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Oxidation of both termini of *p*- and *m*-xylene by *Escherichia coli* transformed with xylene monooxygenase gene

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Abstract

Xylene monooxygenase (XMO) from *Pseudomonas putida* mt-2 catalyzes oxidation of methyl group of toluene and xylenes. While it has been postulated that this enzyme oxidizes one methyl group of xylene, we observed that both methyl groups in *p*- and *m*-xylene were oxidized to alcohol and aldehyde when the relevant genes (*xylM* and *xylA*) were co-expressed in *Escherichia coli* C600 and MC4100. When *p*-xylene was used as a substrate, *p*-hydroxymethylbenzaldehyde and *p*-xylyleneglycol were identified, in addition to *p*-methylbenzylalcohol and *p*-tolualdehyde. When *m*-xylene was used as a substrate, *m*-hydroxymethylbenzaldehyde and *m*-xylyleneglycol were identified, in addition to *m*-methylbenzylalcohol and *m*-tolualdehyde. Ratio of the products varied significantly according to the reaction condition and host strain, presumably reflecting the relative activity of XMO and host-derived dehydrogenase(s). Using various oxidized compounds as substrates, it was indicated that dialcohol (*p*- or *m*-xylyleneglycol) was formed via *p*- or *m*-hydroxymethylbenzaldehyde, respectively, rather than directly from corresponding monoalcohol (*p*- or *m*-methybenzylalcohol).

Keywords: Xylene monooxygenase; *Pseudomonas putida*; Xylene; Xylyleneglycol; Hydroxymethylbenzaldehyde

1. Introduction

Monooxygenase-catalyzed oxidation is often a primary and rate-limiting step in degradation of various inactive hydrocarbons by aerobic microorganisms. Among handful of monooxygenase systems xylene monooxygenase (XMO) system can be characterized by its two-component property, one is a membrane-integrated hydroxylase component (XylM) and the other is a cytoplasmic soluble NADH:acceptor reductase component (XylA). Genes for these component proteins were identified in *Pseudomonas putida* mt-2 [1–4] and other gram-negative bacteria such as *Sphingomonas aromaticivorans* F199 [5] and *P. putida* CM23 [6].

Most extensively studied XMO from *P. putida* mt-2, either as native form or as recombinant form expressed in *Escherichia coli*, exhibits rather wide substrate specificity in that it acts on various methyl-substituted aromatic hydrocarbons and styrene to give corresponding alcohol/aldehyde and styrene oxide, respectively [7,8]. Most favorable substrates are *p*- and *m*-xylene, followed by toluene [8]. In the case of toluene used as a substrate, product benzylalcohol was shown to undergo further oxidation to give benzaldehyde. In the course of second oxidation (oxygenation), transitional *gem*-diol formation was proposed

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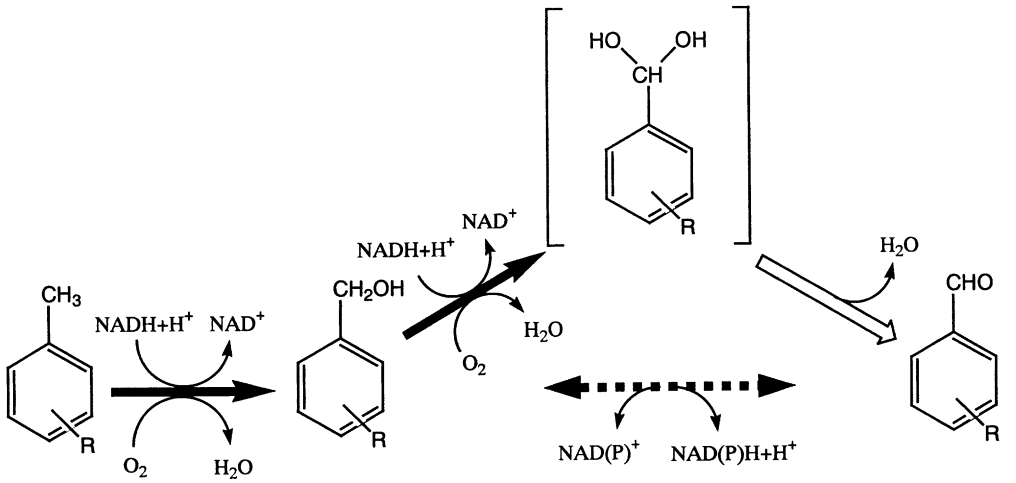


Scheme 1. Reactions carried out in the *xylMA*-transformed *E. coil* cells. Filled arrows indicate reactions catalyzed by *P putida* xylene monooxygenase (XMO). Open arrow indicates spontaneous dehydration reaction. Dashed arrow indicates reaction catalyzed by host dehydrogenase(s).

