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Host-microbiota-insect interactions drive emergent virulence in a complex tree disease

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Abstract

Forest declines caused by climate disturbance, insect pests and microbial pathogens threaten the global landscape, and tree diseases are increasingly attributed to the emergent properties of complex ecological interactions between the host, microbiota and insects. To address this hypothesis, we combined reductionist approaches (single and polyspecies bacterial cultures) with emergentist approaches (bacterial incoluations in an oak infection model with the addition of insect larvae) to unravel the gene expression landscape and symptom severity of host-microbiota-insect interactions in the Acute Oak Decline (AOD) pathosystem. AOD is a complex decline disease characterised by predisposing abiotic factors, inner bark lesions driven by a bacterial pathobiome, and larval galleries of the bark-boring beetle *Agrilus biguttatus*. We identified expression of key pathogenicity genes in *Brenneria goodwinii*, the dominant member of the AOD pathobiome, tissue-specific gene expression profiles, cooperation with other bacterial pathobiome members in sugar catabolism, and demonstrated amplification of pathogenic gene expression in the presence of *Agrilus* larvae. This study highlights the emergent properties of complex host-pathobiota-insect interactions that underlie the pathology of diseases that threaten global forest biomes.

Introduction

Global forests provide essential ecological, economic and cultural services, but their capacity for carbon storage and climate regulation is increasingly threatened by altered climatic conditions and increased attack by pests and pathogens [1,2]. In recent decades, devastating outbreaks of tree disease such as chestnut blight [3], Dutch elm disease [4], and ash dieback [5], have changed the global landscape, and tree pests and diseases therefore represent a major future threat to forest biomes. Such diseases often involve the activity of both insect pests and microbial pathogens, and ultimately arise from complex interactions between the host, environment, pests and pathogens [6–8].

Acute Oak Decline (AOD) is a complex decline disease mediated by abiotic predisposing factors (temperature, rainfall, nutrients) [9] and biotic contributing factors (insect and bacterial) [8] that are a major threat to native oak in the UK, with similar declines described in continental Europe [10–12], Asia [13] and America [14]. The characteristic disease symptoms are outer bark cracks with dark exudates (bleeds), which overlie necrotic tissue in the inner bark, and larval galleries and exit holes of the two-spotted buprestid beetle *Agrilus biguttatus* [10]. Previously, we demonstrated that tissue necrosis on AOD affected trees is caused by a polybacterial complex (pathobiome) which macerates pectin connective tissue within the cells, resulting in inner bark lesions on oak stems [8,15]. The pathobiome is a complex assemblage of organisms that combine to cause disease in host organisms and challenge strict adherence to Koch's postulates [16]. It has previously been shown that AOD is not caused by a single pathogen, but results from interactions between the pathobiome, *A. biguttatus*, the host and it's environment [8]. Within the AOD pathobiome several bacteria are consistently identified, primarily *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Rahnella victoriana*, and occasionally, *Lonsdalea britannica*.

Brenneria goodwinii, is the most active member of the lesion pathobiome, and is thought to be the primary agent of bacterial canker in AOD [8,15]. *Agrilus* larvae are also associated with AOD lesions, and spread necrogenic members of the pathobiome through the inner bark tissue, amplifying the area of tissue necrosis in the inner bark [8].

Unravelling the mechanistic processes and complex multidimensional interactions between the host, environment, insects, and the pathobiome that underlie the aetiology of complex tree diseases is challenging, but represents a major knowledge gap. Considering pathobiome virulence as an emergent property [17], where emerging properties cannot be explained by

their individual components and are greater than the sum of their individual components, is therefore an attractive framework in conceptualising complex tree diseases. Here, we hypothesise that host-microbiota-insect interactions combine to cause emergent properties of pathobiome virulence in AOD. To investigate this, we combined reductionist approaches (interactions with oak tissue in single and polyspecies bacterial culture) with emergentist approaches (bacterial inoculations in an oak infection model with the addition of insect larvae) to unravel the gene expression landscape of host-microbiota-insect interactions in the Acute Oak Decline (AOD) pathosystem.

