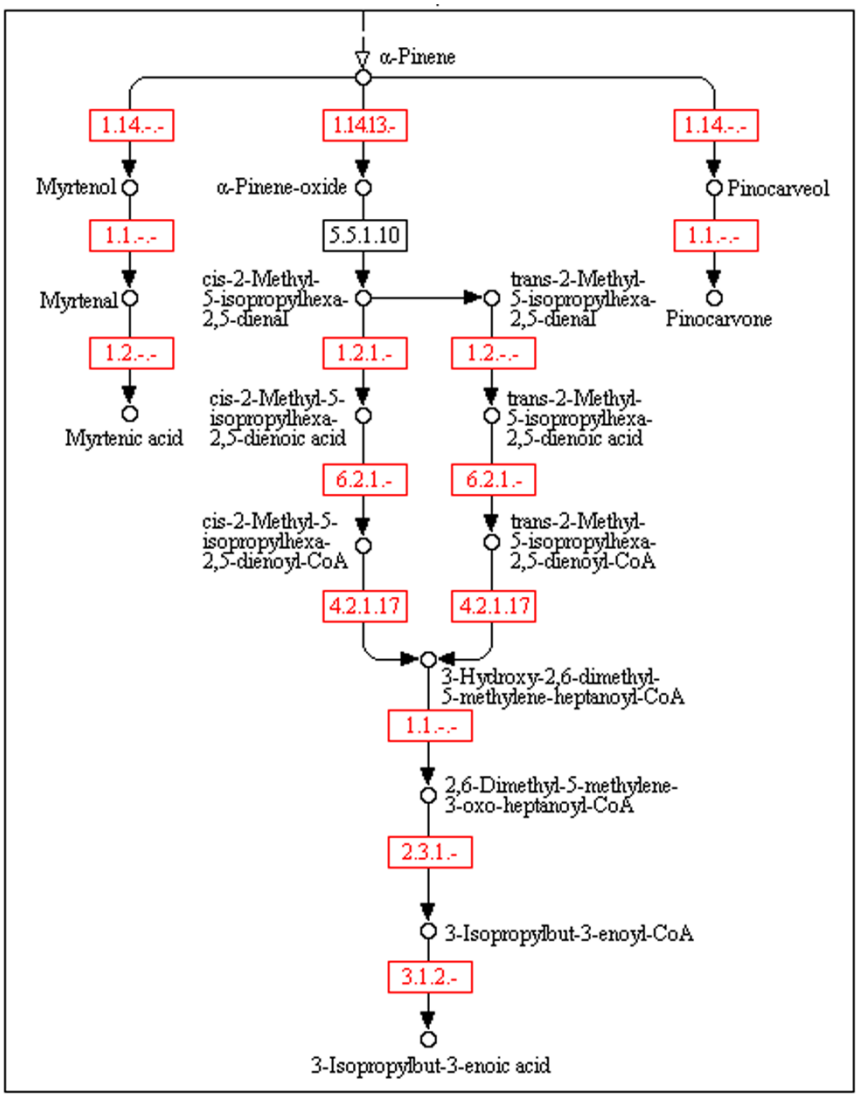

Figure 4 | Enzymes involved in metabolic pathway of α-pinene degradation in the bacterial metagenomes based on KEGG functional annotation. Map was downloaded from the KEGG server with our data mapping to the pathway (http://www.kegg.jp/kegg/tool/color_a_pathway.html).

acquired from the directly sequencing metagenome (Figure 3). Pinenes are monoterpenoid compounds and no pathway of monoterpenoid biosynthesis (map00902) was found in both the bacterial metagenome and the PWN genome, indicating that both the nematode and its symbiotic bacteria could not synthesize monoterpenoid compounds. However, host pine tree can synthesize a large amount of monterpenoids (especially α-pinene) as defensive compounds, to PWN and its symbiotic bacteria, these compounds are toxic and must be degraded by detoxification metabolism. Thus, enzymes involved in monoterpenoid degradation in bacteria metagenome are likely directed at compounds synthesized by the host tree.

Experimental assessment on the capacity of symbiotic bacteria surviving and utilizing α-pinene and benzoate. We then attempted to identify which bacterium or bacteria could produce enzymes involved in terpenoids degradation. First, we isolated bacterial strains from PWN by culture-dependent method and identified 12 bacterial strains from 188 clones based on 16S rRNA gene sequences. Among them, *Stenotrophomonas* was the most abundant strain, followed by *Cytophaga* and *Sphingobacterium*. *Herbaspinillum*, *Agrobacterium* and *Pseudomonas* were richer than *Pedobacter, Achromobacter, Sphingomonas, Variovorax, Ralstonia* and *Dyella* (supplemental Figure S1D).

