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Isolation and characterisation of metaldehyde-degrading bacteria from domestic soils

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1 Isolation and characterisation of metaldehyde-degrading bacteria from domestic soils

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10 Running title: metaldehyde-degrading bacteria in soils

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3 12 **Summary**
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6 13 Metaldehyde is a common molluscicide, used to control slugs in agriculture and
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8 14 horticulture. It is resistant to breakdown by current water treatment processes, and its
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10 15 accumulation in drinking water sources leads to regular regulatory failures in drinking water
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12 16 quality. To address this problem, we isolated metaldehyde degrading microbes from
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14 17 domestic soils. Two distinct bacterial isolates were cultured, that were able to grow
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16 18 prototrophically using metaldehyde as sole carbon and energy source. One isolate belonged
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18 19 to the genus *Acinetobacter* (strain designation E1) and the other isolate belonged to the
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20 20 genus *Variovorax* (strain designation E3). *Acinetobacter* E1 was able to degrade
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22 21 metaldehyde to a residual concentration less than 1 nM, whereas closely related
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24 22 *Acinetobacter* strains were completely unable to degrade metaldehyde. *Variovorax* E3 grew
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26 23 and degraded metaldehyde more slowly than *Acinetobacter* E1, and residual metaldehyde
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28 24 remained at the end of growth of the *Variovorax* E3 strain. Biological degradation of
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30 25 metaldehyde using these bacterial strains or approaches that allow *in situ* amplification of
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32 26 metaldehyde degrading bacteria may represent a way forward for dealing with
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34 27 metaldehyde contamination in soils and water.
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30 Introduction

31 Metaldehyde (CH_3CHO)₄ is an ether, formed from a cyclic tetramerisation of acetaldehyde
32 (Fig. 1A) (Kekulé and Zincke, 1872). Metaldehyde was initially used as a solid fuel firelighter
33 “Meta-fuel” (Miller, 1928), but its major contemporary use is as a molluscicide in agriculture
34 and horticulture. Its application in controlling slugs was known as early as 1934 (Gimingham,
35 1940) and it is now widely used in both agricultural fields and domestic gardens. It is applied
36 as a pelleted bran bait that inhibits slug feeding after exposure (Wedgwood and Bailey,
37 1988), causing effects such as the distention and disintegration of the Golgi apparatus and
38 endoplasmic reticulum in the mucus cells of slugs (Triebkorn et al., 1998).

39 In 2014, Metaldehyde accounted for 87 % of all recorded molluscicide applications on
40 agricultural fields in the UK (Garthwaite et al., 2015). 112 tonnes were applied over 920
41 thousand hectares (21 % of surveyed arable land used to grow crops) in Britain in 2014;
42 primarily on wheat, oilseed rape and potato crops (Garthwaite et al., 2015). The vast
43 majority of failures in drinking water quality in the UK, due to pesticide contamination, are
44 caused by metaldehyde exceeding the regulatory limit of 0.1 µg/L (≈ 0.6 nM) (European
45 Union Council Directive 98/83/EC) (Fig. 1B).

46 The recalcitrance of metaldehyde to degradation at ambient temperature (Fleischmann et
47 al., 2000) is problematic for water treatment, as metaldehyde is not removed by
48 conventional water treatment processes (Kay et al., 2014). Researchers are pursuing a
49 variety of chemical and physical approaches to deal with the problem of metaldehyde
50 contamination (Autin et al., 2013; Doria et al., 2013; Tao and Fletcher, 2013; Tao and
51 Fletcher, 2014). But currently, no economical method exists to degrade or remove
52 metaldehyde from water.

