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Brenneria goodwinii growth *in vitro* is improved by competitive interactions with other bacterial species associated with Acute Oak Decline

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ABSTRACT

Brenneria goodwinii, Rahnella victoriana and *Gibbsiella quercinecans* are three bacterial species frequently isolated together from oak displaying symptoms of Acute Oak Decline (AOD), which include weeping patches on trunks. All three bacterial species play a role in lesion formation in the current episode of AOD in Britain, although *B. goodwinii* is the most dominant. The ongoing research into stem lesion formation characteristic of this polybacterial syndrome has been focussed primarily on the pathogenicity, identification and taxonomy of these bacteria. As all three species were newly classified within the past ten years, there are many unanswered questions regarding their ecology and interactions with each other. To determine the effect of bacterial interactions on fitness *in vitro*, we examined pairwise (diculture) and multispecies (triculture) interactions between *B. goodwinii, R. victoriana* and *G. quercinecans* in oak leaf media microcosms. Additionally, the effect of co-culturing on the evolution of these species was determined and the evolved *B. goodwinii* strains were examined further by whole genome sequencing. Our results indicate that *B. goodwinii* thrived in monoculture with significantly higher viable cell counts than the other two species. Additionally, *B. goodwinii* performed well in pairwise culture with mutually competitive interactions observed between *B. goodwinii* and *R. victoriana*, and between *B. goodwinii* and *G. quercinecans*. In the multispecies triculture, *B. goodwinii* and *R. victoriana* appeared to exhibit co-ordinated behaviour to outcompete *G. quercinecans*. After four weeks *B. goodwinii* grown in co-culture with the other two species developed greater evolved fitness than the strain grown in monoculture as reflected by the increased viable cell counts. The competitive interactions taking place between the threes species indicated evolving improved fitness of *B. goodwinii in vitro*, that gave it a growth advantage over both *R. victoriana* and *G. quercinecans* which showed no significant changes in fitness. Overall, *B. goodwinii* gains greater benefit in terms of fitness from *in vitro* competitive interaction with the other two species.

1. Introduction

Over the last 100 years, episodes of oak decline have been reported sporadically in Britain and Europe (Brady et al., 2017). However, from 2008/2009 onwards, a pattern of disease outbreaks on susceptible, mature (> 50 years old) native oak species (*Quercus robur* and *Q. petraea*) has been noted in Britain. This current episode, termed Acute Oak Decline (AOD) (Denman et al., 2014), is ongoing with affected trees showing a rapid decline over three to five years. The first cases were observed in the south east of England, but in recent years AOD has been noted in the midlands, the Welsh borders and the south west of England. AOD is characterised by stem bleeds and weeping patches active in spring and autumn. The tissues underlying the outer bark are stained and necrotic, leading to the formation of cavities in the inner bark. Galleries of the two-spotted oak borer (*Agrilus biguttatus*) are often found in close proximity to the necrotic lesions. Both biotic and abiotic factors contribute to the decline of symptomatic oak, with a bacterial component recently proven to be the cause of the necrotic lesions that characterise AOD (Denman et al., 2018). In the decade since research into the current episode of AOD in the UK began in earnest, numerous isolation campaigns from symptomatic oak have taken place resulting in an abundance of novel species and even genera that have been validly classified (Brady et al., 2017; Bueno-Gonzalez et al., 2019, 2020). Due to their novel status, our knowledge concerning the biology, genetics and

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pathogenicity of these bacteria has, until recently, been quite limited.

The immense diversity of the oak microbiome of both symptomatic and healthy trees has been examined (Denman et al., 2016; Sapp et al., 2016), with a noticeable shift observed in the culturable microbiome of affected oak and members of the families *Enterobacteriaceae, Pectobacteriaceae, Erwiniaceae, Yersiniaceae* and *Pseudomonadaceae* dominating the lesion microbiome. A polymicrobial disease complex has been implicated as the cause of AOD lesions (Denman et al., 2018) with two species, *Brenneria goodwinii* (Denman et al., 2012) and *Gibbsiella quercinecans* (Brady et al., 2010), responsible for tissue necrosis while a third species, *Rahnella victoriana* (Brady et al., 2014), is thought to contribute to symptom development. Genomic and proteomic analysis of the whole genome sequences of these bacteria have yielded new insights into their possible roles in AOD (Broberg et al., 2018; Doonan et al., 2019). The concept of a polybacterial disease (more than one bacterial agent responsible for symptoms) of plants and trees has become more acceptable in recent years, with numerous studies focusing on the pathobiome (Bass et al., 2019; Doonan et al., 2020). Olive knot disease is one of the best studied examples of polybacterial disease, where the severity of symptoms caused by the pathogen, *Pseudomonas savastanoi* pv. *savastanoi*, is significantly enhanced by *Erwinia toletana*, a common endoyphyte (da Silva et al., 2014).

The complexity of the bacterial community of AOD symptomatic oak creates a limitless possibility for microbial interactions. It is well-known that bacteria are social microorganisms that do not live in isolation, but co-exist with both positive and negative interactions taking place to influence the microbial community structure. These interactions (positive or negative) can be inter- and intra-species, and are influenced by the niche they occupy and the external environment. In a niche occupied by a microbial community, cells are not evenly dispersed but instead spatially distributed according to their metabolic and physiological needs (Stubbendieck et al., 2016) which have evolved over time in response to the metabolic actions of those they interact with (Freilich et al., 2011). More specifically, short-range interactions have been found to dominate in microbial communities (i.e. species that are in the immediate vicinity) (Co et al., 2020). Therefore to understand a bacterial species, it should be placed into an ecological context with other members of its community (Stubbendieck et al., 2016).

Brenneria goodwinii, Gibbsiella quercinecans and *Rahnella victoriana* are the three most frequently isolated bacterial species from oak suffering from AOD (Denman et al., 2018), therefore it is logical to hypothesise that bacterial interactions between these three species occur in the process of symptom development. These interactions could range from mutualism or cooperation to competition or even syntrophy (Oliveira et al., 2014). The aims of our study were to explore the pairwise (diculture) and multispecies (triculture) interactions between these bacteria *in vitro,* to determine the effect of repeated co-culturing on bacterial fitness and to further examine this effect on evolved *B. goodwinii* strains by analysing their whole genome sequences.

