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Anaerobic O-demethylation of chlorinated guaiacols by *Acetobacterium woodii* and *Eubacterium limosum*

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Abstract

The acetogenic bacteria *Eubacterium limosum* and *Acetobacterium woodii* are strict anaerobes with the ability to metabolize *O*-methyl substituents of aromatic compounds. In investigating the versatility of the O-demethylating activity of these acetogens, we examined the anaerobic O-demethylation of chlorinated guaiacols (2-methoxyphenols). Anaerobic cell suspensions of both *E. limosum* and *A. woodii* were able to O-demethylate di-, tri- and tetrachloroguaiacols to the corresponding catechols. The chlorocatechols accumulated and were not further metabolized. A chlorine substituent in the position *ortho* to the methoxyl-group hindered, but did not completely inhibit O-demethylation. Similar O-demethylation of chloroguaiacols has been observed in anaerobic sediments. The O-demethylating enzyme(s) of both strains seem to be fairly non-specific.

Abbreviations: DCC – Dichlorocatechol; DCG – Dichloroguaiacol; TCC – Trichlorocatechol; TCG – Trichloroguaiacol; TeCC – Tretrachlorocatechol; TeCG – Tetrachloroguaiacol

Introduction

A range of chlorinated aromatic compounds are produced during chlorine bleaching of pulp and include chlorinated phenolic compounds, such as chloroguaiacols (2-methoxyphenols) (for references see Häggblom et al. 1986; Häggblom & Salkinoja-Salonen 1991). Approximately 100 to 300 g of chlorinated phenolic compounds arise per ton of conventionally chlorine-bleached pulp (Salkinoja-Salonen et al. 1981). These compounds are poorly degraded in existing biological treatment plants (Leuenberger et al. 1985; Bryant & Amy 1989; Gergov et al. 1988), and are thus discharged with the bleaching effluents into receiving waters. Chlorinated guaiacols have been detected in water samples from areas receiving pulp-bleaching effluents at concentrations of several µg/l (Salkinoja-Salonen et al. 1981; Paasivirta et al. 1985; Xie et al. 1986), and accumulate in sediments where they may attain concentrations of several milligrams per kg of dry weight (Salkinoja-Salonen et al. 1981; Xie et al. 1986).

Under aerobic conditions polychlorinated guaiacols have been shown to be degraded by strains of

Rhodococcus chlorophenolicus and by a strain of *Mycobacterium fortuitum* via initial hydroxylation to chlorinated *para*-hydroquinones (Häggblom et al. 1986, 1988a, b). Additionally, chlorinated guaiacols undergo O-methylation to chloroveratroles in aerobic sediments (Remberger et al. 1986) and this biotransformation has been shown to be catalyzed by several aerobic bacterial species (Allard et al. 1985; Neilson et al. 1983; Häggblom et al. 1988b). However, there is limited information on the anaerobic degradation or transformation of chlorinated guaiacols. Chloroguaiacols were shown to be removed during anaerobic treatment of pulp bleaching effluents (Häggblom & Salkinoja-Salonen 1991) and O-demethylation to chlorocatechols has been shown to occur in anaerobic sediments and enrichment cultures (Remberger et al. 1986; Neilson et al. 1987; Allard et al. 1991) as well as in an anaerobic sludge blanket reactor (Woods et al. 1989). Chlorocatechols tend to be recalcitrant, although in some cases they have been reported to undergo further anaerobic reductive dechlorination (Neilson et al. 1987; Allard et al. 1991, 1992; Woods et al. 1989). A hypothetical 'guaiacol cycle' involving anaerobic O-demethylation and aerobic O-methylation has been proposed to explain the fate of chloroguaiacols in aquatic sediments (Remberger et al. 1986).

Eubacterium limosum and *Acetobacterium woodii* are among the acetogenic bacteria able to O-demethylate the methoxyl substituents of various aromatic compounds and use the methyl group as a C-one carbon source (Bache & Pfennig 1981; Genthner & Bryant 1987; Young & Frazer 1987; DeWeerd et al. 1988; Cocaign et al. 1991). *A. woodii* has also been shown to catalyze dechlorination of tri- and tetrachloromethane (Egli et al. 1990). However, transformation of chlorinated methoxylated aromatic compounds by acetogens has not been studied. In investigating the fate of chlorinated aromatic compounds in anaerobic environments we examined whether these acetogenic bacteria are able to transform chlorinated guaiacols.

