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1 **Title: Microbiome and infectivity studies reveal complex polyspecies**
2 **tree disease in Acute Oak Decline**

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23 **Abstract**

24 Decline-diseases are complex and becoming increasingly problematic to tree health
25 globally. Acute Oak Decline (AOD) is characterised by necrotic stem lesions and
26 galleries of the bark-boring beetle, *Agrilus biguttatus*, and represents a serious threat to
27 oak. Although multiple novel bacterial species and *Agrilus* galleries are associated with
28 AOD lesions, the causative agent(s) are unknown. The AOD pathosystem therefore
29 provides an ideal model for a systems-based research approach to address our hypothesis
30 that AOD lesions are caused by a polymicrobial complex. Here we show that three
31 bacterial species, *Brenneria goodwinii*, *Gibbsiella quercinecans* and *Rahnella victoriana*,
32 are consistently abundant in the lesion microbiome, and possess virulence genes used by
33 canonical phytopathogens that are expressed in AOD lesions. Individual and polyspecies
34 inoculations on oak logs and trees demonstrated that *B. goodwinii* and *G. quercinecans*
35 cause tissue necrosis, and in combination with *A. biguttatus* produce the diagnostic
36 symptoms of AOD. We have proved a polybacterial cause of AOD lesions, providing
37 new insights into polymicrobial interactions and tree disease. This work presents a novel
38 conceptual and methodological template for adapting Koch's postulates to address the
39 role of microbial communities in disease.

40 **Introduction**

41 Trees are essential to landscape function and aesthetics, supporting diverse ecologies
42 (Rackham, 2008) and providing key ecosystem services (Boyd *et al.*, 2013). However,
43 significant areas of forest have been lost due to increasing outbreaks of disease and pest
44 attack, and tree health is a current global concern (Cohen *et al.*, 2016). Tree diseases,
45 including decline-diseases, are rising in profile due to an increased risk of introduction
46 and spread through international plant trade, and amplification effects of current and
47 future climate change (Millar and Stephenson, 2015; McDowell *et al.*, 2011; Oliva *et al.*,
48 2014). Emerging evidence suggests that complex biotic interactions, including
49 polymicrobial and insect activity affect disease occurrence and severity (Adams *et al.*,
50 2013; Buonauro *et al.*, 2015; Lamichhane and Venturi, 2015), yet little progress has been
51 made in applying the latest advances in sequencing and culture-based methodologies to
52 characterize pathosystems in trees. The prevailing paradigm of infection biology
53 contends that one organism causes one disease (proved using Koch's postulates). In
54 contrast, there is increasing recognition of the importance of polymicrobial interactions in
55 human disease, following developments in sequencing technologies that allow
56 microbiome-wide association studies to identify the role of microbial communities in
57 disease (Gilbert *et al.*, 2016). In the medical field this is leading to adaptations of Koch's
58 postulates to include complex interactions between the environment, host, and microbial
59 communities (Fredricks and Relman, 1996; Falkow, 1988; Hill, 1965). However,
60 progress in characterising polyspecies interactions in plant disease has been limited,
61 although there is clearly a need for contemporary approaches to investigating complex
62 tree diseases (Lamichhane and Venturi, 2015). Analysis of such complex biotic

63 interactions requires an integrated, systems approach, particularly in the case of decline-
64 diseases where both complex abiotic and biotic interactions underpin disease
65 development.

66 Decline-diseases, first formally described as a specific disease in the United States
67 (Sinclair, 1965; Manion, 1981) but well documented elsewhere (Delatour, 1983; Thomas,
68 2008; Sinclair and Lyon, 2005), are of global concern (Pautasso *et al.*, 2015). Unlike
69 most common tree diseases, decline-diseases are not caused by single primary pests or
70 pathogens; instead they are complex syndromes, involving the sequential, combined and
71 cumulative effects of (often secondary) biotic and abiotic agents (Brown *et al.*, 2016;
72 Sallé *et al.*, 2014; Thomas, 2008; Manion, 1981). Currently the UK is facing an episode
73 of Acute Oak Decline (AOD), which occurs widely in southern and midland England,
74 extending into Wales (Brown *et al.*, 2016; Denman *et al.*, 2016) and represents a
75 significant threat to oak, particularly native species *Quercus robur* and *Q. petraea*
76 (Denman *et al.*, 2014). First recognized in Britain in the 1980s (Denman and Webber,
77 2009), similar declines have occurred in continental Europe (Gibbs and Greig, 1997;
78 Hartmann *et al.*, 1989; Biosca *et al.*, 2003; Vansteenkiste *et al.*, 2004). AOD-affected
79 trees show discrete, weeping stem patches (stem bleeds), signifying areas of necrosis and
80 fluid filled cavities in the underlying inner bark (Denman *et al.*, 2014) (see Figure 1a-c),
81 which disrupt vascular flow of nutrients and water essential to tree survival
82 (Vansteenkiste *et al.*, 2004). Larval galleries of the buprestid beetle *Agrilus biguttatus* are
83 found in conjunction with lesions (Brown *et al.*, 2015, 2017) (Figure 1d), and can also
84 impact tree condition by girdling the tree when colonization is intense, leading to tree

85 death (Sallé *et al.*, 2014). AOD typically affects mature oaks, but has also been reported
86 in young trees (Brown *et al.*, 2016).

87 First steps to determine the causes of stem bleeds led to the isolation of several novel
88 bacterial species from AOD lesions, with three novel species, *Gibbsiella quercinecans*
89 (*Enterobacteriaceae*) (Brady *et al.*, 2010), *Brenneria goodwinii* (*Pectobacteriaceae*)
90 (Adeolu *et al.*, 2016; Denman *et al.*, 2012), *Rahnella victoriana* (*Yersiniaceae*) (Adeolu
91 *et al.*, 2016; Brady *et al.*, 2014), and an un-named *Pseudomonas* (Denman *et al.*, 2016;
92 Sapp *et al.*, 2016) consistently isolated. Some of these species, e.g. *G. quercinecans* as
93 *Serratia* sp. (Biosca *et al.*, 2003; Poza-Carrión *et al.*, 2008) on *Quercus pyrenacia* and *Q.*
94 *ilex* are implicated as causative agents of stem bleeding on other oak species in Europe
95 (Biosca *et al.*, 2003; Poza- Carrión *et al.*, 2003; Denman *et al.*, 2016). There was less
96 consistent isolation of various other bacterial species, for example *Lonsdalea quercina*
97 ssp. *britannica* (Brady *et al.*, 2012), which is closely related to *Lonsdalea quercina* ssp.
98 *quercina*, the causative agent of acorn gummosis on *Q. agrifolia* and *Q. wislizenii* in
99 America (Hildebrand and Schroth, 1967), and several other novel species that are in the
100 process of being formally described. Although correlation of certain bacterial species
101 with AOD symptomology has been observed, empirical evidence on the causative
102 agent(s) of AOD lesions is lacking and remains a barrier to developing informed
103 management strategies for this disease. In the absence of a single putative primary
104 pathogen as the causal agent of AOD lesions, we hypothesised that a polymicrobial
105 complex is responsible for AOD lesion formation. However, demonstrating causation by
106 a polymicrobial complex with associated insect activity on mature oak trees or logs is

107 challenging in the context of fulfilling Koch's postulates and requires a multi-faceted
108 methodological and conceptual approach.

