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CHLOROMETHANE BIOSYNTHESIS IN POROID FUNGI

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Key Word Index—*Phellinus*; *Inonotus*; Hymenochaetaceae; fungi; biosynthesis; halogenated compounds; chloromethane; methyl esters.

Abstract—A survey of the distribution of chloromethane (MeCl), biosynthesis amongst polypores indicates that the trait is expressed widely in genera of the Hymenochaetaceae. Of 63 species examined in this family, including species from the genera *Phellinus*, *Inonotus*, *Hymenochaete*, *Onnia*, *Phaeolus* and *Fomitoporia*, 34 (54%) were capable of MeCl biosynthesis. The magnitude of MeCl production was dependent on the nature of the medium and the fungal species; however 21 species were capable of converting more than 10% of chloride ion in the medium to MeCl on at least one of the media employed. Of 27 species screened from the Ganodermataceae and the Polyporaceae, only one *Fomitopsis cytisina* was capable of MeCl biosynthesis.

Of the 23 MeCl biosynthesizing species in the genus *Phellinus*, 12 were also capable of producing methyl benzoate and, in most instances, methyl esters of salicylic and 2-furoic acid. Ester production was not observed in *Phellinus* species not possessing the MeCl biosynthesis trait. It is postulated that the biosynthesis of esters and MeCl is biochemically linked; ester biosynthesis being a more advanced trait in evolutionary terms. The results suggest that the potential for MeCl production in nature by wood-rotting fungi is considerable. The environmental implications of a significant fungal contribution to the atmospheric MeCl burden are considered.

INTRODUCTION

Cowan *et al* [1] first established that chloromethane (MeCl) was a natural product of several species placed in the classical genus *Fomes*, a widespread group of perennial wood-rotting fungi, although no quantitative measurements were made at the time. Of 32 species of *Fomes* examined, six were reported to produce MeCl although GC–MS confirmation of the presence of the compound was obtained only for *F. ribis*. Later investigations by Harper [2,3] adapted headspace techniques, designed for determination of volatile bacterial secondary metabolites, to the fungal system and demonstrated that chloride ion was methylated with high efficiency by *Phellinus pomaceus* particularly on cellulosic substrates. Conversion of chloride to MeCl exceeded 90% at concentrations of 50 mM chloride in the growth medium. Bromo- and iodomethanes were also formed in high yield from the corresponding halide ions. Reinvestigation [3] of four other species of *Phellinus* originally reported [1] to produce MeCl revealed that both *P. ribis* and *P. occidentalis* also converted chloride ion to MeCl with high efficiency. However, *P. robiniae* only produced relatively small quantities of MeCl and formation of the compound was not detected in cultures of *P. conchatus*. No comprehensive survey has yet been mounted to determine whether the MeCl biosynthesis trait is confined merely to the four species of *Phellinus* described above or is more widely distributed in *Phellinus* and related genera of the Hymenochaetaceae and even possibly in other families of polypores.

Analysis of headspace volatiles from *P. pomaceus* [3] by GC–MS has demonstrated that methyl esters of benzoic, salicylic and 2-furoic acids are major components. This observation together with the finding that the pseudohalide ion, thiocyanate, acted as a powerful inhibitor not only of halomethane biosynthesis but also of methyl benzoate formation suggests that the methylations of halide and benzoate are closely linked, perhaps even mediated by the same biochemical system. Methyl benzoate and methyl salicylate have also been reported to be important constitutents in *P. igniarius*, *P. laevigatus* and *P. tremulae* [4], species not yet screened for MeCl production. It has been suggested [3] that a comparison of methyl ester and MeCl production by a number of *Phellinus* species may be useful in elucidating the relationship between the biosyntheses of the two classes of compound.