Results and Discussion

Inoculation of *B. goodwinii, G. quercinecans* and *L. britannica* onto oak logs with *A. biguttatus* eggs

Oak logs were inoculated with either *B. goodwinii*, *G. quercinecans* or *L. britannica* (single, bacteria-only treatments), or in combination with *A. biguttatus* eggs (single bacterial species plus *Agrilus* treatments) (i.e. six treatments). RNAseq analysis of the resultant stem lesions (or 'clean' stem tissue, for control treatments) revealed that apart from host genes, *B. goodwinii* genes were the most actively expressed amongst the bacterial species tested (figure 1 and electronic supplementary material table, S1), concurring with previous results [8,15]. The complete genome of *B. goodwinii* FRB141 contains 4869 genes and the highest levels of *B. goodwinii* gene activity in the log infection tests were detected in treatments where *B. goodwinii* was co-inoculated with *A. biguttatus* eggs (515, 3924, and 2464 genes expressed in each replicate, respectively) and there was positive detection of *B. goodwinii* via RT-qPCR (please see materials and methods for our definition of active genes briefly, these are genes which were not differentially expressed, but were deemed 'active' as they passed expression filters e.g. Transcripts per Million, but differ from subsequent analyses that focussed on differential gene expression). By comparison, only one of the three *B. goodwinii* only inoculations produced necrosis, with 3819 active genes detected, while the other two inoculations did not show appreciable lesion development and only 88 and 96 active genes were detected.

Lesions barely developed in *L. britannica* inoculations, with low activity (11 +/- 3 active genes) detected, although this species was previously isolated from naturally symptomatic material and has the genomic potential to cause tissue necrosis [18]. By comparison, when co-inoculated with *A. biguttatus* eggs, two of the three inoculations developed dramatic,

typical AOD lesions, with 46 and 1607 *L. britannica* genes active (figure 1), but both *B. goodwinii* and *G. quercinecans* were also reisolated via RT-qPCR, and 852 and 2942 *B. goodwinii* genes and 579 and 320 *G. quercinecans* genes were found to be active. Notably, *G. quercinecans* which has been consistently isolated from environmental AOD lesions and can cause necrotic lesions on oak [8], had low activity in log inoculations (143 +/- 71 active genes), but had higher gene activity and significant lesion formation when combined with *A. biguttatus* (444 +/- 225).

Thus, with the exception of a single *B. goodwinii* inoculation, none of the single isolate inoculations created significant lesions or demonstrated high gene expression, which supports our hypothesis that although these organisms can be pathogenic, emergent virulence is dependent upon complex host-pathobiome-insect interactions. However, when co-inoculated with *A. biguttatus* eggs that developed into larvae, typical AOD symptoms were developed and *B. goodwinii* gene activity was highly increased. This suggests that the presence of *A. biguttatus* larvae provides a stimulus for enhanced *B. goodwinii* pathogenicity. Furthermore, the biggest lesions formed when genes of all three bacterial species were detected. Despite the fact that only single species inoculations were made, the occurrence of *B. goodwinii* and *G. quercinecans* in the *L. britannica* plus *Agrilus* treatment could be explained either by the bacteria already being present as endosymbionts of the non-symptomatic oak logs, or by them gaining entry through wound inoculations, or that *A. biguttatus* is a vector of *B. goodwinii*, either incidentally or that it resides within *A. biguttatus* as part of the microbiome and is deposited when feeding or egg laying [10]. This suggests that the presence of *A. biguttatus* larvae provides a stimulus for enhanced *B. goodwinii* pathogenicity. However, there is no previous evidence showing that *A. biguttatus* is a vector of *B. goodwinii*, *G. quercinecans or L. britannica* and the bacteria-beetle relationship may be as co-infecting agents taking advantage of declining oak trees [19].

Our results demonstrate that the driver of variation between non-symptomatic and symptomatic oak trees was bacterial inoculum ($P = 0.031$) and the prescence of *A. biguttatus* larvae ($P = 0.005$) (figure 1). Possible sources of variation in gene activity between symptomatic and non-symptomatic trees were tested in a multivariate model, these were: actual lesion size, presence or absence of *A. biguttatus*, bacterial inoculum, and between replicate differences. Biological replicates and lesion size did not account for significant variation in gene activity ($P > 0.05$). Furthermore, differential gene expression analysis revealed that the number of genes expressed in *G. quercinecans* and *L. britannica* was

relatively small, whereas in *B. goodwinii* inoculations, a substantial portion of the *B. goodwinii* geneset (electronic supplementary material table, S2) was differentially expressed. Therefore, the following differential gene expression analysis of *B. goodwinii* was directly compared against control treatments and *B. goodwinii* when co-inoculated with *A. biguttatus* larvae.