109 Here, we applied a systems-level approach to determine cause(s) of necrosis in AOD
110 using microbial isolation and culture, phenotypic tests, genomic analyses of *G.*
111 *quercinecans*, *B. goodwinii*, and *R. victoriana*, metagenome and metatranscriptome
112 analysis of healthy and diseased trees, and inoculation tests comprising both polybacterial
113 inoculations and the addition of live *A. biguttatus* eggs to recreate the symptoms of AOD.
114 This combined sequencing and cultivation-based approach provides a contemporary
115 adaptation of Koch's postulates to address the biotic components of a complex decline-
116 disease, and represents a conceptual model for future analyses of polymicrobial infections
117 in trees and other systems.

118

119 **Methods**

120 **Isolation of bacteria from healthy and diseased oak trees**

121 In the search for putative causal agents of stem lesions we wanted to determine the
122 veracity of differences in occurrence and composition of bacterial communities in lesions
123 and visually healthy trees, as the bacterial microbiome has previously been identified as
124 the likely causal agent of stem lesions (Denman *et al.*, 2016). Conventional isolation and
125 culture and microbiome analyses were used. Samples were acquired through citizen
126 science reports (CSR), as well as structured studies (Denman *et al.*, 2016; Sapp *et al.*,
127 2016) ensuring the broadest possible coverage of AOD sites (Supplementary Table S1
128 and Supplementary Table S2). Eighteen CSR sites were sampled (Supplementary Figure

129 S1 and Supplementary Table S1), together with those from structured studies (Denman *et*
130 *al.*, 2016; Sapp *et al.*, 2016), as well as five sites that had no history of AOD (N-AOD).
131 Trees were sampled by forest pathologists who made site visits following the enquiry. In
132 total, isolations from 66 trees were analysed. Destructive sampling of trees, by removing
133 panels of diseased oak tissue, as well as healthy oak tissue from apparently healthy trees
134 on the same sites, was carried out where permitted, and isolations were made using
135 PYGA medium as described in Denman *et al.* (2014; 2016) and Sapp *et al.* (2016).
136 Owing to the nature of the CSR studies, the number of tissue pieces plated out was
137 variable, dependent upon the sample. Bacterial colonies emerging from chips of tissue
138 were purified using standard streak-plating techniques; single visually representative
139 colonies were selected, cultivated in Luria Bertani broth (LB) and identified with PCR
140 and DNA amplicon sequencing as described in (Denman *et al.*, 2016).

141

142 **Statistical analysis of isolation study datasets**

143 Bacterial yield between healthy and diseased trees was tested by fitting a generalized
144 linear mixed effects model with logit link function and binomial error distribution. Fixed
145 effects were fitted for tree health and tissue position and random effects fitted for sites
146 and trees within sites. Over-dispersion in the model was taken into account by including
147 an additional dispersion parameter.

148 Differences in bacterial communities were analyzed by detrended correspondence
149 analysis, down weighting bacterial species occurring in less than 5% of tissue
150 combinations. Monte Carlo permutation tests were used to test for significant differences

151 in bacterial communities between healthy and diseased trees and to test the effect of
152 tissue position within the tree. Finally, Jaccard's similarity index was used to identify any
153 significant associations between bacteria across the 66 trees of the study.

154

155 **Genome sequencing of bacterial strains isolated from Acute Oak Decline affected**
156 **trees**

157 **Maintenance of bacterial strains used in genome analyses**

158 *Gibbsiella quercinecans* FRB97 (T) (Brady *et al.*, 2010), *Brenneria goodwinii* FRB141
159 (T) (Denman *et al.*, 2012), and *Rahnella victoriana* BRK18a (T) (Brady *et al.*, 2014)
160 were isolated by Forest Research in the CSR studies, from oak trees affected by AOD.
161 Strains were stored in glycerol stocks at -80°C and maintained on nutrient agar (Oxoid) at
162 20°C.

163

164 **DNA preparation and genome sequencing on Pacific Biosciences RSII sequencing**
165 **platform**

166 The whole genomes of *G. quercinecans* FRB97, *B. goodwinii* FRB141 and *R. victoriana*
167 BRK18a were sequenced using the Single Molecule Real-Time (SMRT) technology of
168 the Pacific Biosciences RSII platform. A single colony of each isolate was sampled from
169 nutrient agar (Oxoid) and used as inoculum for liquid culture, which was grown
170 overnight in nutrient broth (Oxoid) at 28°C, and shaken at 150 rpm. Total genomic DNA
171 was isolated using the Genra Puregene Yeast/Bact. kit (Qiagen) and quantified using a

172 Qubit fluorometer (Life Technologies, Paisley, UK). DNA integrity was assessed using
173 1% agarose gel electrophoresis. *G. quercinecans* and *B. goodwinii* DNA libraries were
174 prepared using 10 µg of genomic DNA and sequenced by DUGSIM at Duke University,
175 NC, USA, using P4/C2 chemistry and six SMRT cells per isolate. The *R. victoriana* DNA
176 library was prepared and sequenced by the Centre for Genomic Research, University of
177 Liverpool, UK using P6/C4 chemistry and one SMRT cell. All sequence data described
178 in this study is available under BioProject PRJNA323828, (Supplementary Table S3).
179 Genome assembly and annotation is described in Supplementary Methods.

180

181 **Metagenome sequencing of diseased and healthy oak trees**

182 **Collection of samples for metagenome sequencing**

183 Tissue samples were collected from Runs Wood, Ross-on-Wye, and two sites in
184 Attingham park (Supplementary Table S2). More than half of the trees sampled for
185 metagenome analysis were also sampled in the isolation study. A panel comprising all
186 layers of stem tissue (outerbark, innerbark, sapwood and heartwood) was removed from
187 the visible bleed area on each diseased tree, or from stem areas at similar height on
188 healthy trees, according to previously described methods (Denman *et al.*, 2014, 2016;
189 Sapp *et al.*, 2016). Samples were immediately flash frozen on dry ice and stored at -80°C
190 prior to processing.

191

192 **Metagenome assembly, annotation and mapping to the genomes of *G. quercinecans***