Materials and methods

Culture conditions and experimental setup

Cultures were grown under strict anaerobic conditions in a defined mineral salts medium (Berman & Frazer 1992) under an atmosphere of N_2/CO_2 (70%/30%). *Acetobacterium woodii* (Type strain, ATCC 29683) was grown on ferulate (2.0 mM) at 30° C and *Eubacterium limosum* (Type strain, ATCC 8486) was grown on guaiacol (2.0 mM) or glucose (0.2% wt/vol) at 37° C.

Experiments with chloroguaiacols were done with resting cell suspensions. Cells were harvested by anaerobic centrifugation (11000× g, 40 min, 4° C; JA-10 rotor, Beckman J-21C centrifuge, San Ramon, CA) and resuspended in fresh medium to an optical density of 0.8–1.0 units at 600 nm in an anaerobic glove box with an atmosphere of N_2/H_2 (97:3 vol/vol). The cell suspensions were divided into aliquots of 5 or 10 ml, fed one of the chloroguaiacol isomers at a concentration of 100 µM, and sealed with teflon coated butyl rubber stoppers and aluminum crimp seals. Induction of O-demethylation by guaiacol in *E. limosum* was studied with similarly prepared resting cell suspensions at an optical density of 0.3 units. All incubation mixtures were established in duplicate with uninoculated media as controls. Cultures were incubated at 30° C without shaking, in the dark. Transformation rates were determined by linear regression analysis from the change in substrate concentration over time.

Induction experiments with *E. limosum* were performed with cells grown on glucose. Cells were harvested and resuspended in fresh medium as described above and guaiacol added to 1 mM. To inhibit protein synthesis streptomycin was added to a concentration of 1 mg/ml. This concentration inhibited growth on glucose.

Chemicals

Ferulic acid (3,4-dimethoxycinnamic acid), catechol and streptomycin were from Sigma Chemical Co. (St. Louis, MO, USA), guaiacol was from Fluka AG (Buchs, Switzerland), and tetrachlorocatechol

and 2,4,6-tribromophenol (used as an internal standard in gas-liquid chromatography [GLC] analysis) were from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chlorinated guaiacols and catechols used in this study were synthesized by J. Knuutinen (University of Jyväskylä, Jyväskylä, Finland) as described (Knuutinen 1984). One of the dichloroguaiacol preparations was a mixture (wt/wt) of 4,6-DCG (70%) and 3,5-DCG (30%), designated 4,6/3,5-DCG. One of the trichloroguaiacol preparations was a mixture (wt/wt) of 3, 4, 6-TCG (50%) and 3, 5, 6-TCG (50%), designated 3, 4, 6/3, 5, 6-TCG. These two trichloroguaiacols coeluted in GLC analysis and could not be quantified separately (Häggblom et al. 1988b). All other guaiacols and chlorocatechols were of >99% purity.

Analysis

Liquid samples were taken periodically for analysis with sterile syringes, which had been flushed with N_2/CO_2. Guaiacol, catechol and ferulate were monitored by high-performance liquid chromatography. Samples (0.5 to 1.0 ml) were centrifuged and filtered (0.45 µm; Millipore, Bedford, MA, USA). Analyses were performed with a Beckman 332 LC chromatograph or a Beckman Bioseparation Gradient System (NEC PC-8201 controller, Model 126 Solvent Delivery Module, Model 166 Programmable Detector Module) (Beckman Instruments, Palo Alto, CA, USA) equipped with an automatic injector (Model 231-401, Autosampling Injector, Gilson, Medical Electronics, Middelton, WI, USA) and a Spherisorb C-18 column (250× 4.6 nm, particle size 5 µm; Supelco Inc., Bellefonte, PA, USA), with UV detection at 280 nm, and using a solvent system of 60% 5 mM aqueous formic acid and 40% of 5:1 mixture (vol/vol) of methanol/acetonitrile at a flow rate of 1 ml/min. Data was collected and analyzed on a Chromjet integrator (Spectra Physics, San Jose, CA, USA). Compounds were quantified by comparison of their retention times and peak areas to authentic reference compounds.

