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## Genomic basis for pesticide degradation revealed by selection, isolation and characterisation of a library of metaldehyde-degrading strains from soil

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### ABSTRACT

Metaldehyde, a xenobiotic cyclic ether, is used as molluscicide of choice in agriculture and horticulture, but recently its detection in drinking water sources has become a major cause of concern. We isolated eight new metaldehyde-degrading bacterial strains from allotment and agricultural soils and identified a highly-conserved gene cluster shared amongst one gamma and five beta-proteobacteria, and absent from closely-related, non-degrading type strains. Chemical mutagenesis, and heterologous expression in *E. coli*, confirmed that this gene cluster is responsible for metaldehyde degradation. Other metaldehyde-degrading isolates that lack this pathway indicate that multiple degradation mechanisms have evolved. We demonstrated accelerated biodegradation of metaldehyde in multiple soils, highlighting the importance of the biological component in metaldehyde degradation in nature. We confirmed that the metaldehyde-degrading population in soil is proliferating in response to metaldehyde, but no bulk changes in the composition of the community as a whole were detected, indicating the process is governed by a few rare taxa. Here, we identified the first genetic determinants for the biological degradation of metaldehyde in soil paving the way for targeted bioremediation strategies.

### 1. Introduction

Soils contain extremely diverse microbial communities with versatile metabolic capabilities. The acquisition of new traits occurs by diversification of the existing genetic material from the metagenome and is further enabled by horizontal gene transfer (Maheshwari et al., 2017). Thus, it is possible for novel metabolic activities to emerge and be evolutionarily reinforced via selection within soil microbial communities (Fierer, 2017; Kuzyakov and Blagodatskaya, 2015). Such processes are important to agriculture as pesticides are used to improve crop productivity, but also impact soil microbes, which may degrade them. Genes whose products degrade novel chemicals and confer a competitive nutritional advantage on their microbial hosts have potential to be selected for in soils (Arbeli and Fuentes, 2007).

Metaldehyde ( $\text{CH}_3\text{CHO}$ )<sub>4</sub> is a pesticide used worldwide as a molluscicide to control snails and slugs that damage agricultural crops and domestic gardens (Eckert et al., 2012). It is a xenobiotic that has been used as a pesticide since the 1930s (Gimingham, 1940); it is hydrolytically and photolytically stable (Carpenter, 1989; Kegley et al., 2016). Metaldehyde is applied to many crops, including oilseed rape, wheat and potatoes, and accounted for 84.5% of all molluscicide applications in the UK in 2016

(Garthwaite et al., 2018). It is applied as a pelleted bran bait in the autumn, when molluscs prosper due to the humid conditions. Rain-fall can dissolve metaldehyde and carry the compound to watercourses which may be used for drinking water abstraction (Lazartigues et al., 2013).

Metaldehyde is recalcitrant to removal using conventional drinking water treatment processes based on adsorption of substances to activated carbon (Castle et al., 2017). Moreover, detections of metaldehyde have been the main cause for already treated water not meeting pesticide standards since its monitoring began (Chief Inspector of Drinking Water, 2017). Hence, water companies have been exploring alternative solutions for metaldehyde detection and elimination. Therefore, the idea of using biological strategies to detect and degrade metaldehyde has recently emerged. Metaldehyde can be quickly degraded in soils (Lewis et al., 2016; Zhang et al., 2011) and is oxidised to carbon dioxide under aerobic conditions (European Food Safety Authority, 2010), in comparison to its long half-life in sterile conditions (Simms et al., 2006). This suggests a strong involvement of microorganisms in its degradation. The biotic degradation of metaldehyde in treated soil or sediments proceeds at very different rates in different experiments; with  $\text{DT}_{50}$  values of between <1 and 67 d (Cranor, 1990; Möllerfeld et al., 1993; Zhang and Dai, 2006).

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Very few studies have investigated further the mechanism underpinning the microbial degradation of metaldehyde. Rolph (2016) found improved metaldehyde removal in slow sand filter material following a period of acclimation with relatively elevated metaldehyde concentrations. Thomas and collaborators (Thomas et al., 2017) were the first to isolate bacterial strains capable of metaldehyde degradation (*Acinetobacter calcoaceticus* E1 and *Variovorax* E3). Even so, the diversity of metaldehyde degraders found in nature, the biochemical mechanisms responsible for its degradation, and the effect metaldehyde inputs have in shaping microbial communities in biological habitats remain largely unexplored. Here we examine the diversity of organisms capable of degrading metaldehyde using culture-based and culture-independent molecular methods, focusing on the impact of historical and experimental application of the pesticide. We isolate a set of genes, shared amongst several independent isolates, responsible for metaldehyde degradation.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Metaldehyde (99%) was purchased from Acros Organics, NJ; sodium sulfate ( $\geq 99\%$ ) was purchased from Honeywell-Fluka, Bucharest; all other chemicals were purchased from Sigma-Aldrich, St. Louis, MO.

### 2.2. Analytical methods for metaldehyde

#### 2.2.1. Metaldehyde extraction and quantification in aqueous matrices

Aqueous samples were centrifuged (4000 g, 10 min) and 0.4 mL supernatant added to 0.5 mL dichloromethane in glass chromatography vials (Thermo Scientific), vortexed for 30 s and stabilized for 20 min. 5  $\mu$ L of organic phase were injected into an Agilent 7820A gas chromatograph (Stockport, UK) fitted with a HP-5 column and Flame Ionization Detector. Chromatographic parameters were described previously (Tao and Fletcher, 2013). Limit of detection and limit of quantification were 0.05 mg L<sup>-1</sup> and 0.15 mg L<sup>-1</sup> respectively. Calibration curves were constructed with standard metaldehyde solutions in minimal salts media (MSM) (Thomas et al., 2017) and sample peak area was interpolated in the calibration curves.

#### 2.2.2. Metaldehyde extraction and quantification in soil

Each sample for extraction consisted of 10 g of soil in glass Falcon tubes (Kimble-Chase, NJ). 5 g anhydrous sodium sulfate were added, contents homogenized, and 15 mL dichloromethane added. Tubes were vortexed for 30 s and placed in ultrasonic bath for 20 min. Centrifugation (2000 rpm, 10 min) followed. 2 mL of supernatant were withdrawn from each system, passed through sodium sulfate/silanized glass wool column and filtered through a 0.45  $\mu$ m PTFE filter. Chromatographic analysis was as described for aqueous samples. Peak area for samples was interpolated in a calibration curve. Limit of detection and limit of quantification were 0.25 mg kg<sup>-1</sup> soil and 0.75 mg kg<sup>-1</sup> soil respectively.

### 2.3. Soil sampling and characterization

Three soil sample collections were performed. First collection, January 2017: two council-run allotment plots (Hob Moor Allotments, York, UK; 53.946113, -1.103654). Plot J-Met had been regularly treated with metaldehyde for at least 5 years. Plot J-NoMet had not been treated with metaldehyde for at least 5 years. Second collection, June 2017: a lettuce and cilantro-producing agricultural farm (Caballo Blanco, Cartago, Costa Rica; 9.854884, -83.901134) periodically exposed to metaldehyde for at least 3 years (C-Met). Third collection, October 2017: two different allotment sites: Scarcroft Allotments (S) (York, UK; 53.950840, -1.092036) and Hob Moor Allotments (H). For each site, two strawberry plots were chosen: one treated with metaldehyde during the July–August period (S-Met and H-Met) and another plot with no metaldehyde applications for at least 5 years (S-NoMet, H-NoMet). In each plot, triplicate 300 g sub-samples were taken from the

top 10 cm of the soil and stored in plastic bags. These were combined into a composite sample and stored in loosely tied plastic bags in a temperature-controlled room at 23 °C until analysed. Soil samples were air dried overnight and sieved (2 mm mesh). Physical and chemical parameters for soils are shown in Table S1.

### 2.4. Microbiological analyses

Fig. 1 shows an overview of methods used for the microbiological analyses of the collected soil samples. Soils J-Met, J-NoMet and C-Met were used for isolation of metaldehyde-degrading bacterial strains only. Soils S-Met, S-NoMet, H-Met, H-NoMet were additionally used for constructing soil metaldehyde degradation profiles and analysing associated changes in the microbial communities.