To detect the capacities of bacteria surviving the stress of α-pinene, we used 10 cultivable bacterial strains to test their potential abilities by culturing them on LB medium containing 0.4% α-pinene, contrasted to those grown on LB medium without α-pinene. The result showed that seven out of ten bacterial strains could grow on LB medium containing α-pinene (Figure 5). *Pseudomonas* and *Cytophaga* could grow very well under the stress. *Agrobacterium*, *Stenotrophomonas*, *Achromobacter*, *Herbaspirillum* and *Sphingobacterium* showed slow growth at the early stage. The other three, *Sphingomonas*,*Variovorax* and *Pedobacter*, however, could not grow under the stress of 0.4% α-pinene.

Then, we tested the capacity of those strains utilizing α-pinene as carbon source for their growth by culturing them in M9 buffer containing 0.4% α-pinene. Six tolerant strains could also take α-pinene as carbon source for their growth (with OD_{600} of > 0.01 in 24 h), except *Sphingobacterium*. Among them, *Pseudomonas* could grow much better than others in the M9 buffer containing 0.4% α-pinene (Figure 6A). It means that *Pseudomonas* is more efficiently utilizing α-pinene as the carbon source for its growth.

Moreover, we tested the capacity of those strains utilizing benzoate as the carbon source for their growth by culturing them in M9 buffer containing 0.1% benzoate. The result showed that *Pseudomonas* grew very well in the M9 buffer, and then it was followed by