B. goodwinii **has a high number of significantly upregulated genes in log inoculations when inoculated with** ***A. biguttatus***

Differential gene expression analysis of *B. goodwinii* log inoculations (bacteria only) compared against wound and water controls revealed 191 genes were significantly differentially upregulated (electronic supplementary material table, S2). Comparison of the *B. goodwinii* and *A. biguttatus* treatment with the wound control resulted in 552 upregulated *B. goodwinii* genes. Variance between expressed genes within transcriptomic datasets was measured using principal component analyses (PCA) (figure 2a). This PCA collapsed 73% of the variance and revealed clear separation between transcipt abundance in *B. goodwinii* infected oak logs compared to the control (figure 2a, bottom). The same pattern was found in *B. goodwinii* and *A. biguttatus* inoculated oak logs where 81% of the variance was captured in a PCA and revealed distinct expression patterns in comparison to oak control logs (figure 2a, top). Analysis of differential expression of gene families, revealed signifcant upregulation of putative pathogenic families in *B. goodwinii* and *A. biguttatus* egg inoculations when compared to *B. goodwinii* only oak logs. These gene families were identified using geneset enrichment analysis and revealed that gene families were upregulated in *B. goodwinii* by the presence of *A. biguttatus* eggs. Significantly upregulated functional groups include bacterial pathogenicity homologs, such as bacterial secretion systems (P=0.04, KEGG family 03070), terpenoid biosynthesis (P=0.04, KEGG family 00130), biofilm formation (P=0.007, KEGG family 02026), and quorum sensing (P=0.01, KEGG family 02024) (figure 2b). Differential gene expression analysis between oak log incoulations revealed significant upregulation of pathogenicity associated genes in *B. goodwinii* and *A. biguttatus* oak logs compared to control, in comparison to differential expression of the same gene in *B. goodwinii* only oak logs when compared to contol. Genes were functionally annotated using homologs in closely related bacteria (see methods). Significantly upregulated functional homologs included a biofilm formation gene, exoglucanase B – *chvB* (*Padj* < 0.0001 in *B. goodwinii* + *A. biguttatus* vs. healthy, compared to *B. goodwinii* only vs. healthy, which had no *P* value due

to low transcript expression), an adherence gene – *fhaB* (*Padj*=0.03 in *B. goodwinii* + *A. biguttatus* vs. healthy, compared to *P* = 0.02, N.B *Padj* was NA as the mean read count was low in *B. goodwinii* only v healthy), poly(β-D-mannuronate) C5 epimerase 1, a biofilm formation and quorum sensing gene - *algG* (*Padj* < 0.0001 in *B. goodwinii* + *A. biguttatus* v healthy, compared to *Padj* = 0.0006 *B. goodwinii* only vs. healthy). Poly(β-D-mannuronate) C5 epimerase 1 is a large, type I secreted adhesin which is found in shiga toxin producing *E. coli* strains and in disease formation of the bacterial phytopathogen *Pectobacterium atrosepticum* [20,21]. Both exoglucanase B and poly(β-D-mannuronate) C5 epimerase 1 were significantly upregulated in *B. goodwinii* and *G. quercinecans* only live log inoculations indicating that *A. biguttatus* may not be the only stimulus for its expression. The actual stimulus may be carried by *A. biguttatus* or may reside in the wider environment. Similar to the type I secreted proteins, two copies of the two-partner secreted filamentous hemagglutinin (*fhaB*), a bacterial virulence gene were expressed by *B. goodwinii* across live log transcriptomes. As described above, the number of genes expressed in *B. goodwinii* when *A. biguttatus* was present was greater than *B. goodwinii* only inoculations (191 vs. 552, respectively), but in addition the number of pathogenic gene homologs expressed increased when *A. biguttatus* eggs were combined with *B. goodwinii* (figure 2c).

The T3SS is a primary virulence factor in seven of the top ten bacterial plant pathogens [22]. *B. goodwinii* encodes a complete T3SS and multiple effectors, which is likely to be a key pathogenicity component within AOD tissue necrosis [18]. Within *B. goodwinii* and *A. biguttatus* live log inoculations, four T3 effectors are significantly differentially expressed, only one of which is expressed in *B. goodwinii* only inoculations (figure 2c). Significantly expressed T3 effectors are; HopPtoL (*Padj* = 0.02), SrfB (*Padj* = 0.02), AvrE_2 (*Padj* = 0.015), in addition to AvrE_1 which is significantly differentially expressed in *B. goodwinii* only and with *A. biguttatus* inoculations (*Padj* = 0.04, *B. goodwinii* inoculation only; *Padj* = 0.0001, *B. goodwinii* and *A. biguttatus* co-infection). The AvrE T3 effector is found in a wide number of bacterial plant pathogens due to its proclivity for horizontal gene transfer [23]. Notably, within the plant pathogen *Pseudomonas viridflava*, AvrE is the primary virulence factor [24].