#### 2.4.1. Selective enrichment for the isolation of metaldehyde-degrading strains (a)

A modified selective-enrichment procedure (Abraham and Silambarasan, 2013) was performed to obtain metaldehyde-degrading bacterial strains from the soils. For the first enrichment passage (P1), 1 g of soil was inoculated in 100 mL of MSM with added metaldehyde as sole source of carbon at 100 mg L<sup>-1</sup> in 250 mL Erlenmeyer flasks. All metaldehyde-containing media was filter sterilized (0.22  $\mu$ m) prior to use and supplemented with 2.0 mL L<sup>-1</sup> trace elements (Vishniac and Santer, 1957). P1 was incubated with orbital shaking (25 °C, 150 rpm) in the dark for 72 h. Subsequently, 1.0 mL was transferred to 100 mL fresh medium (P2) and incubated as described for P1. Third and fourth passages (P3 and P4) were carried out similarly. Aliquots from P3 and P4 were streaked onto solid supplemented MSM (0.75% agarose) with 75 mg L<sup>-1</sup> metaldehyde in triplicate and incubated for 72 h at 25 °C in the dark. Different colony morphotypes that grew on this media were purified by restreaking. Pure cultures were also streaked on MSM plates with no added carbon source to identify possible agarose degraders and oligotrophs, which were discarded after their inability to degrade metaldehyde was corroborated in liquid media. Strains that grew on metaldehyde plates and not in no added carbon plates were presumptively identified as metaldehyde degraders. Isolates were preserved by freezing at -80 °C in 15% glycerol.

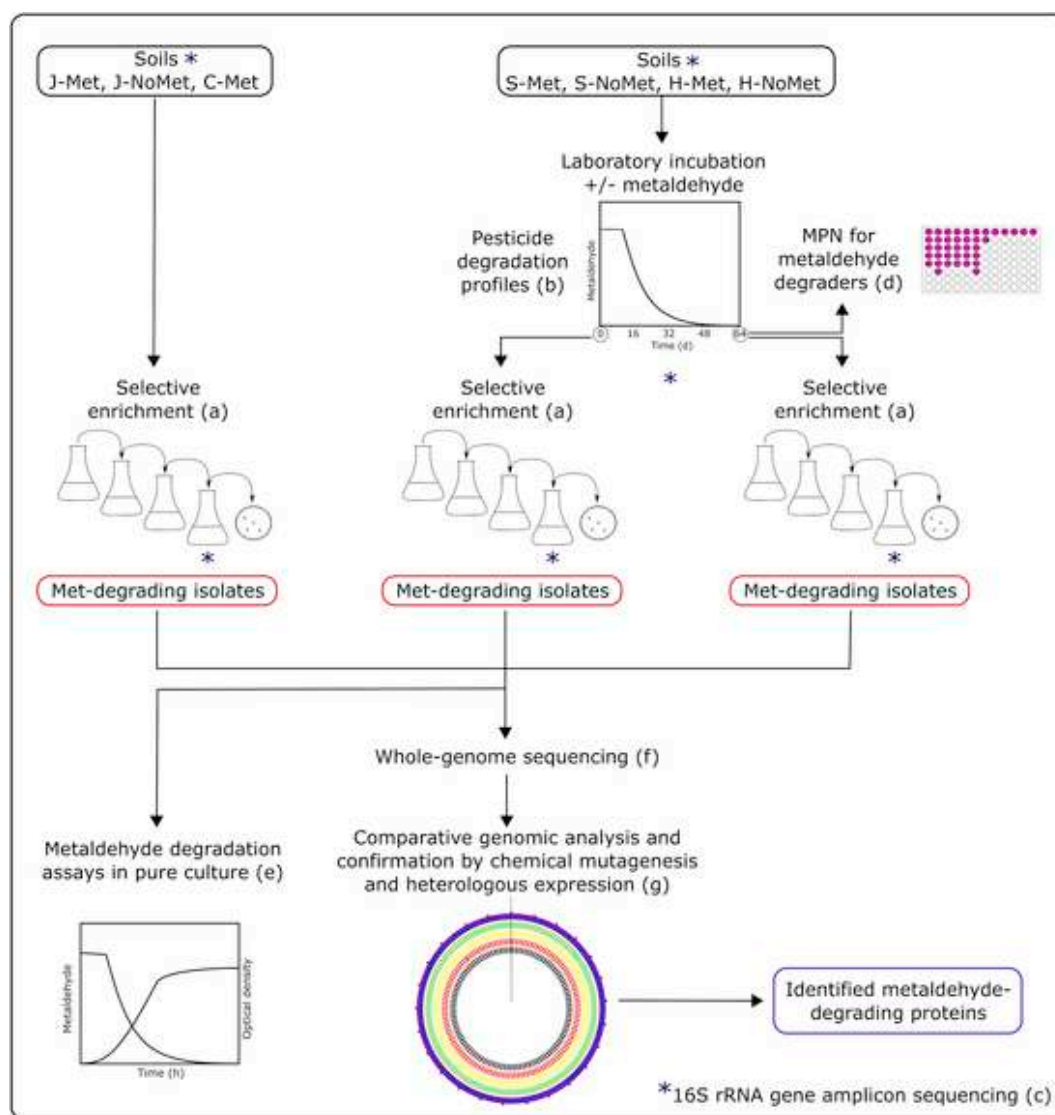
#### 2.4.2. Metaldehyde degradation profiles and microbial community changes in allotment soil microcosms (b)

Metaldehyde degradation was followed in allotment soil samples S-Met, S-NoMet, H-Met, H-NoMet after single and repeated metaldehyde applications at 15 mg kg<sup>-1</sup> soil to investigate accelerated degradation of metaldehyde. Samples were taken for soil genomic DNA extraction to determine changes in soil microbial community in response to metaldehyde addition and incubation of sieved soils. Controls with no metaldehyde addition were labelled cS-Met, cS-NoMet, cH-Met and cH-NoMet. Details are described in Supplementary Materials and Methods.

#### 2.4.3. 16S rRNA gene amplicon sequencing analyses (c)

Genomic DNA was extracted from the original soils, from soils during metaldehyde degradation assays and from passage P4 of all selective enrichments for community 16S rRNA gene amplicon sequence analyses, as well as from the pure cultures of metaldehyde-degrading strains for identification. Details of DNA extraction, amplification and sequencing of 16S rRNA genes are described in Supplementary Materials and Methods. A relative 16S rRNA gene copy number quantification technique (Smets et al., 2016) was carried for soils by spiking them before extraction with an internal DNA standard (*Thermus thermophilus* DSMZ 46338) at an estimated 1% of total DNA. Whole-community 16S rRNA gene amplicon sequence information was analysed using QIIME2 v2017.12 (Caporaso et al., 2010) as described in further detail in Supplementary Materials and Methods.

PRIMER7 (Primer-E Ltd., Auckland, New Zealand) was used for all statistical analyses. Abundance data was standardized by samples, transformed



**Fig. 1.** Overview of microbiological analyses performed for soil samples. All soils were subjected to a selective enrichment process in minimal medium with metaldehyde as the only source of carbon for the isolation of metaldehyde-degrading strains (a). Specific soils were also incubated in the laboratory with and without an initial addition of metaldehyde; remaining pesticide concentrations were periodically quantified for the construction of soil metaldehyde degradation profiles (b). 16S rRNA gene amplicon sequencing analyses (c) were performed for the allotment soils before, during and after the laboratory incubations with and without metaldehyde to evaluate changes in the microbial communities as a consequence of metaldehyde exposure. After incubation, the number of metaldehyde-degrading microorganisms was determined using a most-probable number technique (d). Soils subjected to laboratory incubation were also used for selective enrichment to isolate metaldehyde-degrading strains. Metaldehyde-degrading capabilities of the isolated strains were tested through metaldehyde degradation assays in pure culture (e). Whole-genome sequencing was performed for the metaldehyde-degrading strains (f); comparative genomic analysis (g) was carried out for the identification of candidate genes involved in metaldehyde-degradation pathways and confirmed by chemical mutagenesis and heterologous expression assays.

by fourth root and a Bray-Curtis similarity matrix was constructed. Principal coordinates analysis (PCO) was used for data ordination. Permutational MANOVA (PERMANOVA) was used to assess the influence of different factors in community composition (9999 permutations). PERMDISP was used to test for homogeneity of multivariate dispersions.

