Niche Separation in Methanol Consuming Bacteria isolated from Trees and Plants

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Abstract

Similar organisms are consistently sampled from the same habitats. How does habitat heterogeneity determine local biological diversity? There are many ecological parameters (temperature, pH, redox substrates, resource competition, etc.) which may simultaneously determine the suitability of a particular organism, and therefore the local organismal diversity, making this a difficult question to answer. The surfaces of plants and trees constitute a large potential habitat for microbial life providing diverse, energy rich substrates but also varied environmental challenges to potential colonisers. A genetically and phenotypically diverse group of organisms was isolated from leaves, tree bark and soil by an oxic enrichment strategy providing methanol as the only electron donor. Each inoculum was dominated by a particular group, so given that they can all grow on methanol, which ecological factors determined this spatial heterogeneity? Carbon substrate useage versatility, tolerance of ultra violet radiation and dessication and coloration were investigated as potential determinants.

Part I

Introduction

Why is there biological diversity? Hutchinson popularised the ecological niche concept [2] as a hyper volume in high dimensional space where each dimension represents an environmental parameter. The volume is defined by the range in each dimension in which the organism can grow. Two different organisms cannot occupy an identical niche space in a limited system and in this context the concept can be applied to fitness determinants for evolving organisms. Biological evolution is one way of defining life: a change of inherited traits over generations. An alternative is that of metabolism: the set of chemical reactions allowing maintainence of the low entropy associated with living matter. However, due to the inherent heterogeneity of the Universe, this metabolism has to be achieved in different ways in different places.

Almost all metabolic reactions involve the transfer of electrons between different chemical species and so metabolism depends on the local quality and quantity of electron acceptors and donors. As this availability along with other niche parameters varies through time and space, biological evolution can fill these niches by innovating different solutions to the different problems resulting in a corresponding level of diversity. Costs associated with specific adaptations and physical limitations are among the likely reasons that there is not a single organism able to live in all niches. By characterising which niche parameters are important factors in determining the biological diversity in easily observable systems, predictions can be made about the potential for life and its diversity in less easily observed systems such as other planets, the deep ocean or where there are difficult to cultivate microorganisms.

Given the vast array of potential niche parameters in a specific situation, determining those that are different among a set of organisms can be difficult. Green and purple sulfur bacteria in a phototrophic microbial mat both require hydrogen sulfide as an electron donor, an overlapping niche parameter, but have evolved to harvest energy optimally from light of different wavelengths. This difference in light utilisation differentiates their niche spaces thus both types of organisms avoid competition with each other and increase the diversity of the mat as a whole. More subtle differences are likely to exist among, for example, distinct groups of co-existing purple sulfur bacteria. One approach for inferring important niche parameter differences is to isolate several organisms from the habitat and test their growth under different conditions.

An oxic isolation strategy involving methanol as a sole carbon source and taking inocula from plant leaf surfaces, tree bark and soil yielded isolates. Each inocula was dominated by phylogenetically distinct groups of facultative methylotrophs. Growth on methanol is a common niche parameter among these organisms, but why did each inoculum yield distinct organisms? What aspects of life on a leaf surface are sufficiently different from life in soil or tree bark to prevent the same organism from dominating the community in all three places? An array of potentially varying niche parameters exist from the phyllosphere zones of the leaves, stems and branches, down to the rhizosphere and into the soil including differing carbon substrate availability, light exposure and moisture fluctuations. Hypotheses tested in this study are that the leaf isolates have adapted to be more tolerant of UV radiation and dessication than the bark or soil organisms but that the organisms isolated from the soil can grow on a wider range of carbon substrates. Leaf exudates probably consist of a less diverse mixture than substrates found in soil due to the high diversity of microorganisms in the latter and may be dominated by methanol from pectin methylesterase activity associated with leaf growth [4]. Moisture from rain followed by rapid evaporation due to wind and sunlight means dessication is likely a common event on leaf surfaces.

