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

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Running title: metaldehyde-degrading bacteria in soils

**Summary**

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

## Introduction

Metaldehyde $(CH_3CHO)_4$ is an ether, formed from a cyclic tetramerisation of acetaldehyde (Fig. 1A) (Kekulé and Zincke, 1872). Metaldehyde was initially used as a solid fuel firelighter "Meta-fuel" (Miller, 1928), but its major contemporary use is as a molluscicide in agriculture and horticulture. Its application in controlling slugs was known as early as 1934 (Gimingham, 1940) and it is now widely used in both agricultural fields and domestic gardens. It is applied as a pelleted bran bait that inhibits slug feeding after exposure (Wedgwood and Bailey, 1988), causing effects such as the distention and disintegration of the Golgi apparatus and endoplasmic reticulum in the mucus cells of slugs (Triebskorn et al., 1998).

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

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

It has been shown that the xenobiotic metaldehyde can be quickly degraded in soils (Zhang et al., 2011) and is oxidised to carbon dioxide under aerobic conditions in unsterilised soils (EFSA, 2010). This strongly suggests the involvement of microbes in its degradation, although no microorganisms have been isolated to date that degrade metaldehyde. The degradation of metaldehyde to $CO_2$ is strongly exothermic (heat of combustion 3370 $kJ.mol^{-1}$ (Fleischmann et al., 2000)), suggesting that it has the potential to be a carbon and energy source to support microbial growth. Soils are home to a vast array of microbes and represent a source of metabolic activities that may be of use in industrial and medicinal applications (Delmont et al., 2011). Here we enriched microbes from soils, and report the first isolation and identification of microbial isolates capable of using metaldehyde as a sole source of energy and carbon for growth.

## Results and Discussion

*Two distinct metaldehyde degrading strains were isolated from domestic soils*

Metaldehyde degrading bacteria were selected in a mineral medium consisting of salts $Na_2HPO_4$ (55 mM), $KH_2PO_4$ (11 mM), $NH_4Cl$ (6 mM) and $MgSO_4$ (0.4 mM) (pH 7). This was supplemented with 2 ml/l of a trace elements solution (Vishniac and Santer, 1957). Metaldehyde was provided as sole carbon source and control cultures lacked metaldehyde. Ability to grow using metaldehyde was tested in both liquid enrichment cultures and on solid media, containing 1.5 % agarose. 100 ml liquid cultures were inoculated with 1 g of soil obtained from domestic gardens in York, UK. Cultures were incubated at 30°C for 3 days, 1 ml of enrichment media was sub-cultured into fresh media and incubated for a further 3 days, and subsequently samples were spread onto agarose plates containing 2800 µM (500 mg/L) metaldehyde. 50-200 colonies were obtained on plates when the enrichments were carried out in liquid culture in the presence of 570 µM (100 mg/L) metaldehyde, but not following control enrichments in the absence of metaldehyde. 1 g samples of the same domestic soils were re-suspended in 10 ml of sterile water and 100 µL aliquots spread directly onto agarose plates containing metaldehyde. 2-5 colonies grew on these plates. The morphology of all the colonies was white, round and glossy. Ten isolates were picked for further analysis, and named E1-E6 and M1-M4, to designate the source soils used. Soil E had a recent history of metaldehyde utilization, whereas soil M had not been treated with metaldehyde for at least 5 years. In each case the isolated strains grew on agarose plates supplemented with metaldehyde, but not in its absence, suggesting they were utilizing metaldehyde as a carbon and energy source.

On subculturing the metaldehyde-degrading strains, each strain appeared to be a pure culture, except strain E4 which yielded two distinct colony morphologies, and was subsequently subdivided into E4a and E4b. Colonies from strains E1, E3, E4a, E4b, E5, M1 and M4 were used for amplification of 16S rDNA as described previously with primers U8F and U1492R (Eden et al., 1991). Amplification was achieved using GoTaq polymerase (Promega) with a standard programme of: 98°C for 30 s; 35 cycles of 98°C for 10 s, 50°C for 30 s, 72°C for 60 s; 72°C for 10 min. PCR products were purified using QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions. For Restriction Fragment Length Polymorphism (RFLP) analysis, 1 µg of purified DNA was digested for 1 or 3 hours at 37°C using restriction enzyme HhaI. RFLP revealed two distinctly different ribotypes (see Supporting Information). Two examples of each ribotype were sequenced. Sanger sequencing was used to obtain the nucleotide sequences of the U8F-U1492R amplicons of E1, M1, E3 and E4a using U8F as sequencing primer. Sequences from E1 and M1 were aligned using ClustalX V2.1 and found to be identical across the >900 base region where the base sequence could be confidently assigned. Similarly, the sequences from E3 and E4a were found to be identical across a >900 base region.