#### 2.4.4. Enumeration of metaldehyde-degrading microorganisms in allotment soils (d)

The number of aerobic culturable metaldehyde-degrading microorganisms in allotment soil samples after laboratory incubation  $\pm$  metaldehyde was determined using a most probable number (MPN) enumeration technique in microtitre plates (Dinamarca et al., 2007). Allotment soils S-Met, S-NoMet, H-Met and H-NoMet exposed to metaldehyde during metaldehyde degradation assays and their respective no metaldehyde controls (cS-Met, cS-NoMet, cH-Met and cH-NoMet) were analysed.

#### 2.4.5. Metaldehyde degradation assays in pure culture (e)

To confirm the ability of isolated strains to degrade metaldehyde, bacterial growth and metaldehyde degradation assays were performed in MSM with  $150 \text{ mg L}^{-1}$  metaldehyde. Inocula were prepared by growing each strain on nutrient agar for 72 h and resuspending growth in MSM to initial  $\text{OD}_{600\text{nm}} = 0.1$ .  $0.625 \text{ mL}$  of this inoculum were added to  $100 \text{ mL}$  metaldehyde-supplemented MSM in triplicate  $250 \text{ mL}$  Erlenmeyer flasks. Abiotic controls with an equal volume of MSM instead of inoculum were also prepared. Cultures were incubated in an orbital shaker at  $25 \text{ }^\circ\text{C}$  and  $150 \text{ rpm}$  in the dark. Samples ( $1 \text{ mL}$ ) were withdrawn from the triplicate independent cultures at different time points for  $\text{OD}_{600\text{nm}}$  measurement and metaldehyde quantification.

#### 2.4.6. Whole-genome sequencing of metaldehyde-degrading strains (f)

Confirmed metaldehyde-degrading strains were sent for whole-genome sequencing at MicrobesNG (Birmingham, UK). Sequencing was performed on Illumina MiSeq platform using 2x250bp paired-end reads. *De novo* assembly and quality assessment were performed using SPAdes (Bankevich et al., 2012) and QUAST (Gurevich et al., 2013) respectively. Automated fast annotation was performed using Prokka (Seemann, 2014).

#### 2.4.7. Identification of candidate genes involved in metaldehyde-degradation pathways (g)

To identify candidates for proteins involved in the metaldehyde-degradation pathway, the annotated genomes from newly isolated metaldehyde-degrading strains (*Acinetobacter bohemicus* JMETH-C, *Acinetobacter lwoffii* SMETH-C, *Pseudomonas Vancouverensis* SMETH-B, *Caballeronia jiangsuensis* SNO-D), that of the previously identified degrader *A. calcoaceticus* strain E1 (Thomas et al., 2017) and closely related non metaldehyde-degrading reference strains (*A. calcoaceticus* RUH2202 and *A. bohemicus* ANC3994) were compared. Inability of these latter reference strains to degrade metaldehyde was corroborated experimentally. Reference strains were purchased from the Leibniz Institute DSMZ culture collection. A previously developed Python script (JC Thomas; available at <https://pypi.org/project/blast-score-ratio/>) was used to identify proteins shared between degrading strains but absent from the non-degrading strains. Using this tool, BLAST score ratio (BSR) (Rasko et al., 2005) was calculated for each of the annotated proteins of *A. calcoaceticus* E1 against the most similar proteins present in each of the other degrading and non-degrading *Acinetobacter* strains, *P. Vancouverensis* SMETH-B, and *C. jiangsuensis* SNO-D. Proteins with a BSR value equal to 0.9 or more (shared between the degrading strains) and with a BSR value lower than 0.45 in the non-degrading strains were chosen as candidate proteins and listed. The results of the proteome comparison analyses were corroborated, and figures generated using the BSR-based PATRIC Proteome Comparison Service (Wattam et al., 2017).

For confirmation, chemical mutagenesis was carried out by culturing *A. calcoaceticus* E1 in 20 mL LB media to an  $OD_{600nm} = 0.5$ , adding 180  $\mu$ L of ethyl methanesulfonate (EMS) and incubating at 30 °C for 3 h without shaking. 500  $\mu$ L of sample were inoculated into 40 mL of LB media and incubated at 30 °C for 4 h. Samples were then plated onto LB agar at various dilutions and incubated for 2 days at 30 °C (Geißdörfer et al., 1999). Single colonies were picked and transferred to MSM plates containing either acetate or metaldehyde. Mutants unable to grow on metaldehyde were sent for whole-genome sequencing (as above). Mutations in genes shared between *A. calcoaceticus* E1 and *A. bohemicus* JMETH-C but absent from the respective type strains were identified by BLASTN.

Heterologous expression of putative metaldehyde degradation genes was carried out in *E. coli*. Genomic regions from *A. calcoaceticus* E1 containing *mahX*, *mahX* + *mahY*, and *mahX* + *mahY* + *aldH* were amplified with various sets of primers (Table S8) and inserted into the *EcoRI* site from pBR322. Plasmids were transformed into *E. coli* DH5 $\alpha$ , cultured in liquid LB media, and metaldehyde disappearance measured using Gas Chromatography, as described above.

#### 2.5. Accession numbers

Raw reads for 16S rRNA amplicons and draft whole-genome sequencing data for metaldehyde-degrading strains were deposited in the European Nucleotide Archive under study PRJEB30540. Specific sequences for metaldehyde-degrading genes are available as Supplementary Material.

### 3. Results

#### 3.1. Initial enrichment cultures and sporadic isolation of metaldehyde-degrading strains

An overview of the experimental approach used in this work is shown in Fig. 1. A selective enrichment procedure in liquid media with metaldehyde as sole source of carbon was applied to soil samples from three separate soil collections to isolate metaldehyde-degrading strains. Changes in the abundance of taxa generated by this process were assessed by 16S rRNA gene amplicon sequence analysis (Table S2). The complete list of metaldehyde-degrading strains successfully isolated by the enrichment culture procedure throughout the whole study and their respective identification is presented in Table 1. Not surprisingly, the taxa corresponding to the subsequently isolated metaldehyde-degrading strains tended to dominate the composition of the final stage of the enrichment cultures. However, in some cases other taxa made up a considerable proportion of the community. These may constitute metaldehyde-degrading strains that cannot be isolated in solid media using this approach or non-degrading strains that are using metaldehyde degradation products or other by-products of degrading strains as sources of carbon (Neilson and Allard, 2012).

For soils from first collection, isolation of a metaldehyde-degrading strain (*A. bohemicus* strain JMETH-C) was successful from metaldehyde-exposed soil (J-Met) (Table 1), while no metaldehyde-degrading strains were isolated from the non-exposed soil (J-NoMet). For the second soil collection, performed in a previously-exposed agricultural soil from Costa Rica (soil C-Met), metaldehyde-degrading strain *Sphingobium* sp. strain CMETH-H was successfully isolated. For allotment soils from the third collection, which included metaldehyde exposed and non-exposed soils (H-Met, S-Met, H-NoMet, S-NoMet), no metaldehyde-degrading strains were initially isolated. Thus, at that point no degraders could be isolated from non metaldehyde-ex-

**Table 1**  
Metaldehyde-degrading strains isolated by enrichment culture procedure and identification based on 16S rRNA gene sequences against the NCBI database (limited to sequences from type material).

Soil of origin	Strain code	Closest relative	Closest relative GenBank Accession No.	Similarity (%)	No. of bases compared	Identification of the isolate
J-Met	JMET-C	<i>Acinetobacter bohemicus</i> ANC 3994(T)	KB849175	99.9	1406	<i>Acinetobacter bohemicus</i>
C-Met	CMETH-H	<i>Sphingobium chlorophenolicum</i> NBRC 16172	NR_113840.1	98.5	1329	<i>Sphingobium</i> sp.
H-Met	HMET-A	<i>Rhodococcus globerulus</i> NBRC 14531(T)	BCWX01000023	100.0	1366	<i>Rhodococcus globerulus</i>
H-Met	HMET-G	<i>Sphingobium chlorophenolicum</i> NBRC 16172	NR_113840.1	98.4	1356	<i>Sphingobium</i> sp.
H-NoMet	HNO-A	<i>Rhodococcus globerulus</i> NBRC 14531(T)	BCWX01000023	100.0	1370	<i>Rhodococcus globerulus</i>
S-Met	SMETH-B	<i>Pseudomonas Vancouverensis</i> ATCC 700688(T)	AJ011507	98.9	1387	<i>Pseudomonas Vancouverensis</i>
S-Met	SMETH-C	<i>Acinetobacter lwoffii</i> NCTC 5866(T)	AIEL01000120	98.7	1394	<i>Acinetobacter lwoffii</i>
S-NoMet	SNO-D	<i>Burkholderia jiangsuensis</i> MP-1 <sup>a</sup>	NR_133991.1	99.9	1398	<i>Caballeronia jiangsuensis</i>

<sup>a</sup> The taxon *Burkholderia jiangsuensis* has been reclassified as *Caballeronia jiangsuensis* (Dobritsa and Samadpour, 2016).

posed soils and the isolation of metaldehyde degraders from soils previously exposed to metaldehyde was sporadic.