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3 53 It has been shown that the xenobiotic metaldehyde can be quickly degraded in soils (Zhang
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5 54 et al., 2011) and is oxidised to carbon dioxide under aerobic conditions in unsterilised soils
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7 55 (EFSA, 2010). This strongly suggests the involvement of microbes in its degradation,
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9 56 although no microorganisms have been isolated to date that degrade metaldehyde. The
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11 57 degradation of metaldehyde to CO₂ is strongly exothermic (heat of combustion 3370 kJ.mol⁻¹
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13 58 ¹ (Fleischmann et al., 2000)), suggesting that it has the potential to be a carbon and energy
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15 59 source to support microbial growth. Soils are home to a vast array of microbes and
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17 60 represent a source of metabolic activities that may be of use in industrial and medicinal
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19 61 applications (Delmont et al., 2011). Here we enriched microbes from soils, and report the
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21 62 first isolation and identification of microbial isolates capable of using metaldehyde as a sole
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23 63 source of energy and carbon for growth.
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3 66 **Results and Discussion**
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6 67 *Two distinct metaldehyde degrading strains were isolated from domestic soils*
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8 68 Metaldehyde degrading bacteria were selected in a mineral medium consisting of salts
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10 69 Na₂HPO₄ (55 mM), KH₂PO₄ (11 mM), NH₄Cl (6 mM) and MgSO₄ (0.4 mM) (pH 7). This was
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12 70 supplemented with 2 ml/l of a trace elements solution (Vishniac and Santer, 1957).
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14 71 Metaldehyde was provided as sole carbon source and control cultures lacked metaldehyde.
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16 72 Ability to grow using metaldehyde was tested in both liquid enrichment cultures and on
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18 73 solid media, containing 1.5 % agarose. 100 ml liquid cultures were inoculated with 1 g of soil
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20 74 obtained from domestic gardens in York, UK. Cultures were incubated at 30°C for 3 days, 1
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22 75 ml of enrichment media was sub-cultured into fresh media and incubated for a further 3
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24 76 days, and subsequently samples were spread onto agarose plates containing 2800 µM (500
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26 77 mg/L) metaldehyde. 50-200 colonies were obtained on plates when the enrichments were
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28 78 carried out in liquid culture in the presence of 570 µM (100 mg/L) metaldehyde, but not
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30 79 following control enrichments in the absence of metaldehyde. 1 g samples of the same
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32 80 domestic soils were re-suspended in 10 ml of sterile water and 100 µL aliquots spread
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34 81 directly onto agarose plates containing metaldehyde. 2-5 colonies grew on these plates. The
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36 82 morphology of all the colonies was white, round and glossy. Ten isolates were picked for
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38 83 further analysis, and named E1-E6 and M1-M4, to designate the source soils used. Soil E had
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40 84 a recent history of metaldehyde utilization, whereas soil M had not been treated with
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42 85 metaldehyde for at least 5 years. In each case the isolated strains grew on agarose plates
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44 86 supplemented with metaldehyde, but not in its absence, suggesting they were utilizing
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46 87 metaldehyde as a carbon and energy source.
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3 89 On subculturing the metaldehyde-degrading strains, each strain appeared to be a pure
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5 90 culture, except strain E4 which yielded two distinct colony morphologies, and was
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8 91 subsequently subdivided into E4a and E4b. Colonies from strains E1, E3, E4a, E4b, E5, M1
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10 92 and M4 were used for amplification of 16S rDNA as described previously with primers U8F
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12 93 and U1492R (Eden et al., 1991). Amplification was achieved using GoTaq polymerase
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15 94 (Promega) with a standard programme of: 98°C for 30 s; 35 cycles of 98°C for 10 s, 50°C for
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17 95 30 s, 72°C for 60 s; 72°C for 10 min. PCR products were purified using QIAquick PCR
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19 96 purification kit (Qiagen) following the manufacturer's instructions. For Restriction Fragment
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21 97 Length Polymorphism (RFLP) analysis, 1 µg of purified DNA was digested for 1 or 3 hours at
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23 98 37°C using restriction enzyme HhaI. RFLP revealed two distinctly different ribotypes (see
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25 99 Supporting Information). Two examples of each ribotype were sequenced. Sanger
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29 100 sequencing was used to obtain the nucleotide sequences of the U8F-U1492R amplicons of
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31 101 E1, M1, E3 and E4a using U8F as sequencing primer. Sequences from E1 and M1 were
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34 102 aligned using ClustalX V2.1 and found to be identical across the >900 base region where the
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36 103 base sequence could be confidently assigned. Similarly, the sequences from E3 and E4a
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38 104 were found to be identical across a >900 base region.
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41 105 Subsequent investigation focused on the strains E1 and E3. The sequences of E1 and E3 (see
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43 106 Supporting Information) type strains of *A. pittii*, *A. oleivorans*, and *A. seifertii* also had 99%
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45 107 identity to E1. The E3 sequence has 99% identity to type strains of *Variovorax*
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48 108 *boronicumulans*, *V. paradoxus*, *V. guangxiensis*, *V. ginsengisoli*. Based on these analyses, the
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50 109 isolates have been assigned genera and designated *Acinetobacter* E1 and *Variovorax* E3.
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3 110 The disappearance of metaldehyde from minimal media is proportional to the growth of
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5 111 *Acinetobacter* E1 and *Variovorax* E3 in pure cultures
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7 112 Triplicate cultures of *Acinetobacter* E1 and *Variovorax* E3 were grown in minimal media with
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9 113 850 μM (150 mg/L) metaldehyde, incubated at 30°C with shaking at 200 rpm. An additional
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11 114 3 flasks of media were not inoculated. Periodic samples were taken from each culture and
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13 115 an uninoculated media flask and OD₆₀₀ measurements were made. Contemporaneously,
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15 116 cellular material was removed from samples by centrifugation at 5,000 \times g, the supernatant
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17 117 aspirated and stored at -20°C for later analysis of metaldehyde content. Growth curves are
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19 118 shown in Fig. 2A. During the exponential growth phase, *Acinetobacter* E1 had a doubling
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21 119 time of 8.5 hours, and *Variovorax* E3 had a doubling time of c. 22 hours. There was no
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23 120 increase in optical density in the uninoculated control culture. Metaldehyde concentration
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25 121 of culture media samples was quantified by Liquid Chromatography-Mass Spectrometry (for
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27 122 method, see Supporting Information). Metaldehyde disappeared over a similar timescale to
28
29 123 the growth of the E1 and E3 isolates (Fig. 2B). The disappearance of metaldehyde from the
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31 124 cultures was correlated with the growth of the isolates (Fig. 2C & D). As the sole carbon and
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33 125 energy source present in the culture medium it can be concluded that the strains were
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35 126 catabolising metaldehyde for growth. *Variovorax* E3 catabolises metaldehyde more slowly,
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37 127 has a longer lag time, lower maximum optical density, longer doubling time and higher final
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39 128 concentration of residual metaldehyde compared to *Acinetobacter* E1.
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3 131 Utilization of metaldehyde by *Acinetobacter* E1 is a property not shared by other