[9]. Further oxidation giving rise to benzoic acid was also reported [9,10]. The last step, formation of carboxylic acid, was observed only when the substrate hydrocarbon was nearly consumed [9,10]. In any case oxidation of only one methyl group in aromatic hydrocarbon has been identified.

Bioconversion using living microorganism (*E. coli* in this case) is expected to develop complex oxidation profile as the host cell has inherent dehydrogenase activities [9] (Scheme 1). In this report, we present evidence of XMO-catalyzed oxidation of both termini of *p*- and *m*-xylene, in conjunction with complex oxidation profile affected by host factors.

2. Experimental

2.1. Biological and chemical materials

P. putida mt-2 (ATCC 33015) was used as reference and source of XMO gene. *E. coli* strains C600 (ATCC 23724), MC4100 (ATCC 35695), JM109 (ATCC 53323), RB791 (ATCC 53622), JM101 (ATCC 33876) and W3110 (ATCC 27325) were used as hosts of recombinant plasmid.

Toluene, *p*- and *m*-xylene were purchased from Kanto Kagaku (Tokyo, Japan). Benzylalcohol, benzaldehyde, benzoic acid, *p*-, *m*-methylbenzylalcohol, *p*-tolualdehyde, *m*-tolualdehyde and *n*-dodecane were purchased from Wako Pure Chemical Industries (Osaka, Japan). *p*-, *m*-Xylyleneglycol, terephthalaldehyde, isophthalaldehyde, *p*- and *m*-toluic acid were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). *p*-Hydroxymethylbenzaldehyde and *m*-hydroxymethylbenzaldehyde were chemically synthesized by partial reduction of terephthalaldehyde and isophthalaldehyde, respectively, by adding $NaBH_4$ methanol solution. Yield of each compound after purification by silica gel chromatography was 61.3 and 62.5%, respectively.

2.2. Genetic engineering procedure

TOL plasmid was prepared from *P. putida* mt-2 by alkali-SDS procedure [11]. Genes coding for XMO (*xylM* and *xylA*) were PCR-amplified by DNA polymerase from *Pyrococcus furiosus*, following a procedure of Pyrobest (Takara Shuzo, Tokyo, Japan). Two oligodeoxyribonucleotides, d(ATGAATTCCATGGACACGCTTCGTTATTAC) and d(ACCTGCAGCTAGCAAGGAGGTCTATTAT), were used as primers (underlined; restriction endonuclease recognition sites). Amplified DNA fragment (2330 bp) was digested by *Nco*I and *Pst*I, and then inserted into

*Nco*I–*Pst*I site of pTrc99A [12], to give rise to pTrc99A–xylMA (xylMA; *xylM* plus *xylA* in natural colinear arrangement). *E. coli* cells were transformed by electroporation using MicroPulser (Bio-Lad Laboratories, Hercules, CA), following a procedure presented by the vendor.

2.3. General procedure for the enzymatic reaction

Transformed *E. coli* cells were cultured at 37 °C in 2xYT broth [11] containing 50 μg/ml carbenicillin and induced by 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) for 3 h. Reactions were conducted at 30 °C in horizontally laid 8 ml screw-cap glass vials containing 1 ml reaction mixture with reciprocal shaking at 280 strokes per min. Reaction mixture was composed of substrate (around 1.5 mM), washed cells (OD_{600} = 10), 1% glucose and 50 mM phosphate buffer (pH 7.4). Reaction was quenched by adding 80 μl of 10% perchloric acid on ice.

2.4. Analysis of the reaction mixture

To the quenched reaction mixture 500 mg NaCl and 1 ml diethylether containing 0.2 mM *n*-dodecane (as internal standard) were added. After vigorous shaking ether phase was recovered by centrifugation and then analyzed by gas chromatography and/or GC–MS. For GC analysis Hewlett Packard HP5890 gas chromatograph equipped with capillary column HP5 (cross-linked 5% PH ME silicone; length, 30 m; i.d., 0.32 mm; film thickness, 0.25 μm) was employed. Compounds were detected by a flame ionization detector. GC–MS analysis was carried out on Hewlett Packard G1800A GCD, equipped with the same column as mentioned above. Compounds were detected by an electron ionization detector.