3.2. Metaldehyde is degraded faster in soils after metaldehyde treatment

Metaldehyde degradation profiles for allotment soils H-Met, H-NoMet, S-Met and S-NoMet after a single application are shown in Fig. 2a.

quantify the persistence of metaldehyde in the soil samples, data regression for the degradation profiles was performed using Single First Order or modified Hockey-stick models (Table S3) (FOCUS, 2006). With a half-life of 3.9 d, degradation was much faster in soil S-Met than other soils.

Fig. 2b shows metaldehyde degradation profiles for allotment soils subjected to two consecutive pesticide applications after a 6-month storage. A single pesticide application was enough to generate 1.7–6.1-fold reductions

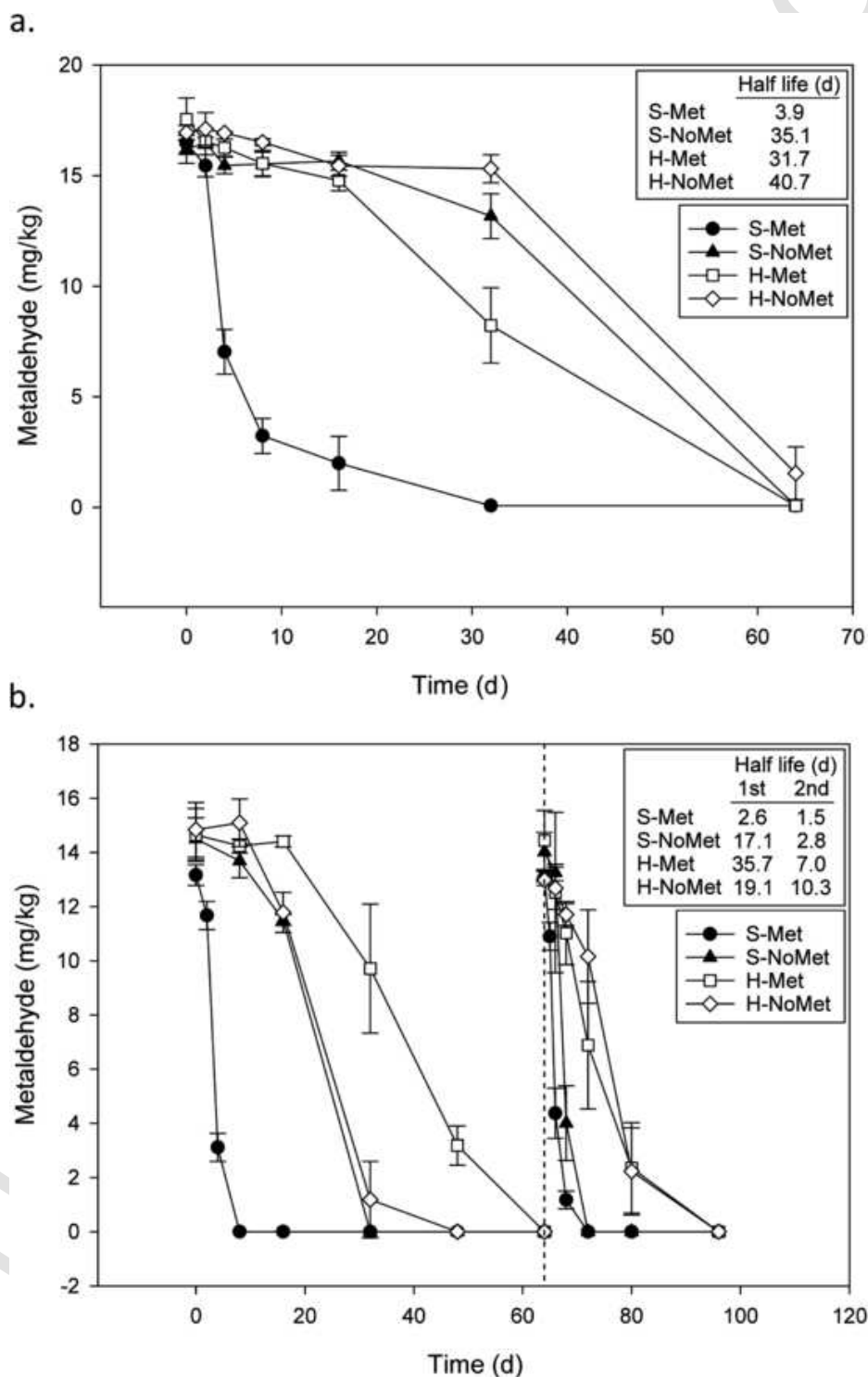


Fig. 2. a. Metaldehyde degradation profiles in freshly collected allotment soils after an initial application of 15 mg kg<sup>-1</sup> soil. b. Degradation of metaldehyde allotment soils after storage (6 months) following an initial application of 15 mg kg<sup>-1</sup> soil and a second application (dashed vertical line) at the same dose. Bars represent standard deviation for 3 replicates. Regression statistics are presented in Table S3. S-Met: Scarcroft Metaldehyde, S-NoMet: Scarcroft No Metaldehyde, H-Met: Hob Moor Metaldehyde, H-NoMet: Hob Moor No Metaldehyde.

in metaldehyde half-lives in all the allotment soil samples on the second application; however, the effect was more evident in the soils in which the half-life was initially higher. The results show accelerated degradation occurred in the samples, which highlights the importance of biological mechanisms for metaldehyde elimination in soil.

### 3.3. Consistent enrichment and isolation of a greater diversity of metaldehyde degraders after incubating soil microcosms with metaldehyde in the laboratory

The accelerated degradation of metaldehyde pointed towards evolutionary selection of biologically-driven degradation of metaldehyde. Hence, we hypothesized that metaldehyde-degrading organisms have been further enriched in soils after exposure to metaldehyde in the laboratory. Thus, to improve the recovery of metaldehyde-degrading isolates, selective enrichment for metaldehyde degraders was performed using the soil samples already incubated with metaldehyde for 64 d. This strategy permitted the isolation of metaldehyde-degrading strains from all four allotment soils in which the selective enrichment procedure had initially failed. Strains from diverse genera, including Gram-negative (*Acinetobacter*, *Pseudomonas*, *Sphingobium*, *Caballeronia*) and Gram-positive (*Rhodococcus*) isolates were successfully obtained. The identified metaldehyde-degrading strains are listed in Table 1. For all soils, in total, six Gram-negative and two Gram-positive strains were isolated. Whole-genome sequencing was performed for all the distinct taxa. Quality statistics for sequencing runs and assemblies are shown in Table S4.

The most abundant taxa in the final stage of these successful enrichment cultures, and their respective percentages in these original soils are also shown in Table S2. In the original soils, the genera with the highest abundances that could be confidently assigned taxonomy across all samples included the archaea *Candidatus Nitrososphaera* (9.6% of the initial community on average, SD: 3.1%) *Bacillus* (4.3%, SD: 2.5%) and *Kaistobacter* (2.4%, SD:1.7%), all which are commonly reported as some of the main genera found in soil (Zhalnina et al., 2013) and whose numbers decreased to undetectable levels at the end of the enrichment cultures. On the other hand, even though some genera such as *Acinetobacter*, *Sphingobium* and *Burkholderia*, all well-known xenobiotic degraders, were undetectable in the original soils with the sequencing depth used, their numbers increased to be amongst the dominant members of the enrichment cultures, which highlights both the strong selection pressure the populations were subjected to, and their ability to respond rapidly to it (Kurm et al., 2017).