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5 132 *Acinetobacter*

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7 133 The remainder of the work focused on *Acinetobacter* E1 which has faster growth kinetics,

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9 134 and a more rapid and complete utilization of metaldehyde, compared to *Variovorax* E3.

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11 135 *Acinetobacter* E1 was unable to grow using glucose, fructose, arabinose or glycerol as

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13 136 alternative carbon substrates.

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17 137 It was desirable to identify other strains related to *Acinetobacter* E1 for comparative

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19 138 purposes. *A. calcoaceticus* RUH 2202 (Nemec et al., 2011) was purchased from the Belgian

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21 139 Coordinated Collection of Microorganisms, *A. calcoaceticus* ANC3678 (Nemec et al., 2011),

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23 140 *A. calcoaceticus* NIPH1 (Nemec et al., 1999), *A. pittii* ANC3678 (Nemec et al., 2011) *A. pittii*

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25 141 70.29 (Seifert et al., 1994), and *A. baylyi* DSM14961 (Carr et al., 2003) from the CIP culture

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27 142 collection (Pasteur Institute, Paris). The ability of these *Acinetobacter* to use metaldehyde

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29 143 was assessed by streaking colonies from an LB plate onto a MSM + metaldehyde plate and

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31 144 inoculating into liquid media containing 850 μ M metaldehyde. There were no signs of

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33 145 growth in either media after 4 days' incubation at 30 °C. *Acinetobacter* E1, unlike strain RUH