Subsequent investigation focused on the strains E1 and E3. The sequences of E1 and E3 (see Supporting Information) type strains of *A. pittii*, *A. oleivorans*, and *A. seifertii* also had 99% identity to E1. The E3 sequence has 99% identity to type strains of *Variovorax boronicumulans*, *V. paradoxus*, *V. guangxiensis*, *V. ginsengisoli*. Based on these analyses, the isolates have been assigned genera and designated *Acinetobacter* E1 and *Variovorax* E3.

The disappearance of metaldehyde from minimal media is proportional to the growth of *Acinetobacter* E1 and *Variovorax* E3 in pure cultures

Triplicate cultures of *Acinetobacter* E1 and *Variovorax* E3 were grown in minimal media with 850 µM (150 mg/L) metaldehyde, incubated at 30°C with shaking at 200 rpm. An additional 3 flasks of media were not inoculated. Periodic samples were taken from each culture and an uninoculated media flask and $OD_{600}$ measurements were made. Contemporaneously, cellular material was removed from samples by centrifugation at 5,000 × g, the supernatant aspirated and stored at -20°C for later analysis of metaldehyde content. Growth curves are shown in Fig. 2A. During the exponential growth phase, *Acinetobacter* E1 had a doubling time of 8.5 hours, and *Variovorax* E3 had a doubling time of c. 22 hours. There was no increase in optical density in the uninoculated control culture. Metaldehyde concentration of culture media samples was quantified by Liquid Chromatography-Mass Spectrometry (for method, see Supporting Information). Metaldehyde disappeared over a similar timescale to the growth of the E1 and E3 isolates (Fig. 2B). The disappearance of metaldehyde from the cultures was correlated with the growth of the isolates (Fig. 2C & D). As the sole carbon and energy source present in the culture medium it can be concluded that the strains were catabolising metaldehyde for growth. *Variovorax* E3 catabolises metaldehyde more slowly, has a longer lag time, lower maximum optical density, longer doubling time and higher final concentration of residual metaldehyde compared to *Acinetobacter* E1.

Utilization of metaldehyde by *Acinetobacter* E1 is a property not shared by other *Acinetobacter*

The remainder of the work focused on *Acinetobacter* E1 which has faster growth kinetics, and a more rapid and complete utilization of metaldehyde, compared to *Variovorax* E3. *Acinetobacter* E1 was unable to grow using glucose, fructose, arabinose or glycerol as alternative carbon substrates.

It was desirable to identify other strains related to *Acinetobacter* E1 for comparative purposes. *A. calcoaceticus* RUH 2202 (Nemec et al., 2011) was purchased from the Belgian Coordinated Collection of Microorganisms, *A. calcoaceticus* ANC3678 (Nemec et al., 2011), *A. calcoaceticus* NIPH1 (Nemec et al., 1999), *A. pittii* ANC3678 (Nemec et al., 2011) *A. pittii* 70.29 (Seifert et al., 1994), and *A. baylyi* DSM14961 (Carr et al., 2003) from the CIP culture collection (Pasteur Institute, Paris). The ability of these *Acinetobacter* to use metaldehyde was assessed by streaking colonies from an LB plate onto a MSM + metaldehyde plate and inoculating into liquid media containing 850 µM metaldehyde. There were no signs of growth in either media after 4 days' incubation at 30 °C. *Acinetobacter* E1, unlike strain RUH 2202, was able to grow on phenol, whereas *A. calcoaceticus* RUH 2202 grew on 1 % ethanol as a carbon source, but strain E1 could not grow with ethanol. Both *Acinetobacter* strains E1 and RUH 2202 grew on acetate as a carbon source, which allowed for comparative analysis of metaldehyde utilization under the same growth conditions. Following growth on acetate as sole carbon source, *Acinetobacter* E1 utilized 40 µM metaldehyde over a 30 minute period, whereas there was no loss of metaldehyde in cultures of *A. calcoaceticus* RUH 2202 (Fig. 3A).