2. Material and methods

2.1. Bacterial strains and growth media

Brenneria goodwinii FRB 186 (*Q. robur*, UK), *Gibbsiella quercinecans* BH1/65b (rifampicin resistant strain) (*Q. robur*, UK) and *Rahnella victoriana* BRK18a *(Q. robur*, UK), isolated from necrotic lesions of AOD symptomatic oak, were used in this study (strains supplied by Forest Research). The rifampicin (Rif) resistant *G. quercinecans* strain was generated by exposure to 10 mg/ml of the antibiotic (Sigma-Aldrich). Briefly, an overnight culture was washed and re-suspended in ¼ Ringers (Oxoid). The suspension was spread onto LB + Rif, incubated at 28 °C for 24–48 h and resulting colonies were subcultured twice on LB + Rif (no difference in growth rate was observed between the Rif resistant and wild type strains). For general growth and maintenance, strains were grown on Luria-Bertani (LB, Oxoid) agar at 28 °C and stored in glycerol stocks at −80 °C. For preparation of overnight cultures, strains were grown with maximum aeration in LB broth at 28 °C (shaking incubation at 200 rpm). The oak leaf broth for the microcosm growth experiment was prepared according to Fiegna et al. (2015) using 50 g of autoclaved oak tree leaves (picked from a healthy, mature *Q. robur* in July 2018).

2.2. Microcosm growth experiment

Microcosm communities were created by inoculating *B. goodwinii* FRB 186 (Bg), *G. quercinecans* BH1/65b (Gq) and *R. victoriana* BRK18a (Rvic) into oak leaf broth singly, in monoculture, and in all possible combinations, in di- and triculture (Fig. 1a). Overnight cultures of the bacterial strains in LB broth were washed, re-suspended in ¼ Ringers solution (Oxoid) and the OD adjusted to $OD_{600} = 0.3$. One ml of the suspension was used to inoculate 9 ml of oak leaf broth to a starting OD of 0.03 in triplicate, for each species. Additionally, all possible combinations of the three species (Bg + Rvic, Bg + Gq, Rvic + Gq and Bg + Rvic + Gq) were inoculated in equal volumes (500 μl per 2 species and 330 μl per 3 species) in 9 ml of oak leaf broth in triplicate. The inoculated microcosms were incubated static at 25 °C. After 7 days the community biomass (in optical density, OD) of each microcosm was measured and 100 μl of the culture was used to re-inoculate 9.9 ml fresh oak leaf broth. Serial dilutions up to 10^{-9} were performed on the week-old microcosms and plated on LB agar, where colonies of *B. goodwinii* and *R. victoriana* can be easily distinguished from each other (Fig. S1), and on LB agar + rifampicin for those microcosms containing *G. quercinecans*. Viable colonies were counted (in colony forming units, CFUs) and compared after 48 h incubation at 28 °C. Biomass measurement, re-inoculation, serial dilution and viable colony counts were repeated every 7 days for 4 weeks. Pairwise species interactions were derived for the weekly measurements by comparing the sum of the CFUs from monoculture ($A_{mono} + B_{mono}$) to the sum of the total CFUs from each constituent species of the corresponding diculture ($A_{di} + B_{di}$) (Foster and Bell, 2012).

2.3. Growth assay of evolved species

The three constituent species were re-isolated from each replicate of the final microcosms (mono-, di- and tricultures from week 4) and their identity confirmed by species-specific high resolution melt analysis (Brady et al., 2016). Overnight LB broth cultures of the individual re-isolated strains and ancestral strains (the strains used to initiate the microcosm experiment) were washed, re-suspended in ¼ Ringers solution and the OD adjusted to $OD_{600} = 0.3$. One ml of the suspension was used to inoculate 9 ml of standard M9 minimal media (containing glucose as the carbon source, Sigma-Aldrich) (Fig. 1b). The inoculations were incubated under static conditions for 7 days at 25 °C. Serial dilutions up to 10^{-9} were performed and plated onto LB agar plates which were incubated for 48 h at 28 °C. Viable colonies were counted and comparisons were made between the ancestral and re-isolated strains.

2.4. Statistical analyses

General linear models were used to compare bacterial growth across the microcosms. For the microcosm growth experiments, the response variable was either the biomass (measured by optical density) or the $\log_{10}$ of the viable colony counts. The factors considered were time (weeks 1 to 4), microcosm composition (mono-, di- and tri-cultures), and their interaction. For the growth assay of evolved species experiment, the response variable was the $\log_{10}$ of the viable colony counts for each of the three constituent species and the factors considered were the strain inoculum origins (ancestral strains and re-isolated strains from microcosms). Post hoc testing was conducted using the Bonferroni pairwise comparisons method (95% confidence level). Calculations were done in Minitab 17 (23). Data generated from the analysis is available from the UWE Research Data Repository (Brady et al., 2021).