193 **FRB97 (T), *B. goodwinii* FRB141 (T) and *R. victoriana* BRK18a (T)**

194 Metagenomic reads were assembled using RAY-meta v2.3.1 (Boisvert *et al.*, 2012) using
195 default parameters. Assemblies were annotated using Prokka v1.11 (Seemann, 2014).
196 Translated annotations were aligned against the translated protein sequences of *G.*
197 *quercinecans* FRB97 (T), *B. goodwinii* FRB141 (T), *R. victoriana* BRK18a (T), and two
198 control genomes *Pectobacterium carotovorum* ssp. *carotovorum* PC1 and *Paenibacillus*
199 *polymyxa* SC2, using BLASTx v2.2.26 (Altschul *et al.*, 1990). Metagenome sequences
200 with greater than 97% homology for at least 50 amino acids to proteins identified in *G.*
201 *quercinecans*, *B. goodwinii* and *R. victoriana* were considered a match (Supplementary
202 Table S4). Control genomes were selected to measure the stringency of alignment. *P.*
203 *carotovorum* ssp. *carotovorum* is a canonical plant pathogen, and a member of the soft-
204 rot *Enterobacteriaceae* (SRE) (N.B. many members have recently been reclassified into
205 novel families (Adeolu *et al.*, 2016; Charkowski *et al.*, 2012)), which had been identified
206 sporadically and at low relative abundance from metagenomic taxonomic surveys within
207 this study (Supplementary Table S5). However, *P. carotovorum* ssp. *carotovorum* had
208 not previously been isolated from necrotic lesions on Acute Oak Decline affected trees.
209 Therefore, it was proposed that coding domain alignments to *P. carotovorum* ssp.
210 *carotovorum* align to conserved genes which are present in many *Enterobacteriaceae*,
211 and their low relative abundance does not signify their presence but is an artefact of the
212 alignment process. Resultant Circos plots agree with this proposal as conserved genes are
213 frequently found in the metagenomic alignment against the *P. carotovorum* ssp.
214 *carotovorum* PC1 genome, which are likely to align to other *Enterobacteriaceae*. Within
215 *G. quercinecans*, *B. goodwinii* and *R. victoriana* there is strong homology to conserved
216 and variant genes. *P. polymyxa* SC2 was identified at low relative abundance in healthy

217 and diseased metagenomic samples (Supplementary Table S5), therefore it was selected
218 to test stringency of the metagenomic alignment and to measure its relative abundance in
219 healthy and diseased microbiomes using an alternative method. Homologous bacterial
220 protein identities and the workflow used for metagenomic analysis is available from
221 GitHub: (https://github.com/clydeandforth/multi_omics_study.git).

222

223 **Taxonomic classification of metagenome sequences**

224 To compare the taxonomic composition of the oak microbiome, raw sequence reads were
225 taxonomically labelled using Kraken v0.10.5 beta (Wood and Salzberg, 2014) and One
226 Codex (Minot *et al.*, 2015) (Figure 2a). Taxonomic labelling using Kraken was
227 performed on the standard RefSeq genome database supported by Kraken, with the
228 addition of the genomes of *B. goodwinii* FRB141 (T), *R. victoriana* BRK18a (T), *G.*
229 *quercinecans* FRB97 (T), and *Lonsdalea quercina* ssp. *quercina* ATCC 29281 (Figure 2d
230 and Supplementary Table S5). Taxonomic analysis using One Codex was conducted
231 against the One Codex March '16 Preview database with the addition of the genomes of
232 *G. quercinecans*, *B. goodwinii* and *R. victoriana*.

233

234 **Functional annotation of metagenome sequences**

235 Metagenome datasets derived from samples AT2, AT3, AT4, AT5, AT6, ROW1, ROW2
236 and ROW3 were analysed via MG-RAST (Meyer *et al.*, 2008) using Hierarchical
237 Classification against the Subsystems database with an *E*-value cut-off of 1e-5, a
238 minimum percentage identity cut-off of 80%, and a minimum alignment length of 50.

239 Descriptions of the taxonomic and functional composition of the metagenomes derived
240 from MG-RAST were comparable with those derived from analysis of the same datasets
241 using One Codex and Kraken, and further validated by mapping of metagenome reads
242 against the finished genomes of *B. goodwinii*, *G. quercinecans* and *R. victoriana*.

243

244 **Statistical analysis of metagenome datasets**

245 Statistical analyses were performed using Primer v7 (Clarke and Gorley, 2015) with
246 PERMANOVA+ add on to explore relationships between community changes (Figure
247 2b). One Codex metagenome data was log (N+1) transformed, to downweight the most
248 abundant genera. Next, dissimilarities were calculated with the S17 Bray-Curtis similarity
249 coefficient. A principal coordinate ordination analysis was performed by plotting the
250 inter-point dissimilarity values for each factor (site and disease status), the variation in
251 community composition was plotted as the first two axes (preserving actual
252 dissimilarities) (Gower, 1966). A correlation was performed between each taxon and
253 each community coordinate. Correlations with each component were deemed significant
254 ($R^2 > 0.5$) and a vector biplot was overlaid to visualise the strength of the correlation. A
255 Welch's *t*-test was performed to test significance of differences between key taxa
256 (identified above) healthy and diseased trees (pooled abundances for each factor).
257 Resultant *p*-values from Welch's *t*-test are overlaid on correlation biplot with significance
258 at >95% ($p < 0.05$) deemed significant. Comparative functional analysis of MG-RAST
259 (Meyer *et al.*, 2008) annotated, metagenome data was performed using Stamp v2.1.3
260 (Parks *et al.*, 2014). Statistically significant functional differences between diseased and
261 healthy communities were calculated using a G-test with Yates' correction. The

262 Newcombe-Wilson test was performed to calculate confidence intervals between two
263 binomial population proportions (Brown and Li, 2005).

264

265 **Metatranscriptome sequencing of AOD diseased oak trees**

266 **Collection of samples for metatranscriptome sequencing and RNA extraction**

267 For RNA sampling, two separate lesions (samples AT11 and AT12) from a single tree
268 were analysed in June 2013. Swabs of the lesion fluid were collected in addition to tissue
269 from the active margins of the lesion and immediately frozen in liquid nitrogen, and
270 transported back to the laboratory in a vessel containing liquid nitrogen. Samples were
271 stored at -80°C prior to processing. Before RNA extraction, samples in liquid nitrogen
272 were ground with a pestle in a mortar to homogenise the tissue. RNA was extracted from
273 2g of tissue using the PowerSoil Total RNA Isolation Kit (MoBio) according to
274 manufacturer's instructions. RNA was quantified using a Qubit fluorometer (Thermo
275 Fisher) and quality assessed using the Bioanalyzer 2100 (Agilent).

276

277 **Metatranscriptome sequencing**

278 Sequencing libraries were prepared from samples of total RNA using the strand-specific
279 ScriptSeq preparation kit (Illumina), and sequenced using 2x100bp paired-end
280 sequencing on the Illumina HiSeq platform. Reads were trimmed using first Cutadapt
281 1.2.1 (Martin, 2011) and additionally Sickle 1.2.00 (Joshi and Fass, 2011). Due to low

282 RNA yields from the lesion samples, total RNA was sequenced and rRNA sequence
283 reads were subsequently depleted *in silico* prior to mRNA transcript analysis.

284

285 **Metatranscriptome assembly and functional annotation**

286 Two *in silico* rRNA depleted metatranscriptome libraries were aligned to the *Gibbsiella*
287 *quercinecans* FRB97 (T), *Brenneria goodwinii* FRB141 (T), *Rahnella victoriana*
288 BRK18a (T), and control genomes (*P. carotovorum* ssp. *carotovorum* PC1 and *P.*
289 *polymyxa* SC2) (Supplementary Figure S2 and S3) with Bowtie2 v2.2.4 (Langmead and
290 Salzberg, 2012), using local mode to maximise alignment score. Aligned reads were
291 converted from Sequence Alignment/Map (SAM) to Binary Sequence Alignment/Map
292 (BAM) format and indexed using SAMtools v1.2 (Li *et al.*, 2009). To avoid false
293 positives in the detection of gene expression, a gene was considered as being expressed if
294 3 or more transcripts were aligned and the combined coverage from both libraries
295 represented more than 20% of the gene, (adapted from (Versluis *et al.*, 2015) (Figure 2a-c
296 and Supplementary Figure S4). A custom Perl script was designed to extract transcript
297 alignments, and is available from GitHub:
298 (https://github.com/clydeandforth/multi_omics_study.git). Aligned transcripts were
299 visualised in Artemis (Carver *et al.*, 2012).