Detoxification genes in *B. goodwinii* are stimulated by the presence of *A. biguttatus*, which may neutralise host defences

As described above, co-infection of oak logs with *A. biguttatus* significantly increases the number of significantly differentially expressed genes within *B. goodwinii* and stimulates expression of putative pathogen genes. In addition, homologs of genes which neutralise tree defences were expressed. In previous studies, these homologs have been shown to create a desirable environment for pupation and bacterial persistence [25]. The number of significantly differentially expressed genes in *B. goodwinii* inoculated logs increased from 191 to 552 when *A. biguttatus* eggs were co-inoculated. Genes upregulated by *A. biguttatus* eggs and not in *B. goodwinii* only log inoculations included host defence detoxification genes; catalase peroxidase (*Padj* < 0.0001; E.C. 1.11.1.21), glutathione reductase (*Padj* = 0.02; E.C. 1.8.1.7), and glutathione regulated potassium efflux system (*Padj* = 0.02). Catalase peroxidase and glutathione reductase are encoded on the same operon; catalase peroxidase (*katG*) protects against hydrogen peroxide released by host defences [26] and glutathione is a metabolite of isoprene and its derivative terpene, both of which are common in oak trees and used to combat abiotic stress and in high quantities are toxic to bark boring beetles [7,27,28]. *B. goodwinii* mediated terpene reduction may exhaust terpene synthesis similar to that of drought stressed oaks which initially produce abundant amounts of terpenes but upon severe drought stress are no longer able to synthesise the volatiles, leaving them open to herbivores [29].

The oak host up-regulates more defence-associated genes during co-inoculation with *A. biguttatus*

Examination of oak host transcripts within infection tests revealed differential gene expression when challenged with *B. goodwinii* only compared to *B. goodwinii* with *A. biguttatus* eggs. This analysis revealed 25 significantly up-regulated genes in logs inoculated with *B. goodwinii* and *A. biguttatus* eggs compared to 12 up-regulated genes with only *B. goodwinii*. This result provides futher evidence of an increase in activity of *B. goodwinii* when co-infected with *A. biguttatus*. For both *B. goodwinii* treatments we discovered the up-regulation of genes encoding the calcium sensor protein CML38. This protein, and calcium signalling proteins in general are reportedly induced during, wounding, stress and pathogen infection [30,31]. Furthermore, during inoculation with *B. goodwinii* only, and with *G. quercinecans* and eggs, there was significant up-regulation of a NDR1/HIN1 like protein, which is associated with senescence and pathogen infection [32]. Host genes encoding NDR1/HIN1 like proteins have previously been reported as up-regulated when comparing

field AOD lesion bark to that from non-symptomatic trees. During inoculation of *B. goodwinii* and eggs, there was also significant up-regulation of two infection associated genes encoding WUN1, a wound induced protein, and EP3, an endochitinase associated with infection [33–35]. These results support the conclusion that bacterial co-infection with *A. biguttatus* enhances not only bacterial activity but also overall triggering of host defence-associated genes.

In vitro analysis of the *B. goodwinii* and *G. quercinecans* transcriptome response to oak sapwood and phloem tissue

To gain greater understanding of interactions between two key bacteria within the AOD pathobiome, *in vitro* transcriptome assays were designed to measure gene expression changes of *B. goodwinii* and *G. quercinecans* in pure cultures and co-cultures containing oak phloem and sapwood (figure 3 and see methods for recipe). A key unanswered question in AOD pathology relates to the nature of pathobiome interactions between *B. goodwinii* and *G. quercinecans*, and whether they represent competitive or cooperative strategies.

Gene expression of *B. goodwinii* within phloem and sapwood *in vitro* cultures varies substantially between single inoculations and co-cultures

Gene expression analysis revealed that *B. goodwinii* has a substantial transcriptomic response to oak sapwood tissue two hours post inoculation, significantly differentially expressing 39 genes ($P < 0.05$; 35 upregulated and 4 downregulated) (figure 3a). Upregulated genes were mostly sugar transport/catabolism ($n = 11$) and general metabolism genes but also included an anti-bacterial gene, the type I secretion protein colicin V (attacks closely related bacteria) [36]. This effect is not found in oak phloem tissue (figure 3b), indicating that *B. goodwinii* is stimulated by glucose and xylose rich sapwood tissue which it can utilise as a sugar source.

In co-culture, two hours post inoculation with *G. quercinecans*, *B. goodwinii* significantly differentially expressed genes which were not expressed in axenic *B. goodwinii* culture ($n = 14$ in phloem; $n = 13$ in sapwood) (figure 3i). This response was found in both oak phloem and sapwood tissue (figure 3i – 3l), with upregulated genes including those associated with sugar depolymerisation, which hydrolyse long chain sugar polymers such as α-N-arabinofuranosidae (E.C. 3.2.1.55), bacterial α-L-rhamnosidase (E.C. 3.2.1.40), and β-galactosidase (E.C. 3.2.1.23). These enzymes degrade plant tissue by breaking glycosidic

linkages in the pectic polysaccharide, rhamnogalacturonan-II [37] and hemicellulose [38]. In sapwood at two hours post inoculation (figure 3a), flagellar motility genes (n = 2) were upregulated including the motility regulator *fliA* [39] indicating that sapwood and *G. quercinecans* stimulate the flagellar apparatus of *B. goodwinii*.