*Acinetobacter* E1 degrades metaldehyde to completion, and this degradation is followed by oxygen consumption

Following growth on metaldehyde, *Acinetobacter* E1 utilized 40 µM metaldehyde over a 12 minute period (Fig. 3A). This suggests a c. 2-fold increase in activity of the metaldehyde degrading enzyme following culturing with metaldehyde. Furthermore, suspensions of *Acinetobacter* E1 utilize oxygen in a metaldehyde-dependent manner after growth on metaldehyde, but not after growth on acetate (Fig. 3B). This oxygen consumption is delayed compared to metaldehyde disappearance, indicating that the metaldehyde catabolism involves metaldehyde degradation, followed by an oxygen-dependent metabolic step. The apparent $K_M$ of cell suspensions of *Acinetobacter* E1 for metaldehyde was c. 50 µM, and it is noted that metaldehyde was degraded to below the limit of detection in these experiments (<1 nM metaldehyde) in 30 minutes (Fig. 3C), which suggests that this or similar strains may have value in future bioremediation strategies.

Metaldehyde is a xenobiotic (*i.e.* only in existence due to human activity via chemical synthesis) that has been in widespread use for about 100 years. The metaldehyde degrading strains *Acinetobacter* E1 and *Variovorax* E3 share evolutionary heritage with other bacteria with versatile metabolism (Fewson, 1967; Willems et al., 1991) and a demonstrated ability to degrade xenobiotics (Mirgain et al., 1993; Greene et al., 2000; Sorensen et al., 2005; Wang and Gu, 2006; Bruland et al., 2009; Carbajal-Rodriguez et al., 2011; Zhang et al., 2012; Rajoo et al., 2013; Murdoch and Hay, 2015) and other potentially recalcitrant chemicals (Reisfeld et al., 1972; Abbott et al., 1973; Koh et al., 1985; Hwang and Draughon, 1994; Singh and Lin, 2008; Zhao et al., 2009). The metabolic versatility of *Acinetobacter* and *Variovorax* isolates varies between isolates, presumably due to horizontal acquisition of

genetic traits, selected in particular environments. Future work will focus on identifying the mechanistic basis for metaldehyde degradation.

To conclude, here we have demonstrated the first isolation of bacteria capable of degrading the commonly used molluscicide metaldehyde. Metaldehyde is a stable polymer of acetaldehyde which consists of a ring structure in which the bonds are aliphatic C-C single bonds and C-O ethers. Biological degradation of metaldehyde via the metabolic processes in bacteria such as *Acinetobacter* E1 and *Variovorax* E3 may prove valuable in dealing with metaldehyde contamination in natural environments and drinking water sources.