Fungal MeCl prduction may be important in an environmental context. Globally at least five million tonnes of MeCl must originate from natural sources according to estimates based on ambient environmental concentrations; man-made emissions of 26 thousand tonnes are of little consequence in this context [5–7]. Atmospheric concentrations of MeCl have latterly attained particular significance because of the importance now attached to MeCl and man-made halocarbons in controlling the rate of destruction of ozone in the stratosphere. Currently MeCl accounts for 25% of the chlorine in the stratosphere [7], the remainder being provided mainly by the chlorofluorocarbons **11** and **12**. The origin of atmospheric MeCl is not clear although both the combustion of vegetation [8] and reaction of chloride ion in seawater with iodomethane produced by marine macroalgae [9] have been proposed as major natural sources. However, recent work [6, 10] suggests that such

Table 1. Chloromethane production by Phellinus species and the volatile aromatic methyl esters identified in each species

Species	Culture collection accession number	Host species or genus from which culture isolated	Percentage Cl^- converted to MeCl: Glucose/ mycol. peptone/ agarose 9.5 mM Cl^-	Malt extract/ agarose 9.5 mM Cl^-	Filter paper/ mycol. peptone 10 mM Cl^-	Methyl esters of aromatic acids present at concn > 10 ng/vial
P. badius (Berk.) G. H. Cunn.	CBS 449.76	*Acacia catechu*	0.8	5.1	0	None
P. caryophylli (Raciborski) G. H. Cunn.	CBS 448.76	*Shorea robusta*	0	0	0	None
P. chrysoloma (Fr.) Dnk	CBS 180.24	*Picea*†	0	10	15	None
P. cinchonensis (Murr.) Ryvarden	CBS 447.76	*Quercus dilatata*	0	0	0	None
P. conchatus (Pers.:Fr.) Quél	NCWRF-FPRL 142	*Fraxinus americana*	0	0	0	None
P. everhartii (Ellis & Gall) Pilát	CBS 176.34	*Quercus*†	0	0	0	None
P. fastuosus (Lév.) Ahmad	CBS 213.36	*Gliricidia sepium*	0	0	0	None
P. ferreus (Pers.) Bourd. & Galzin	CBS 444.48	*Pseudotsuga menziesii*	0	0	0	None
P. ferruginosus (Schrad.:Fr.) Pat.	CBS 168.29	*Quercus*	0	0	0	None
P. gilvus (Schw.:Fr.) Pat.	CBS 387.54	*Quercus*†	0	0	0	None
P. hartigii (Allesch. & Schnabl) Pat.	CBS 382.72	*Abies sachalinensis*	0	0	0	None
P. hippophäecola H. Jahn	CBS 168.31	*Hippophäe rhamnoides*	0	1.5	1.9	Benzoate, salicylate, 2-furoate, 3-furoate
P. igniarius (L.:Fr.) Quél. var igniarius	CBS 381.72	*Pyrus malus*	16	39	63	Benzoate, salicylate, 2-furoate, cinnamate
P. igniarius (L.:Fr.) Quél. var igniarius	CBS 380.72	*Quercus petraea*	21	37	56	Benzoate, salicylate, 2-furoate
P. laevigatus (Fr.) Bourd. & Galzin	CBS 256.30	*Betula verrucosa*	0.2	17	1.0	Benzoate
P. lamaensis (Murr.) Sacc. & Trott.	NCWRF-FPRL 383	*Hevea*	0	0	0*	None
P. linteus (Berk. & Curt.) Teng	CBS 454.76	*Corylus colurna*	0.5	0.9	0*	None
P. lundellii Niemelä	CBS 540.72	*Betula*	55	54	27	Benzoate, salicylate, 2-furoate
P. nigricans (Fr.:Fr.) Karst.	*CBS* 213.48	*Alnus*†	0.3	9.2	0.3	Benzoate, salicylate
P. nigrolimitatus (Romell) Bourd. & Galzin	CBS 599.82	*Picea*†	1.6	0.8	1.2	None
P. noxius (Corner) G. H. Cunn.	CBS 170.32	*Elaeis guineensis*	0	0	0	None