300

301 **Log inoculations**

302 Pathogenicity tests were set up to reproduce lesions characteristic of AOD under
303 controlled conditions. The following hypotheses were tested: 1) Key species consistently

304 isolated from AOD symptomatic oak can cause necrosis of oak stem tissue, 2)
305 Combinations of key bacterial species cause more severe tissue necrosis (reflected in
306 larger lesions), than individual species alone, 3) The interaction between *A. biguttatus*
307 larvae (derived from eggs) and bacteria, leads to the development of AOD symptoms.

308 Four experiments were carried out over three consecutive years as testing could only be
309 done annually when beetle eggs were available. Three trials were carried out on oak logs
310 in growth chambers, and the fourth trial was set out in the field, where stems of young
311 plantation-oak (25 years old), were used instead of logs.

312

313 **Growth Chamber Log trials**

314 Logs used in the trials were obtained from freshly felled *Q. robur* trees, with diameter at
315 1.3m (DBH) = 16 - 20 cm. The trees were located in the Straits Enclosure of the Alice
316 Holt Forest, Hampshire, England, UK, and logs were transported to the laboratory after
317 felling where they were prepared for inoculation. Logs measuring 40 cm (mini-logs), or
318 130 cm (long logs) in length had the uppermost cut-surface sealed with isoflex liquid
319 rubber (Ronseal Ltd, Sheffield) to prevent desiccation. Logs were placed, lower cut
320 surface down, in saucers containing water. Logs were inoculated in August or September,
321 and incubated at 25°C in the growth chamber with a 12h photoperiod. Mini-logs were
322 used for all treatments involving *A. biguttatus* eggs, in each case only one bacterial +
323 eggs treatment type per mini-log was tested to guard against cross-contamination through
324 larval spread (Supplementary Table S2). All the bacterial treatments without eggs (either
325 single species or combinations of species) were placed on the same long log, with

326 inoculation points marked out along its length. Eggs of *A. biguttatus* were produced in
327 Forest Research's laboratories at Alice Holt (Reed, 2016). In a single experiment (2014
328 NW; Supplementary Table S2), non-wound inoculations were carried out; the remaining
329 two growth chamber trials and the field trial were inoculated using shallow wounds to the
330 outer surface of the innerbark, made by a 10 mm cork-borer. Half a loop of inoculum
331 scooped from 24h-old inoculum plates using disposable plastic loops was inserted into
332 the wound and rubbed to dislodge the bacterial cells around the wound surface. The
333 outerbark plug was replaced on the inoculation point, wounds covered with parafilm and
334 damp cotton wool, and sealed with duct tape. After 4 months incubation, experiments
335 were terminated, outer bark stripped from inoculation points to expose lesions, which
336 were hand traced onto tracing paper, and back isolations were cultured onto peptone yeast
337 glucose agar. Mass bacterial colonies that developed were tested for the presence of *B.*
338 *goodwinii*, *G. quercinecans*, *Rahnella* and *Lonsdalea* using a multiplex Taqman qPCR
339 assay. Lesion areas were calculated using ASSESS V2 (APS, Minnesota).

340 **Statistical analyses**

341 **Log lesion areas**

342 The four pathogenicity trials were used to establish the impact of different bacterial
343 species tested individually, in combination (polybacterial inoculations), and with or
344 without the addition of *A. biguttatus* eggs (Supplementary Table S2) on tissue necrosis
345 (assessed by the size of the lesion area associated with each inoculation point).

346 Lesion area data were refined in a hierarchical fashion, such that the following data sets
347 were used for lesion area analysis:

348 1) Non-contaminated samples fulfilling Koch's postulates, with galleries present (for
349 *A. biguttatus* samples indicating that eggs had hatched)

350 2) Non-contaminated samples fulfilling Koch's postulates, with or without galleries
351 present (for *A. biguttatus* samples)

352 3) Non-contaminated samples

353 Further information on statistical analyses of log lesion areas are described in
354 Supplementary Methods.

355

356

357

358 **Results**

359 **Isolation study**

360 In the isolation study, analysis of 38 diseased trees from 23 sites, plus 13 healthy trees in
361 11 of these sites, and 15 healthy trees from five sites with no history of AOD, identified
362 159 bacterial taxa. Higher yields of bacteria were obtained from lesion margin tissue of
363 symptomatic trees compared with healthy tree tissue ($F_{1,28}$, $p < 0.001$), and the lesion
364 margin bacterial profile was significantly different to healthy tissues (Monte-Carlo
365 permutation test of 1000 permutations, $p < 0.001$) (Supplementary Figure S5 and
366 Supplementary Table S6). Key genera isolated included *Pseudomonas* (comprising
367 multiple taxa and occurring in healthy as well as diseased trees); but *Gibbsiella*,
368 *Brenneria* and *Rahnella* dominated lesion margins (Supplementary Table S6). *G.*
369 *quercinecans* occurred on all disease sites, and was isolated from 83% of diseased and
370 4% of healthy trees sampled, comprising 17% of total diseased samples, and $< 0.1\%$ of
371 total healthy samples. *B. goodwinii* was more difficult to isolate, but was obtained from
372 15 sites, accounting for 16% of total diseased and $< 0.1\%$ of total healthy samples.
373 Members of the genus *Rahnella* were obtained from 65% of diseased sites, but *R.*
374 *victoriana* was isolated only from diseased trees (37%), on 9 of the sites. *L. quercina* ssp.
375 *britannica* (Brady *et al.*, 2012) was isolated sporadically on four sites, and a
376 *Pseudomonas* species (*P. fulva*-like) not yet formally identified, was isolated at eight sites
377 from diseased trees only (Supplementary Table S6). There was a significant co-
378 occurrence of *G. quercinecans* and *B. goodwinii* in diseased tissue ($J=0.56$, $p < 0.001$), but
379 neither was isolated from trees on sites with no history of AOD.

380

381 **Oak microbiome analysis**

382 Taxonomic analysis of the metagenome using unassembled metagenome sequence reads
383 against the One Codex (Minot *et al.*, 2015) March '16 Preview database (with the
384 addition of the genomes of *G. quercinecans*, *B. goodwinii* and *R. victoriana*) also
385 revealed a shift in microbiome composition between healthy stem tissue and AOD lesions
386 (Figures 2a and 2b). *Periglandula*, *Burkholderia*, *Streptomyces*, *Bacillus* and *Auriemonas*
387 were the most abundant genera in healthy trees, whereas *Brenneria* dominated diseased
388 tissue (mean read abundance, 37.5%) (Figure 2a). The mean read abundance of
389 *Gibbsiella* (0.9%) and *Rahnella* (3.7%) was also greater in diseased tissue when
390 compared with healthy tissue (both 0.1%). *Pseudomonas*, a diverse genus comprising
391 both endophytic and phytopathogenic bacteria (Vinatzer *et al.*, 2014), had similar mean
392 abundances in both diseased (4.8%) and healthy trees (3.3%). Correlation coefficients
393 and Welch's unequal variances *t*-tests revealed that *Streptomyces* ($t_{\text{Welch's}} = 49.7$,
394 $p=0.004$) and *Periglandula* ($t_{\text{Welch's}} = 821.8$, $p<0.001$) were significantly associated with
395 healthy trees, whereas *Brenneria* ($t_{\text{Welch's}} = 12.4$, $p=0.006$) and *Gibbsiella* ($t_{\text{Welch's}} = 4.7$,
396 $p=0.05$) were strongly correlated with the lesions of diseased trees (Figure 2b and
397 Supplementary Table S7). *Pseudomonas* and *Rahnella* were not strongly correlated with
398 either health state (Supplementary Table S7).