3. Results

3.1. Selection of host strains

E. coli strains harboring pTrc99A–xylMA were analyzed with respect to the ability to oxidize toluene and xylenes. All of the tested transformants (C600-MA, MC4100-MA, JM109-MA and RB791-MA) exhibited production of corresponding monoalcohol after 30 min reaction (Fig. 1). Monoaldehyde was also detected in C600-MA and MC4100-MA. In a separate experiment JM101-MA and W3110-MA were shown to oxidize toluene and *p*-xylene in a comparable degree to JM109-MA (data not shown). C600-MA and MC4100-MA were then selected for further analyses.

3.2. Identification of further oxidized compounds

In a prolonged reaction using *p*- or *m*-xylene as a substrate, we observed several additional peaks on gas chromatography. In C600-MA and MC4100-MA-catalyzed reactions, these peaks were identified by GC–MS as follows: *p*-hydroxymethylbenzaldehyde and *p*-xylyleneglycol (*p*-xylene as a substrate), and *m*-hydroxymethylbenzaldehyde and *m*-xylyleneglycol (*m*-xylene as a substrate). Trace amounts of carboxylic acids (*p*- and *m*-methylbenzoic acid) were also detected (data not shown).

3.3. Reaction of p-xylene and its oxidized derivatives

To elucidate the oxidation route(s) of *p*-xylene, various oxidized compounds in addition to *p*-xylene were used as substrates (Fig. 2). Influence of host factors was assessed by inclusion of C600-V and MC4100-V (vacant cells, i.e. transformed with pTrc99A). Analysis of the compounds formed in the course of 1 and 2 h reactions showed complex time-dependent product profile (Fig. 2A and B). C600-V and MC4100-V exhibited significant reducing activities, converting *p*-tolualdehyde (–CHO) to *p*-methylbenzylalcohol (–OH) (Sections 5 and 6) and terephthalaldehyde [$(–CHO)_2$] to *p*-xylyleneglycol [$(–OH)_2$] via *p*-hydroxymethylbenzaldehyde [(–OH)(–CHO)] (Sections 9 and 10) (Fig. 2C and D). In both C600-MA and MC4100-MA, *p*-xylyleneglycol was formed likely from *p*-hydroxymethylbenzaldehyde, not from *p*-methylbenzylalcohol (Sections 3–6). A separate experiment with shorter reaction period (10 min) using C600-MA as a catalyst and *p*-hydroxymethylbenzaldehyde as a substrate, terephthalaldehyde (0.15 mM) was detected, along with *p*-hydroxymethylbenzaldehyde (0.76 mM). These findings were interpreted as is shown in Scheme 2. Assuming that NAD(P)-dependent dehydrogenase catalyzes reversible reaction and that the