Part II
Materials and Methods

1 Isolation

Maple tree leaves, bark and soil from nearby were used as inocula. Each were pressed onto agar containing only methanol as a growth substrate as described in the MBL Microbial Diversity Laboratory Manual. After replating and streaking colonies, PCR amplification of a fragment of the small subunit ribosomal rRNA was performed using 1492r and 8f primers. The product was sequenced and similarity with sequences already in the National Center for Biological Information non-redundant database was calculated using BLAST. The sequences were also added to a SILVA rRNA database phylogeny using parsimony as implemented in Arb.

2 Stress tolerance

2.1 UV radiation.

Isolates were grown overnight in 0.5% yeast extract liquid medium at 30°C in a shaking incubator. Cells were diluted in cell buffer (10ml 100x Fresh Water Base, 5ml 1M NH4Cl, 1ml 1M K phosphate, 1ml 1M NaSO4, 5ml 1M MOPS pH 7.2, 1ml Trace minerals in 1000 ml, with 1 ml x1000 trace vitamins added after autoclaving) to 1 in 10000 concentration. Nutrient agar plates were dried in a laminar flow hood for 40 mins after which the cell suspensions were spread on duplicate plates per strain per treatment. Plates were exposed to 0, 1, 5 and 20 minutes of ultra violet radiation (40 cm from a Philips G36T6L 39 watt sterilamp emitting 13.8 watts of light in the 100-280 nm range). Colony forming units were counted after 48 hours incubation at 30°C.

2.2 Dessication.

Isolates were grown overnight in 0.5% yeast extract liquid medium at 30°C in a shaking incubator. Cells were diluted in cell buffer (10ml 100x Fresh Water Base, 5ml 1M NH4Cl, 1ml 1M K phosphate, 1ml 1M NaSO4, 5ml 1M MOPS pH 7.2, 1ml Trace minerals in 1000 ml, with 1 ml x1000 trace vitamins added after autoclaving) to 1 in 10000 concentration. Ten 7.5ul drops were applied on sterile 47mm AP10 MF support pads without visible contact between patches of cell suspension in duplicate per strain per treatment. The pads were placed in air tight jars containing dessicant (blue Dririte in a 90mm petri dish) for 0, 1, 4 and 26 hours. After the desication treatment, each pad was placed on a nutrient agar plate (pre-dried in a laminar flow hood for 40 mins to prevent spreading of cells across the agar surface) and growth of each area with added cells checked after 48 hours incubation at 30°C [1].

3 C substrate useage

For each strain to be tested, enough cells to provide a density with absorption of 0.1 in 15 ml of cell buffer (10ml 100x Fresh Water Base, 5ml 1M NH4Cl, 1ml 1M K phosphate, 1ml 1M NaSO4, 5ml 1M MOPS pH 7.2, 1ml Trace minerals in 1000 ml, with 1 ml x1000 trace vitamins added after autoclaving) were washed three times and inoculated into a Phenotype Microarray 1 plate (Biolog, Inc., Hayward, CA, USA). Absorbance readings were taken at a wavelength of 600 nm 0, 24, 48 and 72 hours after inoculation. For each well, the absorbance at t = 0 was substracted to correct for pipetting error. For each time point across wells, the absorbance of the blank was subtracted to correct for growth over the first 24 hours on stored or carried over substrates from before innoculation into the plate as shown in figure 1. The variance of readings at 24, 48, and 72 hours in the blank well was used as a measure of absorbance reading error. The Z score for each well at 72 hours was calculated and those with a score greater than 5 were interpretted as a positive result.