### 3.4. Variation in the bacterial community due to metaldehyde addition and laboratory incubation

The addition of metaldehyde to the soil samples and the incubation in-soil increased the chances of isolating metaldehyde degraders. To further explore the variations in the microbial community occurring because of these procedures, changes were assessed by two different techniques: Most-probable number (MPN) of metaldehyde degraders and 16S rRNA amplicon sequencing of DNA from soil.

Fig. 3a shows the results of the MPN assay of metaldehyde-degrading bacteria at the end of the degradation assay. For all the allotment soils tested metaldehyde degraders were significantly more abundant on exposure to metaldehyde compared to untreated controls. For the soils except S-Met (which showed the fastest metaldehyde degradation even without metaldehyde exposure) this was a difference of several orders of magnitude.

The abundances of 16S rRNA gene copies were compared between soil samples during the metaldehyde degradation time course (Fig. 3b). Quality statistics for sequencing runs are shown in Table S5. Irrespective of the addition of metaldehyde, there is a consistent marked increase in bacterial abundance throughout the incubation period for all soils, which may also increase the chances of successful isolation of metaldehyde degraders. The fraction of the population at the Phylum level corresponding to Proteobacte-

ria showed an increase for all soils between the 0 d and 16 d time points and showed a slow decline thereafter.

To explore the differences in the composition of bacterial communities during the in-soil metaldehyde degradation assay, a PCO ordination of whole bacterial community 16S rRNA gene amplicons was constructed (Fig. 3c). Principal coordinates 1 and 2 accounted for 42.7% of the variation. PERMANOVA analyses revealed that soil of origin (Pseudo-F = 6.26,  $p = 0.0001$ ,  $df = 31$ ) and time (Pseudo-F = 2.6058,  $p = 0.0001$ ,  $df = 31$ ) but not metaldehyde addition in the laboratory (Pseudo-F = 0.26951,  $p = 0.9999$ ,  $df = 31$ ) significantly influenced the soil community composition. Sample group dispersions were homogeneous for all factors as revealed by PERMDISP analyses.

### 3.5. Growth of isolated strains using metaldehyde as a sole source of carbon

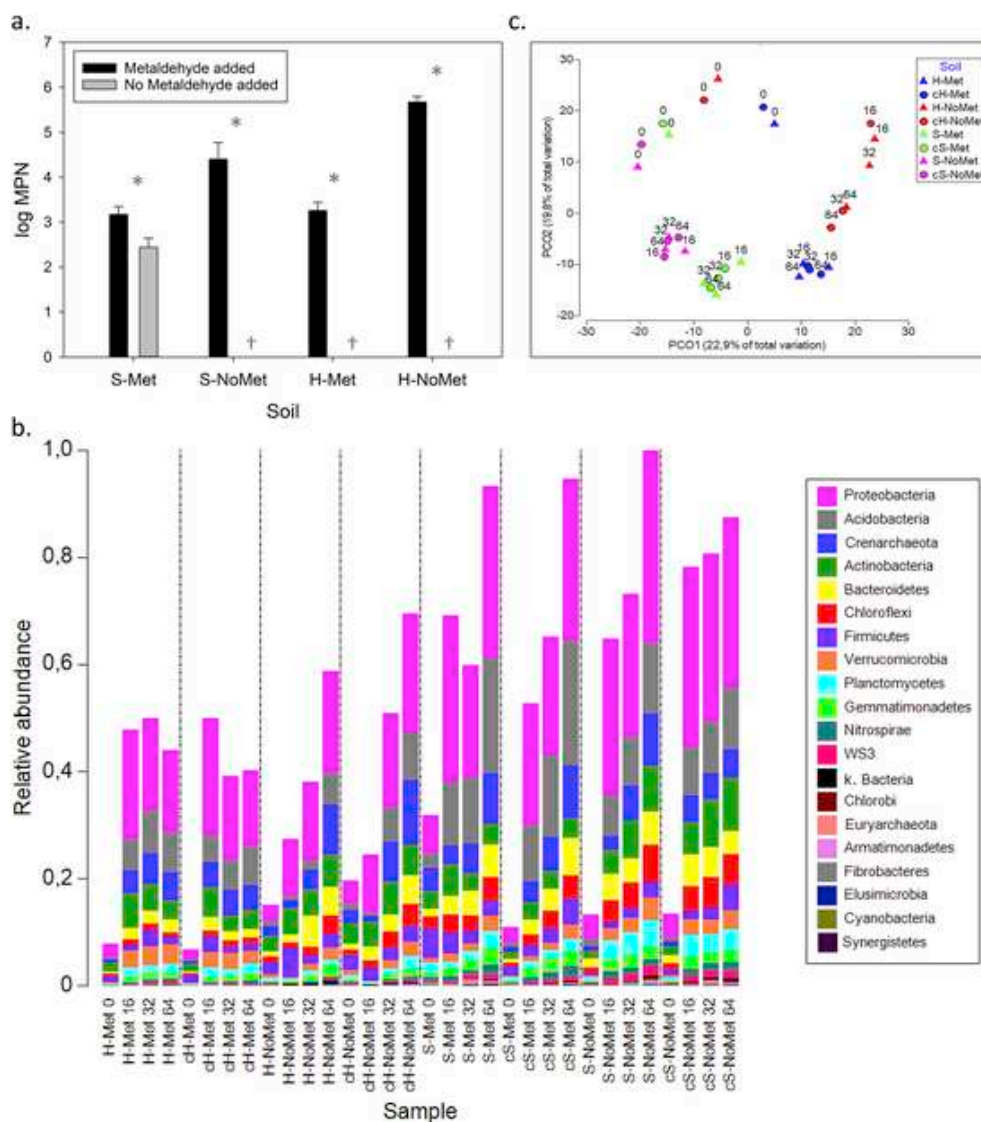
Growth of all the isolated degrading strains (and the previously isolated *A. calcoaceticus* E1) on metaldehyde (150 mg L<sup>-1</sup>) as the sole source of carbon was achieved, and a strong correlation with the disappearance of metaldehyde was observed, supporting the conclusion that they use metaldehyde as a carbon and energy source (Fig. 4, Table S6). *A. bohemicus* JMET-C stood out as the strain with the shortest lag phase, doubling time, time required for metaldehyde removal below the limit of detection and the highest maximum compound degradation rate (Fig. 4). The rest of the *Acinetobacter* strains followed with respect to the time needed for metaldehyde elimination; *P. Vancouverensis* SMET-B came next. The *Sphingobium* CMET-H/HMET-G strains were intermediate in this regard, while the *Rhodococcus guberulus* HMET-A/HNO-A and *C. jiangsuensis* SNO-D strains were slowest.

### 3.6. Comparative genomic analysis of metaldehyde-degrading strains

Diverse metaldehyde-degrading strains were isolated, and a draft genome for each one was obtained. Comparative genomic analyses focused on identifying shared genes encoding proteins needed for metaldehyde degradation.

A previous study from our group had identified an *Acinetobacter* strain (E1) capable of degrading metaldehyde (Thomas et al., 2017); additionally, two other strains of *Acinetobacter* (*A. bohemicus* JMET-C and *A. lwoffii* SMET-C) were isolated in this study. The predicted proteomes from these strains, along with those of the metaldehyde-degrading strains *P. Vancouverensis* SMET-B, *C. jiangsuensis* SNO-D, and the non-degrading strain *A. calcoaceticus* RUH2202 were compared through Blast Score Ratio (BSR) analysis using *A. calcoaceticus* E1 as reference. A total of 65 candidate proteins were identified as present in all metaldehyde-degrading strains of *Acinetobacter* but absent from *A. calcoaceticus* RUH2202. Inclusion of the genomes from more evolutionarily divergent strains SNO-D and SMET-B decreased the number of predicted proteins shared amongst the metaldehyde-degrading strains and absent from RUH2202 to four (Table 2). Identical results were obtained when the analysis was repeated using *A. bohemicus* ANC3994 as non-degrading strain. A graphical representation of the results is displayed in Fig. 5.