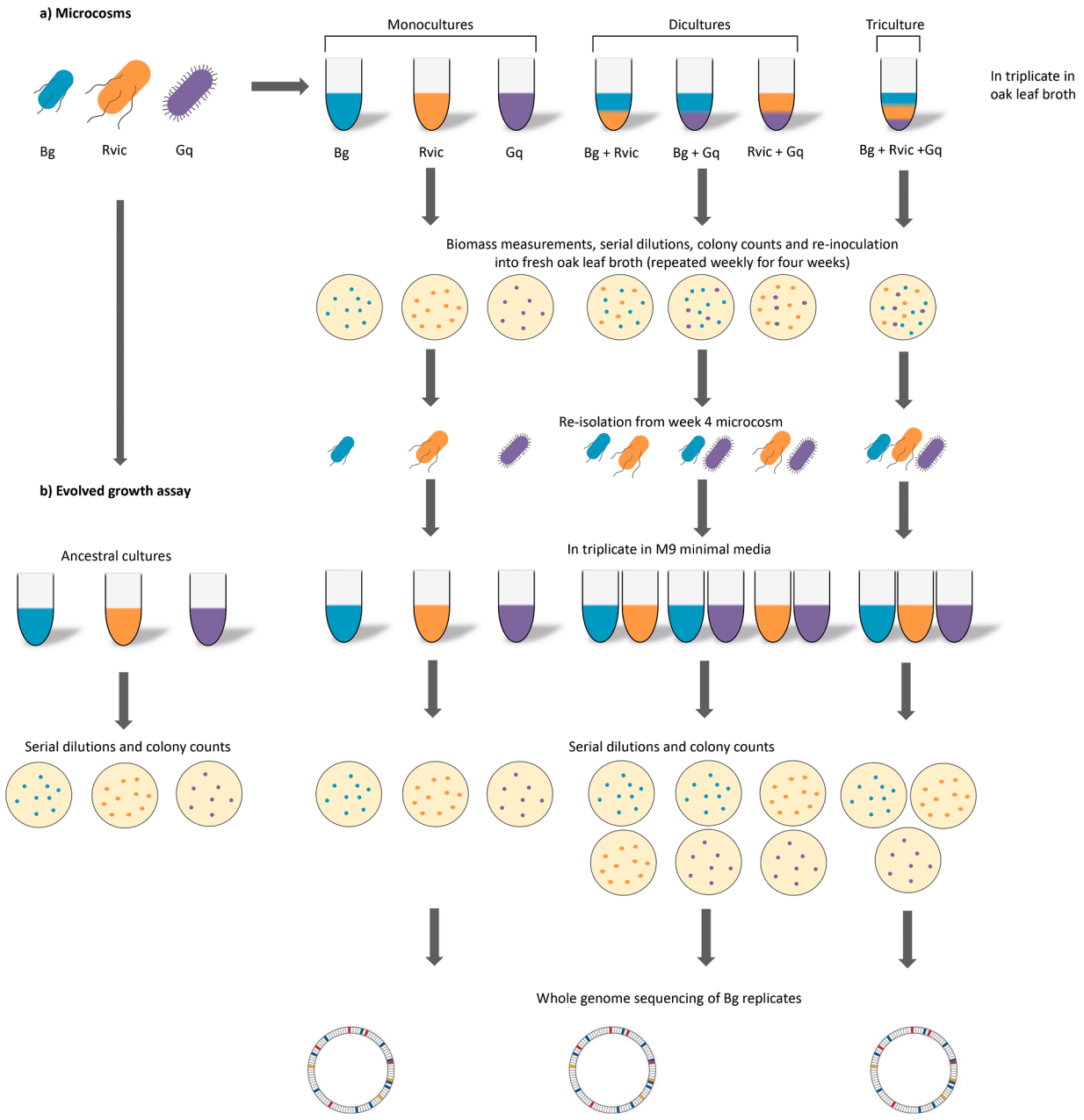

Fig. 1. (a) Summary of microcosm inoculations. Pure cultures of *Brenneria goodwinii* – Bg (blue), *Rahnella victoriana* – Rvic (orange) and *Gibbsiella quercinecans* – Gq (purple) were used to inoculate oak leaf broth in monoculture, as well as in all different combinations in di- and tricultures in triplicate. Biomass measurements, serial dilutions and viable colony counts were performed weekly on all microcosms which were then used to inoculate fresh oak leaf broth. The experiment ran for four weeks. (b) Summary of evolved growth assay. Re-isolated colonies from the week 4 microcosms as well as the ancestral cultures were used for the evolved growth assay, where they were inoculated individually into M9 minimal media. Serial dilutions and viable colony counts were performed after one week. Whole genome sequencing was performed on the *B. goodwinii* strains from the Bg monoculture, Bg + Rvic diculture and Bg + Rvic + Gq triculture.

2.5. Whole genome sequencing and SNP determination

Whole genome sequencing was performed on the *B. goodwinii* FRB 186 strain from each replicate of the evolved growth assays for the Bg monoculture, Bg + Rvic diculture and Bg + Rvic + Gq triculture ($n = 9$). Strains were sequenced by MicrobesNG (Birmingham, UK) using the Illumina HiSeq platform, following DNA extraction by cell lysis, DNA purification with SPRI (Solid Phase Reversible Immobilization) beads and library preparation with the Nextera XT Library Prep Kit (Illumina). Whole genome assemblies were annotated using Prokka v1.14 (Seemann, 2014). Resultant annotations were aligned against the ancestral *B. goodwinii* FRB 186 strain (JAESVZ000000000) using Roary v3.12.0 (Page et al., 2015) and SNPs subsequently identified within coding regions using SNP-sites v2.5.1 (Page et al., 2016). Alignments were visualised using the R package (R core team) msa v1.16.0 (Bodenhofer et al., 2015).

3. Results

3.1. Comparison of AOD-associated bacterial growth in microcosms

Over four weeks, the evolving AOD-associated bacteria were grown individually or in combination in static microcosms with a weekly transfer into fresh media. The average community biomass (measured by optical density) was calculated from each set of microcosms (mono-, di- and tricultures), along with the standard error (Fig. 2a).