Part III
Results

4 Isolation

Several organisms were isolated from each inoculum. Each inoculum was dominated by a phylogenetically distinct group. The leaf isolates had greatest small subunit ribosomal rRNA sequence similarity with members of the *Methylobacterium*, the bark isolates with *Oxalobacteraceae*, and the soil isolates with *Paenibacillaceae*. Representatives of each group were selected for study and are listed in 1. One soil isolate was affiliated to the *Cohnella* genus [3] but had only 95% sequence identity with the nearest cultured organism, *Cohnella panacarvi* [6]. According to convention, a less than 97% identity is indicative of different species [5] although DNA-DNA hybridisation is a more reliable indicator.

5 Stress tolerance

5.1 UV radiation.

The hypothesis that the pink leaf isolated methylotrophs were most tolerant of UV light followed by the bark isolates and then the soil isolates was tested. The least tolerant were the *Oxalobacteraceae* bark isolates showing no growth after 1.5 minutes of UV exposure followed by the *Methylobacteria* with only one strain showing some growth after 1.5 minutes while the *Cohnella* soil isolate was the least adversely affected with a few cfus surviving 3 minutes of exposure. Overall the treatment was harsh with the majority of cfus for all strains killed

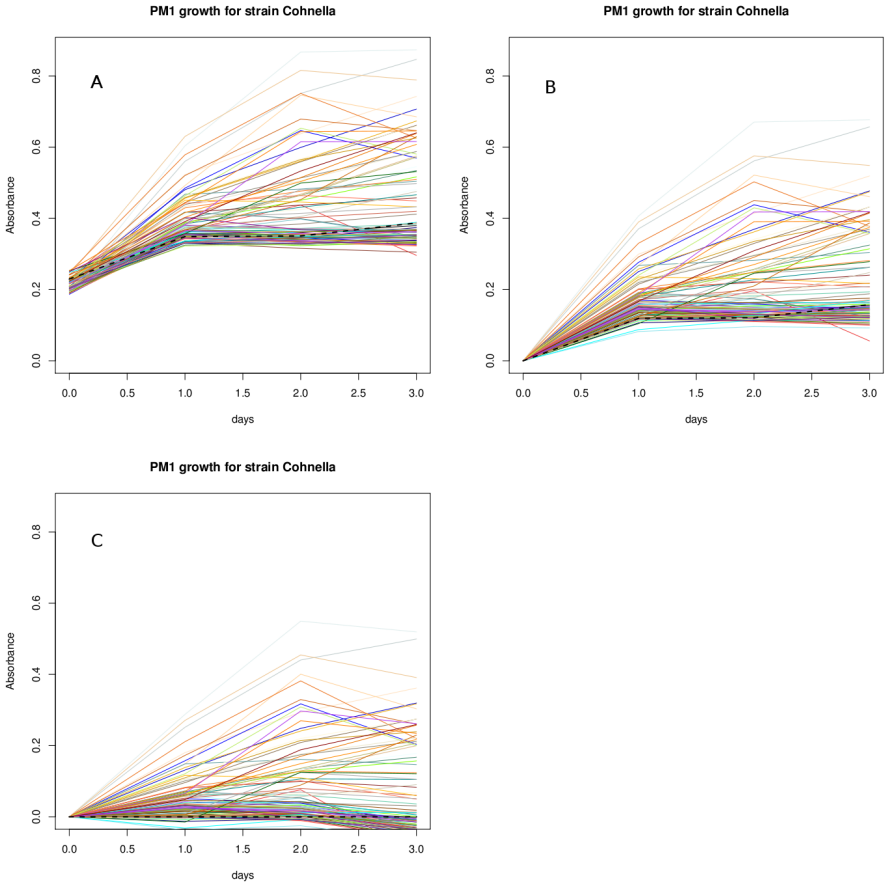

Figure 1: Data processing of Phenotype Microarray 1 plates: A, raw data, B, data corrected for pippetting error, C, data corrected for residual growth (see Materials and Methods fo more details). The blank control well is shown with a thick dashed line.