Chlorinated guaiacols and catechols were derivatized by acetylation in buffer solution with 2, 4, 6-tribromophenol as an internal standard as previously described (Häggblom et al. 1986, 1988b). Acetylated derivatives were analyzed by GLC using a HP 5890 Series II gas chromatograph (Hewlett Packard, Co., Palo Alto, CA, USA) equipped with a HP 7673A Automatic Injector, an electron capture ^{63}Ni detector, and a DB-5 capillary column (30 m × 0.32 mm, film thickness 0.25 µm; J&W Scientific, Folsom, CA, USA), with data analysis using the HP 3365 ChemStation software. The relative retention times of chloroguaiacols and chlorocatechols using comparable capillary columns are listed in Häggblom et al. (1988b).

Results and discussion

O-Demethylation of di-, tri and tetrachloroguaiacols by E. limosum *and* A. woodii

For studying the substrate specificity of O-demethylation, *E. limosum* and *A. woodii* were grown on guaiacol and ferulate, respectively, and were thus induced for O-demethylation. The O-demethylation of the chloroguaiacols by resting cell suspensions of *E. limosum* and *A. woodii* is shown in Figs 1A and B, respectively. For *E. limosum,* 20 to 95% of the chloroguaiacols was transformed after 5 hours with formation of nearly stoichiometric amounts of the corresponding chlorocatechol. The cultures of *A. woodii* showed less activity, with 5 to 60% of the chloroguaiacols transformed with stoichiometric accumulation of the corresponding chlorocatechol. The chloroguaiacols were stable in media controls, with less than 3% loss observed in 23 hours. Both *E. limosum* and *A. woodii* were thus able to O-demethylate all di-, tri- and tetrachloroguaiacols studied. In general the dichloroguaiacols were transformed more readily than the trichloroguaiacols or TeCG. O-Demethylating activity was highest with 4,5-DCG. *E. limosum* and *A. woodii* also O-demethylated guaiacol to catechol, with 100% and 59%, respectively, of guaiacol transformed in 5 h (results not shown).

A further examination of the effect of the position of the chlorine substituents on the O-demethylating system can be seen in Fig. 2, in which the mixture of 3,5-DCG and 4,6-DCG is transformed to

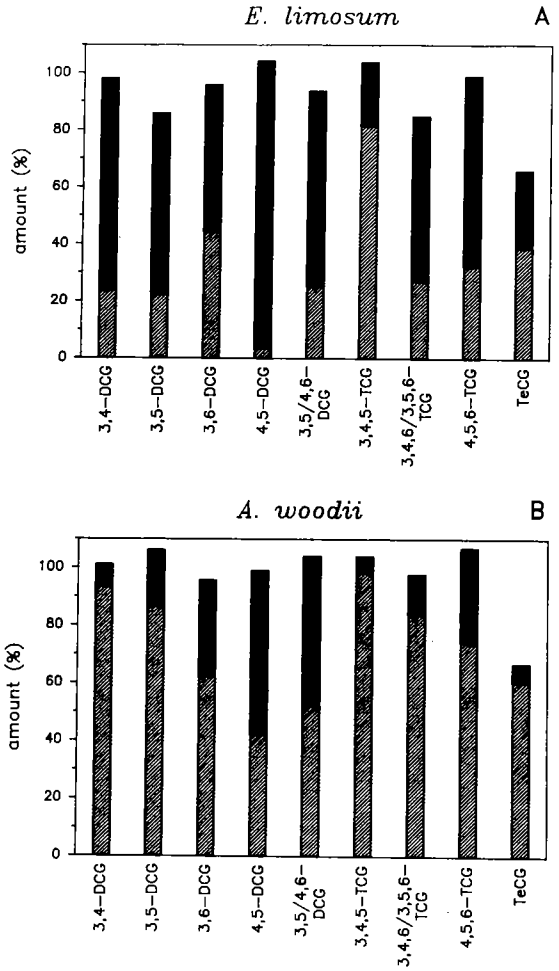

Fig. 1. O-demethylation of chloroguaiacols by induced cells of *E. limosum* (A) or *A. woodii* (B). Bars indicate the percent of the initial substrate recovered as chloroguaiacol (▨) or chlorocatechol (■) after 5 hours.