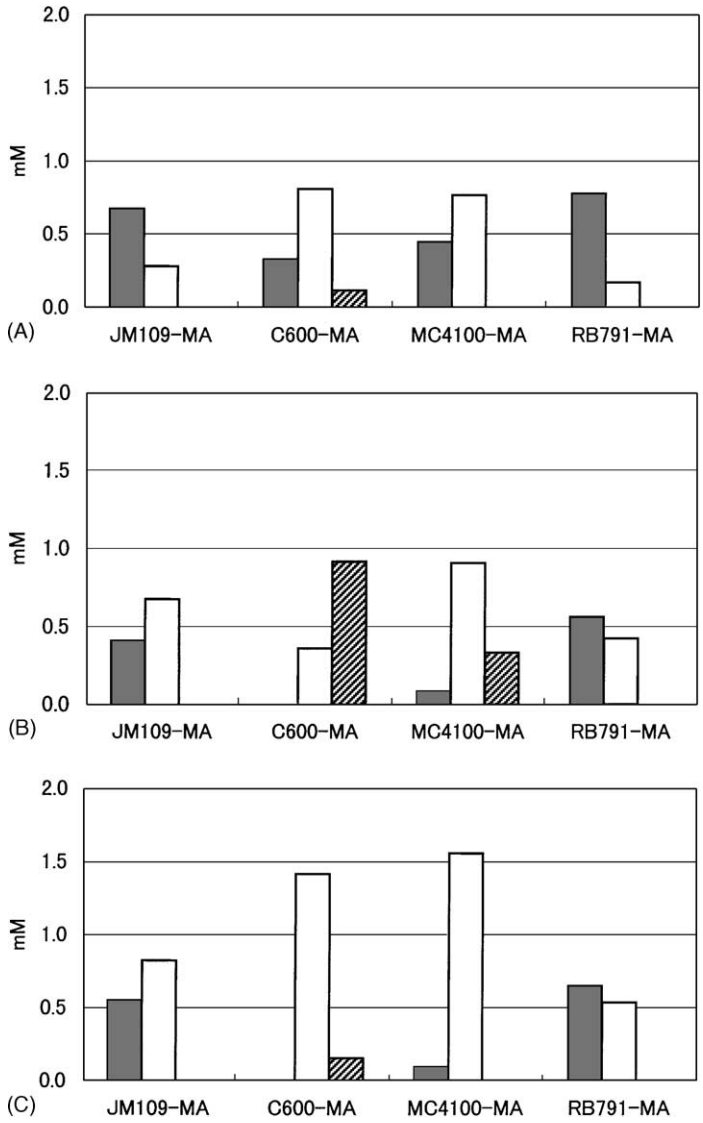


Fig. 1. Oxidation of toluene, *p*- and *m*-xylene by *xylMA*-transformed *E. coil* strains. (A) Toluene was oxidized by *xylMA*-transformed JM109, C600, MC4100 and RB791. Amounts of toluene (filled bar), benzylalcohol (open bar) and benzaldehyde (hatched bar) were quantified by measuring gas chromatography area. (B) *p*-Xylene was oxidized by various *E. coil* strains expressing *xylMA*. Amounts of *p*-xylene (filled bar), *p*-methylbenzylalcohol (open bar) and *p*-tolualdehyde (hatched bar) are shown. (C) *m*-Xylene was oxidized by various *E. coli* strains expressing *xylMA*. Amounts of *m*-xylene (filled bar), *m*-methylbenzylalcohol (open bar) and *m*-tolualdehyde (hatched bar) are shown.

equilibrium between alcohol and aldehyde is inclined to alcohol (NAD/NADH ratio is 10.6 under aerobic conditions with glucose excess) [9,13], host-derived dehydrogenase(s) most likely gives a net effect to convert aldehyde to alcohol.

3.4. Reaction of m-xylene and its oxidized derivatives

Similar analyses were carried out for the *m*-xylene oxidation (Fig. 3). Again in this case significant