399 To identify the most abundant species in the lesion microbiome, raw metagenome reads
400 were aligned using Kraken (Wood and Salzberg, 2014) against reference genome
401 databases (see methods), revealing seventeen genomes that were commonly detected in
402 diseased tissue (Figure 2d and Supplementary Table S7). *B. goodwinii*, *G. quercinecans*
403 and *R. victoriana* were detected in the lesion metagenome of all trees with AOD. Overall,

404 *B. goodwinii* was the most abundant genome in the lesion microbiome (range, 0.3-49% of
405 metagenome sequence reads; mean, 12%), but was also detected in much lower
406 proportions in healthy trees (0.01% in all samples). *R. victoriana* (range, 0.01-15%;
407 mean, 2.1%) and *G. quercinecans* (range, 0.02-0.8%; mean, 0.3%) were the second and
408 fourth most abundant genomes in the lesions of diseased trees, respectively. Functional
409 metagenome analysis (Figure 2c and Supplementary Figure S6) revealed that genes
410 associated with carbohydrate metabolism, membrane transport, and virulence, defence
411 and disease, are key features of the lesion microbiome, suggesting that many of these
412 functions are encoded in the genome of *B. goodwinii*, *G. quercinecans* and *R. victoriana*.

413

414 **Genome analysis of AOD-associated bacteria**

415 Whole genome sequencing of *B. goodwinii* FRB141, *G. quercinecans* FRB97, and *R.*
416 *victoriana* BRK18a (Supplementary Table S8) revealed that they are phylogenetically
417 related to opportunistic phytopathogens belonging to the SRE (Charkowski *et al.*, 2012;
418 Adeolu *et al.*, 2016) and possess a catalogue of virulence genes associated with canonical
419 phytopathogens (Supplementary Table S9). The SRE alternate their lifestyle from benign
420 commensals to brute force necrotrophic-pathogens, which macerate cell wall
421 polysaccharides by releasing plant cell wall degrading enzymes (PCWDEs) (Toth *et al.*,
422 2006). A genome-wide search for PCWDEs in *G. quercinecans*, *B. goodwinii* and *R.*
423 *victoriana* revealed the presence of pectinases, cellulases and tannases (Figure 3b,
424 Supplementary Table S9) whose activity has been confirmed phenotypically
425 (Supplementary Figure S7). Furthermore, *G. quercinecans* and *R. victoriana* possess a
426 type II secretion system, an operon which releases most PCWDEs and is therefore the

427 central virulence facilitator of necrotrophic plant pathogens. *B. goodwinii* encodes a type
428 III secretion system, the principal virulence factor of established hemibiotrophic
429 pathogens such as *Pseudomonas syringae*, which use the operon to evade immune
430 surveillance, allowing bacteria to increase their numbers before releasing necrotic
431 enzymes as nutrients are depleted (Tampakaki *et al.*, 2010).

432 To address the role of *B. goodwinii*, *G. quercinecans*, and *R. victoriana* in the aetiology
433 of AOD, we aligned metagenome sequences and transcripts recovered from necrotic
434 lesions of AOD-affected trees against their genomes (Figure 3a). Alignment of six AOD
435 lesion metagenomes, revealed an average of 2225 homologous proteins in *B. goodwinii*
436 FRB141, 858 in *G. quercinecans* FRB97 and 396 in *R. victoriana* BRK18a
437 (Supplementary Table S4). Furthermore, annotated genes from the assembled
438 metagenome of a healthy oak revealed only two homologous proteins in *B. goodwinii*
439 FRB141, one in *G. quercinecans* FRB97, and two in *R. victoriana* BRK18a. Lesion
440 transcripts were aligned to coding regions within *B. goodwinii* FRB141, *G. quercinecans*
441 FRB97, and *R. victoriana* BRK18a; this revealed that the transcripts aligned significantly
442 to virulence genes including PCWDEs, secretion system machinery, and regulators of
443 PCWDEs (Figure 3c and Supplementary Figure S4). *G. quercinecans* FRB97 expressed
444 the pectic enzymes, polygalacturonase and rhamnogalacturonate lyase, and β -glucosidase
445 (cellulase), pectate exo-lyase, oligogalacturonide lyase (which cleaves polygalacturonic
446 acid, the by-product of pectin lyase) (Moran *et al.*, 1968). *B. goodwinii* FRB141
447 expressed phosphocellosbiose, β -galactosidase and several type III secretion system
448 effectors, *R. victoriana* BRK18a expressed many general secretory pathway (GSP) genes,
449 a β -glucosidase, a tannase and carbohydrate esterase enzyme (Yao *et al.*, 2013). Key

450 regulators of pectinolysis were expressed in all three bacteria including *kdgR*, *phoP*,
451 *pecT*, *rsmA/rsmB*, and *gacA* (Figure 3a).

452

453 **Log lesion areas**

454 Figures 1e-l show lesions caused by bacteria with or without *A. biguttatus* inoculated into
455 logs in pathogenicity tests. Notable results for lesion area analysis are presented in
456 Figures 1m and 1n (non-contaminated samples fulfilling Koch's postulates, with galleries
457 present for *A. biguttatus* samples). In bacteria only inoculations, lesions significantly
458 bigger than wound controls (Figure 1m) were made by the combination of bacterial
459 species *B. goodwinii* + *G. quercinecans* ($t_{dunnetx} = 2.97$, $p=0.044$); the bacterial species *G.*
460 *quercinecans* had larger lesion areas versus the controls, although this was not significant
461 and the $p<0.05$ level ($t_{dunnetx} = 2.79$, $p=0.068$); *B. goodwinii* lesions were smaller and not
462 significantly different from the controls in terms of mean lesion area ($t_{dunnetx} = 1.30$,
463 $p=0.796$), but the necrosis was clearly different to the control wound response (see
464 Figures 1e vs 1f). Inoculation with *Erwinia billingiae*, a known ubiquitous non-
465 pathogenic bacterium, served as a negative control demonstrating lesion area no different
466 to the control wound response. The pattern strengthened when bacteria plus *A. biguttatus*
467 (applied as eggs) were co-inoculated (Figure 1n). Lesions created by *B. goodwinii* and *A.*
468 *biguttatus* eggs (larvae) were significantly bigger than wound controls ($t_{dunnetx} = 3.40$,
469 $p=0.0125$), as were *G. quercinecans* and *A. biguttatus* ($t_{dunnetx} = 4.65$, $p=0.0002$) and the
470 combination of *B. goodwinii* + *G. quercinecans* + *A. biguttatus* ($t_{dunnetx} = 3.92$, $p=0.0027$)
471 (Figure 1n). Bacteria were re-isolated from lesion margins and at intervals along the
472 larval galleries, which were becoming necrotic forming part of the lesion. Apart from

473 demonstrating the necrogenic ability of the bacteria, these results showed that the larvae
474 and larval galleries have an important part in increasing lesion area, implicating them in
475 the spread of bacteria within infected trees.