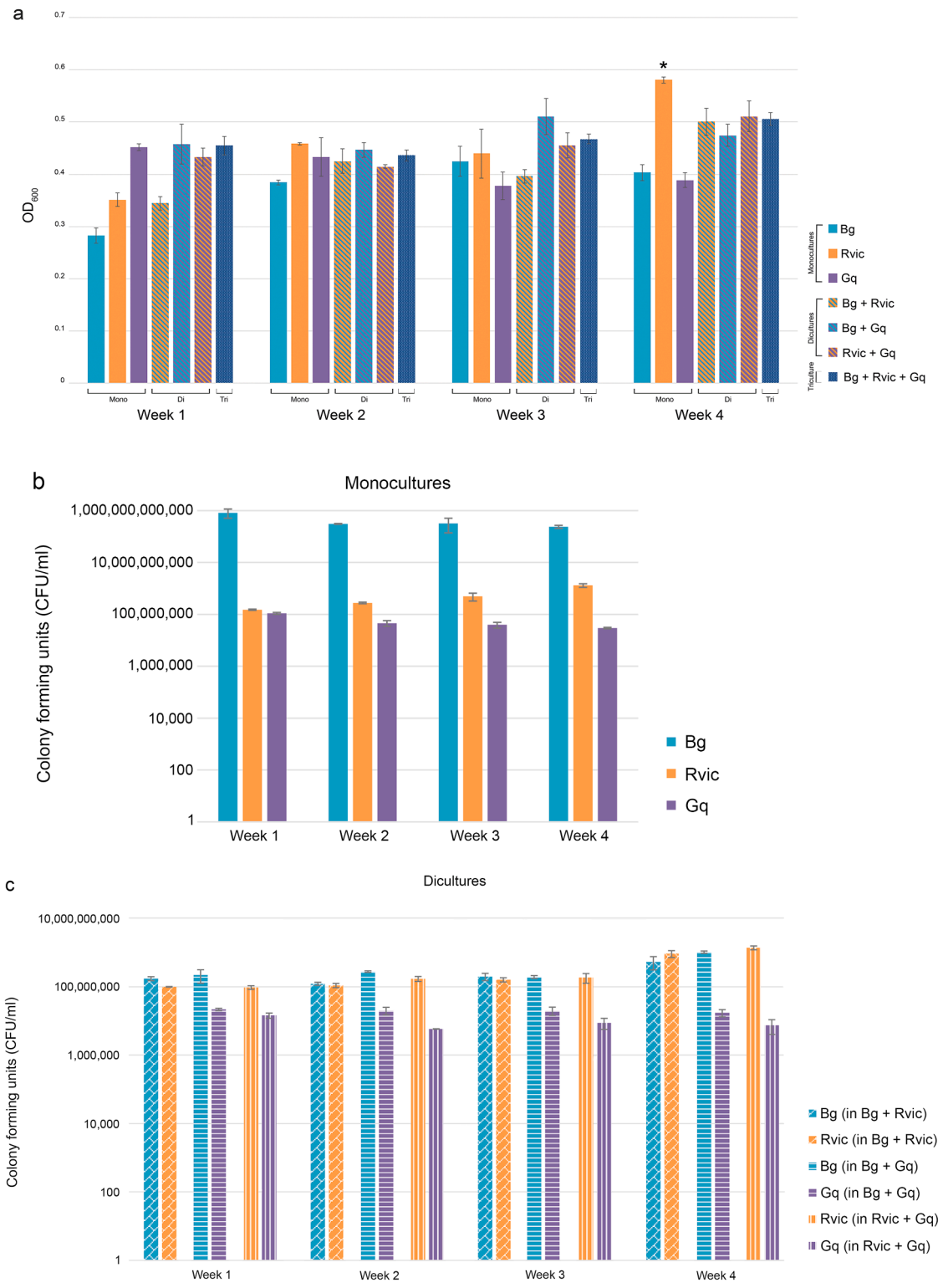

Fig. 2. (a) Comparison of average community biomass. Comparison of the biomass (measured in optical density) of monocultures, dicultures and tricultures of *Brenneria goodwinii* FRB 186 (Bg – blue), *Rahnella victoriana* BRK18a (Rvic - orange) and *Gibbsiella quercinecans* BH1/65b (Gq – purple) in oak leaf broth microcosms across a four-week incubation period. Error bars represent standard error of the mean of three biological experimental replicates. * indicates statistically significant differences as assessed with ANOVA. Bg = *B. goodwinii* monoculture, Rvic = *R. victoriana* monoculture, Gq = *G. quercinecans* monoculture, Bg + Rvic = *B. goodwinii* and *R. victoriana* diculture, Bg + Gq = *B. goodwinii* and *G. quercinecans* diculture, Rvic + Gq = *R. victoriana* and *G. quercinecans* diculture, Bg + Rvic + Gq = *B. goodwinii, R. victoriana* and *G. quercinecans* triculture. **(b)** *Comparison of viable cell counts for monocultures*. Comparison of viable cell counts (measured in colony forming units, CFUs) of *Brenneria goodwinii* FRB 186 (Bg - blue), *Rahnella victoriana* BRK18a (Rvic - orange) and *Gibbsiella quercinecans* BH1/65b (Gq - purple) from monocultures grown in oak leaf broth microcosms across a four-week incubation period. Values are reported on a logarithmic scale. Error bars represent standard error of the mean of three biological experimental replicates. Statistically significant differences are described in the results. Bg = *B. goodwinii* monoculture CFUs, Rvic = *R. victoriana* monoculture CFUs, Gq = *G. quercinecans* monoculture CFUs **(c)** *Comparison of viable cell counts for dicultures*. Comparison of viable cell counts (measured in colony forming units, CFUs) of *Brenneria goodwinii* FRB 186 (Bg - blue), *Rahnella victoriana* BRK18a (Rvic - orange) and *Gibbsiella quercinecans* BH1/65b

(Gq - purple) from corresponding dicultures grown in combinations in oak leaf broth microcosms across a four-week incubation period. Values are reported on a logarithmic scale. Error bars represent standard error of the mean of three biological experimental replicates. Statistically significant differences are described in the results. Bg (in Bg + Rvic) = *B. goodwinii* CFUs from *B. goodwinii* and *R. victoriana* diculture, Rvic (in Bg + Rvic) = *R. victoriana* CFUs from *B. goodwinii* and *R. victoriana* diculture, Bg (in Bg + Gq) = *B. goodwinii* CFUs from *B. goodwinii* and *G. quercinecans* diculture, Gq (in Bg + Gq) = *G. quercinecans* CFUs from *B. goodwinii* and *G. quercinecans* diculture, Rvic (in Rvic + Gq) = *R. victoriana* CFUs from *R. victoriana* and *G. quercinecans* diculture, Gq (in Rvic + Gq) = *G. quercinecans* CFUs from *R. victoriana* and *G. quercinecans* diculture (**d**) *Comparison of viable cell counts for tricultures*. Comparison of viable cell counts (measured in colony forming units, CFUs) of *Brenneria goodwinii* FRB 186 (Bg - blue), *Rahnella victoriana* BRK18a (Rvic - orange) and *Gibbsiella quercinecans* BH1/65b (Gq - purple) from corresponding tricultures grown collectively in oak leaf broth microcosms across a four-week incubation period. Values are reported on a logarithmic scale. Error bars represent standard error of the mean of three biological experimental replicates. Statistically significant differences are described in the results. Bg (in Bg + Rvic + Gq) = *B. goodwinii* CFUs from *B. goodwinii, R. victoriana* and *G. quercinecans* triculture, Rvic (in Bg + Rvic + Gq) = *R. victoriana* CFUs from *B. goodwinii, R. victoriana* and *G. quercinecans* triculture, Gq (in Bg + Rvic + Gq) = *G. quercinecans* CFUs from *B. goodwinii, R. victoriana* and *G. quercinecans* triculture.