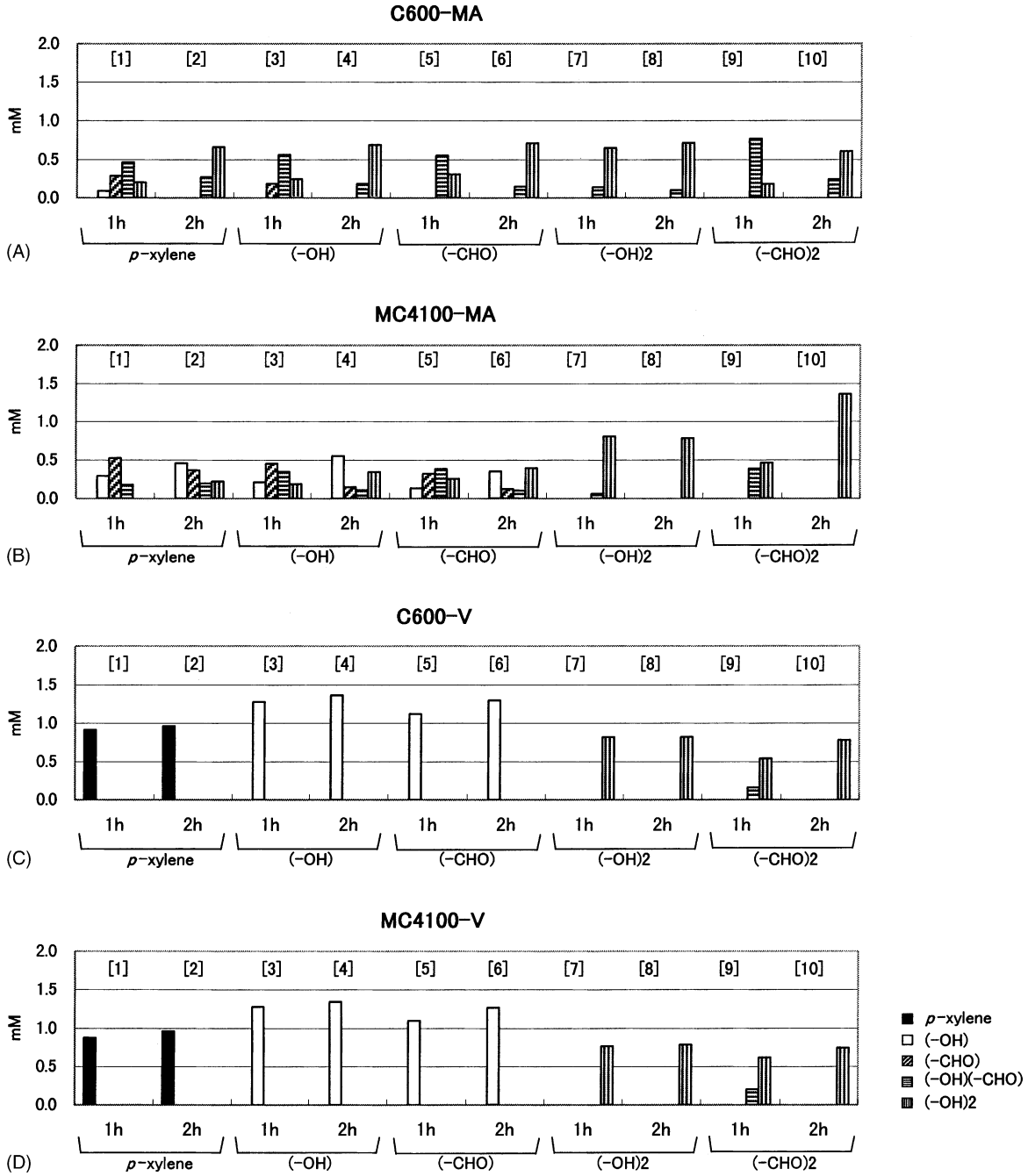
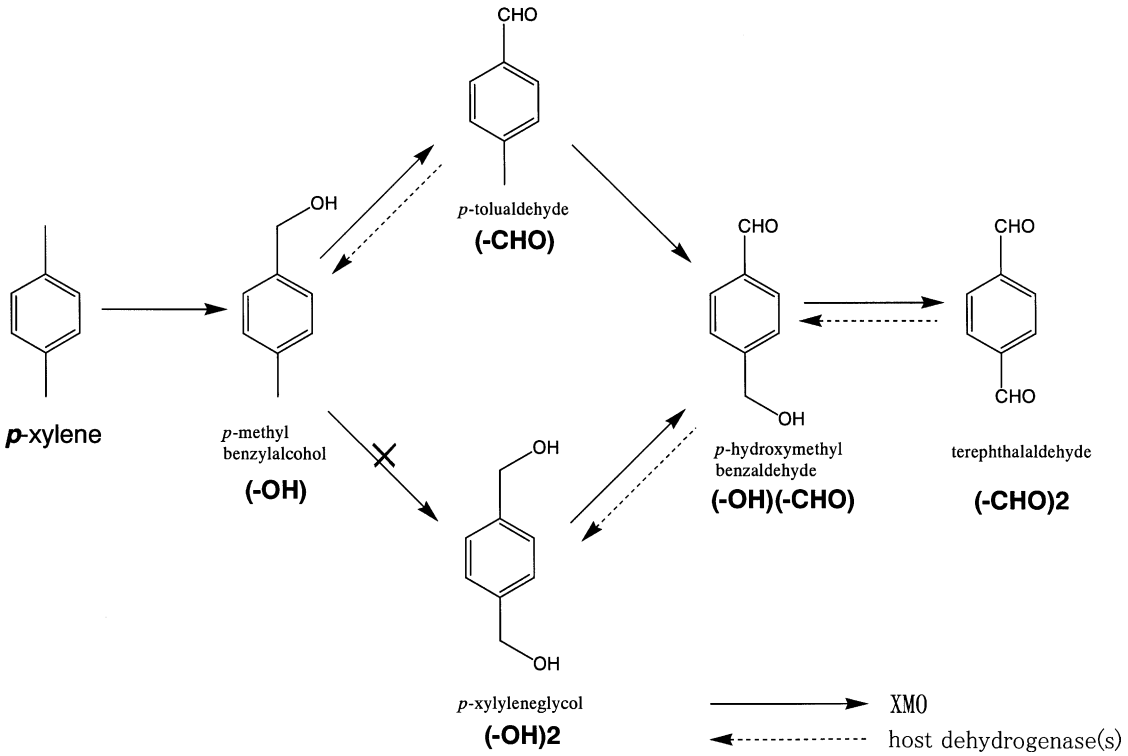


Fig. 2. Oxidation of *p*-xylene and related (oxidized) compounds by *xylMA*-transformed *E. coil* cells (C600-MA and MC4100-MA) as compared with cells without *xylMA* (vacant cells; C600-V and MC4100-V). *p*-Xylene, *p*-methylbenzylalcohol (–OH), *p*-tolualdehyde (–CHO), *p*-xylyleneglycol [$(–OH)_2$] and terephthalaldehyde [$(–CHO)_2$] were used as substrates. After 1 and 2 h reaction periods *p*-xylene (filled bar), *p*-methylbenzylalcohol (open bar), *p*-tolualdehyde (hatched bar), *p*-hydroxymethylbenzaldehyde (bar with horizontal stripe) and *p*-xylyleneglycol (bar with vertical stripe) were analyzed.