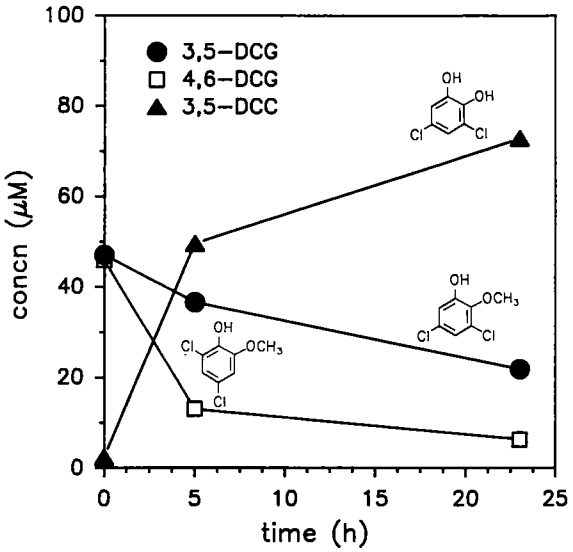

Fig. 2. O-demethylation of a mixture of 3,5-DCG and 4,6-DCG by induced cells of *A. woodii.*

3,5-DCC by induced cells of *A. woodii.* 4,6-DCG was rapidly depleted from an initial concentration of 45 μM to 15 μM in 5 hours, after which very little transformation occurred. Removal of 3,5-DCG was slower with only 50% depleted in 24 hours. The O-demethylation product of both dichloroguaiacols is 3,5-DCC, which accumulated to nearly stoichiometric amounts, and was not transformed further. The faster transformation of 4,6-DCG suggests that a chlorine substituent next to the methoxyl group hinders O-demethylation. This is also seen when comparing the rate and extent of O-demethylation of the trichloroguaiacols 3,4,5-TCG and 4,5,6-TCG by *A. woodii.* Only 5% of 3, 4, 5-TCG was O-demethylated to 3,4,5-TCC in 23 hours, while 30% of 4,5,6-TCG was transformed to 3,4,5-TCC in 5 hours (Fig. 1B). A similar substrate specificity was also observed with *E. limosum* (Fig. 1A).

Various methoxy-substituted aromatic acids have been found to serve as substrates for anaerobic O-demethylation and a number of bacterial strains seem to have a broad substrate specificity (Bache and Pfennig 1981; Deweerd et al. 1988; Wu et al. 1988; Daniel et al. 1991). Sequential O-demethylation has also been reported with multi-methoxylated compounds (Cocaign et al. 1991; Daniel et al. 1991). Whether the broad substrate range is due to multiple enzymes or a broad enzyme specificity is not clear. Anaerobic O-demethylation of methoxyphenols has not been extensively studied and has previously been reported only for *C. thermoaceticum* (Wu et al. 1988; Daniel et al. 1991) and a strain tentatively identified as *Acetobacterium* sp. (Sembiring & Winter 1990). We found that *E. limosum* and *A. woodii* O-demethylated both guaiacol and chloroguaiacols, although the *A. woodii* strain was unable to grow on guaiacol (2 mM), perhaps due to guaiacol toxicity. To our knowledge this is the first report on anaerobic O-demethylation of chlorinated substrates by pure cultures. Anaerobic cell suspensions of *A. woodii* and *E. limosum* O-demethy-