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35 146 2202, was able to grow on phenol, whereas *A. calcoaceticus* RUH 2202 grew on 1 % ethanol

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37 147 as a carbon source, but strain E1 could not grow with ethanol. Both *Acinetobacter* strains E1

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39 148 and RUH 2202 grew on acetate as a carbon source, which allowed for comparative analysis

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41 149 of metaldehyde utilization under the same growth conditions. Following growth on acetate

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43 150 as sole carbon source, *Acinetobacter* E1 utilized 40 μ M metaldehyde over a 30 minute

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45 151 period, whereas there was no loss of metaldehyde in cultures of *A. calcoaceticus* RUH 2202

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47 152 (Fig. 3A).

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3 154 *Acinetobacter* E1 degrades metaldehyde to completion, and this degradation is followed by
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5 155 oxygen consumption
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7 156 Following growth on metaldehyde, *Acinetobacter* E1 utilized 40 μM metaldehyde over a 12
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10 157 minute period (Fig. 3A). This suggests a c. 2-fold increase in activity of the metaldehyde
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12 158 degrading enzyme following culturing with metaldehyde. Furthermore, suspensions of
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14 159 *Acinetobacter* E1 utilize oxygen in a metaldehyde-dependent manner after growth on
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16 160 metaldehyde, but not after growth on acetate (Fig. 3B). This oxygen consumption is delayed
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18 161 compared to metaldehyde disappearance, indicating that the metaldehyde catabolism
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20 162 involves metaldehyde degradation, followed by an oxygen-dependent metabolic step. The
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22 163 apparent K_M of cell suspensions of *Acinetobacter* E1 for metaldehyde was c. 50 μM , and it is
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24 164 noted that metaldehyde was degraded to below the limit of detection in these experiments
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26 165 (<1 nM metaldehyde) in 30 minutes (Fig. 3C), which suggests that this or similar strains may
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28 166 have value in future bioremediation strategies.

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31 167 Metaldehyde is a xenobiotic (*i.e.* only in existence due to human activity via chemical
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33 168 synthesis) that has been in widespread use for about 100 years. The metaldehyde degrading
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35 169 strains *Acinetobacter* E1 and *Variovorax* E3 share evolutionary heritage with other bacteria
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37 170 with versatile metabolism (Fewson, 1967; Willems et al., 1991) and a demonstrated ability
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39 171 to degrade xenobiotics (Mirgain et al., 1993; Greene et al., 2000; Sorensen et al., 2005;
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41 172 Wang and Gu, 2006; Bruland et al., 2009; Carbajal-Rodriguez et al., 2011; Zhang et al., 2012;
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43 173 Rajoo et al., 2013; Murdoch and Hay, 2015) and other potentially recalcitrant chemicals
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45 174 (Reisfeld et al., 1972; Abbott et al., 1973; Koh et al., 1985; Hwang and Draughon, 1994;
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47 175 Singh and Lin, 2008; Zhao et al., 2009). The metabolic versatility of *Acinetobacter* and
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49 176 *Variovorax* isolates varies between isolates, presumably due to horizontal acquisition of
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3 177 genetic traits, selected in particular environments. Future work will focus on identifying the
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5 178 mechanistic basis for metaldehyde degradation.
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9 179 To conclude, here we have demonstrated the first isolation of bacteria capable of degrading
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11 180 the commonly used molluscicide metaldehyde. Metaldehyde is a stable polymer of
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13 181 acetaldehyde which consists of a ring structure in which the bonds are aliphatic C-C single
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15 182 bonds and C-O ethers. Biological degradation of metaldehyde via the metabolic processes in
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17 183 bacteria such as *Acinetobacter* E1 and *Variovorax* E3 may prove valuable in dealing with
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19 184 metaldehyde contamination in natural environments and drinking water sources.
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26 186 **Acknowledgments**