Scheme 2. Proposed oxidation route of *p*-xylene and related compounds.

reducing activities were observed in C600-V and MC4100-V, converting *m*-tolualdehyde (–CHO) to *m*-methylbenzylalcohol (–OH) (Sections 5 and 6) and isophthalaldehyde [$(–CHO)_2$] to *m*-xylyleneglycol [$(–OH)_2$] (Sections 9 and 10) (Fig. 3C and D). It was, however, not clear whether *m*-xylyleneglycol was formed from *m*-hydroxymethylbenzaldehyde or from *m*-methylbenzylalcohol.

Reactions with shorter incubation periods (10 and 30 min) were carried out to further analyze this oxidation route (Fig. 4). Sections 4–6 clearly showed that *m*-tolualdehyde and *m*-hydroxymethylbenzaldehyde were formed when *m*-methylbenzylalcohol was used as a substrate, indicating that *m*-xylyleneglycol was formed from *m*-hydroxymethylbenzaldehyde, not directly from *m*-methylbenzylalcohol. A separate experiment with 10-min reaction using C600-MA as a catalyst and *m*-hydroxymethylbenzaldehyde as a substrate, isophthalaldehyde (0.28 mM) was detected, along with *m*-hydroxymethylbenzaldehyde (0.61 mM). These findings were interpreted as is shown in Scheme 3. Also in this case host-derived dehydrogenase was expected to give a net effect to convert aldehyde to alcohol.

4. Discussion

We in this report demonstrated that *E coli* cells expressing XMO from *P. putida* mt-2 oxidizes both termini of *p*- and *m*-xylene. While methyl group of xylene is oxidized also by other enzymes such as methane monooxygenase from *Methylococcus capsulatus* (Bath) [14] and alkane hydroxylase (alkane-1-monooxygenase) from *Pseudomonas oleovorans* GPo1 [15], enzymatic oxidation of both termini of xylene has not been reported elsewhere. Among various oxidation products, *p*- and *m*-xylyleneglycol were of particular interest. Formation of *p*- and *m*-xylyleneglycol was found interplay of XMO and host-derived reducing