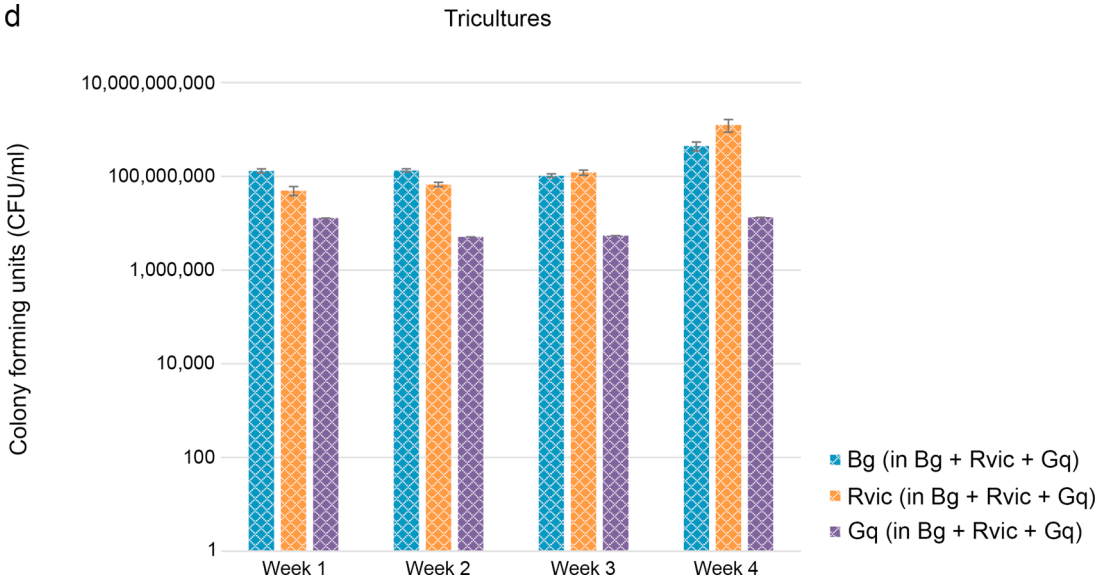

Fig. 2. (*continued*).

Monocultures: Compared to the starting inoculum (OD_{600} = 0.03), the monocultures all showed a marked increase in yield after one week's incubation. The biomass of *B. goodwinii* and *R. victoriana* generally increased every week (*B. goodwinii* always lower than *R. victoriana*), with *R. victoriana* displaying a sharp increase in week 4 compared to week 3 ($p = 0.01$). In contrast, *G. quercinecans* biomass began to decrease after week 1.

Dicultures: The biomass measurements of all three dicultures fluctuated over the course of the experiment often alternately increasing and decreasing, or vice versa. In weeks 1 and 2, the biomass measurements for each diculture were comparable to those of one (or both) of its constituent species. By week 4 the yield of Bg + Gq was greater than that of its corresponding monocultures, while the Bg + Rvic and Rvic + Gq biomass were greater than that of Bg and Gq in monoculture, but less than Rvic in monoculture.

Triculture: The Bg + Rvic + Gq triculture showed a gradual increase in biomass each week, except in week 2 where it decreased compared to the measurement from week 1. The biomass yield of the triculture across the experiment was greater than that of its constituent species, with the exception of the Rvic monoculture in weeks 2 and 4.

In addition to biomass measurements, viable cell counts for each constituent species from the mono-, di- and triculture microcosms (ie. *B. goodwinii* and *R. victoriana* from Bg + Rvic) from each weeks' microcosm were compared and plotted on a logarithmic scale (Fig. 2b–d).

Monocultures: The colony forming units (CFUs) for the *B. goodwinii* monoculture were significantly greater ($p < 0.0001$) than those observed for *R. victoriana* or *G. quercinecans* (Fig. 2b). Each week values exceeding 10^{11} CFU/ml were recorded with the starting inoculum determined as 10^{10} CFU/ml (OD_{600} = 0.03). While the *R. victoriana* monoculture significantly increased in colony numbers from week 1 to week 4 ($p = 0.005$) where it reached 10^9 CFU/ml (from a starting inoculum of 10^7 CFU/ml, OD_{600} = 0.03), the *G. quercinecans* monoculture decreased each week from a starting inoculum of 10^6 CFU/ml (OD_{600} = 0.03) with the highest measurement noted in week 1 (10^8 CFU/ml).

Dicultures: The colony counts for *B. goodwinii* and *R. victoriana* from the Bg + Rvic diculture were nearly equal for each week (~10^8 CFU/ml), whereas in the Bg + Gq and Rvic + Gq dicultures, significantly lower CFUs (10^7 and 10^6 CFU/ml) were determined for *G. quercinecans* compared to *B. goodwinii* or *R. victoriana* ($p < 0.0001$) (Fig. 2c).

Triculture: The weekly counts for *B. goodwinii* and *R. victoriana* from the Bg + Rvic + Gq triculture microcosm were similar to each other, and to the counts from the Bg + Rvic diculture, exponentially outnumbering the *G. quercinecans* CFUs by week 4 ($p < 0.0001$ and $p < 0.0001$) (Fig. 2d).

Mutually competitive species interactions were indicated between *B. goodwinii* and *R. victoriana*, and between *B. goodwinii* and *G. quercinecans*, as the sum of the monoculture CFUs was greater than that of the dicultures ($A_{mono} + B_{mono} > A_{di} + B_{di}$; see Supplementary data). No species interaction was indicated between *R. victoriana* and *G. quercinecans*, with the sum of the monoculture counts equal to those of the constituent species counts of the diculture ($A_{mono} + B_{mono} = A_{di} + B_{di}$).

3.2. Comparison of evolved AOD-associated bacterial growth

To determine if the three AOD-associated species evolved in terms of fitness during the microcosm experiment, each constituent species was re-isolated from all microcosms (mono-, di- and triculture) and their individual growth monitored in M9 minimal media. The statistical analysis of the microcosm data revealed that the microcosm itself was a significant factor ($p < 0.0005$), therefore the evolved growth rate study was performed in M9 minimal media to exclude this influence. When the

growth (in viable CFUs) of evolved *B. goodwinii* from the monoculture and triculture microcosms is compared to that of ancestral *B. goodwinii*, their yields are significantly lower ($p < 0.0001$ and $p = 0.009$, respectively) (Fig. 3a). *B. goodwinii* from the monoculture microcosm had the lowest evolved yield (10^9 CFU/ml), while *B. goodwinii* from the Bg + Rvic + Gq microcosm had the lowest yield of the di- and tri-cultures, although this was still approx. 10^{11} CFU/ml. The CFUs of evolved *R. victoriana*, re-isolated from all microcosms, are in the same region (~10^9 CFU/ml) as ancestral *R. victoriana*. (Fig. 3b). The exception is *R. victoriana* from the Rvic + Gq microcosm, which is significantly lower compared to the ancestral strain ($p < 0.0001$), displaying a viable colony count more than 10-fold lower than those of the other evolved *R. victoriana* strains. Similarly, the counts for evolved *G. quercinecans* are all in the same range as those for ancestral *G. quercinecans* (10^9 CFU/ml) (Fig. 3c).