P. occidentalis (Overh.) Gilbertson	CBS 196.55	*Crataegus douglasii*	43	29	79	None
P. pachyphloeus (Pat.) Pat.	CBS 446.76	*Mangifera indica*	42	3.8	18	None
P. pectinatus (Klotzsch) Quél.	CBS 445.76	*Murraya*	0	0	0	None
P. pini (Thurc.:Fr.) Pilát	CBS 210.36	*Tsugu heterophylla*	4.9	70	47	None
P. pomaceus (Pers.) Maire	NCWRF-FPRL33A	*Prunus*	57	46	82	Benzoate, salicylate, 2-furoate
P. pomaceus (Pers.) Maire	NCWRF-FPRL33B	*Prunus*	48	47	85	Benzoate, salicylate, 2-furoate
P. populicola Niemelä	CBS 638.75	*Populus tremulae*	6.0	43	22	Benzoate
P. pseudolaevigatus Parm.	CBS 351.80	*Betula manshurica*	2.2	0	3.2	Benzoate, salicylate
P. punctatus (Fr.) Pilát	CBS 301.33	*Salix*†	0	0	0	None
P. rhabarberinus (Berk.) G. H. Cunn.	CBS 282.77	*Rhus wallichii*†	3.0	6.1	12	None
P. ribis Karst. var ribis	NCWRF-FPRL 42	*Crataegus*	21	50	82	Benzoate, salicylate, 2-furoate
P. robiniae (Murr.) A. Ames	NCWRF-FPRL 180	*Robinia*	0.3	24	0	Benzoate, salicylate
P. robustus (Karst.) Bourd. & Galzin	CBS 175.34	*Quercus*†	9.8	14	4.7	None
P. senex (Nees & Mont.) Imazeki	CBS 442.76	*Cedrus toona*	0	0.2	0*	None
P. spiculosus (Campbell & Davidson) Niemelä	CBS 345.63	*Carya ovata*	0.1	6.9	1.2	None
P. tremulae (Bond.) Bond. & Borisov	CBS 123.40	*Populus*†	0.4	6.9	0	Benzoate, salicylate
P. trivialis Bres.	CBS 512.63	*Salix caprea*	22	19	47	Benzoate, salicylate
P. viticola (Schw. apud Fr.:Fr.) Rehm	CBS 214.36	*Picea*†	0	0	0*	None

*Little or no growth on medium.

†If host species from which isolated not recorded, typical host genus or species given where known.

sources probably do not account for more than a small proportion of total atmospheric MeCl.

If, however, the MeCl biosynthesis trait is widespread and strongly expressed in *Phellinus* and related wood-rotting fungi [3], forest habitats could be a major source of natural MeCl as such species are common in both the temperate and tropical regions of the world showing vegetative growth not only on wood but also on plant litter and soil organic matter. As the normal chloride content of plant material ranges between 0.2 and 10 g/kg and the affinity of the methylating system is high, the potential MeCl production in forest habitats could be very large. Consequently, the extensive worldwide deforestation which has occurred in recent history could have significantly reduced the efflux of MeCl into the environment and hence the prevailing concentration in the atmosphere. Such a possibility would have profound implications for the global atmospheric models employed to predict the effect of man-made halocarbons on the upper atmosphere which currently assume a constant natural production of MeCl.

In this paper we describe a survey for the MeCl biosynthesis trait in species of various families of the sub-order Polyporineae, as defined by Pegler [11], in particular the Hymenochaetaceae. Where evidence of MeCl biosynthesis has been found we have quantified production of MeCl on three different media. In addition all *Phellinus* and *Inonotus* species examined have been screened for the presence of methyl esters of benzoic, salicylic and 2-furoic acids. The results are discussed in terms of their biochemical, environmental and chemotaxonomic implications.