476

477 **Bacterial positive back-isolation and *Agrilus biguttatus* contamination**

478 There was a high success of back-isolation for both *B. goodwinii* and *G. quercinecans*
479 when these species were included as treatments (70% and 80% respectively); both
480 species were also identified as contaminants (i.e. back-isolated when not part of the
481 treatment inoculation; *B. goodwinii* = 9%; *G. quercinecans* = 16%), although the
482 contamination rate was significantly lower than treatment back-isolation in both cases (*B.*
483 *goodwinii*: $z = 7.98, p < 0.001$; *G. quercinecans*: $z = 6.65, p < 0.001$). For *G. quercinecans*,
484 the rate of contamination was affected by the presence of *A. biguttatus* eggs: when eggs
485 were absent *G. quercinecans* contamination was 3%; when eggs were present this rose to
486 38% contamination, a significant increase ($z = 3.59, p < 0.001$). There was no significant
487 effect of *A. biguttatus* eggs on *B. goodwinii* contamination (eggs absent, *B. goodwinii*
488 contamination = 2%; eggs present, *B. goodwinii* contamination = 7%, $z = 1.60, p = 0.11$).

489 Discussion

490 Fulfilling Koch's postulates represents the traditional paradigm for proving disease
491 causation. However, contemporary molecular approaches have transformed our
492 appreciation of the role of microbial communities and polymicrobial interactions in
493 disease. There are situations where the one pathogen causes one disease model is not
494 suitable to prove causality and must be adapted to accommodate polymicrobial
495 infections. For example, in decline-diseases of trees, application of Koch's postulates in
496 the strictest sense cannot fully address proving causality of lesion-formation as more than
497 one necrogenic agent is involved in the disease syndrome. Here, causation of AOD
498 lesions was demonstrated using a combined cultivation-based and sequencing approach
499 in a dynamic adaptation of Koch's postulates analogous to that described by Byrd and
500 Segre (2016). Koch's first postulate is fulfilled by the consistent isolation and
501 metagenomic detection of *G. quercinecans*, *B. goodwinii*, and *R. victoriana* in trees
502 affected by AOD.

503 This combined approach delivered an improved understanding of lesion microbiome
504 composition, as some species were difficult to isolate and culture. For example, *B.*
505 *goodwinii* and *Lonsdalea quercina* ssp. *britannica* were under-represented, whereas *G.*
506 *quercinecans*, which is more amenable to isolation and cultivation, was over-represented,
507 with the opposite trend evident in metagenomic studies. Furthermore, *G. quercinecans*, *B.*
508 *goodwinii* and *R. victoriana* were absent on sites with no history of AOD, were rarely
509 isolated from healthy oak on diseased sites, and had negligible abundance in healthy
510 metagenome samples at AOD sites, complying with Koch's second postulate. Ultra-low
511 levels of *G. quercinecans*, *B. goodwinii* and *R. victoriana* were detected in some of the

512 healthy trees, but only from sites where AOD is present, raising interesting questions
513 about their existence in the wider environment. Possible explanations for the presence of
514 *G. quercinecans*, *B. goodwinii* and *R. victoriana* in healthy trees on AOD sites may
515 include; (1) the tree is in early stages of lesion formation, where visible symptoms have
516 not yet developed, or that (2) *G. quercinecans*, *B. goodwinii* and *R. victoriana* are
517 endophytes or epiphytes that opportunistically multiply after tissue necrosis is initiated by
518 another organism. In the first situation, the chances of detecting asymptomatic lesion
519 formation seem fairly remote, especially as crown condition is not a reliable indicator of
520 tree predisposition status in the early stages of decline development. In the second case,
521 several pieces of evidence counteract the possibility that these organisms multiply
522 opportunistically after tissue necrosis occurs, as (1) we observed the expression of
523 putative necrogenic enzymes and virulence factors of these bacteria in AOD lesions *in*
524 *planta*, implicating them in having an active role in tissue degradation, and (2) *G.*
525 *quercinecans* and *B. goodwinii* caused significant lesion formation in log inoculation
526 trials, demonstrating actual lesion forming capabilities. It is important to note that the oak
527 logs and trees used in our trials were selected from areas with no history of the disease.

528 Furthermore, all three bacterial species possess the genomic capability to cause tissue
529 necrosis, as determined through genomics, *in situ* functional metagenomics and
530 metatranscriptomics. Inoculation onto live oak logs confirmed significant necrogenic
531 ability of *B. goodwinii* and *G. quercinecans*, but further work with *R. victoriana*
532 inoculation is required. Thus, the pathogenic phenotype of *B. goodwinii* and *G.*
533 *quercinecans* has been confirmed, broadly fulfilling Koch's third postulate, that
534 inoculation tests can cause the disease anew. However, bacterial species combinations

535 caused even greater necrosis indicating cumulative effect and possible synergism, this is
536 a contemporary adaptation of Koch's postulates *sensu stricto*. Finally, once inoculated
537 onto live oak panels, *B. goodwinii* and *G. quercinecans* were re-isolated to fulfil Koch's
538 fourth postulate (although back-isolation of some species was difficult, and there is a
539 need for developing rapid, cost-effective tools to detect pathogens that are difficult to
540 isolate). Consequently, we propose that the biotic component of the AOD lesions is a
541 polybacterial complex comprised primarily of *G. quercinecans* and *B. goodwinii*, which
542 are now established as key causal agents of tissue necrosis, the primary symptom of
543 AOD. Our studies also indicated that other members of the microbiome may contribute to
544 the pathology of AOD. Microbiome analysis suggested that *R. victoriana* is abundant and
545 also important in AOD lesions; however, back-isolation of *R. victoriana* was variable,
546 and further tests to characterize its role and interactions with *B. goodwinii* and *G.*
547 *quercinecans* are required. In addition, *Lonsdalea quercina* ssp. *britannica* in particular
548 demonstrated variable, but at times virulent pathogenicity in log inoculations, although it
549 was not consistently present in AOD lesions. Our results indicate that *Agrilus* larvae
550 potentiate the spread of these necrogenic bacterial species within tree tissue, generating
551 new break-out points of tissue necrosis, and replicating the observed aetiology of AOD.
552 Ultimately, vascular degradation arising from a combination of bacterial tissue necrosis
553 and inner bark damage from larval galleries exacerbates and accelerates the decline by
554 interrupting carbon resource allocation, preventing accumulation in roots, and reducing
555 water availability.

556 It is clear that, where possible, microbiome analysis methods together with inoculation
557 assays should become the accepted standard for profiling disease complexes, particularly

558 when considering complex interactions between microorganisms, insects, the
559 environment, and the host. This phenomenon has never previously been addressed in
560 arboreal systems using the approaches described here. Our work therefore highlights the
561 importance of a systems-level approach for characterizing the pathology of complex
562 diseases, and represents a conceptual and methodological template for adapting Koch's
563 postulates to address the role of microbial communities in disease. In recent years,
564 microbiome studies have transformed our understanding of the role of human gut
565 microbiota in a variety of conditions, including bowel and cardiovascular disease (Frank
566 *et al.*, 2007; Koeth *et al.*, 2013), and consequently, microbiome-wide association studies
567 that link microbial consortia to disease will play an important role in the development of
568 future diagnostics and therapies for disease (Gilbert *et al.*, 2016). For decline-diseases in
569 particular, further adaptation may be required to include the role of host predisposition.