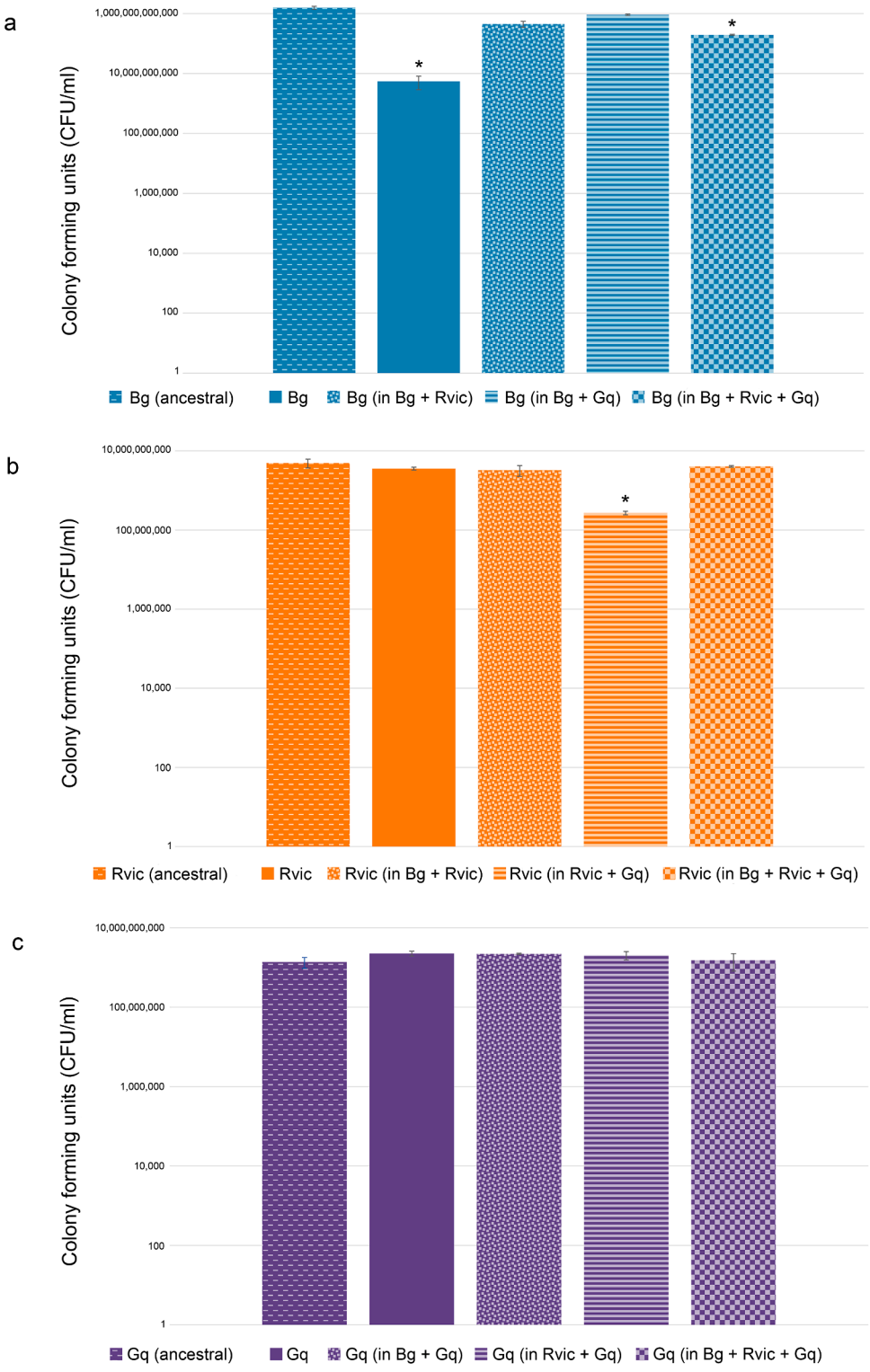

Fig. 3. Comparison of evolved viable cell counts. Comparison of viable cell counts (measured in colony forming units, CFUs) of evolved (a) *Brenneria goodwinii* FRB 186 (Bg - blue), (b) *Rahnella victoriana* BRK18a (Rvic - orange) and (c) *Gibbsiella quercinecans* BH1/65b (Gq - purple) re-isolated from each microcosm (monoculture, diculture and triculture) and ancestral strains (original culture used to inoculate microcosms) following 7 day static incubation in M9 minimal media. Values are reported on a logarithmic scale. Error bars represent standard deviation of the mean of three biological experimental replicates. * indicates statistically significant differences compared to ancestral strain assessed with ANOVA. (a) Bg (ancestral) = *B. goodwinii* ancestral strain, Bg (in Bg + Rvic) = *B. goodwinii* re-isolated from *B. goodwinii* and *R. victoriana* diculture, Bg (in Bg + Gq) = *B. goodwinii* re-isolated from *B. goodwinii* and *G. quercinecans* diculture, Bg (in Bg + Rvic + Gq) = *B. goodwinii* re-isolated from *B. goodwinii, R. victoriana* and *G. quercinecans* triculture. (b) Rvic (ancestral) = *R. victoriana* ancestral strain, Rvic (in Bg + Rvic) = *R. victoriana* re-isolated from *B. goodwinii* and *R. victoriana* diculture, Rvic (in Rvic + Gq) = *R. victoriana* re-isolated from *R. victoriana* and *G. quercinecans* diculture, Rvic (in Bg + Rvic + Gq) = *R. victoriana* re-isolated from *B. goodwinii, R. victoriana* and *G. quercinecans* triculture. (c) Gq (ancestral) = *G. quercinecans* ancestral strain, Gq (in Bg + Gq) = *G. quercinecans* re-isolated from *B. goodwinii* and *G. quercinecans* diculture, Gq (in Rvic + Gq) = *G. quercinecans* re-isolated from *R. victoriana* and *G. quercinecans* diculture, Gq (in Bg + Rvic + Gq) = *G. quercinecans* re-isolated from *B. goodwinii, R. victoriana* and *G. quercinecans* triculture.