RESULTS AND DISCUSSION

Of 37 species of *Phellinus* investigated, 23 (62%) exhibited MeCl biosynthesis on at least one of the three media used (Table 1). In general, the cellulosic medium (filter paper/mycological peptone) was the most favourable to the formation of MeCl. The yield of the compound (expressed as the percentage of Cl^- originally present in the medium converted to MeCl) was very high in some species, e.g. *P. pomaceus*, 85%; *P. ribis*, 82%; *P. occidentalis*, 79%; and *P. igniarius*, 63%. Yields tended to be somewhat lower on malt extract medium. Nevertheless some species again gave relatively high conversion rates, e.g. *P. pini*, 70% *P. lundellii*, 50%. Glucose-based medium was the least conducive to MeCl biosynthesis but even so yields in the region of 50% were observed with a number of species. Lower rates of MeCl production by *P. pomaceus* on glucose-based medium were also noted by Harper and Kennedy [3]. The biosynthesis of secondary metabolites is frequently suppressed by glucose or other readily utilizable carbon sources.

The MeCl biosynthesis trait was not always expressed very strongly. Consequently several species showed very low conversion rates. Thus *P. nigrolimitatus* did not methylate more than 1.6% of available chloride ion on any medium although the presence of MeCl was definitely confirmed during culture on all three media. As previously observed in *P. pomaceus* [3] there is evidence of quantitative differences between various isolates of some other species in the degree of expression of the MeCl biosynthesis trait. Thus *P. igniarius* var. *igniarius* CBS 380.72 yielded 30% more MeCl on glucose/mycological peptone medium than a different isolate of the same species, CBS 381.72. It is of some interest environmentally that collectively *Phellinus* species capable of MeCl biosynthesis can invade the wood of a wide range of host tree species including not only angiosperms and gymnosperms but also species of both temperate and tropical origin (see Table 1).

Of the 23 MeCl biosynthesising species of *Phellinus*, 12 (52%) were capable of methyl benzoate production. Many of the latter species were also found to synthesize methyl esters of salicylic and 2-furoic acids. In cultures of *P. hippophaëcola* in addition to the three aforementioned esters, methyl 3-furoate was detected whilst in one strain of *P. igniarius*, methyl cinnamate was present. Ester production did not occur in any of the *Phellinus* species not possessing the MeCl biosynthesis trait. This observation tends to support the suggestion [3] that the biosynthesis of esters and MeCl are biochemicaly linked. Ester biosynthesis in evolutionary terms may simply represent the consequence of an extension of the substrate range of the methylating system involved in MeCl production.

Inonotus is generally regarded as the genus most closely related to *Phellinus* in the Hymenochaetaceae separated taxonomically only by its monomitic hyphal system and hence its more short-lived and softer fruiting bodies. Accordingly a number of *Inonotus* species were screened for MeCl and methyl ester biosynthesis. Of 14 species investigated, eight (57%) exhibited MeCl biosynthesis (Table 2) although the quantities produced in culture were on the whole substantially less than with the *Phellinus* species examined. The highest yield (43%) was shown by *I. hispidus* on malt extract medium. The widespread occurrence of the MeCl biosynthesis trait in *Inonotus* confirms the close taxonomic relationship between the genus and *Phellinus*. Interestingly in no *Inonotus* species investigated was the presence of methyl esters detected implying that if ester production is an evolutionary advance on MeCl biosynthesis, *Inonotus* is rather more primitive biochemically than *Phellinus*.

Species from other genera in the Hymenochaetaceae have been screened for MeCl production (Table 3). The non-poroid genus, *Hymenochaete*, is probably the most important genus in the family other than *Phellinus* and *Inonotus*. Of four *Hymenochaete* species investigated, only one, *H. corrugata* exhibited significant MeCl biosynthesis. However, representative species of the genera, *Onnia*, *Phaeolus* and *Fomitoporia* also gave significant levels of MeCl production indicating that the trait is widely distributed throughout the family Hymenochaetaceae. Again differences in MeCl biosynthesis between strains of the same species were evident but unlike the situation in *Phellinus* where differences were merely of magnitude, in *Onnia* qualitative differences were apparent in *Onnia circinata*. Thus strain CBS 420.48 appeared completely incapable of MeCl production at levels above the limit of detection of the techniques employed whilst strains CBS 246.30 showed biosynthesis of substantial quantities of MeCl.