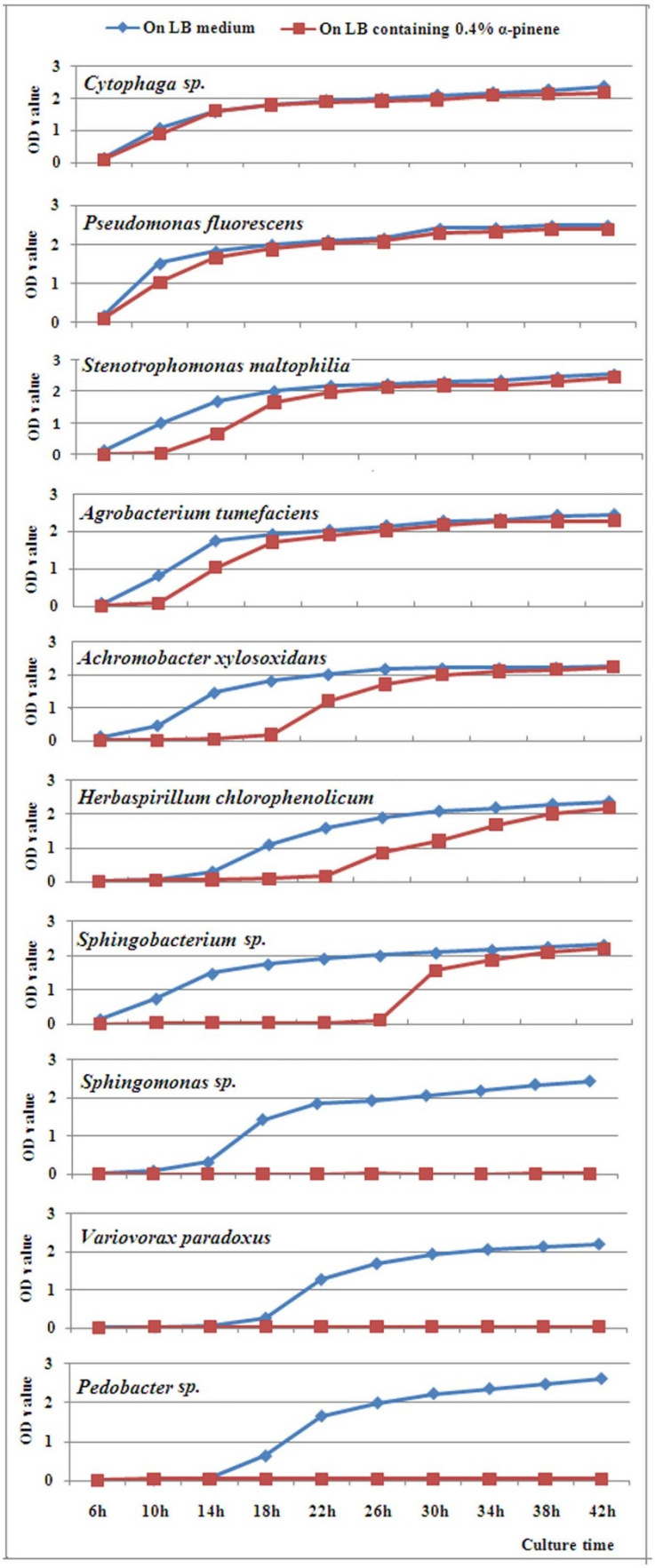

Figure 5 | Growth of bacterial strains in LB nutrient solution with or without 0.4% α-pinene. OD values indicate propagation levels of bacteria at different timzes.

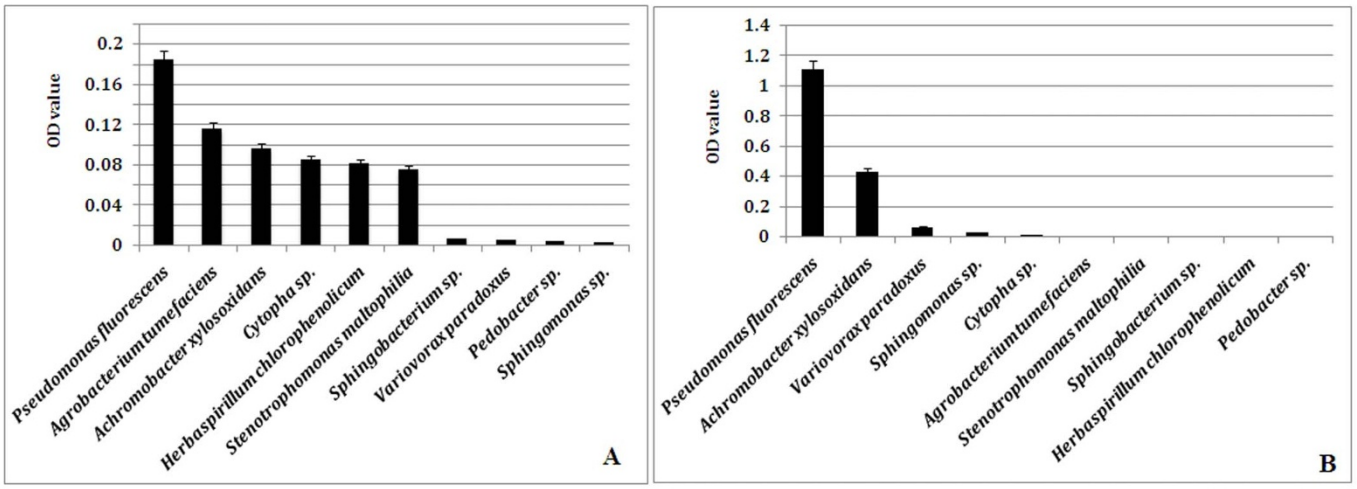

Figure 6 | Growth of bacterial strains in M9 buffer containing 0.4% α-pinene (A) or 0.1% benzoate (B) at 25°C for 24 h.

Achromobacter. It means these bacteria could perfectly utilize benzoate as the carbon source for their growth. *Variovorax*, *Sphingomonas* and *Cytopha* could also grow in the M9 buffer (with OD_{600} of > 0.01 in 24 h). However, *Agrobacterium*, *Stenotrophomonas*, *Spingobacterium*, *Herbaspirillum* and *Pedobacter* could not grow in the M9 buffer (with OD_{600} of < 0.01 in 24 h), meaning that they could not utilize benzoate as the carbon source for growth (Figure 6B).

We also assigned those metagenes involved in the pathway of α-pinene degradation to a taxonomic affiliation by MEGAN. But only a small part of them (174 metagenes, 13% of total hits) in the dataset of the bacteria enriched metagenome had a definite taxonomic affiliation. The identified genera were mainly *Pseudomonas*, *Achromobacter*, *Agrobacterium*, *Cupriavidus*, *Stenotrophomonas*, *Caulobacter*, *Sphingobacterium*, *Rhizobium*, etc. (supplemental Table S2). Among them, 40 metagenes were assigned to *Pseudomonas*, 23 metagenes were assigned to *Achromobacter* and *Agrobacterium*. These bacteria might be the main strains for α-pinene degradation.