G. quercinecans has a substantial upregulation of genes towards oak phloem tissue but not sapwood

The environmental reservoir and ecological niche of *G. quercinecans* is unconfirmed. However, it is a robust bacterium that can survive in harsh environments [40] and is consistently isolated from AOD lesions where it may contribute to tissue necrosis [18]. Evidence provided here reveals that *G. quercinecans* can is differentially stimulated by oak phloem (figure 3f) and may assist *B. goodwinii* in colonising this environment by inducing expression of hitherto unexpressed genes (figure 3j & 3l).

Here, *G. quercinecans* significantly differentially expressed 42 genes in single inoculations with phloem tissue at two hours post inoculation (32 upregulated and 10 downregulated) (figure 3f). A large number of upregulated genes are involved in sugar catabolism/transport (n = 10), but also upregulated were general metabolism genes, the type IV secretion system (T4SS) component *virB4* and a key PCWDE - rhamnogalacturonan lyase (E.C. 4.2.2.23). The *in vitro* environment, containing oak phloem and sapwood, may mirror the environmental habitat of *G. quercinecans*, which has previously been isolated from rotting wood and has many saprophytic properties [18,40].

Sugar consumption by _G. quercinecans_ in oak sapwood is stimulated by _B. goodwinii_

Compared to axenic growth of *G. quercinecans* in sapwood (figure 3e & 3g), co-culture with *B. goodwinii* induced significant differential expression of 21 genes (14 upregulated and 7 downregulated) (figure 3i & 3k). Upregulated gene function included sugar catabolism/transport (n = 5), iron transporters (n = 3) and two secondary PCWDEs (n = 2). It was anticipated that co-culture could potentially induce expression of anti-bacterial effectors but similar to *B. goodwinii* in phloem, *G. quercinecans* catabolises and transports sugars from sapwood when *B. goodwinii* is present (figure 3i – 3l). Despite the encoding of multiple toxin-antitoxin systems and type VI secretion systems, there was no evidence of competitive behaviour between *B. goodwinii* and *G. quercinecans*. These are closely related bacteria,

isolated from the same environmental niche and these experiments suggest that they assist each other to metabolise oak tissue. Anti-bacterial effectors may be expressed at later stages of co-culture, when resources are reduced, but this was not tested here.

RNA-seq validation using RT-qPCR analysis of *G. quercinecans* FRB97 and *B. goodwinii* FRB141 putative pathogenicity genes

Two RT-qPCR gene expression assays were used to validate RNA-seq data using the same RNA extracts as the *in vitro* RNA-seq experiment. In *G. quercinecans tssD* was selected, as homologs of this gene form part of the T6SS injectosome [41], and in *B. goodwinii fliA* was selected, which is an alternative sigma factor and controls flagella filament synthesis, chemotaxis machinery, and motor switch complex genes in *E. coli* [42].

RT-qPCR assays revealed that gene expression was highest at 6 HPI for *tssD* (an average of $2.2x10^5$ absolute transcript copies at 2 HPI, $3.5x10^6$ at 6 HPI, $2.7x10^5$ at 12 HPI, $4.1x10^4$ at 24 HPI), and 2 HPI for *fliA* (an average of $5.5x10^4$ absolute transcript copies at 2 HPI, $8.7x10^3$ at 6 HPI, and $2.6x10^3$ at 12 HPI) (electronic supplementary material figure, S1). RNA-seq data revealed high gene expression of *fliA* in axenic *B. goodwinii* culture at 2 HPI, and differential upregulation in co-culture with *G. quercinecans*, in nutrient broth (NB) & sapwood (NBS) and nutrient broth & phloem (NBP) cultures at 2 HPI only, with gene expression being suppressed with the addition of *G. quercinecans* in Nutrient Broth (NB). *tssD* was highly expressed at 6 HPI, concurring with the RT-qPCR data (electronic supplementary material figure, S1), and was differentially upregulated at 2 HPI in NBS and NBP compared to NB. Within the *G. quercinecans* transcriptome *tssD* was upregulated in NBS and NBP, suggesting that it is part of a wider virulence transcription cascade, and may respond to eukaryotic stimuli. Transcriptomic expression data of *tssD* and *fliA*, data correlates with the RT-qPCR data, however, small variations may be explained by the high sensitivity of RT-qPCR [43,44].

Conclusions

Here we investigated the emergent properties of pathobiome virulence in AOD. We used gene expression analysis of axenic and co-cultures of bacteria supplemented with oak inner bark tissue, and oak infection tests using combinations of the *A. biguttatus* bettle and microbial pathobionts. We demonstrated that the pathogenic potential of the dominant bacterial species within the AOD lesion pathobiome, *B. goodwinii*, is stimulated by a co-invading native beetle, *A. biguttatus*, and also potentially induced by other microorganisms in the AOD pathobiome associated with either the host or *A. biguttatus*. Furthermore, *B. goodwinii* genes induced by the presence of *A. biguttatus* may confer nutrient acquisition benefits to beetle eggs and larvae.