To assess whether the MeCl biosynthesis trait existed in polypores outside the Hymenochaetaceae, representative species of the other major families of the sub-order Polyporineae [11], i.e. the Ganodermataceae and the Polyporaceae were examined (Table 4). Of 27 species screened only one, *Fomes fraxineus* (now more commonly known as *Fomitopsis cytisina*) was unambiguously confirmed as possessing the MeCl biosynthesis trait. This

Table 2. Chloromethane production by Inonotus species

Species	Culture collection accession number	Host species or genus from which cultures isolated	Percentage Cl^- converted to MeCl: Glucose/mycol. peptone/agarose 9.5 mM Cl^-	Malt extract/ agarose 9.5 mM Cl^-	Filter paper/ mycol. peptone 10 mM Cl^-
I. andersonii (Ellis & Everh.) Czerny	CBS 312.39	*Quercus*	18	18	8
I. cuticularis (Bull.:Fr.) Karst.	CBS 445.50	*Fagus sylvatica*	0	1.7	4.2
I. dryadeus Pers.:Fr.) Murr.	CBS 948.70	*Quercus*	0	0	0
I. glomeratus (Peck) Murr.	CBS 359.34	*Acer*†	0	14	0
I. hispidus (Bull.:Fr.) Karst.	CBS 386.61	*Fraxinus*†	3	43	11
I. nothofagi G. H. Cunn.	CBS 476.72	*Nothofagus*†	1.0	0	0*
I. obliquus (Pers.:Fr.) Pilát	CBS 314.39	*Betula*†	0	0	18
I. patouillardii (Rick) Imazeki	CBS 364.34	*Quercus gilva*	0	0	0
I. porrectus Murr.	CBS 296.56	*Gleditschia triacanthos*	0	0	0
I. radiatus (Sow.:Fr.) Karst.	CBS 578.81	*Alnus*	0	3.0	0*
I. rheades (Pers.) Karst.	CBS 127.71	*Populus alba*	15	1.1	8.3
I. tabacinus (Mont.) Karst.	CBS 311.39	*Shorea robusta*	0	0	0
I. tamaricis (Pat.) Maire apud Pilát	CBS 288.33	*Tamarix*	0	0	0
I. weirii (Murr.) Kotl. & Pouzar	CBS 163.40	*Thuja plicata*	0	0	0

*Little or no growth on medium.

†If host species from which culture isolated not recorded, typical host genus or species given where known.

Table 3 Chloromethane production by the family Hymenochaetaceae excluding the genera Phellinus and Inonotus

Species	Culture collection accession number	Percentage Cl^- converted to MeCl: Glucose/mycol. peptone/agarose 9.5 mM Cl^-	Malt extract/ agarose 9.5 mM Cl^-	Filter paper/ mycol. peptone 10 mM Cl^-
Coltricia perennis (L.:Fr.) Murr.	CBS 372.52	0	0	0*
Coltriciella dependens (Berk. & Curt.) Murr.	CBS 247.50	0	0	0
Fomitoporia flavomarginata Murr.	CBS 218.48	11	20	16
Hymenochaete corrugata (Fr.:Fr.) Lév.	CBS 133.40	1.3	3.4	6.4
Hymenochaete mougeotti (Fr.) Massee	CBS 596.87	0	0	0*
Hymenochaete rubiginosa (Dicks.:Fr.) Lév.	CBS 237.39	0	0	0*
Hymenochaete tabacina (Sow.:Fr.) Lév.	CBS 134.40	0	0	0
Onnia ciricinata (Fr.) Karst.	CBS 246.30	12	2.3	22
Onnia circinata (Fr.) Karst.	CBS 420.48	0	0	0*
Onnia orientalis (Lloyd) Imazeki	CBS 323.29	0	0	0
Phaeolus schweinitzii (Fr.:Fr.) Pat.	CBS 326.29	0	0	0.4*
Pycnoporellus alboluteus (Ellis & Everh.) Kotlaba & Pouzar	CBS 418.48	0	0	0*
Pycnoporellus fulgens (Fr.) Donk	CBS 285.78	0	0	0*