570 While AOD has been formally described as a major threat to UK oak, similar decline-
571 diseases have been reported in mainland Europe (Biosca *et al.*, 2003; Poza-Carrión *et al.*,
572 2008; Hartmann *et al.*, 1989; Vansteenkiste *et al.*, 2004; Delatour, 1983), the Middle
573 East, and the Americas (Lynch *et al.*, 2014) indicating that AOD is a global concern that
574 has likely evaded attention due to the complexity of its causative agents. The
575 polybacterial nature of AOD exhibits similarities to other economically important tree
576 diseases such as olive knot disease (Buonaurio *et al.*, 2015) but may also be applied to
577 other complex diseases. Generally, our findings highlight the importance of
578 understanding polymicrobial interactions in the context of future-proofing plant health to
579 protect important but increasingly disease-prone forests and crops that are a fundamental
580 part of our global landscape.

581 **Accession codes**

582 All sequence data described in this study is available under BioProject PRJNA323828
583 and PRJNA321868.

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756

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767

768 **Fig 1.** Symptoms of Acute Oak Decline observed in the field (a-d), reproducing the symptoms of
769 AOD through log inoculations with combinations of bacteria and *Agrilus* larvae associated with
770 AOD (e-l), and statistical analysis of lesion formation in log inoculations (m-n). (a) Stem bleeds
771 symptomatic of Acute Oak Decline (AOD) on a mature *Quercus robur* in the field. (b) Close up
772 view of stem bleeds and bark cracks characteristic of AOD. (c) Cross-section through AOD stem
773 bleeds, showing degradation of vascular tissue (red arrow). (d) Longitudinal section through an
774 AOD stem bleed showing the association between bacterial lesions (blue arrow) and *Agrilus*
775 *biguttatus* galleries (red arrow). (e) Wound response in the innerbark of the control treatment
776 (inoculation of a wound with sterile water) of log inoculation trials. (f) Lesion formed in the
777 innerbark of oak logs inoculated with *Brenneria goodwinii* in log inoculation trials. (g) Lesion
778 formed in the innerbark of oak logs inoculated with *Gibbsiella quercinecans* in log inoculation
779 trials. (h) Lesion formed in the innerbark of oak logs inoculated with a combination of *B.*
780 *goodwinii* and *G. quercinecans* in log inoculation trials. (i) Wound response in the innerbark of
781 the *A. biguttatus* treatment (inoculation of a wound with live eggs of *A. biguttatus*). Note the
782 wound response (blue arrow) and clean gallery created by a larva (red arrow). (j) Lesion formed
783 in the innerbark of oak logs inoculated with *B. goodwinii* and eggs of *A. biguttatus*. Note the
784 lesion developing from the inoculation point (blue arrow), and from the galleries (red arrow). (k)
785 Lesion formed in the innerbark of oak logs inoculated with *G. quercinecans* plus eggs of *A.*
786 *biguttatus*. Note the lesion developing from the inoculation point (blue arrow), and from the
787 galleries (red arrow). (l) Lesion formed in the innerbark of oak logs inoculated with *B.*
788 *goodwinii*, *G. quercinecans* plus eggs of *A. biguttatus*. Note the lesion developing from the
789 inoculation point (blue arrow), and from the galleries (red arrow). (m) Mean lesion area formed
790 in the inner bark of logs inoculated with bacteria. Colour indicates statistical significance (see
791 key). The bacterial species inoculated were back-isolated in fulfilment of Koch's fourth
792 postulate. Error bars = - standard error. (n) Mean lesion area formed in the inner bark of logs
793 inoculated with bacteria and *A. biguttatus* eggs. Colour indicates statistical significance (see key
794 in m). The bacterial species inoculated were back-isolated in fulfilment of Koch's fourth
795 postulate. *Eb* – *Erwinia billingiae*, *Bg* – *Brenneria goodwinii*, *Gq* – *Gibbsiella quercinecans*.
796 Error bars = - standard error.

797

798 **Fig 2.** Comparative metagenomic analysis of the taxonomic composition and function of the oak
799 microbiome in healthy tissue (from trees without AOD) and diseased tissue (from trees with
800 AOD). (a) The plot depicts the genera represented in all metagenomes based on One Codex
801 binning of raw reads, demonstrating a clear shift in the microbial community between healthy
802 and diseased trees. (b) *Brenneria* and *Gibbsiella* are statistically correlated to diseased tissue, as
803 shown by a principal coordinate ordination analysis based on statistics calculated by Primer v7
804 and PERMANOVA+ using One Codex binning data. The first two axes depict the plotted
805 community composition. Correlation vectors in the graph are significant using an $R^2 > 0.05$. A
806 Welch's *t*-test was performed to test significance of differences between key taxa (identified
807 above) between healthy and diseased trees (pooled abundances for each factor). Resultant *p*-
808 values from Welch's *t*-test are overlaid on the correlation biplot. (c) Gene groups involved in
809 bacterial phytopathogenic activity are significantly increased in AOD diseased trees, as shown
810 by comparative functional analysis of SEED subsystem categories as annotated by MG-RAST on
811 assembled metagenome contigs. The analysis of SEED subsystem metagenome data was
812 performed using Stamp. Statistically significant functional differences between diseased and
813 healthy communities were calculated using a G-test with Yates' correction. The Newcombe-
814 Wilson test was performed to calculate confidence intervals between two binomial population
815 proportions. (d) The genomes of 17 species were found to be common across all AOD
816 metagenomes. Visualization of Kraken metagenome analysis of stem samples demonstrates the
817 shifts in bacterial microbiome compositions. Bubble sizes are categorized based on the relative
818 percentage frequencies of Kraken species-level alignment of raw metagenome sequencing reads,
819 and are depicted in the figure as 1 (read frequency <0.01), 2 (read frequency 0.01-0.1), 3 (read
820 frequency 0.1-1), 4 (read frequency 1-10) and 5 (read frequency >10). Red bubbles signify
821 samples from diseased trees, while blue bubbles signify samples from healthy trees. The 17
822 common species were determined based on common occurrence across all diseased tissue
823 samples. Additionally, six species more abundant among the healthy trees were included to
824 provide a contrasting shift.