The co-operative behaviour of *B. goodwinii* and *G. quercinecans* in a nutrient rich environment may differ from the AOD lesion environment where resources are scarce. However, both bacteria persisted in oak phloem and sapwood when combined, and when resources were plentiful there was no significant upregulation of interbacterial competition genes. It was also revealed that *G. quercinecans* favours sugar metabolites from oak phloem tissue, whereas *B. goodwinii* favours oak sapwood as a carbon source. The role of *L. britannica* in the lesion environment is unclear but merits further investigation due to its encoded pathogenic potential and high expression activity in combination with *B. goodwinii* and *A. biguttatus*. It is possible that AOD pathobiome constituents each contribute degradative enzymes to systematically macerate oak tissue, thereby co-operating to provide ingestible sugars as a public good. To fully characterise the molecular processes uncovered in this study will require tractable genetic manipulations of single gene effects in appropriate model systems.

In conclusion, we identified expression of key pathogenicity genes in *Brenneria goodwinii*, the dominant member of the AOD pathobiome, tissue-specific gene expression profiles, cooperation with other bacterial pathobiome members in sugar catabolism, and demonstrated amplification of pathogenic gene expression in the presence of *Agrilus* larvae. These data highlight the emergent propereties of complex multidimensional interactions between host plants, insects and the microbiome that underpin complex tree decline diseaeses that threaten the global landscape.

Methods

In vitro culture-based assay

Strains, growth medium and conditions

Strains of *Gibbsiella quercinecans* FRB97 and *Brenneria goodwinii* FRB141 were obtained by Forest Research (Surrey, UK) from AOD affected trees. Isolates were maintained on nutrient agar (Oxoid) at room temperature. To simulate growth on sapwood and phloem, cells were cultured in nutrient broth (Oxoid) containing 1% (w/v) milled sapwood (NBS), nutrient broth with 1% (w/v) milled phloem (NBP) and a control consisting of nutrient broth (NB). Initially, a 10 ml starter culture from a single colony was incubated overnight to stationary phase at 28°C on a shaking incubator at 100 rpm. 1% of the overnight culture, was centrifuged and re-suspended, before addition to three replicate culture flasks containing 150 ml volumes of NB, NBS, and NBP (figure 3). The flasks were incubated at 28°C and 100 rpm, for 6 HPI, with cell suspensions collected at 2 HPI and 6 HPI. At each time point 25 ml of liquid was collected in a 50 ml Falcon tube and centrifuged for 5 mins at 3000 rpm. The supernatant was discarded, and pelleted cells were frozen in liquid nitrogen.

Log infection assay

Log trials were established in 2015 (electronic supplementary table, S3 for list of log inoculation treatments, resultant lesion sizes and futher information). Owing to the high cost of transcriptomics when the trial was terminated and samples processed, only a sub-set of 3 inoculations points in each of the above described treatments were sampled, at random, from the log test, except where there were exceptional cases of typical AOD lesions i.e. two *Lonsdalea* inoculations, which were specifically included in the transcriptomic analyses. Following lesion area measurements and plating lesion margin wood chips [8] the remaining lesion was chiselled out, placed in a labelled ziplock plastic bag and snap-frozen in liquid nitrogen and stored at -80C until RNA extraction too place.

RNA extraction

RNA extraction from bacterial cultures

Total RNA was extracted from cell pellets of bacterial cultures using the RNeasy Mini Kit (Qiagen), according to manufacturer's instructions. Genomic DNA was removed from extracted RNA samples using TURBO DNA-free DNase kit (Ambion). Total RNA was pooled from three biological replicates in equimolar quantities giving a total quantity of 750 ng (electronic supplementary material figure, S2). Total rRNA was depleted to enrich mRNA (transcripts) using the RiboZero rRNA depletion kit (Illumina). The protocol was performed according to manufacturer's instructions. Depleted mRNA concentrations were measured using a Qubit fluorometer (Invitrogen). Remnant rRNA was minimal as confirmed by the Centre for Genomic Research (CGR) (University of Liverpool, UK), using the Agilent 2100 BioAnalyzer.