Source	SSU rRNA phylogeny	Colony morphology	Name
Leaf (top)	Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium	Small, round, pink, 'fluid'	Meth. 1
Leaf (top)	Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium	Small, round, pink, 'fluid'	Meth. 2
Leaf (bottom)	Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium	Small, round, pink, 'fluid'	Meth. 3
Tree Bark	Betaproteobacteria, Burkholderiales, Oxalobacteraceae	Small, very crinkly, pale, 'stiff'	Oxal. 1
Tree Bark	Betaproteobacteria, Burkholderiales, Oxalobacteraceae	Small, very crinkly, very light pink, 'stiff'	Oxal. 2
Soil	Firmicutes, Bacilli, Paenibacillaceae, Paenibacillus	Small, wobbly, very light pink, 'fluid'	Paen.
Soil	Firmicutes, Bacilli, Paenibacillaceae, Cohnella	Small, wobbly, very light pink, 'fluid'	Cohnella

Table 1: Isolation source, phylogeny and colony morphology of faculativte methylotroph isolates.

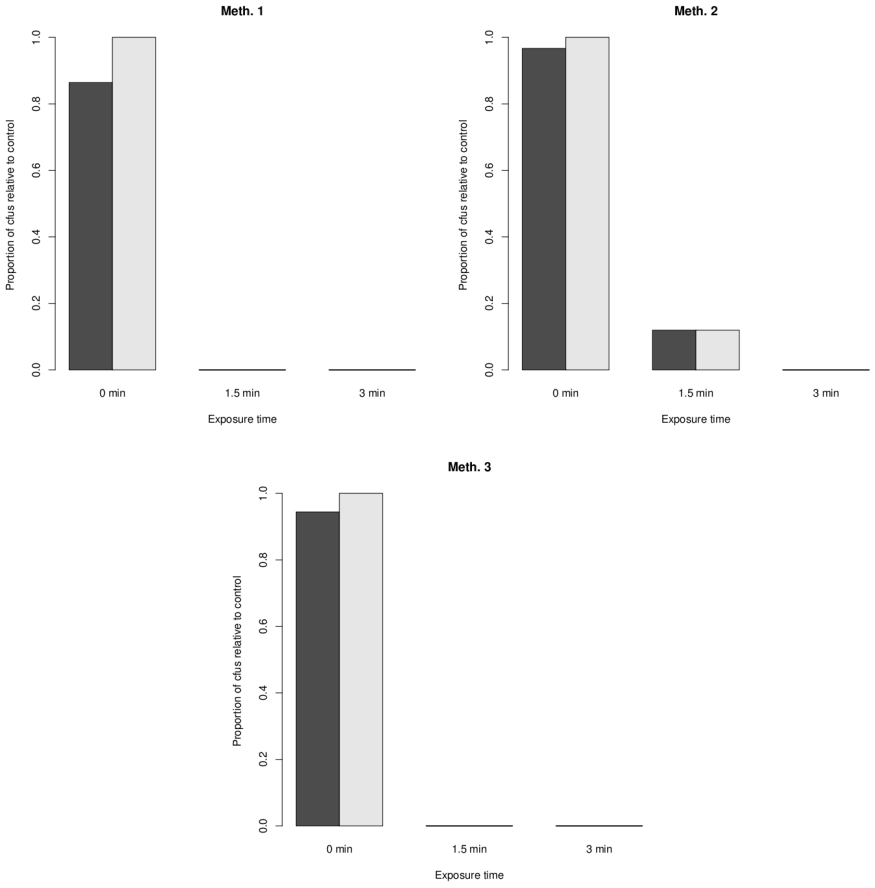

Figure 2: Tolerance of ultra violet light by leaf surface isolated Methylobacterium strains

after 1.5 minutes exposure. The soil strain was unexpectedly tolerant of the UV radiation. Figures 2 to 4 show plots of growing cfus after UV exposure treatments.