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284 **Figure legends.**

285 Figure 1. (A) Skeletal structure of metaldehyde. (B) Frequency of water quality failures per
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47 286 year in the UK due to metaldehyde or all other pesticides. Compiled from the Drinking
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49 287 Water Inspectorate annual regional reports, available from
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51 288 <http://www.dwi.gov.uk/about/annual-report>.
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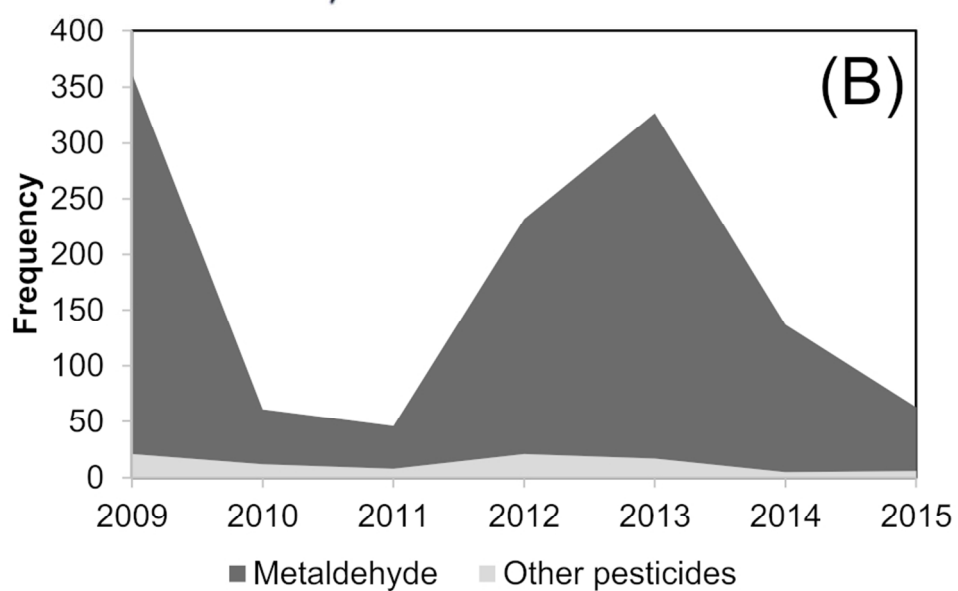
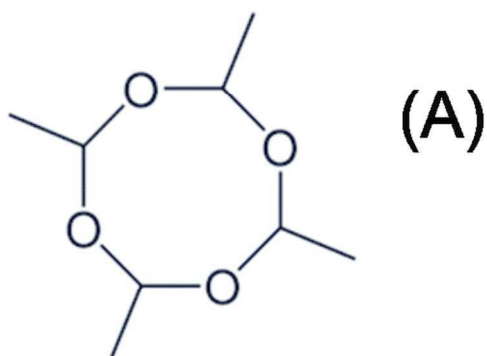
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3 290 Figure 2. Growth and metaldehyde utilization by *Acinetobacter* E1 and *Variovorax* E3. (A)
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5 291 Mean OD₆₀₀ (measured using a Jenway 6300 spectrophotometer) in liquid culture with 850
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7 292 μM metaldehyde as sole carbon and energy source, inoculated with single colonies of
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10 293 *Acinetobacter* E1 (open circles) and *Variovorax* E3 (filled circles), or not inoculated (filled
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12 294 triangles). Error bars give SD of triplicate independent cultures. (B) Mean [metaldehyde] in
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14 295 culture media during growth of *Acinetobacter* E1 (open circles) and *Variovorax* E3 (filled
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16 296 circles), or not inoculated (filled triangles). Error bars give SD of triplicate independent
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18 297 cultures. Correlation between culture optical density and residual metaldehyde
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20 298 concentration during growth of (C) *Acinetobacter* E1 ($R^2 = 0.94$) and (D) *Variovorax* E3 ($R^2 =$
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22 299 0.88) in media containing metaldehyde as the sole energy and carbon source.
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301 Figure 3. Metaldehyde utilization and metaldehyde-dependent oxygen utilization. (A)
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33 302 Metaldehyde utilization in samples of washed *Acinetobacter* cells resuspended to an OD₆₀₀ =
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35 303 1.0 treated with 53 μM metaldehyde following culture of *Acinetobacter* E1 in acetate (filled
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37 304 circles; rate of metaldehyde utilization = $1.5 \pm 0.1 \mu\text{M}\cdot\text{min}^{-1}$) or in metaldehyde (filled
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39 305 triangles; rate of metaldehyde utilization = $3.8 \pm 0.3 \mu\text{M}\cdot\text{min}^{-1}$), or strain RUH 2202 grown
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41 306 with acetate (open circles; rate of metaldehyde utilization = $-0.1 \pm 0.1 \mu\text{M}\cdot\text{min}^{-1}$) as sole
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43 307 carbon source. (B) Metaldehyde-dependent oxygen utilization in samples of washed
44
45 308 *Acinetobacter* cells resuspended to an OD₆₀₀ = 1.0 treated with 53 μM metaldehyde added
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47 309 at time zero. *A. calcoaceticus* RUH2202 (cultured in acetate) (solid thin line; rate of O₂
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49 310 utilization = $1.6 \pm 0.4 \mu\text{M}\cdot\text{min}^{-1}$), *Acinetobacter* E1 cultured in acetate (solid thick line; rate
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51 311 of O₂ utilization = $2.7 \pm 1.1 \mu\text{M}\cdot\text{min}^{-1}$) or in metaldehyde (dashed line; rate of O₂ utilization =
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53 312 $24.5 \pm 3.8 \mu\text{M}\cdot\text{min}^{-1}$). Data is representative of at least three replicates. (C) Three time
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3 313 courses of metaldehyde degradation following culture of *Acinetobacter* E1 with
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5 314 metaldehyde as sole carbon source. Metaldehyde axis is split to show rate of disappearance
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8 315 between 0 – 0.2 μM , and 0.2 – 50 μM metaldehyde.
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Figure 1. (A) Skeletal structure of metaldehyde. (B) Frequency of water quality failures per year in the UK due to metaldehyde or all other pesticides. Compiled from the Drinking Water Inspectorate annual regional reports, available from <http://www.dwi.gov.uk/about/annual-report>.