RNA extraction from log inoculations

RNA was extracted from logs using the method described in our previous multi-omic AOD work and described here [45]. Briefly, inner bark around log inoculation spots was scraped off and snap frozen in liquid nitrogen. Oak tissue was homogenised using a mortar and pestle, and extraction buffer (4 M guanidine thiocyanate, 0.2 M sodium acetate pH 5.0, 25 mM EDTA, 2.5% (w/v) polyvinylpyrrolidone and 1% (v/v) β-mercaptoethanol) was added. The frozen tissue in extraction buffer was further ground until thawed, while additional extraction buffer and 20% sodium lauroyl sarcosinate were mixed into the sample. The sample mixture was shaken vigorously at room temperature and further processed using the RNeasy Plant Mini kit (Qiagen). After centrifugation in the QIAShredder column, 350 µl of the supernatant was mixed with 0.9 volumes of ethanol, and subsequently centrifuged in the RNeasy Mini column. After this centrifugation step, the manufacturer's instructions for the RNeasy Plant Mini kit were followed. The extracted RNA was treated with DNase I (Qiagen) and further concentrated and purified using the RNeasy MinElute Cleanup kit (Qiagen) following the manufacturer's instructions. The purified RNA was checked for quality using 1% agarose gel electrophoresis and a NanoDrop spectrophotometer (LabTech), and the concentration determined using the Qubit RNA HS assay kit (Thermo Fisher) following the manufacturer's instructions. Subsequently, rRNA was depleted from RNA extracts using a 1:1 combination of the Ribo-Zero rRNA Removal kits for plant seed/root and for bacteria (Illumina) according to the manufacturer's instructions. The rRNA depleted samples were again purified using the RNeasy MinElute Cleanup kit (Qiagen) again and stored at -80 ºC before sequencing.

RNA sequencing

Library preparation, transcriptomic sequencing, and post-sequencing QC of depleted RNA samples was performed by Centre for Genomic Research (CGR), University of Liverpool, UK. Samples were assayed for quality using an Agilent 2100 Bioanalyzer. Log infection samples were further assayed for quality using the Eukaryote Total RNA Pico Series II. All libraries were prepared using the strand-specific ScriptSeq kit (Illumina), and subsequently paired-end sequenced (2x125 bp) on one lane (N.B. *in vitro* and log infection samples were sequenced on separate lanes) of the Illumina HiSeq 2500 platform (electronic supplementary material figure, S3 & electronic supplementary material figure, S4).

Transcriptome analysis

RNA-seq QC

Illumina adapter sequences were removed from raw FastQ files containing the sequencing reads using Cutadapt v1.2.1 [46], using the option –O 3, which specifies that at least 3 base pairs have to match the adapter sequences before they were trimmed. Sequences were quality trimmed using Sickle v1.2 [47] with a minimum quality score of 20. Reads shorter than 10 bp were removed. RNA-seq QC was performed by Centre for Genomic Research (CGR), University of Liverpool, UK (electronic supplementary material figure, S3 & electronic supplementary material figure, S4).

Bioinformatic analysis of transcriptome data

Bioinformatic analyses were carried out on SuperComputing Wales, an HPC network, using GNU/Linux Red Hat Enterprise Linux Server release 7.4 (Maipo). A complete list of commands used to perform the below analysis is hosted on GitHub (https://github.com/clydeandforth/Bg_Ab_logs.git).

Transcriptome alignment and differential gene expression analysis

RNA recovered from log inoculations and sequenced on the Illumina HiSeq, was aligned using Bowtie2 v1.1.2 [48] to an in-house database of structurally and functionally annotated coding regions (electronic supplementary methods) used in a previous field AOD

microbiome analysis [15], but with the addition of *Lonsdalea britannica* 477. Transcript counts for each gene were calculated using eXpress v1.5.1 [49]. To give an overview of species activity in the lesion environment, an active gene was defined as those with transcripts per million (TPM) >1 and a total transcript count of three. TPM rather than raw read counts was used to normalise the number of transcripts across samples and remove sequencing depth as an experimental artefact. Subsequently, in a separate test, to get a statistically robust understanding of transcriptional activity, significantly differentially expressed genes were identified using DESeq2 v1.2 [50]. Genes which had *P*-adjusted values <0.05 between conditions were considered as significantly differentially expressed. Principal coordinate analyses based on dispersion of mean normalised gene count data between samples was calculated and plotted using DESeq2 v1.2.

Gene-set enrichment analyses of KEGG pathways were used to measure functional upregulation of gene families between samples using the R packages gage v2.30.0 [51] and clusterProfiler v3.8.1 [52]. GAGE uses a two sample t-test to compare expression level changes between genesets. KEGG datasets were compiled from KEGGREST v1.20.1 (accessed 04/02/2019) and comprised pathways from the following bacteria: *Dickeya dadantii* 3937, *Pectobacterium carotovorum* subsp. *carotovorum* PC1, *Escherichia coli* K12, *E. coli* 0157:H7 Sakai, *Rahnella aquatilis* CIP 78.65, *Serratia proteamaculans* 568, and plants: *Phoenix datylifera*, *Arabidopsis thaliana*, *Methylorubrum populi* BJ001.