5.2 Dessication.

The hypothesis tested was that leaf *Methylobacteria* isolates are most tolerant of dessication. No growth of the bark isolated *Oxalobacteraceae* was observed even on the non-dessicated control. It is likely that growth on the filter paper was difficult for these strains and so data was not collected for them. Table 2 shows how growth was affected after desiccation treatments for each strain. The leaf and soil strains were similarly tolerant of dessication: both soil strains were slightly affected after 45 hours of dessication, Meth. 2 was unaffected after

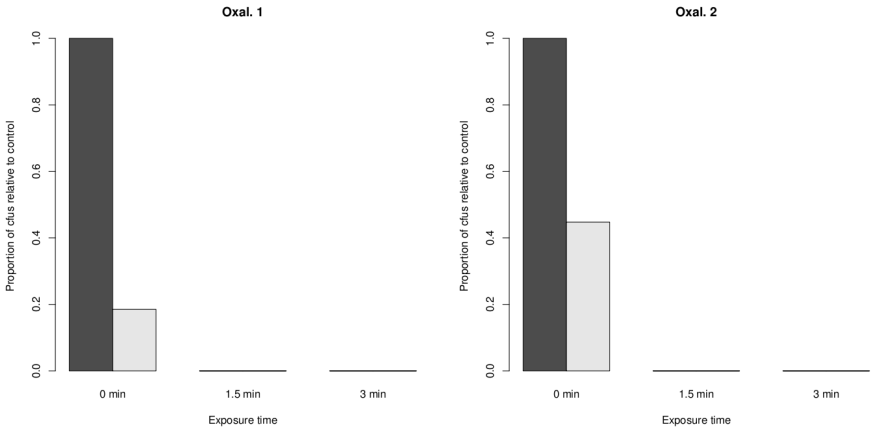

Figure 3: Tolerance of ultra violet light by tree bark isolated Oxalobacteraceae strains

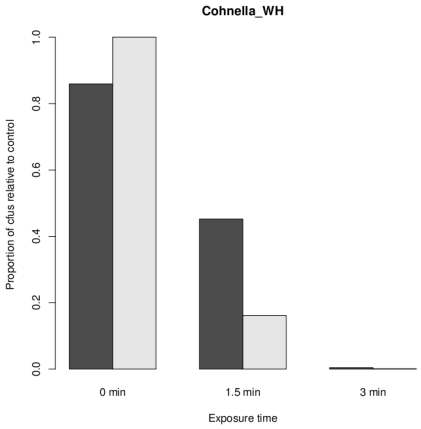

Figure 4: Tolerance of ultra violet light by soil isolated strain 'Cohnella'

Strain	0 hours	1 hour	12 hours	45 hours
Meth. 1	++	+	+	
Meth. 2	++	++	++	++
Meth. 3	++	++	+	
Paen.	++	++	++	+
Cohnella	++	++	++	+

Table 2: Desiccation tolerance of tree associated facultative methylotrophs. Two '+' symbols represents growth unaffected by the treatment (the same as the control), one '+' symbol represents less growth than the control, no symbols represents no growth.

45 hours while Meth. 1 and 3 were adversely affected after 1 and 12 hours respectively.

6 C substrate useage

The hypothesis to be tested was that the leaf strains are specialised to a more narrow range of C based substrates with the bark isolates showing more versatility and the soil isolates the most versatility. Meth. 1 could grow on 16 of 95 substrates, Meth. 2 on 33, Oxal. 1 on 56, Oxal. 2 on 50, Paen. on 75 and Cohnella on 43. The leaf isolates were the most specialised growing on the smallest different number of tested substrates while the soil and bark isolates demonstrated more versatiliy.