The identified proteins corresponded to a single cluster of four apparently horizontally-transferred genes. Predicted protein sequences were used to search NCBI's conserved domain database (Marchler-Bauer et al., 2015), using the cluster from *A. bohemicus* JMET-C as query. Thresholds of 50% and 70% identity have been proposed for assignment of third and full Enzyme Commission numbers at the domain level (Addou et al., 2009), and these values were used to support predicted functional assignment and naming of genes. Where only lower sequence identity to existing genes was observed, genes were denoted *mah* (for *metaldehyde*). The first gene in the cluster (*mahX*) contains a main domain classified into the 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily, with the highest sequence identity (49%) to a protein involved in biosynthesis of mitomycin an-



**Fig. 3.** a. Most probable number of culturable metaldehyde-degrading bacteria per g of soil 64 d after the addition of metaldehyde at 15 mg kg<sup>-1</sup> or no addition at all. Dagger indicates <math>< 23\text{ MPN g}^{-1}</math> soil. Bars represent standard deviation for 3 replicates. Asterisk indicates significant difference ( $p < 0.05$ ). b. Abundance and classification by phyla of 16S rRNA gene copies in individual allotment soil samples (relative to the sample with the highest count) with and without an initial addition of metaldehyde (15 mg kg<sup>-1</sup>) throughout a 64 d incubation period. Only the most abundant 20 phyla are listed in the key. c. Principal coordinates analysis plot of whole bacterial community 16S rRNA gene amplicons from individual allotment soil samples with and without an initial addition of metaldehyde (15 mg kg<sup>-1</sup>) throughout a 64 d incubation period. Labels indicate day of sampling. Samples were rarefied to 56749 sequences each after denoising and quality control.

tibiotics from *Sphingobium japonicum*. The second gene (*mahY*) is related to the vicinal oxygen chelate family, which is found in a variety of structurally related metalloproteins, including the type I extradiol dioxygenases, glyoxalase I and a group of antibiotic resistance proteins, with the highest sequence identity (41%) to a hypothetical unannotated protein from *Mycolicibacterium moriokaense*. The third gene (*aldH*) could be confidently assigned as an NAD(P)<sup>+</sup>-dependent aldehyde dehydrogenase due to high identity (78%) to an aldehyde dehydrogenase from *Solimonas* sp. The fourth gene (*tnpA*), annotated as Y2-transposase, is almost identical (99%) to a transposase from *Pseudomonas pseudoalcaligenes*. All these functions are consistent with a potential horizontally-transferable metaldehyde degradation pathway. In *P. vancouverensis* SMET-B the *tnpA* was found to be located elsewhere in the genome. In *C. jiangsuensis* SNO-D, *aldH* was truncated.

A possible horizontal gene transfer event to the *Acinetobacter* taxon was supported by the presence of the transposase gene in the cluster, IS6 family insertion sequences at both ends of the respective contigs and different GC

nucleotide percentages between the gene cluster (59.81–62.74%) and the draft whole genome sequences of metaldehyde-degrading *Acinetobacter* strains (38.7–40.2%), all parametric indicators of horizontal gene transfer (Ravenhall et al., 2015).

Chemical mutagenesis of *A. calcoaceticus* E1 led to isolation of four strains unable to degrade metaldehyde. In each case, the deletion of part of the gene encoding the putative oxygenase (*mahX*) was the only shared difference from the wild-type (Fig. S1), which further supports the involvement of the gene cluster in metaldehyde degradation.

### 3.7. Heterologous expression of *mahX* confers ability to degrade metaldehyde on *E. coli*

*mahX*, *mahX* + *mahY*, and *mahX* + *mahY* + *aldH* were cloned into pBR322 and transformed into *E. coli*. Fig. 6 illustrates that *mahX* was necessary and sufficient to confer the ability to degrade metaldehyde on *E. coli*, confirming the inferences from comparative genomics and chemical mutagenesis. The rate of metaldehyde degradation is faster in *E. coli* containing



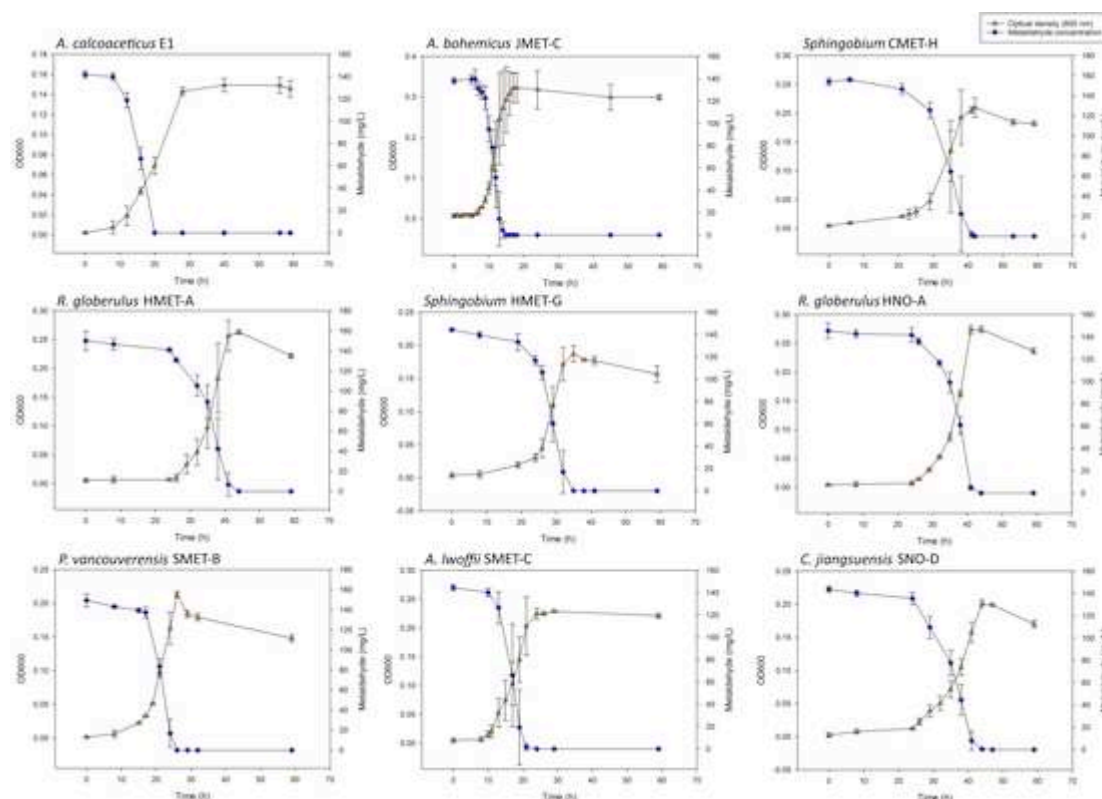


Fig. 4. Growth of degrading strains on metaldehyde ( $150 \text{ mg L}^{-1}$ ) as the sole source of carbon. The inoculum was substituted by an equal volume MSM in the abiotic controls. Bars represent standard deviation for 3 replicates. Final metaldehyde concentration in the abiotic controls was 99.9% (CV = 3.0%) of the starting concentration.

Table 2

Predicted proteins shared between metaldehyde-degrading strains, absent from non-degrading strains and their respective BSR values when compared against the reference genome (*A. calcoaceticus* E1).

	MahX		MahY		AldH		TnpA	
	aa length	BSR <sup>a</sup>	aa length	BSR <sup>a</sup>	aa length	BSR <sup>a</sup>	aa length	BSR <sup>a</sup>
<i>A. calcoaceticus</i> E1	314	1.000	149	1.000	231	1.000	503	1.000
<i>A. calcoaceticus</i> RUH2202	NP <sup>b</sup>	-	NP <sup>b</sup>	-	495	0.441	NP <sup>b</sup>	-
<i>A. bohemicus</i> JMET-C	314	1.000	149	1.000	231	1.000	503	1.000
<i>A. lwoffii</i> SMET-C	314	0.994	149	0.993	231	1.000	387	0.997
<i>P. vancouverensis</i> SMET-B	314	0.975	149	0.993	231	0.995	327	0.997
<i>C. jiangsuensis</i> SNO-D	314	0.984	149	0.993	88	1.000	262	1.000

<sup>a</sup> BSR: Blast-Score Ratio.

<sup>b</sup> NP: not present.

*mahX* only, than in strains also expressing *mahY*. The reasons for this are unknown, but may be related to difference in gene expression between these different constructs. *E. coli* bearing *mahY* (under the *mahX* promoter) cloned into pBR322 is unable to degrade metaldehyde (data not shown).