825

826

827 **Fig 3.** Functional genome analysis of *Gibbsiella quercinecans* FRB97 (T), *Brenneria*
828 *goodwinii* FRB141 (T), and *Rahnella victoriana* BRK18a. (a) Circular representations of *B.*
829 *goodwinii* FRB141, *G. quercinecans* FRB97, and *R. victoriana* BRK18a genomes. From
830 outside to inside, circles represent: (1) Assembled bacterial genomes, outermost (orange)
831 circle, with encoded secretion system annotated at their genomic loci. (2) Metatranscriptome
832 heatmap. Alignment of two *in silico* combined metatranscriptomes recovered from two
833 necrotic lesions of AOD-affected trees, against the bacterial genomes. Blue saturation
834 represents increasing transcript alignments. (3-9) Seven metagenomes from necrotic lesions
835 on AOD affected trees and one healthy tree (metagenomes were extracted from two sites,
836 Attingham and Runs Wood), were aligned through their coding domains to homologous
837 regions in the bacterial genomes. (3) Attingham healthy (AT1) aligned metagenome coding
838 domains (light purple). (4) Attingham diseased (AT7) aligned metagenome coding domains
839 (aqua). (5) Attingham diseased (AT8) aligned metagenome coding domains (blue). (6)
840 Attingham diseased (AT9) aligned metagenome coding domains (orange). (7) Runs Wood
841 diseased (RW1) aligned metagenome coding domains (green). (8) Runs Wood diseased
842 (RW2) aligned metagenome coding domains (pink). (9) Runs Wood diseased (RW3) aligned
843 metagenome coding domains (grey). (10) G+C content across the bacterial genome. (11)
844 G+C skew across the bacterial genome. (b) Schematic diagram of transporters and
845 transported proteins recovered from lesion metatranscriptomes and aligned against each
846 bacterial genome. Number of significantly expressed (>3 aligned transcripts, covering >20%
847 of the gene) genes is shown in parentheses. (c) Number of transcripts aligned against selected
848 virulence genes encoded within *B. goodwinii* FRB141, *G. quercinecans* FRB97, and *R.*
849 *victoriana* BRK18a. Gene categories are represented by the following colours, red - plant cell
850 wall degrading enzymes (PCWDE), purple - general secretory pathway (GSP), yellow - type
851 II secretion system (T2SS), blue - type III secretion system (T3SS), pink - type III secretion
852 system effectors (T3SS effectors), and green - global regulators (GR)

853 **Supplemental Tables:**

854 **Supplementary Table S1. Forest and woodland sites used for bacterial isolation from**
855 **diseased (Acute Oak Decline) and healthy oak trees.** Ordinance survey co-ordinates are
856 provided.

857 **Supplementary Table S2. Annual log inoculation test treatments using single and**
858 **multiple bacterial species inoculations, with or without the addition of eggs of *Agrilus***
859 ***biguttatus*.** Microbiome samples for metagenomic analysis were gathered at three of these
860 sites, two sites in Attingham park were sampled on two dates: Samples AT1, AT7, AT8 and
861 AT9 were collected from OS Eastings 356033, Northings 310372, in November 2013 as part
862 of the CSR framework of sample collection; and samples AT2, AT3, AT4, AT5 and AT6 at
863 OS Eastings 356033, Northings 310372, were collected additionally in June 2015. Samples
864 RW1, RW2 and RW3 were collected from Runs Wood in February 2014 (OS Eastings
865 563207, Northings 310858) as part of the CSR framework, and samples ROW1, ROW2 and
866 ROW3 were additional samples collected from Ross-on-Wye (OS Eastings 357887,
867 Northings 221731), in June 2015. Ordinance survey co-ordinates are provided.

868 **Supplementary Table S3. Accession numbers for genomic, metagenomic,**
869 **metatranscriptomic libraries.** All libraries were submitted to the National Center for
870 Biotechnology Information. *Genome assembly.

871 **Supplementary Table S4. Alignment of metagenome coding domains to the genomes of**
872 ***Brenneria goodwinii* FRB141 (T), *Gibbsiella quercinecans* FRB97 (T), and *Rahnella***
873 ***victoriana* BRK18a (T) and two control genomes, *Pectobacterium carotovorum* ssp.**
874 ***carotovorum* PC1, a known plant pathogen, and the endophyte, *Paenibacillus polymyxa***
875 **SC2.** To assess the abundance of *Brenneria goodwinii*, *Gibbsiella quercinecans*, and
876 *Rahnella victoriana* in the Acute Oak Decline (AOD) lesion metagenome, alignments were
877 made between assembled metagenomic libraries from AOD diseased and a healthy oak tree
878 (AT1), and assembled bacterial coding domains of *B. goodwinii*, *G. quercinecans* and *R.*
879 *victoriana*. In addition, to assess the veracity of our alignment approach, we tested two
880 additional control genomes; *Pectobacterium carotovorum* ssp. *carotovorum* PC1 is a well-
881 characterized bacterial phytopathogen that has not previously been associated with oak. This
882 genome would therefore not be expected to be present within AOD lesion metagenomes, and
883 therefore provides an indication of the potential level of alignment matches from homologous
884 genes found within other members of the *Enterobacteriaceae*. Only a small proportion of

885 metagenome coding domains (44-183) mapped with *P. carotovorum* ssp. *carotovorum* PC1,
886 suggesting that the greater proportion of matches that occur between *B. goodwinii*, *G.*
887 *quercinecans* and *R. victoriana* reflect the actual presence and abundance of those species in
888 AOD lesion metagenomes, rather than them representing metagenome coding domains from
889 other *Enterobacteriaceae* that have been falsely attributed to our focal species (*B. goodwinii*,
890 *G. quercinecans* and *R. victoriana*). *Paenibacillus polymyxa* SC2 is a putative endophyte
891 found at low relative abundance within symptomatic and healthy oak tissue and was included
892 as a control to ensure that the high numbers of metagenome coding domains mapped to *B.*
893 *goodwinii*, *G. quercinecans* and *R. victoriana* are accurate and would not occur in the same
894 way for other *Enterobacteriaceae* that are known to also be present in AOD lesions. The low
895 numbers of coding domains that map against *P. polymyxa* SC2 (0-8) again supports the
896 veracity of our alignment approach, and the abundance of *B. goodwinii*, *G. quercinecans* and
897 *R. victoriana* in AOD lesion tissue.

898 **Supplementary Table S5. Combined Kraken output data table.** Output data of Kraken
899 metagenomic binning of all metagenomes. Where U=unidentified, D=domain, P=phylum,
900 C=class, O=order, F=family, G=genus, S=species. The standard Kraken database was
901 supplemented using the sequenced genomes of *Rahnella victoriana*, *Lonsdalea quercina*,
902 *Gibbsiella quercinecans* and *Brenneria goodwinii*.

903 **Supplementary Table S6. Occurrence of the most commonly isolated bacterial taxa on**
904 **sites and trees.**

905 **Supplementary Table S7. Significance of genera detected in healthy and symptomatic**
906 **metagenome samples.** Welch's unequal variances *t*-tests of genus abundance detected in
907 metagenome datasets derived from healthy and diseased oak trees. ^aAsymptotically F
908 distributed.

909 **Supplementary Table S8. Genome metrics of *Brenneria goodwinii* FRB141 (T),**
910 ***Gibbsiella quercinecans* FRB97 (T), and *Rahnella victoriana* BRK18a (T) sequenced**
911 **bacterial isolates.** Bacteria were isolated from necrotic lesions of Acute Oak Decline
912 affected trees.

913 **Supplementary Table S9. Genomic loci of putative bacterial virulence genes.** Genes
914 encoded within *Brenneria goodwinii* FRB141, *Gibbsiella quercinecans* FRB97, and *Rahnella*
915 *victoriana* BRK18a. PCWDE – plant cell wall degrading enzymes, T2SS – type II secretion
916 system, T3SS – type III secretion system, T4SS – type IV secretion system.