Strain Meth. 1: L-Glutamine, L-Arabinose, D-Glucose-1-Phosphate, M-Inositol, m-Hydroxy Phenyl Acetic Acid, D-Ribose, Tween 80, D-Gluconic Acid, L-Aspartic Acid, D,L-α-Glycerol-Phosphate, D-Alanine, L-Lactic Acid, D-Mannitol, Sucrose, Uridine, 2-Aminoethanol

Strain Meth. 2: D-Serine, L-Asparagine, L-Glutamine, Glycyl-L-Aspartic Acid, Glycyl-Lglutamic Acid, Tricarballylic Acid, Glycerol, D-Glucose-1-Phosphate, M-Inositol, m-Hydroxy Phenyl Acetic Acid, L-Fucose, D-Ribose, Tyramine, Tween 80, D-Gluconic Acid, L-Aspartic Acid, Propionic Acid, Mucic Acid, D-Alanine, L-Lactic Acid, α-D-Lactose, Glycolic Acid, Maltotriose, Glyoxylic Acid, D-Mannose, D-Mannitol, D-Melibiose, Sucrose, L-Glutamic Acid, Uridine, Adenosine, Inosine, 2-Aminoethanol

Strain Oxal. 1: , Glycyl-L-Proline, L-Arabinose, D-Sorbitol, D-Galactonic , D-Aspartic Acid, Citric Acid, Tricarballylic Acid, p-Hydroxy Phenyl Acetic Acid, N-Acetyl-D Glucosamine, Glycerol, D-Glucosaminic Acid, D-Glucose-1-Phosphate, L-Serine, m-Hydroxy Phenyl Acetic Acid, 1,2-Propanediol, D-Fructose-6-Phosphate, L-Threonine, Tyramine, Succinic Acid, D-Glucuronic Acid, Tween 20, Tween 40, Tween 80, L-Alanine, D-Psicose, D-Galactose, D-Gluconic Acid, L-Rhamnose, α-Keto-Glutaric, α-Hydroxy-, Bromo Succinic Acid, L-Alanyl-Glycine, L-Lyxose, L-Aspartic Acid, α-Hydroxy-, Acetoacetic Acid, Glucuronamide, L-Proline, D-Xylose, Acetic Acid, α-Methyl-DGalactoside, β-Methyl-D Glucoside, Mucic Acid, N-Acetyl-β-DMannosamine, Pyruvic Acid, L-Lactic Acid, α-D-Glucose, Glycolic Acid, Mono Methyl Succinate, L-Galactonic, D-Trehalose, Formic Acid, Maltose, Lactulose, Maltotriose, Glyoxylic Acid, Methyl Pyruvate, D-Mannitol, D-Melibiose, Sucrose, 2-Deoxy Adenosine, D-Cellobiose, D-Malic Acid, Phenylethylamine, Dulcitol, Thymidine, Uridine, Adenosine, Inosine, L-Malic Acid, 2-Aminoethanol

Strain Oxal. 2: Glycyl-L-Aspartic Acid, Glycyl-Lglutamic Acid, Glycyl-L-Proline, L-Arabinose, D-Sorbitol, D-Galactonic , Tricarballylic Acid, Glycerol, D-Glucose-1-Phosphate, m-Hydroxy Phenyl Acetic Acid, L-Threonine, Tyramine, Succinic Acid, D-Glucuronic Acid, Tween 40, Tween 80, L-Rhamnose, L-Alanyl-Glycine, L-Lyxose, L-Aspartic Acid, α-Hydroxy-, Acetoacetic Acid, D-Xylose, Acetic Acid, α-Methyl-DGalactoside, β-Methyl-DGlucoside, Mucic Acid, α-D-Glucose, Mono Methyl Succinate, L-Galactonic, D-Trehalose, Mal-

tose, Lactulose, Maltotriose, Glyoxylic Acid, D-Mannitol, 2-Deoxy Adenosine, D-Cellobiose, D-Malic Acid, Phenylethylamine, Dulcitol, Uridine, L-Malic Acid