3.3. SNPs within genome coding regions of evolved Brenneria goodwinii

Whole genome sequence alignments of coding regions of nine *B. goodwinii* genomes (from the evolved growth assays) were compared to the ancestral *B. goodwinii* FRB 186 genome. A total of 1632 SNPs were identified across the evolved genomes, with 459 identified in the Bg monocultures of which 337 were fixed, 543 in the Bg + Rvic dicultures and 630 in the Bg + Rvic + Gq tricultures of which 181 and 106 were fixed, respectively. Fixed SNPs were defined as variable alleles between the ancestral and evolved strains, and which were consistent across the three evolved replicates. Subsequently, fixed SNPs which produced non-synonymous substitutions (i.e. those that result in protein coding substitutions) were identified in two genes across evolved experimental replicates. The first of these was a large type I secreted adhesin of between 3067 and 7689 amino acids. Due to the large size of the gene and the number of indels it is likely that the number of substitutions is a sequencing reaction artefact. The second gene encodes a putative β-lactamase which consists of 420 amino acids and has three substitutions at positions 227 (serine – threonine), 263 (glutamine – leucine) and 386 (leucine - isoleucine) in all evolved strains except one replicate from the Bg + Rvic + Gq triculture which has a truncated form of the gene (115 amino acids) and only the final substitution at position 386 (Fig. S2).

Sites which were not fixed at the same position across the three evolved strains, but where more than one strain varied from the ancestral strain at the same site were further analysed. A single gene, the type VI secretion core gene *vgrG* matched these criteria. The ancestral strain of this gene comprises 506 amino acids which are conserved in all evolved strains from position 243 (Fig. S3). The ancestral protein consists of one protein family (IPR006533; N.B. IPR = InterPro) and three domains (IPR006531, IPR018769, IPR028244) one of which is within the protein family. All replicates of the evolved genomes from the monocultures and Bg + Rvic dicultures are missing the protein family IPR006533, and have only one or two of the three domains. All triculture replicate genomes contain variable copies of the gene. One replicate has protein family IPR006533 and all three domains with an extended copy of the gene (839 amino acids), another replicate has three domains but is missing protein family IPR006533, and the third has protein family IPR006533 and all three domains but has a reduced size (428 amino acids).

4. Discussion

Modelling of bacterial growth has shown that bacteria which undergo constant and rapid growth, regardless of nutrient concentration (termed nutrient-insensitive) will always outcompete other species that are dependant on their environment (termed nutrient-sensitive) (Mao et al., 2015). Previous *in vitro* growth studies (data not shown) of the AOD-associated species indicated *B. goodwinii* is biologically less fit than *R. victoriana* and *G. quercinecans*. It grows slowly in liquid media and is outperformed by *R. victoriana* and *G. quercinecans* in growth assays, suggesting *B. goodwinii* is a nutrient-sensitive bacterial species. However, these previous growth assays were performed on monocultures and did not take into account that community structure affects the fitness of bacterial cells (Stubbendieck et al., 2016) or that interactions can occur in multispecies communities.

The community biomass reflects the resource consumption in a microcosm and the resulting impact on species (Fiegna et al., 2015). The fluctuating weekly biomass measurements for the dicultures is indicative of interactions taking place between the species in the microcosms. Both *B. goodwinii* and *R. victoriana* appear to flourish in the oak leaf broth in a static, oxygen-poor environment, while *G. quercinecans* decreases in yield over time demonstrating a possible negative effect of the oak leaf broth on exponential growth. Although there were differences in the starting inoculum CFUs for each species due to variations in their cell size, the weekly biomass measurements did not reflect this. Despite often obtaining higher biomass measurements for the *R. victoriana* and *G. quercinecans* monocultures, the number of viable colonies (ie. how many bacterial cells are alive in the growth media at each time point) observed for *B. goodwinii* in monoculture were substantially greater. The larger cell size of *R. victoriana* compared to *B. goodwinii* (Denman et al., 2012; Brady et al., 2014) could account for the higher optical density measurements, especially the sharp increase in biomass of *R. victoriana* in week 4, but significantly lower corresponding CFUs suggesting the biomass was composed mostly of cell debris due to rapid growth of *R. victoriana* and limited nutrients. A second explanation is that nutrient-sensitive bacteria that are dependant on their environment (ie. *B. goodwinii*) make better use of their environment by exploiting resources and can likely produce more progeny than nutrient-insensitive bacteria (Stubbendieck et al., 2016).

When *B. goodwinii* and *R. victoriana* were grown in diculture, their viable cell counts were similar in number, indicating that *B. goodwinii* and *R. victoriana* can stably co-exist *in vitro*, despite *R. victoriana* being biologically fitter. Similarly, Ratzke et al. (2020) found a higher co-existence of bacteria in diculture under nutrient-poor conditions compared to nutrient-rich conditions. Microbes can co-exist with a degree of stability at certain nutrient concentrations, but other species can be out-competed when nutrients are limited (Tilman, 1977), as evidenced by the limited growth of *G. quercinecans* in diculture, where it was easily outcompeted by both *B. goodwinii* and *R. victoriana*. Motility, antibiotic production and co-ordinated behaviour are all mechanisms that aid competition and the means for bacteria to gain nutrients (Hibbing et al., 2010). *B. goodwinii* and *R. victoriana* have a competitive advantage as both are motile, which very probably contributes to the competitive exclusion of *G. quercinecans* in the triculture and implies co-ordinated behaviour or mutualism of these two species (Reichenbach et al., 2007). Alternatively, *B. goodwinii* and *R. victoriana* could have evolved to utilise the waste products generated by *G. quercinecans* thereby improving their niche colonisation (Lawrence et al., 2012).

Competition (species consume shared resources) has been shown to prevail over cooperation (metabolites produced by one species are consumed by another) in microbial interactions (Foster and Bell, 2012), as observed in the results from the diculture microcosms. If we classify the pairwise species interactions of the three AOD-associated bacteria used in our study, by comparing the total viable colony count of a diculture to the sum of the constituent species when grown in monoculture, all interactions but one are mutually competitive (Foster and Bell, 2012). The exception is the Rvic + Gq diculture where there is no interaction, suggesting *R. victoriana* and *G. quercinecans* are using non-overlapping resources and possibly occupy different niches in the microcosm. This is not to say that cooperation doesn't exist between these species in their natural environment. The oak leaf microcosm cannot reflect the true nutrient concentration, nor the extent of the microbial diversity, of the natural environment of these three AOD-associated bacteria, which could explain the lack of observed cooperation. Deng and Wang (2016) found that the complexity of carbon source in the environment plays a role in determining which bacterial interactions take place. When substrates are more complex, like lignocellulose, bacteria work synergistically to degrade them. Whereas if a simple substrate, such as glucose, is available, the same bacteria will compete antagonistically for the limiting nutrient. It is also possible that given more time in the microcosms, the species could have evolved and adapted to form a more cooperative interaction (Lawrence et al., 2012).