**Acknowledgments**

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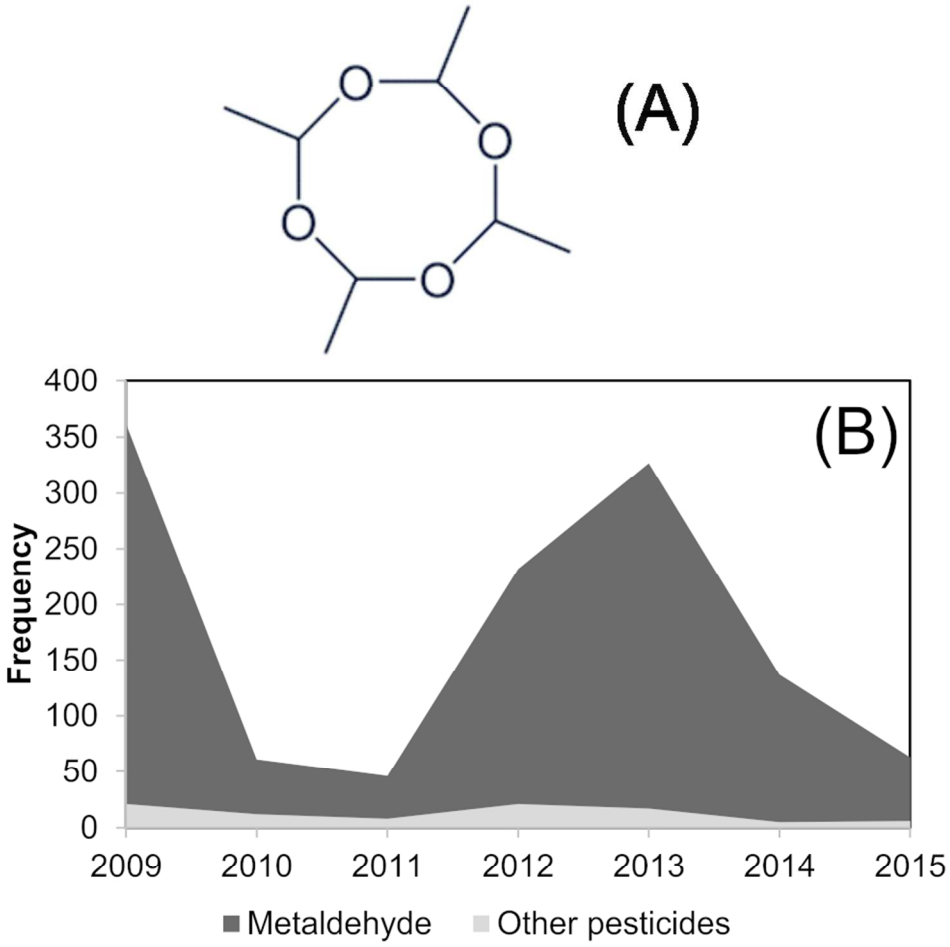
**Figure legends.**

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.

Figure 2. Growth and metaldehyde utilization by *Acinetobacter* E1 and *Variovorax* E3. (A) Mean $OD_{600}$ (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.

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 µM metaldehyde following culture of *Acinetobacter* E1 in acetate (filled circles; rate of metaldehyde utilization = 1.5 ± 0.1 $\mu M.min^{-1}$) or in metaldehyde (filled triangles; rate of metaldehyde utilization = 3.8 ± 0.3 $\mu M.min^{-1}$), or strain RUH 2202 grown with acetate (open circles; rate of metaldehyde utilization = -0.1 ± 0.1 $\mu M.min^{-1}$) 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 µM metaldehyde added at time zero. *A. calcoaceticus* RUH2202 (cultured in acetate) (solid thin line; rate of $O_2$ utilization = 1.6 ± 0.4 $\mu M.min^{-1}$), *Acinetobacter* E1 cultured in acetate (solid thick line; rate of $O_2$ utilization = 2.7 ± 1.1 $\mu M.min^{-1}$) or in metaldehyde (dashed line; rate of $O_2$ utilization = 24.5 ± 3.8 $\mu M.min^{-1}$). 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 µM, and 0.2 – 50 µM metaldehyde.