Table 2 | Enzymes involved in the pathway of limonene and pinene degradation deduced from the bacterial metagenome and the genome of *Serratia* sp. M24T3, comparing to those in genome of the nematode *B. xylophilus* genome

KO	EC	Activity	Reaction	Number of genes in bacterial enriched metagenome	Number of genes in directly sequencing metagenome	Number of genes in *Serratia sp.* M24T3 genome	Number of genes in *B.xyolphilus* genome
K00492	1.14.13.–	alpha-pinene monooxygenase	R06406	146	53	6	0
-	5.5.1.10	alpha-pinene-oxide lyase (decyclizing)	R04040	-	-	-	-
K00155	1.2.1.-	cis-/2-methyl-5-isopropylhexa-2, 5-dienal dehydrogenase, trans-2-methyl-5-isopropylhexa -2, 5-dienal dehydrogenase, myrtenal dehydrogenase	R06407, R06408, R06403	104	28	1	0
K01913	6.2.1.-	cis-2-methyl-5-isopropylhexa-2, 5-dienoate-CoA ligase, trans-2-methyl-5-isopropylhexa-2, 5-dienoate-CoA ligase	R06409 R06410	85	38	0	1
K01692	4.2.1.17	cis-2-methyl-5-isopropylhexa-2, 5-dienoyl-CoA hydro-lyase, trans-2-methyl-5-isopropylhexa -2, 5-dienoyl- CoA hydro-lyase	R06411, R06412	256	72	1	1
K00120	1.1.-.-	3-hydroxy-2,6-dimethyl-5-methylene-heptanoyl-CoA dehydrogenase, myrtenol dehydrogenase, pinocarveol dehydrogenase	R06413, R06402, R06405	128	28	3	0
K00206	1.2.-.-	Myrtenal dehydrogenase	R06403	11	3	0	0
K00680	2.3.1.-	2,6-dimethyl-5-methylene-3-oxo-heptanoyl-CoA C-acetyltransferase	R06414	261	119	30	29
K01076	3.1.2.-	3-isopropylbut-3-enoyl-CoA thioesterase	R06415	42	20	3	0
K00517	1.14-.-	alpha-pinene dehydrogenase	R06401, R06404	70	42	2	2
K10533	3.3.2.8	limonene-1,2-epoxide hydrolase	R09387	1	0	0	0
K00128	1.2.1.3	aldehyde dehydrogenase (NAD^+)	R06366	225	81	5	12
K01726	4.2.1.-	perillyl-CoA hydratase	R06369	74	24	3	0

Xenobiotics degradation in a strain of *Serratia* achieved from *B. xylophylus*. We also downloaded the published genome sequences of *Serratia* sp. M24T3 from NCBI (accession: AJHJ00000000), which was achieved from *B. xylophylus* isolated from *Pinus pinaster* in Portugual[43], and KEGG functional annotation was performed. Similar result was obtained from the genome, with more hits in benzoate degradation (supplemental figure S3). In addition, there are 54 genes encoding 9 enzymes involved in α-pinene degradation in the genome, only one enzyme (EC6.2.1.-) was not found in the genome of *Serratia sp*. (Table 2). The result indicated that bacteria associated with PWN have similar xenobiotic metabolism, even through the association types are different, such as *Serratia sp*. M24T3 was taken as a potential biocontrol agent for PWN[43].

Discussion

Many previous works have studied bacteria associated with PWN, and some bacteria were isolated and identified by culture-dependent methods[24–26], and also by 16S rRNA gene sequences[27,28,44]. Comparing results obtained in this study and previously published results reveals that the composition of bacterial fauna and the abundance of each bacterium associated with each geographical PWN population were different. For example, it was reported that *Pseudomonas* were dominant strains in PWNs isolated in China, but *Bacillus* were dominant in Japan and both genera were dominant in Korea[45]. However, these two genera were not included in the new report of bacteria isolated from Korean PWN by Kwon and his colleagues[46]. In Portugal, the dominant bacteria are different among the three studies[27,28,44]. We think the following factors could be contribute to the different results obtained in different studies including: the material used for bacterial isolation is chips of wood (which tree species) or the nematodes (which nematode species); the culture medium (which type of medium); the isolation methods (colonies collected from the PWN trail or from the crushed PWN suspension); treatments (used or not) and its methods to exclude organisms contaminating the outer surfaces of the nematodes. Moreover, the temporal and spatial dynamics of bacterial populations could also affect the abundance of each strain. It was reported that the amount of bacteria and the predominant species changed with the symptoms procession after inoculation of PWN in the tree *Pinus pinaster*[44], and the major bacterial population associated to the nematodes was different according to the forest area[27]. In our study, *Stenotrophomonas* was the most dominant strain in PWNs isolated in Zhejiang Province in China, which was different from previous reports[25,26]. Except the factors including temporal and spatial variation of bacterial community, it is very difficult to distinguish *Stenotrophomonas maltophilia* from the genus *Pseudomonas* and *Xanthomonas* using regular identification method, which also lead to the ambiguity. For example, *S. maltophilia* was initially classified as *Pseudomonas maltophilia*, but changed to *Xanthomonas maltophilia*, and eventually become the type species belong to the genus *Stenotrophomonas*[47].