*Little or no growth on medium.

species gave yields of 0.8% on glucose/mycological peptone medium, 0.3% on malt extract medium and 9.8% on filter paper/mycological peptone medium. The precise significance of this sole non-Hymenochaetaceous MeCl biosynthesizing species is difficult to evaluate. *Fomitopsis* is placed in the sub-family Fomitoideae of the Polyporaceae by Pegler [11]. Another species of this genus investigated, *Fomitopsis pinicola*, did not display MeCl formation nor did members of other genera in this sub-family namely *Heterobasidion*, *Laricifomes* and *Poria*. Equally *Rigidoporus*, *Daedalea*, *Datronia*, *Lenzites* and *Trametes* in the sub-family Corioloideae lacked the ability to synthesize MeCl. Whether *Fomitopsis cytisina* is an aberrant species atypical of its genus and indeed its family, or whether further investigations of *Fomitopsis* and other genera with a close taxonomic affinity will reveal further MeCl producing species remains to be seen. Nevertheless, it is clear that the MeCl biosynthesis trait is expressed at both a qualitative and quantative level to the greatest extent in the Hymenochaetaceae where the majority of species studied (55%) were capable of MeCl production. In view of the suppression of secondary metabolism by readily utilized C sources it is possible that an even greater proportion of species in this family of

Table 4. Poroid fungi excluding the Hymenochaetaceae screened for chloromethane production

Species	Culture collection accession number
Coriolopsis occidentalis (Klotzsch) Murr.	IMI 79126
Coriolus hirsutus (Wulf.:Fr.) Quél. (= *Trametes hirsutus*)	CBS 320.29
Coriolus versicolor (L.:Fr.)Quél. (= *Trametes versicolor*)	IMI 83026
Daedalea dickinsii (Berk.) Bond.	CBS 452.76
Daedalea quercina L.:Fr.	CBS 202.50
Datronia scutellata (Schw.) Domanski	CBS 459.66
Fomes clelandi Lloyd	CBS 208.36
Fomes fomentarius (L.:Fr.) Fr.	CBS 249.50
Fomes fraxineus (Bull.) Fr. (= *Fomitopsis cytisina*)	NCWRF.FPRL 17A
Fomes officinalis (Vill.) Lloyd (= *Laricifomes officinalis*)	NCWRF.FPRL 81A
Fomes ulmarius (Sow.) Fr. (= *Rigidoporus ulmarius*)	NCWRF.FPRL 241A
Fomitopsis pinicola (Swartz:Fr.) Karst.	CBS 313.82
Ganoderma applanatum (Pers.) Pat.	CBS 250.61
Ganoderma resinaceum Boud. apud Pat.	CBS 194.76
Heterobasidion annosum (Fr.:Fr.) Bref.	CBS 834.72
Hetebobasidion insulare (Murr.) Ryvarden	CBS 451.76
Hydnopolyporus fimbriatus (Fr.:Fr) Reid	CBS 384.51
Laetiporus sulphureus (Bull.:Fr.) Murr.	CBS 343.69
Lenzites betulina (L.:Fr.) Fr.	CBS 245.66
Nigroporus vinosus (Berk.) Murr. (= *Polyporus vinosus*)	CBS 176.29
Polyporus intybaceus Berk. (= *Grifola intybaceus*)	CBS 573.65
Polyporus squamosus (Huds.:Fr.) Fr.	CBS 426.48
Poria carbonica Overh.	CBS 440.48
Poria placenta (Fr.) Cooke (= *Tyromyces placenta*)	CBS 447.48
Poria subacida (Peck) Sacc. (= *Perenniporia subacida*)	CBS 442.48
Rigidoporus microporus (Fr.:Fr.) Overeem	CBS 173.33
Rigidoporus sanguinolentus (Alb. & Schw.:Fr.) Donk	CBS 193.76