Strain Paen.: D-Serine, D-Glucose-6-Phosphate, L-Asparagine, L-Glutamine, Glycyl-L-Aspartic Acid, Glycyl-Lglutamic Acid, Glycyl-L-Proline, D-Sorbitol, D-Galactonic , Tricarballylic Acid, N-Acetyl-DGlucosamine, D,L-Malic Acid, D-Glucosaminic Acid, D-Glucose-1-Phosphate, M-Inositol, m-Hydroxy Phenyl Acetic Acid, D-Saccharic Acid, D-Ribose, 1,2-Propanediol, D-Fructose-6-Phosphate, D-Threonine, L-Threonine, Tyramine, Succinic Acid, D-Glucuronic Acid, Tween 40, Tween 80, D-Galactose, D-Gluconic Acid, L-Rhamnose, L-Lyxose, L-Aspartic Acid, α-Keto-Butyric Acid, α-Hydroxy-, Acetoacetic Acid, D-Xylose, Acetic Acid, α-Methyl-DGalactoside, β-Methyl-D Glucoside, Mucic Acid, D-Alanine, L-Lactic Acid, β-D-Lactose, Mono Methyl Succinate, L-Galactonic, D-Trehalose, Maltose, Lactulose, D-Mannose, D-Mannitol, D-Melibiose, Sucrose, 2-Deoxy Adenosine, D-Malic Acid, Uridine, Inosine

Strain Cohnella: Glycyl-L glutamic Acid, Glycyl-L-Proline, L-Arabinose, D-Sorbitol, D-Galactonic , Citric Acid, Tricarballylic Acid, Glycerol, D-Glucose-1-Phosphate, m-Hydroxy Phenyl Acetic Acid, L-Threonine, Tyramine, Succinic Acid, D-Glucuronic Acid, Tween 40, Tween 80, D-Psicose, D-Galactose, L-Rhamnose, α-Hydroxy-, L-Alanyl-Glycine, L-Lyxose, L-Aspartic Acid, α-Hydroxy-, Acetoacetic Acid, D-Xylose, Acetic Acid, α-Methyl-DGalactoside, β-Methyl-DGlucoside, Mucic Acid, N-Acetyl-β-DMannosamine, Pyruvic Acid, L-Lactic Acid, α-D-Glucose, Mono Methyl Succinate, L-Galactonic, D-Trehalose, Maltose, Lactulose, Maltotriose, Glyoxylic Acid, D-Mannitol, 2-Deoxy Adenosine, D-Cellobiose, D-Malic Acid, Phenylethylamine, Thymidine, Uridine, L-Malic Acid, 2-Aminoethanol

Part IV
Discussion

The characterisation of these strains has revealed some potentially important traits among tree associated facultative methylotrophs with respect to local habitat specificity. The sensitivity to UV radiation of the *Oxalobacteraceae* isolates may inhibit them from colonizing leaves while the narrower substrate useage of the *Methylobacteria* may make them poor competitors in the tree bark niche. Fungal decomposition of lignin in the tree bark niche may provide diverse C substrates for which it is beneficial to be able to grow on. The possibility of endospore formation by *Paenibacillus* and *Cohnella* isolates may have enhanced their stress tolerance. The recovery stage of the stress tolerance experiments were on 0.5 % nutrient agar which may provide unrealistically energy rich conditions for transforming from endospore back to vegetative cells. This means stress tolerance may have been enhanced for this group.

The bottom of the leaves were observed to harbor more potential isolates than the tops and colonies isolated from the tops of leaves were generally more

intensely pigmented (data not shown). It would be interesting to investigate whether pigment intensity correlates with light tolerance and whether the absorbance spectra of extracted carotenoids are the same or whether there is a diversity of types as well as variation in the amount produced among different organisms. Inoculation of leaf facultative methylotrophs onto bark and vice versa with monitoring of population sizes could be done to test the effect of various niche parameters. For example growth of *Oxalobacteraceae* cells on leaves with a treatment of decreased light could test the relative importance of light intensity versus other potential ecological niche parameters.

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