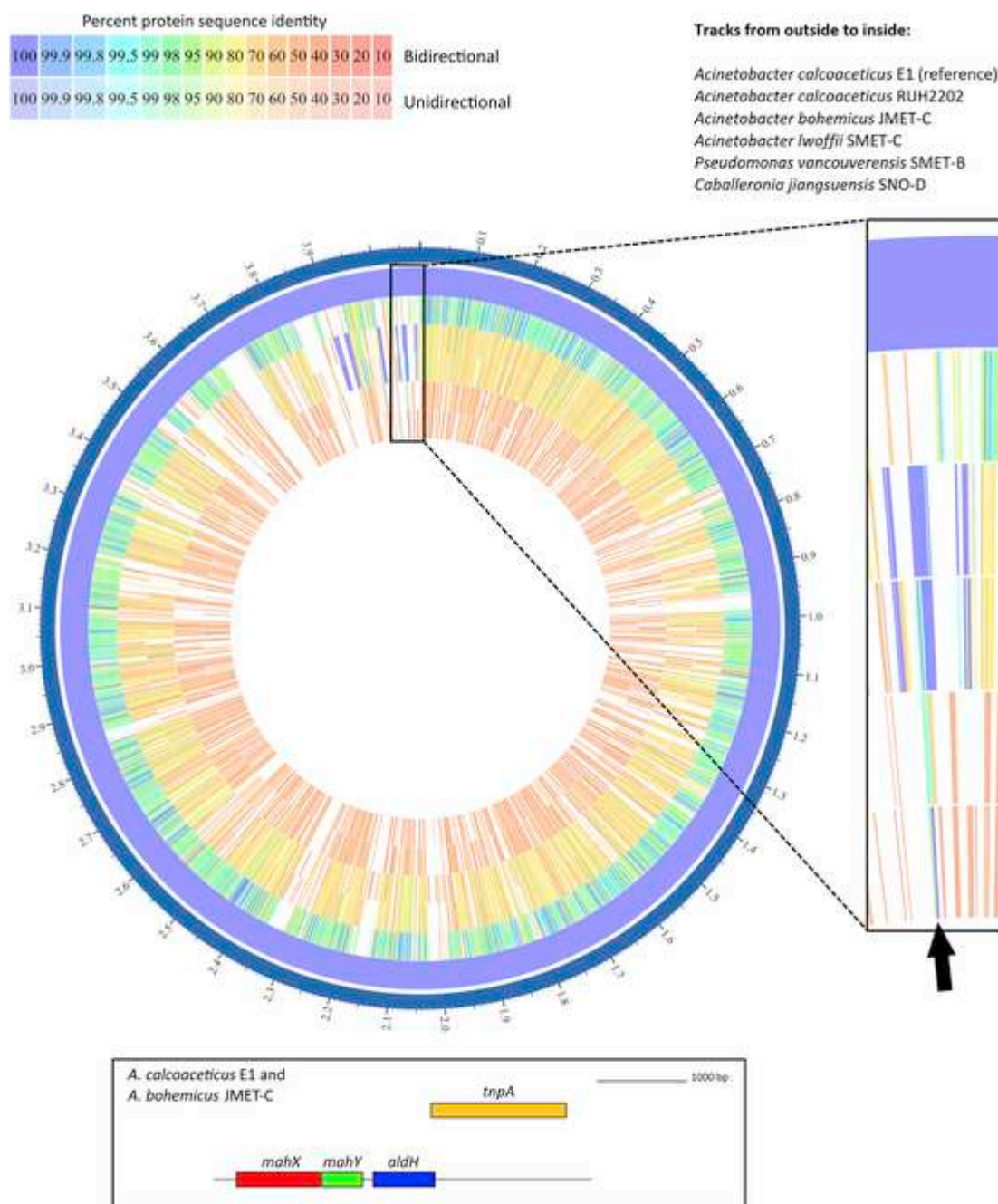
#### 4. Discussion

Previous work has indicated microbial activity is involved in degradation of the xenobiotic pesticide metaldehyde (Simms et al., 2006; Thomas et al., 2017). Here we have used a systematic molecular and microbiological approach to analyse and improve isolation techniques, and gain insight into the abundance, distribution and mechanisms of microbial metaldehyde degradation.

We successfully combined the isolation of diverse metaldehyde-degrading strains with increasingly affordable whole-genome sequencing to, by means of comparative genomics, identify a xenobiotic-degrading gene cluster. This strategy has proven effective for other xenobiotics in the past as

well (Yan et al., 2016), and as whole-genome sequencing becomes increasingly affordable, it has the potential of becoming a very important approach to identifying organic compound-degrading gene clusters. This functional assignment was subsequently confirmed by chemical mutagenesis and heterogeneous expression of the enzyme activity. This illustrates the value of obtaining a broad collection of isolates for the identification of degrading mechanisms.

Availability of labile carbon is considered the main limiting factor for microbial growth in soil (Aldén et al., 2001), and metaldehyde, when added to the soil, constitutes such a source of carbon. In this context, the presence of genes for its degradation provide the host with the ability to utilize this readily available carbon source, constituting a selective advantage. Genes *mahX* and *mahY* have a moderate similarity to other well-characterized genes, so they may share an evolutionary ancestor with them. However, they appear to have diverged sufficiently so that new catalytic properties, such as substrate specificity towards metaldehyde, has emerged, was selected



**Fig. 5.** Proteome comparison between metaldehyde-degrading and non-degrading strains using *Acinetobacter calcoaceticus* E1 as reference. The analysis was performed using PATRIC Proteome Comparison Service (Wattam et al., 2017). A diagram of the shared metaldehyde-degrading gene cluster is shown in the inset. *mahX*: 2-oxoglutarate and Fe(II)-dependent oxygenase; *mahY*: vicinal oxygen chelate protein; *aldH*: NAD(P)<sup>+</sup>-dependent aldehyde dehydrogenase; *tnpA*: Y2-transposase.

for, and transferred to other hosts via transposable elements in plasmids or other vectors. *mahX* encodes a predicted 2-oxoglutarate-dependent oxygenase. Given that this gene is sufficient to bring about at least the initial step of metaldehyde degradation, we propose that MahX is an oxygenase that activates metaldehyde metabolism by oxygenation and ring cleavage. The predicted product is a hemiacetal (1,3,5,7-tetramethyl-2,4,6-trioxo-1-hydroxy-7-octanone) (Fig. 7). Whilst this hemiacetal is unstable, the timescale of its chemical degradation is likely to be minutes to hours (Chiang and Kresge, 1985). We speculate that MahY acts as a lyase that accelerates the iterative breakdown of the hemiacetal intermediate to acetaldehyde (Fig. 7). MahY is most closely related to the vicinal oxygen chelate (VOC) superfamily (He and Moran, 2011). Whilst the predicted substrate here is not a VOC *per se*, VOC family members bind substrates with two oxygen atoms and some

members of the family are lyases in keeping with the predicted function here. Acetaldehyde generated from MahY is predicted to be converted to acetate by AldH (Fig. 7), and subsequently incorporated into central metabolism.

Since the degrading gene cluster identified in *Acinetobacter*, *Caballeronia* and *Pseudomonas* is not present in some of the other metaldehyde-degrading isolates from this study (*Sphingobium* sp. CMET-H and HMET-G, *R. globerulus* HMET-A and HNO-A) and from an earlier study (*Variovorax* sp. E3) (Thomas et al., 2017), as shown by whole-genome sequencing and PCR-based gene detection (data not shown), it is clear that additional metaldehyde-degrading mechanisms are found in nature.