white-rot fungi would have exhibited the trait if the culture medium had been a C source more akin to their natural substrate, i.e. lignin-based or indeed wood of the natural host tree. The possession of the MeCl biosynthesis trait and to a lesser degree the ability to synthesize esters may well be a useful diagnostic character in the classification of the Hymenochaetaceae.

Reference has already been made to the not insignificant contribution of naturally produced MeCl to the chlorine burden of the stratosphere and the profound implications of a demonstration that the magnitude of the contribution had varied in the past. The survey detailed in this paper suggests that the potential for MeCl production by fungi in nature is large. The MeCl biosynthesis trait is possessed by the majority of species in an important family of white rot fungi, the Hymenochaetaceae, which is widely distributed in both temperate and tropical forest habitats. It is conceivable that a major proportion of natural MeCl could be derived from such a biological source. The global deforestation which has occurred in recent history may therefore have had a significant impact on atmospheric MeCl levels. A definitive conclusion regarding this possibility must await measurement of fungal MeCl production *in situ* in forest habitats. In the meantime less confidence can be placed in the conventional assumption employed in current computer models of the atmosphere that natural MeCl production has remained constant at five million tonnes per year throughout previous millenia.

EXPERIMENTAL

Fungal cultures and maintenance media. Cultures were acquired from the National Collection of Wood-Rotting Fungi, Princes Risborough Laboratory, Building Research Establishment, Aylesbury, Bucks, U.K., the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands and the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, U.K.

Fungi were maintained on slants of 5% (w/v) malt extract agar (Oxoid) containing chloramphenicol (25 mg/l). Cultures to be used for inoculation were grown for 3 weeks at 25° in 25 ml vials with loosely fitting screw caps.

Growth media. For investigation of the percentage conversion of chloride ion to MeCl by different species the following media were employed.

Glucose/mycological peptone/agarose medium contained (per l): glucose (30 g), mycological peptone (5 g), BDH agarose '10' (10 g) and NaCl (0.5 g) to give a final chloride concentration of 9.5 mM. The pH was adjusted to 6.5.

Malt extract/agarose medium contained (per l): malt extract (Oxoid, 30 g), BDH agarose '10' (10 g) and NaCl (0.5 g) to give a final Cl^- concn of 9.5 mM.

Filter paper/mycological peptone medium consisted of filter paper (Whatman 540), 6.8 g per flask, to which was added liquid

medium (20 ml) containing per l mycological peptone (5 g) and NaCl (0.5 g) to give a final Cl^- concn in the complete medium of 10 mM. The pH of the liquid medium was adjusted to 6.5 before addition to the filter paper.

As a precaution against bacterial contamination, chloramphenicol (25 mg/l) was routinely added to all media, experiments having indicated that the presence of the compound did not affect MeCl production [2].

Cultural conditions. Cultures were grown in 250 ml conical flasks containing 20 ml medium at 25°. The inoculum which was spread uniformly over the surface of the medium was a mycelial suspension (1 ml) prepared by homogenizing mycelium (60 mg fr. wt) in sterile dist. H_2O (20 ml) for 30 sec with an Ultra Turrax homogenizer. The mycelium used for inoculation was grown on 5% (w/v) malt extract agar containing not more than 1 mM chloride. Flasks were fitted with outlets prepared as previously described [2] permitting a limited exchange of gases between the internal and external atmospheres. Thus each conical flask was provided with a polytetrafluoroethylene-coated rubber bung through which passed a glass tube (10 × 0.8 cm i.d.) packed uniformly with cotton wool fitted so that the lower end was flush with the lower surface of the stopper. The bung was also equipped with a sampling port comprising a glass tube (1 mm i.d.) projecting 5 mm into the interior of the flask and flared at the upper end so as to accommodate a small polytetrafluoroetyhlene coated rubber bung.