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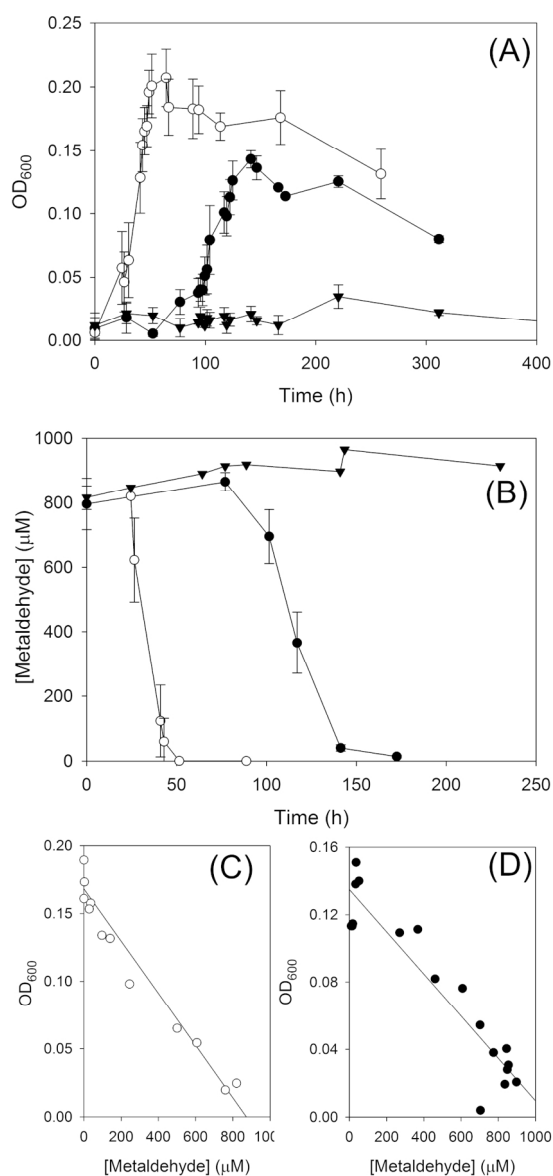