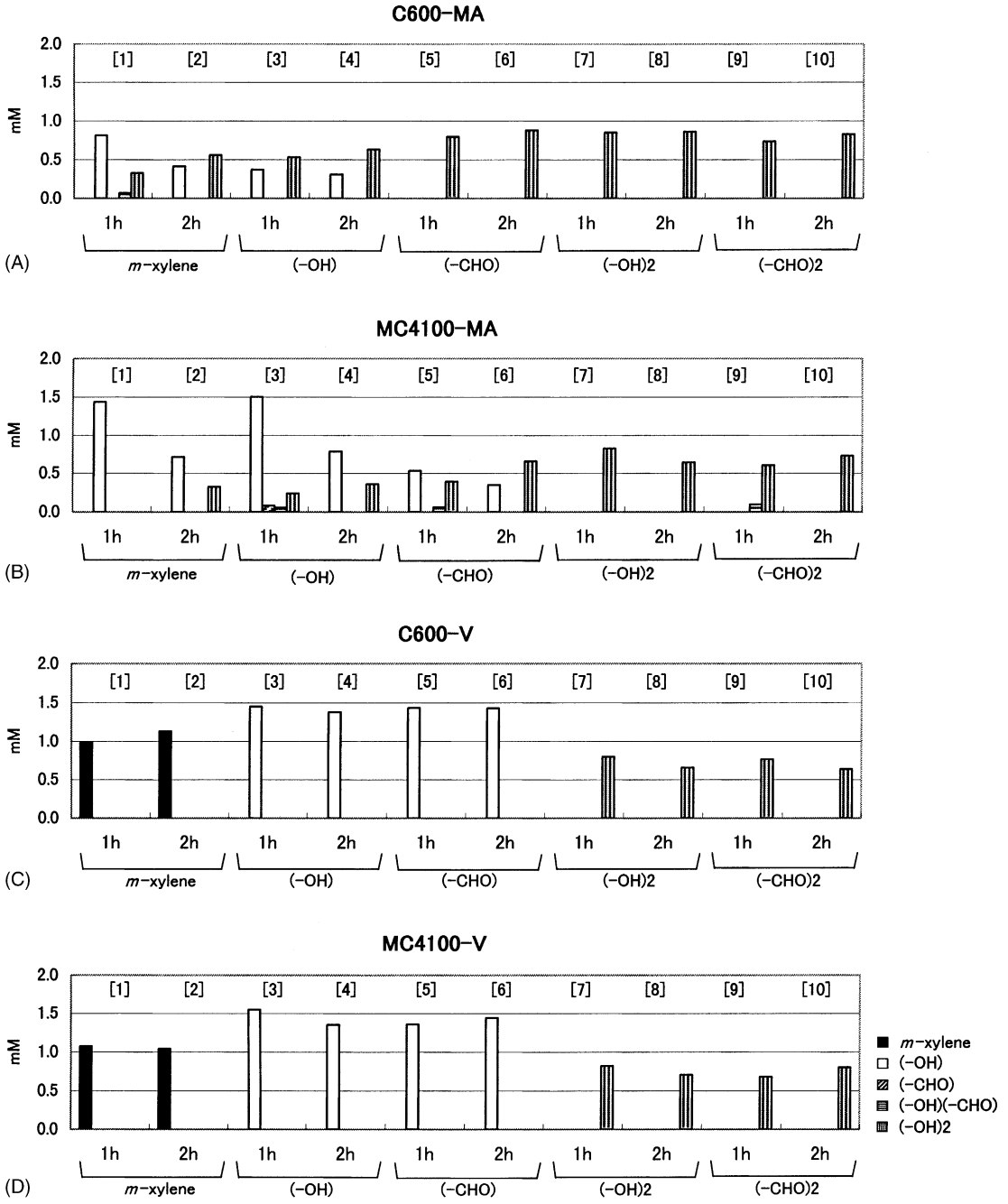


Fig. 3. Oxidation of *m*-xylene and related (oxidized) compounds by *xylMA*-transformed *E. coli* cells (C600-MA and MC4100-MA) as compared with cells without *xylMA* (vacant cells; C600-V and MC4100-V). *m*-Xylene, *m*-methylbenzylalcohol (–OH), *m*-tolualdehyde (–CHO), *m*-xylyleneglycol [$(–OH)_2$] and isophthalaldehyde [$(–CHO)_2$] were used as substrates. After 1 and 2 h reaction periods *m*-xylene (filled bar), *m*-methylbenzylalcohol (open bar), *m*-tolualdehyde (hatched bar), *m*-hydroxymethylbenzaldehyde (bar with horizontal stripe) and *m*-xylyleneglycol (bar with vertical stripe) were analyzed.