Chloromethane determination. Samples of headspace (2 ml) were withdrawn from culture flasks through the sampling port and injected into a GC fitted with an FID detector and equipped with a glass column (1.5 m × 2 mm) packed with Tenax GC operated at N_2 gas flow rate of 20 ml/min. The oven temperature was programmed from 60 to 140° at 24°/min.

Calculation of total MeCl *generated.* The total MeCl generated by a fungal culture was calculated by means of a computer integrating programme as described in refs [2, 3] from measurements of MeCl in the headspace of the flask at regular intervals during incubation and a knowledge of the half life of MeCl in the flask obtained from a previous calibration procedure. Normally the headspace above a fungal culture was monitored for MeCl at intervals of between 24 and 48 hr from the time of inoculation until the headspace concentration in the flask had fallen to less than 0.050 μg/ml or in the case of comparatively low MeCl-yielding cultures until it had decreased to less than 10% of the peak headspace concentration attained by the culture. All fungal species were grown in duplicate flasks on each medium and the mean MeCl yield for each species expressed as the percentage of Cl^- originally present in the medium converted to MeCl.

GC-MS. For confirmation of the presence of MeCl in cultures the following procedure was adopted. The fungus was grown in culture at 25° in a 40 ml vial with loosely-fitting screw cap lined with a Teflon-coated silicone disc on the medium on which MeCl production by the species was apparently most copious. After 10 to 15 days the cap of the vial was screwed down tightly. Following a further 7 days incubation the vial was attached to a Hewlett Packard 7675A Purge and Trap sampler and purged for 5 min at 25° with He at a flow rate of 20 ml/min, the volatile components being adsorbed on a Tenax trap. The Purge and Trap sampler was linked to a GC equipped with a fused silica WCOT capilliary column (10 m × 0.32 mm) coated with Poraplot Q porous polymer. The entrapped volatiles were thermally desorped using a split ratio of 10:1 on to the first 10 cm of the capillary column by cooling the latter in liquid N_2. On complete desorption of the volatiles the oven was programmed from 30 to 150° at 10°/min with He as carrier gas at a flow rate of 2 ml/min. The GC was linked via a direct inlet to a Kratos MS25 RFA mass spectrometer coupled to a Data General DG 30 data system. The mass spectrometer was operated in the electron impact mode at an ionizing voltage of 4 kV. The total ion current between *m/z* 30 and 200 was monitored by scanning at 1 sec/decade. The presence of MeCl was confirmed by the acquisition of a mass spectrum typical of the compound at the normal retention time. The limit of detection was *ca* 100 ng/vial.

For confirmation of the presence of methyl esters of various aromatic acids in the cultures the following procedure was adopted. The fungus was cultured at 25° on the filter paper medium (or, if no growth was exhibited by the species on this substrate, malt extract medium) in 40 ml vials with loosely fitting screw caps. After 2 or 3 weeks incubation the vial was attached to the Purge and Trap sampler and purged for 5 min at 80° with He at 20 ml/min. The trapped volatiles were desorped as described above on to a fused silica WCOT capillary column (25 m × 0.32 mm) with BP10 as bonded phase. On complete desorption the chromatograph oven was programmed from 30° to 250° at 10°/min at a He flow rate of 2 ml/min. The mass spectrometer was operated as described above except that the total ion current between *m/z* 30 and 300 was recorded. Methyl esters of benzoic, salicylic, 2- and 3-furoic and cinnamic acids were recorded as present if a typical mass spectrum was obtained at the expected retention time of the compound. The practical detection limit for the individual esters was 10 ng/vial.

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