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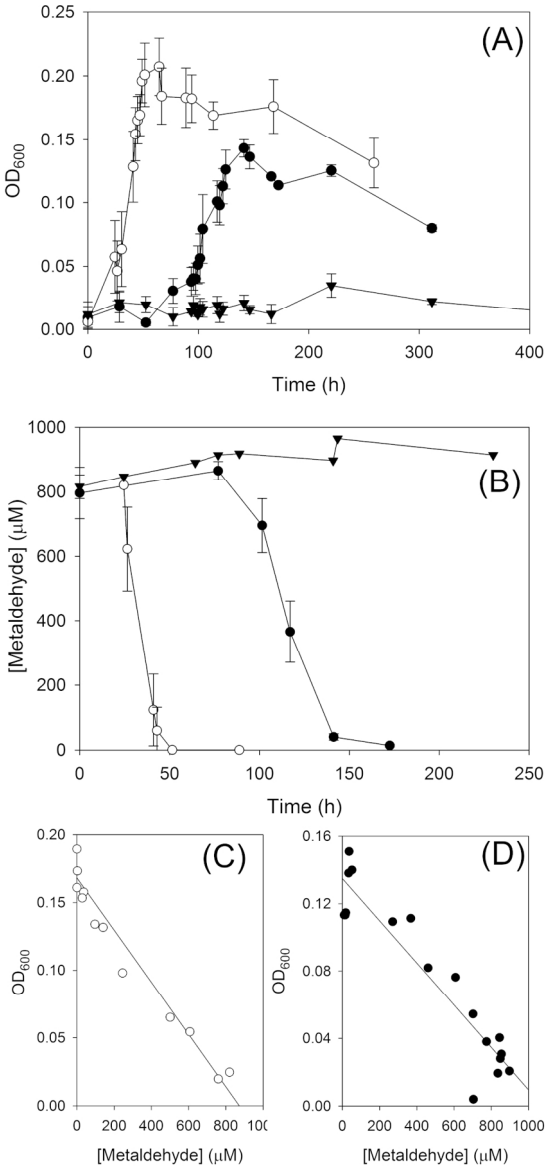


Figure 2. Growth and metaldehyde utilization by Acinetobacter E1 and Variovorax E3. (A) Mean OD600 (measured using a Jenway 6300 spectrophotometer) in liquid culture with 850 μM metaldehyde as sole carbon and energy source, inoculated with single colonies of Acinetobacter E1 (open circles) and Variovorax 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 (R2 = 0.94) and (D) Variovorax E3 (R2 = 0.88) in media containing metaldehyde as the sole energy and carbon source.

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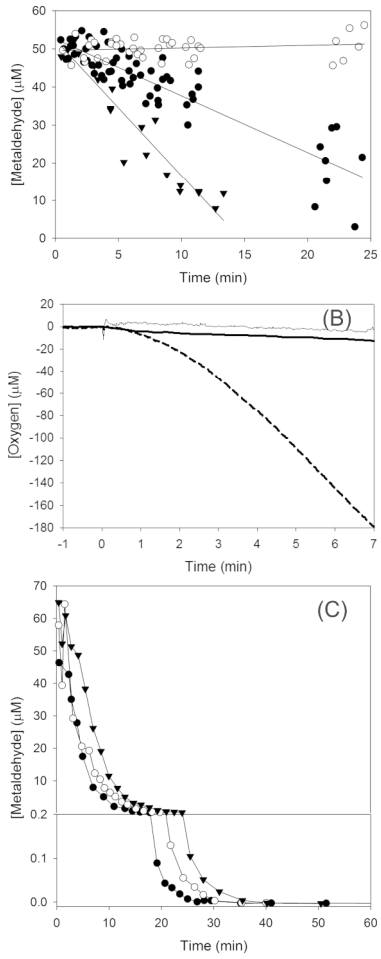


Figure 3. Metaldehyde utilization and metaldehyde-dependent oxygen utilization. (A) Metaldehyde utilization in samples of washed Acinetobacter cells resuspended to an OD600 = 1.0 treated with 53 µM metaldehyde following culture of Acinetobacter E1 in acetate (filled circles; rate of metaldehyde utilization = 1.5 ± 0.1 µM.min-1) or in metaldehyde (filled triangles; rate of metaldehyde utilization = 3.8 ± 0.3 µM.min-1), or strain RUH 2202 grown with acetate (open circles; rate of metaldehyde utilization = -0.1 ± 0.1 µM.min-1) as sole carbon source. (B) Metaldehyde-dependent oxygen utilization in samples of washed Acinetobacter cells resuspended to an OD600 = 1.0 treated with 53 µM metaldehyde added at time zero. A. calcoaceticus RUH2202 (cultured in acetate) (solid thin line; rate of O2 utilization = 1.6 ± 0.4 µM.min-1), Acinetobacter E1 cultured in acetate (solid thick line; rate of O2 utilization = 2.7 ± 1.1 µM.min-1) or in metaldehyde (dashed line; rate of O2 utilization = 24.5 ± 3.8 µM.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 µM, and 0.2 – 50 µM metaldehyde.

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