As the microbiome used for this metagenomic study was isolated from nematodes of a long-cultured strain in our study, one may question that if the microbiome is bias to that in the nature environment. We compared our result with the result of a recent study, in which bacteria were obtained from strains of *B. xylophilus* isolated from naturally wilted pine trees in China[48]. We found that the composition of bacterial genera and the dominant species are similar, suggesting that the bacterial strains acquired in our study can represent the current microbiomes of PWN. Similar result was also obtained in another study, in which by comparing bacterial composition associated to PWN from symptomatic tree *Pinus pinaster* and from long-term preserved cultures, it was found that the main composition and the predominant genera from both source were similar[28]. Thus, some species remain in association with *B. xylophilus*, even after successive nematode generations in the laboratory, suggesting a strong specific bacteria–nematode relationship.

Even though the fauna of bacterial consortia symbiosis with PWN perhaps has a temporal and spatial variation, the ecological functions of the symbiotic bacterial consortia are similar. Comparing the two metagenomes obtained from the nematode kept in laboratory and the nematode from chips of a symptomatic tree, as well as with the bacterial genome from Portuguese PWN, the functional annotations of them were very similar. Enzymes involved in metabolism of xenobiotics degradation are abundant in the symbiotic bacteria, which formed a complex metabolic network. Benzoate (map00362) and its related degradation pathways are likely the main metabolic pathways of detoxification, and each has at least one complete degradation pathway. Moreover, a complete pathway of α-pinene degradation was also observed in the bacterial metagenome (Figure 4, Table 2). Therefore, xenobiotics detoxification is an important ecological function of the bacterial consortia symbiosis with PWN.

Xenobiotics detoxification is equally important to PWN. Analysis of xenobiotic metabolisms in PWN from the genome[49] and the transcriptome[10] published in NCBI showed that pathways of detoxification metabolism in the nematode were obviously different from those in the symbiotic bacteria, and two pathways are complementary. As previously reported, cytochrome P450 enzymes are rich in the nematode and xenobiotics degradation by cytochrome P450 (map00980, map00982) are the main pathways[10,49]. However, a few enzymes were involved in benzoate degradation and its related pathways. Also, only a few enzymes are involved in the pathways of limonene and pinene degradation in the genome of PWN and they could not fulfil a complete pathway of pinene degradation (Table 2). However, pinenes are the most abundant monoterpenoids in pine hosts. It was reported that crude sulphate turpentine from pine trees was mostly composed of α-pinene (60–65%) and β-pinene (25–35%)[50]. The secondary metabolic compounds, such as benzoic acid, benzaldehyde, phenol, phenylethanone, etc., are also relatively rich in pine trees[5]. Therefore, living on pine trees, the nematodes must have effective mechanisms to degrade these compounds. Based on our results, we hypothesize that PWN and its symbiotic bacteria have evolved a complete xenobiotic degradation system by a functional complementary mutualism in order to cope with various compounds produced by the secondary metabolism of the pine host.

Methods

Samples sources. Bacteria were isolated from two PWN isolates, both of which were collected from Zhejiang Province, China. One PWN isolate (ZJSS) was long-term cultured in our library, and another was newly collected from chips of a pine tree that suffered from PWD in Zhejiang Province. Baermann funnel technique was employed for nematodes isolation[51]. Nematodes were sterilized with 5% H_2O_2 for 30 min to get rid of opportunistic bacteria attaching on the surface of nematodes before they were cultured for the first time. Nematodes were cultured on fungal mats of *Botrytis cinerea* grown on potato-dextrose agar (PDA) plates at 25°C for about ten days. Fresh cultured nematodes were collected and washed several times with double distilled water (DDW), and then used for DNA extraction and bacteria isolation.