Multivariate analysis - Generalised linear model

To test for biological variation between samples, the effect of inoculum, beetle presence/absence, lesion size and replicate were included in a generalised linear model (GLM) [53]. Normalised read count data produced using eXpress (described below) were set as the multivariate response variable and the biological predictors were fit using a negative binomial distribution. The ‘manyglm’ function of the R package [54] mvabund [55] was used to carry out the analysis. Inoculum, beetle presence/absence, lesion size, and replicate were included as exploratory variables to allow the model to test our hypotheses.

Transcriptomic analysis of *in vitro* sequence data

Sequenced RNA from *in vitro* tests was aligned to a custom database and counted as described above. The transcript per million (TPM) counts from eXpress analysis were used to

calculate the Generalised Fold Change (GFOLD) [56], which uses the posterior distribution of the raw fold change to calculate differential expression of genes between conditions and is analogous to the *P* value in DESeq2. Genes which had GFOLD values >1.5 or <1.5 between conditions were considered as significantly differentially expressed.

Data availability

Sequence data has been deposited in NCBI under BioProject PRJNA369790.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

JD and MB carried out the molecular lab work, RNA extraction and depletion, statistical and bioinformatic analysis. JD drafted the manuscript and created the figures; JEM supervised the labwork and critically revised the manuscript; SD conducted log tests and critically revised the manuscript; All authors, designed and coordinated the study. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

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Figure legends

Figure 1. Transcriptome analysis of oak log infection tests comprising single bacterial species incoluations and bacteria plus *Agrilus biguttatus* egg inoculations. From left to right: Organisms inoculated into oak logs are shown in the key on the top right, these are *Gibbsiella quercinecans*, *Brenneria goodwinii*, *Lonsdalea britannica* and *Agrilus biguttatus*. There were three biological replicates of each infection test, including replicate water only and wound controls. Each of the three bacterial species were inoculated individually and in combination with eggs of *A. biguttatus*. Exemplary pictures of a single log inoculation replicate from each treatment are shown. The number of expressed genes from log inoculations are shown in the bar chart, with each expressed gene aligned against a custom database and colour coded with the Genus/Species key shown on the bottom left of the figure. Oak transcripts were excluded from the bar chart.

Figure 2. Transcriptome analysis of *Brenneria goodwinii* inoculations on live oak logs. (b) Gene set enrichment analysis (GSEA) of *B. goodwinii* gene families when compared to (left) water and wound control oak logs; (right) *B. goodwinii* inoculated in combination with *A. biguttatus* when compared to *B. goodwinii* only. The lower q-value represents increased magnitude of gene family expression and circle size represents number of genes per family. (a), principal component analysis (PCA) of (top) *B. goodwinii* (n = 3) compared to control (n = 6); (bottom) *B. goodwinii* and *A. biguttatus* compared to control (n = 6). (c) gene expression changes of selected significantly differentially expressed genes, these are anti-toxicity genes (yellow), biofilm and persistence genes (purple), secretion system effectors (blue). (top) *B. goodwinii* compared to control; (bottom) *B. goodwinii* and *A. biguttatus* compared to control. Transcriptome samples were taken from log inoculations of bacterial combinations, wound and water controls, and field samples of AOD lesions and asymptomatic oaks. *Bg* = *Brenneria goodwinii*; *Gq* = *Gibbsiella quercinecans*; eggs = *A. biguttatus*.

Figure 3. *In vitro* transcriptome analysis of *Brenneria goodwinii* and *Gibbsiella quercinecans* in nutrient broth supplemented with oak phloem and oak sapwood. Each panel shows gene expression changes when phloem and sapwood are present, compared with nutrient broth only controls. (a) *B. goodwinii* in sapwood at 2 HPI. (b) *B. goodwinii* in phloem at 2 Hours Post Inoculation HPI. (c) *B. goodwinii* in sapwood at 6 HPI. (d) *B. goodwinii* in phloem at 6 HPI. (d) *G. quercinecans* in oak sapwood at 2 hours post inoculation (HPI). (e) *G. quercinecans* in oak phloem at 2 HPI. (f) *G. quercinecans* in sapwood at 6 HPI. (g) *G. quercinecans* in phloem at 6 HPI. (i) *B. goodwinii* and *G. quercinecans* in sapwood at 2 HPI. (j) *B. goodwinii* and *G. quercinecans* in phloem at 2 HPI. (k) *B. goodwinii* and *G. quercinecans* in sapwood

686 at 6 HPI. (I) *B. goodwinii* and *G. quercinecans* in phloem at 6 HPI. DEG = differentially expressed gene.
687 HPI = hours post inoculation. GFOLD is the generalised fold change.

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