Figure 2. Growth and metaldehyde utilization by *Acinetobacter* E1 and *Variovorax* E3. (A) Mean OD₆₀₀ (measured using a Jenway 6300 spectrophotometer) in liquid culture with 850 μM metaldehyde as sole carbon and energy source, inoculated with single colonies of *Acinetobacter* E1 (open circles) and *Variovorax* E3 (filled circles), or not inoculated (filled triangles). Error bars give SD of triplicate independent cultures. (B) Mean [metaldehyde] in culture media during growth of *Acinetobacter* E1 (open circles) and *Variovorax* E3 (filled circles), or not inoculated (filled triangles). Error bars give SD of triplicate independent cultures. Correlation between culture optical density and residual metaldehyde concentration during growth of (C) *Acinetobacter* E1 ($R^2 = 0.94$) and (D) *Variovorax* E3 ($R^2 = 0.88$) in media containing metaldehyde as the sole energy and carbon source.

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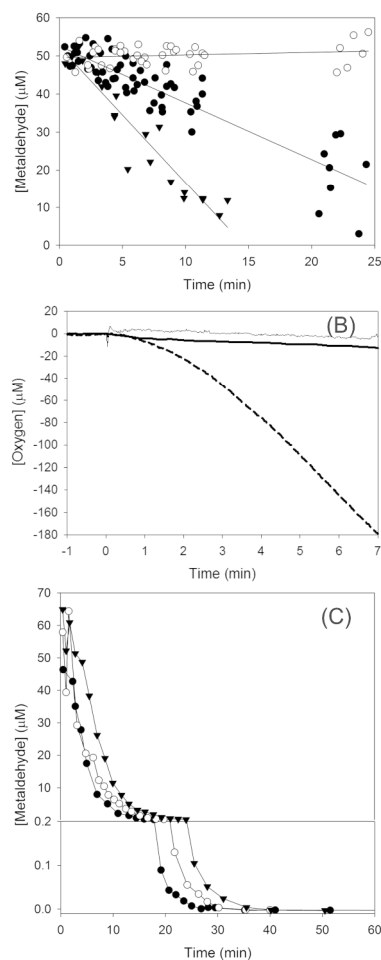


Figure 3. Metaldehyde utilization and metaldehyde-dependent oxygen utilization. (A) Metaldehyde utilization in samples of washed *Acinetobacter* cells resuspended to an $OD_{600} = 1.0$ treated with $53 \mu\text{M}$ metaldehyde following culture of *Acinetobacter* E1 in acetate (filled circles; rate of metaldehyde utilization = $1.5 \pm 0.1 \mu\text{M}\cdot\text{min}^{-1}$) or in metaldehyde (filled triangles; rate of metaldehyde utilization = $3.8 \pm 0.3 \mu\text{M}\cdot\text{min}^{-1}$), or strain RUH 2202 grown with acetate (open circles; rate of metaldehyde utilization = $-0.1 \pm 0.1 \mu\text{M}\cdot\text{min}^{-1}$) as sole carbon source. (B) Metaldehyde-dependent oxygen utilization in samples of washed *Acinetobacter* cells resuspended to an $OD_{600} = 1.0$ treated with $53 \mu\text{M}$ metaldehyde added at time zero. *A. calcoaceticus* RUH2202 (cultured in acetate) (solid thin line; rate of O_2 utilization = $1.6 \pm 0.4 \mu\text{M}\cdot\text{min}^{-1}$), *Acinetobacter* E1 cultured in acetate (solid thick line; rate of O_2 utilization = $2.7 \pm 1.1 \mu\text{M}\cdot\text{min}^{-1}$) or in metaldehyde (dashed line; rate of O_2 utilization = $24.5 \pm 3.8 \mu\text{M}\cdot\text{min}^{-1}$). Data is representative of at least three replicates. (C) Three time courses of metaldehyde degradation following culture of *Acinetobacter* E1 with metaldehyde as sole carbon source. Metaldehyde axis is split to show rate of disappearance between $0 - 0.2 \mu\text{M}$, and $0.2 - 50 \mu\text{M}$ metaldehyde.

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