Shotgun library construction. A shotgun genomic DNA library was constructed from genomic DNA extracted from enriched bacteria, which were isolated from the long-term cultured PWN strain in our laboratory. First, symbiotic bacteria were enriched by Nycodenz density gradient centrifugation[52]. Second, the enriched bacterial cells were suspended and used for DNA extraction. A typical phenol/chloroform/isoamyl alcohol method was used to extract the genomic DNA of bacteria[53]. DNA from triple extractions was mixed together and used for the library construction. Third, about 20 μg genomic DNA was used for a shotgun DNA library construction. DNA library preparation followed the manufacturer's instruction (Illumina), as reported previously[54]. Final, paired-end sequencing was performed (read length 75 bp, insert size 130 bp) using the Illumina Genome Analyzer at the Beijing Genomics Institute (Shenzhen, China).

Metagenome data analysis. The raw sequences obtained from the shotgun library were first cleaned up by trimming adapters, removing low quality reads and PCR duplication. High quality short reads were then assembled by the SOAPdenovo assembler[55], with -M 3 -d 1 -u parameters. Different K values (from 21 to 63) were

tested and the value 23 was selected based on the largest N50 of the assembled contigs. Contigs more than 200 bp were reserved to form a dataset, which was used for the later analysis. For functional annotation, MetaGene[56] was employed to predict the ORFs of the assembled contigs. The predicted ORFs were grouped using CD-HIT-EST with the coverage over 90% and minimum identity 95%, and finally the non-redundant gene set was obtained. The ORFs were translated into protein sequences using NCBI Genetic Codes11. We used BLASTp to search for the protein sequences of the predicted Genes in KEGG databases (http://www.genome.jp/kegg/), with *e*-value $< 1e^{-5}$. The genes with KEGG annotation were assigned into KEGG pathways. For taxonomic assignment we firstly used the Illumina GA reads aligned against the known bacteria genomes (ftp.ncbi.nih.gov/genomes/Bacteria/) using SOAPaligner. Two mismatches at most were allowed in the first 35-bp region and 90% identity over the read sequence, to get the information of taxa and their reads number. Then, we used the assembled contigs to search against the dataset including all of the microbe genome sequences deposited in GenBank by BLASTn, with 1×10^{-8} and minimal 90% identity cut-off. The taxonomical level of each contig was then determined by the lowest common ancestor (LCA)-based algorithm that was implemented in MEGAN4[57]. The taxonomic abundance was evaluated by reads number of each taxon, which was obtained by mapping all of the reads to the assembled contigs using SOAP2 with default parameters[55].

To examine whether genomic DNA harvested from enriched bacteria is biased against the bacterial species that are hard to dissociate from the nematodes, we have constructed another shotgun library from the total DNA extracted from a newly isolated PWN strain directly, with no treatment of bacterial enrichment or any effort to disassociate bacteria from their host. Nematodes were first pulverised by successive freeze-thaw cycles in liquid N_2 for five to six times, then the total DNA was extracted using phenol-chloroform extraction method. Library construction and sequencing was finished in the Beijing Genomics Institute (Shenzhen, China) with a Roche/454 Genome Sequencer 20 System. The clean reads were first assembled by SOAPdenovo software. Then, the assembled contigs were filtrated by eliminating the nematode sequences, with reference to the genome sequences of *B. xylophilus*[49]. The remaining assembled contigs were searched against all of the microbe genome sequences deposited in NCBI using BLASTn. Taxonomic assignment and KEGG function annotation were performed as above.