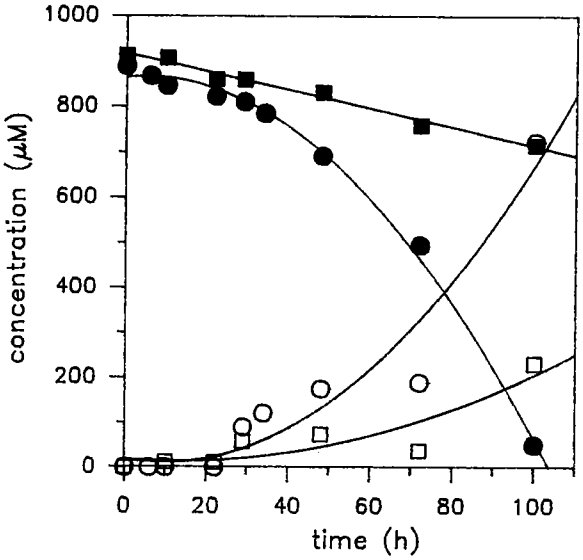

Fig. 3. Transformation of guaiacol by glucose grown cells of *E. limosum* in the presence (squares) or absence (circles) of streptomycin (1mg/ml). Symbols: guaiacol (■ ●), catechol (□ ○).

lated to a different degree all the di-, tri- and tetrachloroguaiacols studied with formation of the corresponding chlorocatechols (Fig. 1A and B). It appears that a chlorine substituent next to the methoxyl group hinders O-demethylation (Fig. 2). The effect of other substituents on O-demethylation of aromatics has not been systematically studied.

Constitutive and induced chloroguaiacol O-demethylation in E. limosum

Previous experiments (Berman, unpublished) had shown that O-demethylating activity in *E. limosum* was induced by veratric acid (3,4-dimethoxybenzoic acid) and that guaiacol as a sole carbon source supported growth. As seen from Fig. 3 guaiacol induced O-demethylation in glucose grown cells within 30 hours. Streptomycin (1mg/ml) inhibited induction, but a low constitutive level of O-demethylation was observed.

To study the rates of O-demethylation of chloroguaiacols, *E. limosum* was grown on either guaiacol (induced) or glucose (non-induced) and transformation of chloroguaiacols was studied. Cells were

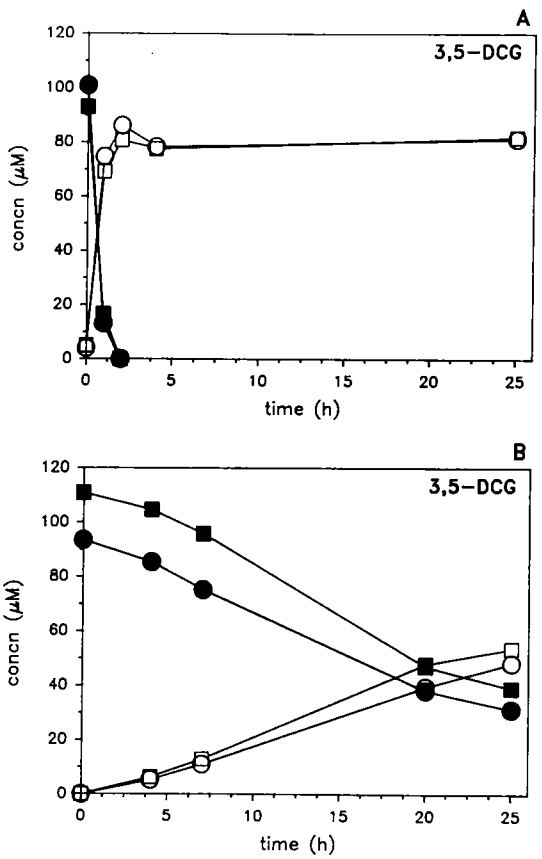

Fig. 4. O-demethylation of 3,5-DCG to 3,5-DCC by (A) induced (guaiacol grown) or (B) non-induced (glucose grown) cells of *E. limosum* in the presence or absence of streptomycin. Symbols: 3,5-DCG (■ ●) to 3,5-DCC (□ ○), with streptomycin (circles), without streptomycin (squares).

harvested, resuspended in fresh medium and 3,5-DCG or TeCG added to 100μM in either the absence or presence of streptomycin (0.5mg/ml) to inhibit protein synthesis. Cells grown on guaiacol rapidly O-demethylated 3,5-DCG to 3,5-DCC without a lag (Fig. 4, Table 1). Streptomycin did not inhibit O-demethylation by induced cells. 3,5-DCG was completely transformed in 2 hours at a rate of 77±4 and 88±12μmol l^{-1} h^{-1} in the absence and presence of streptomycin, respectively (Fig. 4A). Uninduced cells grown on glucose expressed a low O-demethylating activity, and slowly transformed 3,5-DCG. Approximately 60% of 3,5-DCG was O-demethylated to 3,5-DCC in 25 hours (Fig. 4B). The addition of streptomycin did not inhibit this low level of O-demethylation. No induction of the aryl O-demeth-

ylating system in the cell suspensions of *E. limosum* was observed during the 25 h incubation.