Static liquid cultures are seen as environments with multiple ecological niches (Hibbing et al., 2010), and as such provide the opportunity for variants to evolve which are better suited to colonise these alternative niches. As forcing the three different bacterial species to grow together *in vitro* under static conditions is not the natural environment for these microbes, we were interested to discover that the evolutionary effect on growth rate was most pronounced for *B. goodwinii* while there was little effect on *R. victoriana* and *G. quercinecans*. The lower yield of evolved *B. goodwinii* from the microcosms compared to

that of ancestral *B. goodwinii* (original strain used for inoculation), suggests that adaptation to the new environment of the oak leaf media had an overall negative effect on growth rate of *B. goodwinii*. However, comparison of evolved *B. goodwinii* strains from the di- and tricultures to that from monoculture reveals a positive effect of diversity as these isolates display enhanced growth rates. As evidenced in similar studies (Fiegna et al., 2015; Lawrence et al., 2012), it's possible that *B. goodwinii* evolved to inhabit alternative niches in the presence of competition from *R. victoriana* and *G. quercinecans* as predicted in the niche simulation model (De Mazancourt et al., 2008).

The effect of species interactions on the evolved *B. goodwinii* strains was examined further by analysing their whole genome sequences for single nucleotide polymorphisms (SNPs). Molecular analysis of base and amino acid substitutions revealed selective pressure at multiple sites within the *B. goodwinii* genomes. The sites of interest were those alleles containing fixed, non-synonymous substitutions across evolved strains compared to the ancestral strain, or where the evolved strains had more than one allele which differed from the ancestral strain. One of the genes of interest with fixed, non-synonymous substitutions codes for a β-lactamaseadhesin. β-lactams are found widely in soil bacteria and in plant pathogenic bacteria such as *Pseudomonas syringae* (Demanèche et al., 2008), and are hydrolysed by β-lactamase genes which are common in Gram negative bacteria (Bush and Bradford, 2019). Evolution of the β-lactamase gene may indicate reduced competitive pressure compared to that which is faced in the natural environment by the ancestral *B. goodwinii* strain. This is most likely as the evolved *B. goodwinii* strains from the monocultures had the same evolved alleles as that of the evolved *B. goodwinii* strains from dicultures and tricultures containing *R. victoriana* and *G. quercinecans*. This indicates a lack of utility for the β-lactamase gene in the evolved environments possibly leading to its transition into a pseudogene before its eventual loss through the gradual forces of genome purging (Ochman and Davalos, 2006).

Several copies of the type VI secretion system (T6SS) component *vgrG* (TssI) are encoded in the *B. goodwinii* genome, one of which had multiple insertion/deletion (indel) substitutions at the 5′ gene region. None of the evolved alleles were an exact match to the ancestral strain. The ancestral allele consists of three domains, the first two of these domains (where the insertion and deletions occur) are Rhs regions or rearrangement hotspots (Pei et al., 2020). The Rhs domain is part of the envelope spanning the *vgrG* needle spike component of the T6SS, which is responsible for puncturing eukaryotic and prokaryotic cells to deliver toxic effectors, and can sometimes act as a structural effector (Coulthurst, 2019). The first Rhs domain is deleted in all three evolved *B. goodwinii* alleles from the Bg + Rvic dicultures. It is retained in all three evolved *B. goodwinii* alleles from the Bg + Rvic + Gq tricultures (one strain contained an extended copy of the domain). It has previously been shown that deletion of the Rhs domain abolishes secretion of the T6SS (Pei et al., 2020), suggests that the deleted Rhs components in evolved *B. goodwinii* strains from Bg + Rvic dicultures prevent toxic effectors being delivered into a co-operative bacterium (*R. victoriana*). Interestingly, four copies of the *vgrG* gene were previously found to be upregulated during *in planta* infections *of B. goodwinii* in necrotic oak lesions, contributing to the hypothesis that *vgrG* is central to persistence of *B. goodwinii* in the environmental niche of a necrotic oak lesion (Doonan et al., 2020). Taken together, these findings show that the *vgrG* gene is integral to environmental persistence of *B. goodwinii*. Further work to confirm the effects of *vgrG* on competitive growth of *B. goodwinii* would need to be undertaken with knockout mutant strains.

5. Conclusion

In this study, we demonstrated for the first time that *in vitro* growth of *B. goodwinii* can be more robust and prolific than *R. victoriana* or *G. quercinecans* and that competition prevails in pairwise and multispecies microcosms. Over time, the mutually competitive interactions between *B. goodwinii* and both *R. victoriana* and *G. quercinecans* seem to enhance its growth rate and fitness. This *in vitro* study mimics the species behaviour we observe in lesion development with *B. goodwinii*, the primary causal agent of necrosis, as the most abundant species in the AOD lesion and *R. victoriana* and *G. quercinecans* as the possible 'secondary invaders'. Indeed live oak inoculations have demonstrated that a combination of *B. goodwinii* and *G. quercinecans* causes greater necrosis than single species (Denman et al., 2018), and only combined inoculations which included *B. goodwinii* yielded lesions (Ruffner et al., 2020), illustrating the probable synergistic relationships observed *in vitro* in some pairwise and multispecies microcosms. Because of the immense diversity of the oak microbiome, it can't be discounted that other frequently isolated bacteria from oak lesions (ie. *Pseudomonas* species (Bueno-Gonzalez et al., 2019, 2020) play a role in the interactions between *B. goodwinii, R. victoriana* and *G. quercinecans*. Indeed, further work could include the expansion of the present study with more complex inoculation media and more combinations of bacterial species frequently isolated from AOD symptomatic oak. Controlled combined growth experiments will be key to improved understanding of bacterial ecological relationships and host-microbe interactions (Nawy, 2017), such as those occurring between AOD bacteria and their oak host.

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CRediT authorship contribution statement

Carrie Brady: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization, Project administration. **Mario Orsi:** Validation, Formal analysis, Writing – review & editing. **James M. Doonan:** Formal analysis, Data curation, Writing – original draft. **Sandra Denman:** Writing – review & editing, Funding acquisition. **Dawn Arnold:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2021.100102.

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