Previous attempts to isolate metaldehyde-degrading strains from soils resulted in varying degrees of success. Thomas and collaborators (Thomas et al., 2017) et

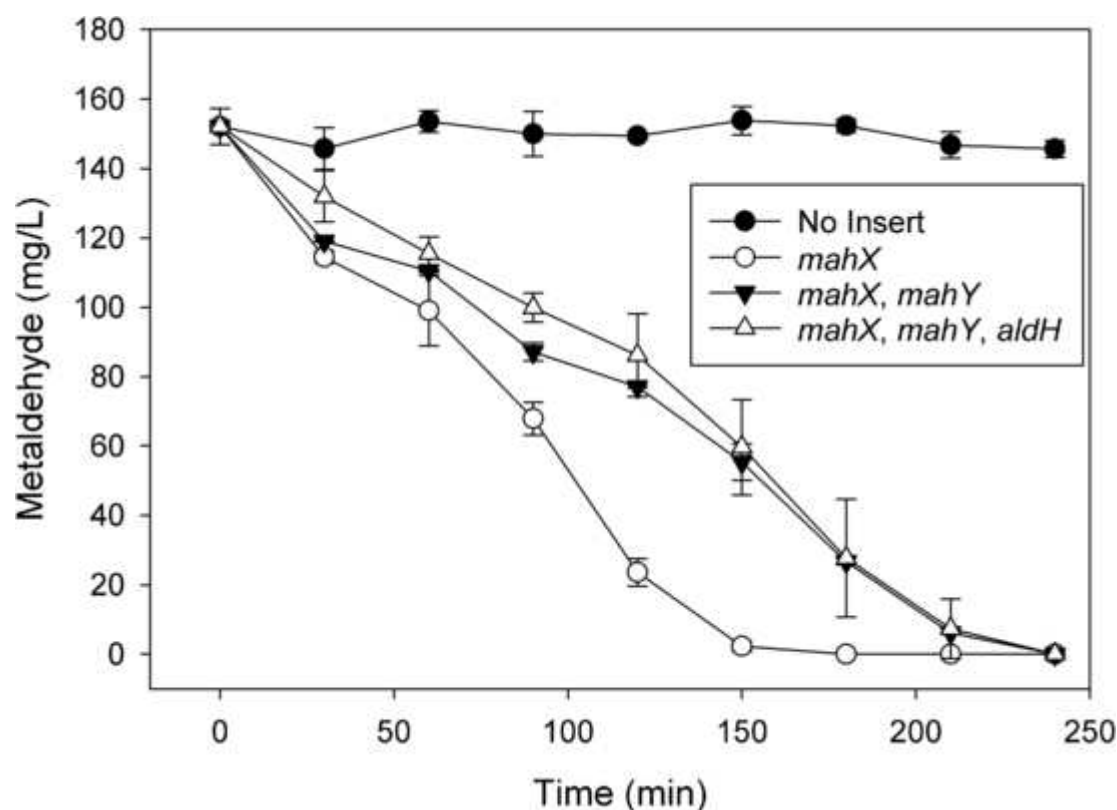


Fig. 6. *mahX* is sufficient to confer degradation of metaldehyde. Degradation of metaldehyde by *E. coli* was measured following growth in LB liquid media. *E. coli* DH5 $\alpha$  carried plasmid pBR322 (solid circles), and derivatives of this plasmid containing *mahX* (open circles), *mahX* & *mahY* (filled triangles), or *mahX*, *mahY* and *aldH* (open triangles).

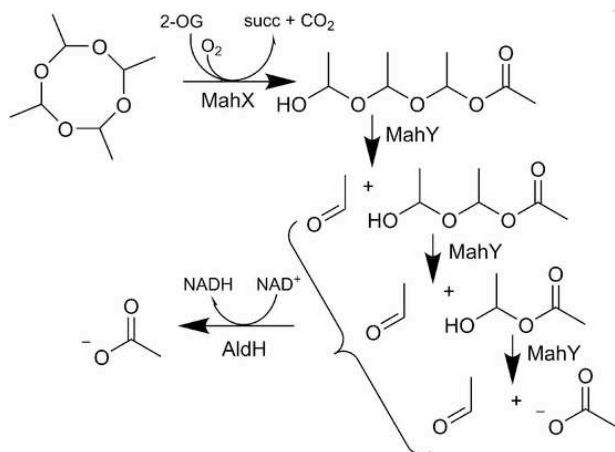


Fig. 7. Predicted pathway for metaldehyde degradation. MahX is related to 2-oxoglutarate (2-OG)-dependent oxygenases that generate succinate (succ) and CO<sub>2</sub>. MahX oxygenates metaldehyde to release a linear hemiacetal that is cleaved iteratively into acetaldehyde + a shorter chain hemiacetal, and eventually acetate. AldH oxidises acetaldehyde to acetate in an NAD<sup>+</sup>-dependent reaction.

al., 2017) isolated two metaldehyde-degrading strains from domestic soils, nevertheless, additional attempts using three different previously-exposed agricultural soils failed. Initially, we obtained similar results; we could only sporadically isolate degraders from previously-exposed soils. Even though previous applications of metaldehyde to the soil in the field seemed to aid in the subsequent isolation of degraders, it was far from a guarantee. Many variables such as the length of time between application and soil sampling, the dose of pesticide, its formulation (and thus distribution and fate in the field), and the sampling regime, may all influence the abundance of metaldehyde degraders in the soil samples, and thus the success of isolation strategies. Furthermore, laboratory culture conditions cannot fully replicate the

ideal conditions to suit the physiology of many of members of the soil microbial community.

The fact that metaldehyde is normally applied as a pellet in the field (vs. liquid spray forms) influences its behaviour and distribution in soil (Bond, 2018). This is important because a gradient of decreasing concentrations in soil would be expected as distance from the pellet increases. The input of labile C sources to soil increases the abundance and activity of microorganisms generating microbial hotspots (Kuzaykov and Blagodatskaya, 2015). The distribution of metaldehyde after pellet applications may generate defined zones of enhanced metaldehyde degradation in soil. This would pose a challenge when sampling, because high biodegradation hotspots, with elevated numbers of degraders, could easily be missed.

We were able to consistently isolate metaldehyde degraders from horticultural soils, regardless of their metaldehyde exposure history, after adding metaldehyde to soil samples in the laboratory and incubating them for a defined period. This approach minimized the challenges to isolation that would arise from distribution of activity in hotspots. Further analysis revealed that metaldehyde addition increased the number of culturable metaldehyde degraders in the samples, while incubation in laboratory conditions (initial homogenization, 25 °C, 100% relative humidity) also increased the total bacterial biomass, greatly facilitating, in combination, the isolation of degraders. Even though this approach has been used in the past for other pesticides (Goda et al., 2010; Perruchon et al., 2016; Singh et al., 2004) it seems to be of particular importance for the isolation of metaldehyde degraders because the uneven distribution of the pesticide generated from normal field applications makes the direct isolation from freshly collected soils difficult.

In this study we observed that metaldehyde degradation occurred faster in soils previously exposed to metaldehyde in the field compared to soils with very similar physicochemical characteristics but not directly exposed to the pesticide. Furthermore, even a single metaldehyde addition to soil samples in the laboratory led to accelerated degradation of metaldehyde and accumulation of culturable metaldehyde-degrading microbes. This illustrates

that metaldehyde degrading strains are strongly selected by metaldehyde in soil.

Culture-independent analysis during metaldehyde degradation showed that the main factors governing bacterial community composition were soil origin and incubation time, not metaldehyde. Several studies have found important pesticide-driven changes in community structure with other pesticides only when using doses of several times the recommended application rate or after repeated additions (Crouzet et al., 2010; Cycoń et al., 2013; Itoh et al., 2014). Nevertheless, at a finer level, several specific taxa were enriched after metaldehyde application (Table S7). Notably, the lists of most enriched taxa in all soils after metaldehyde application were dominated by anaerobes, which may not reflect the specific groups executing metaldehyde degradation in soil but may instead be related to increased bacterial metabolism and abundance, leading to anaerobic conditions developing in the soils during the incubation due to increased oxygen respiration (Shennan et al., 2018; Streminska et al., 2014). This is consistent with the fact that enrichment of specific anaerobic taxa was observed even in the already metaldehyde-adapted soil S-Met.

A metaldehyde-degrading *Sphingobium* was isolated following enrichment (in soil H-Met), and this genus was identified, through amplicon sequencing, as being enriched > 300-fold by metaldehyde in the soil (Table S7). Beyond that, no other cultured metaldehyde-degraders were identified as enriched in soil amplicon sequencing. This is presumably a result of metaldehyde degradation being a rare trait, possessed by a relatively low proportion of the total microbial community (reflected by  $10^3$ - $10^5$  metaldehyde-degraders (Fig. 3a) out of an estimated  $10^8$ - $10^9$  microbial cells/g of soil in our samples).

This study highlights the continuing value of “traditional” experimental enrichment methods, and their application in coordination with contemporary molecular methods. Here we have provided insight into the diversity and mechanism of biological metaldehyde degradation; this understanding will be essential for predicting the environmental fate of the compound and for optimizing and monitoring the performance of engineered biological systems for metaldehyde removal from drinking water.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2019.107702>.

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