The rates of O-demethylation of 3,5-DCG and TeCG by induced and non-induced cell of *E. limosum* are listed in Table 1. Guaiacol grown cells were distributed uniformly into separate vessels. These cell suspensions completely transformed 100 µM TeCG to TeCC without a lag in 4 hours and as observed for 3,5-DCG, with comparable rates of O-demethylation observed in the presence or absence of streptomycin. However, as seen earlier (Fig. 1) TeCG was a poorer substrate. Uninduced cells, grown on glucose, showed only a low level of O-demethylation, with approximately 20% of TeCG transformed to TeCC in 25 hours, at a rate of 1.3± 0.1 µmol l^{-1} h^{-1} both in the absence and presence of streptomycin.

Our results indicated that *E. limosum* had a low constitutive level of O-demethylating enzymes showing activity with both 3,5-DCG and TeCG. DeWeerd et al. (1988) showed that O-demethylation was induced by methoxylated aromatic acids. We found that guaiacol also acted as an inducer and served as a growth substrate for *E. limosum.* A 20-fold or more difference in the rate of O-demethylation of chloroguaiacols was observed between induced and uninduced cells of *E. limosum* (Fig. 4, Table 1).

The ability to use *O*-methyl substituents of aromatic compounds as a C-one carbon source has been found in numerous acetogenic bacteria, including *Acetobacterium, Eubacterium, Clostridium, Sporomusa* and *Syntrophococcus* (for review see Young & Frazer 1987). Strains capable of anaerobic O-demethylation have been isolated from a variety of habitats. The biochemical mechanism for O-demethylation is not yet characterized, although Berman and Frazer (1992) showed that the O-demethylating system in cell extracts of *A. woodii* is dependent on tetrahydrofolate and ATP, which may be co-substrates in the aryl O-demethylation reaction. Different biochemical features may apply to the O-demethylating activities studied in cell extracts of *Syntrophococcus sucromutans* (Doré & Bryant 1990) and *Clostridium thermoaceticum* (Wu et al. 1988; Daniel et al. 1991).

Even though acetogens attack a variety of ring substituents, they do not cleave the aromatic ring. No further transformation of the chlorocatechols was observed with the pure cultures we studied. However, dechlorination of chlorocatechols has been observed in anaerobic sediments and enrichment cultures (Neilson et al. 1987; Remberger et al. 1986; Allard et al. 1991, 1992). DeWeerd et al. (1986) showed that O-demethylation and dechlorination activities in *Desulfomonile tiedjei* DCB-1 were not related, and that *E. limosum* grown on methoxylated benzoates was not able to transform chlorobenzoates. It seems that anaerobic reductive dechlorination is fairly substrate specific, while aryl O-demethylating enzyme(s) have a broad substrate range. Our results suggest that the observed O-demethylation of chloroguaiacols occurring in anaerobic sediments (Neilson et al. 1987; Remberger et al. 1986; Häggblom & Berman, unpublished results) may be mediated by acetogenic bacteria.

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Table 1. Effect of induction on O-demethylation of 3,5-DCG and TeCG by *E. limosum.*

Substrate	Rate of transformation (µmol l^{-1} h^{-1})[a]			
	Induced		Non-induced	
	– streptomycin	+ streptomycin	– streptomycin	+ streptomycin
3,5-DCG	77± 4	88± 12	3.1± 0.002	2.6± 0.1
TeCG	27	25± 0.4	1.3± 0.1	1.3± 0.1

[a] All data from duplicate cultures except for TeCG with induced cells in the absence of streptomycin.

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