16S rRNA gene library construction and sequences analysis. The diversity of bacteria symbiosis with the PWN strain used for the metagenome was analyzed by construction of a 16S rRNA gene library[30]. The procedure was as following: firstly, the cultured nematodes were pulverised by successive freeze-thaw cycles in liquid N_2 for five to six times, and total DNA was extracted using a phenol-chloroform extraction method. Secondly, the 16S rRNA gene was amplified with primers 27F and 1492R[58]. To reduce the potential bias of a single experiment, DNA was extracted three times from each PWN strain and amplified with five replicas. All PCR products from the same PWN strain were mixed together for a library construction. Thirdly, the purified PCR products were ligated into the PGM-T vectors (TIANGEN, China) and then transformed into competent cells (*Escherichia coli*, TIANGEN, China). Lastly, transformants were plated on LB medium containing 50 μg/ml ampicillin overnight at 37°C. About 200 white colonies were picked out randomly from each transformation plate, and separately grown in LB liquid medium containing ampicillin (50 μg/ml) at 37°C for 6 hours. By PCR identification, 75 positive clones containing the full-length inserts (about 1500 bp) were selected to sequence with primers T7 and SP6 using the 3730 sequencer (sequenced by SinoGenoMax Co., Ltd, China). The obtained sequences were checked for chimeras using the Chimera Check programme from the Ribosomal Database Project (RDP)(http://rdp.cme.msu.edu/). Operational Taxonomic Units (OTUs) were calculated with the software package DOTUR at a 97% similarity[59]. The representative sequences of each OTU were searched against the NCBI database using BLASTN to search matches.

Cultivable bacteria isolation and 16S rRNA gene identification. Nematodes were placed into 200 μl DDW and homogenised. Homogenate was cultured in Petri dishes with a LB (Luria Broth) nutrient medium, and then incubated in a growth chamber at 30°C for 5 days. Then, after a serial ten-fold dilution (10^{-6}), the cultured suspension was spread on LB medium plates. The total numbers of bacterial colonies on each plate were counted, and then preliminarily categorised based on colony morphology (shape, elevation, surface, size, opacity and pigmentation). Five to ten clones of each type of isolates were picked out randomly from each plate and separately put into 400 μl LB culture fluid with a sterilized toothpick, and incubated at 25°C for 24 h. Then, a 4-ml bacterial suspension of each culture was centrifuged at 12,000 rpm for 1 min, and the total DNA was extracted according to a modified alkaline lysis protocol[60]. The 16S rRNA gene was amplified with above primers. The PCR products were purified using the EasyPure quick gel extraction kit (Transgene, China), and then directly sequenced from both ends.

Assessment of potential ability of cultivable bacteria to survive and utilize α-pinene and benzoate. To assess the potential ability of bacteria to survive the stress of α-pinene, ten identified bacterial strains were used to test. Each strain was cultured in a 1-ml liquid LB medium at 30°C for 6 hours. A 10-μl volume of each culture (with equal density) was then added to 50 ml liquid LB medium containing 0.4% α-pinene (based on the acute oral toxicity to rat, LD50 3700 mg/kg, http://www.sciencelab.com), and cultured at 30°C with 200 rpm/min. After 6 hours, 2 ml of each culture was sampled every two hours until 36 hours. The density of bacteria was assayed by ultraviolet spectrophotometer (UNICO Instruments Co., Ltd., Shanghai, China). OD_{600} value of each strain cultured in LB medium with no α-pinene was taken as the control treatment. Experiment repeated three times. Mean OD_{600} values of the three replicates were used to draw the growth curves of each bacterial strain.

Then, we tested the ability of those cultivable bacterial strains to utilize α-pinene and benzoate as the sole carbon source. A 10-μl of each above strain (with the same concentration) was added to a 50-ml M9 buffer (1 litre containing 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, pH7.4; after autoclaving, added 2 ml 1 M $MgSO_4$) containing 0.4% α-pinene or 0.1% benzoate (based on LD50 1714 mg/kg to rat, http://ec.europa.eu) and cultured at 25°C for 24 h (200 rpm/min). OD_{600} value of each culture was detected. Experimental replicates and control treatments were designed as above. OD_{600} value less than 0.01 indicates the bacterial strain did not grow.

Nucleotide sequence accession numbers. Metagenome data have been submitted to NCBI, accession number was SRA048563. 16S rRNA gene sequences were submitted to GenBank (accession: GU563738 - GU563762, GU569098 - GU569161). We also downloaded the genome sequences of the nematode *B. xylophilus* (accession: CADV01000001- CADV01010432) and the genome sequences of bacterium *Serratia sp*. M24T3 isolated from Portuguese *B. xylophilus* (accession: AJHJ00000000) from NCBI for analysis.

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Author contributions

B.Y.X. and X.Y.C. conceived and designed the experiments; X.L.T. and Z.C.M. performed the experiments; Y.S.W., R.M.L., X.L.T. and X.Y.C. analyzed the data; X.Y.C. and X.L.T. wrote the paper; N.C. and B.Y.X. edited and approved the manuscript.

Additional information

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