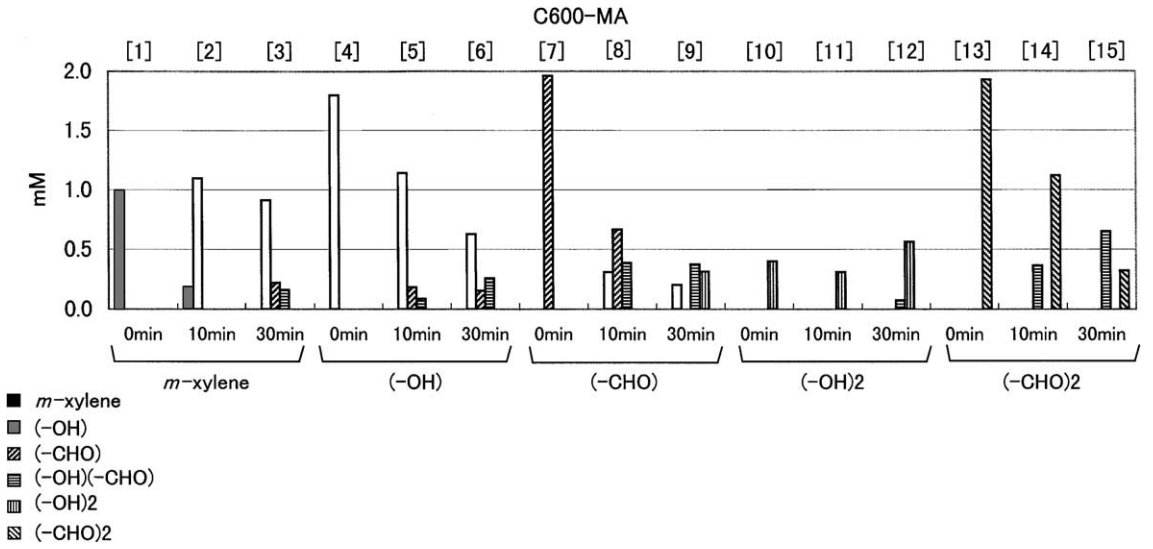
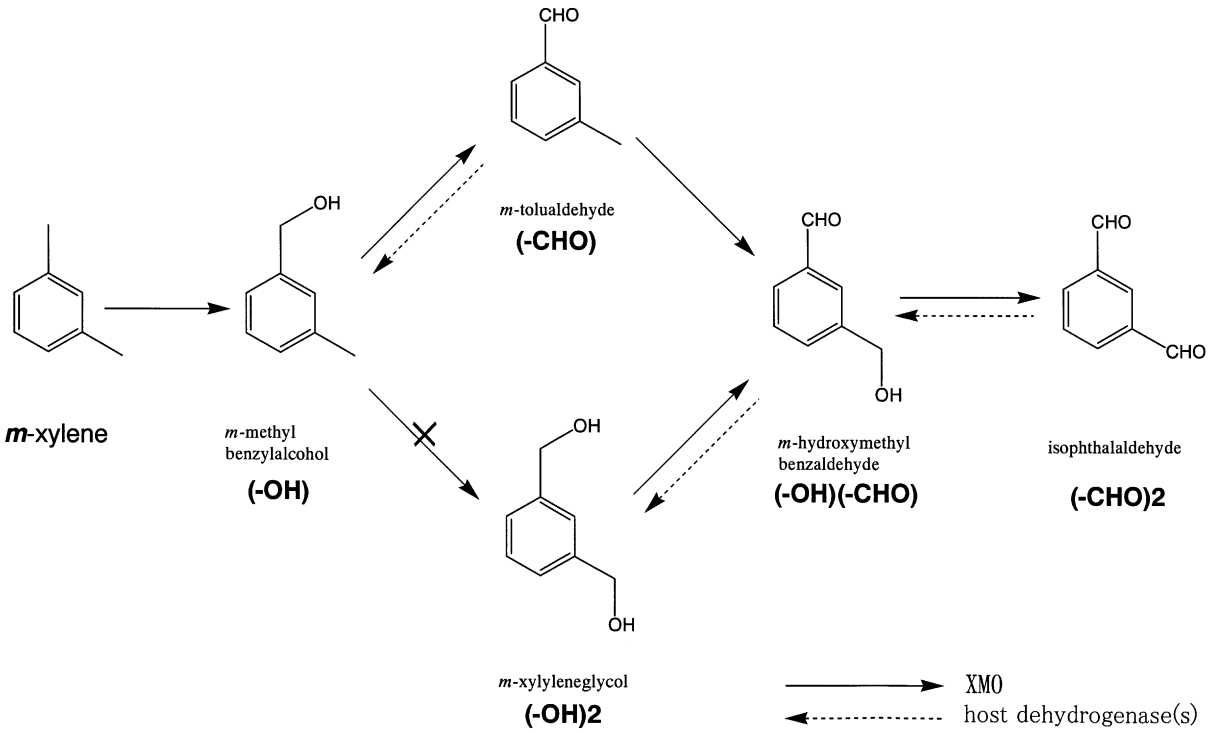


Fig. 4. Oxidation of *m*-xylene and related (oxidized) compounds by *xylMA*-transformed C600 with shorter reaction periods (10 and 30 min). Abbreviation is the same as in Fig. 3 except that another hatched bar representing isophthalaldehyde is included.



Scheme 3. Proposed oxidation route of *m*-xylene and related compounds.

activities. Our finding that these compounds were formed from *p*- or *m*-hydroxymethylbenzaldehyde, not from *p*- or *m*-methylbenzylalcohol, implies that XMO distinguish monoalcohol and monoaldehyde, the latter being accepted for oxidation of both termini. The other interesting aspect is that *p*- and *m*-xylyleneglycol are considered important building blocks in polymer industry. Biotechnological approach presented here may present alternative industrial synthetic routes.

In this study, we employed several *E. coli* host strains, among which C600 and MC4100 presented higher XMO activity than others. It was not possible to relate this fact to specific genetic marker(s) in each strain. Another important unsolved question is what host factor(s) is responsible for the reducing activities. Considering that *xylB* gene product, benzylalcohol dehydrogenase, is physiologically relevant in the toluene degradation pathway [1,7,9,16], *xylB* counterpart of *E. coli* is a likely candidate. Search of the genome database revealed that *adhC* gene product, a type III alcohol dehydrogenase [17], showed significant homology (amino acid identity, 30%) to *xylB* gene product from *P. putida* mt-2. Several less homologous alcohol dehydrogenase (candidate) ORFs in the *E. coli* genome were also identified (data not shown). Deduced amino acid sequence of *adhC* gene product was even more homologous to *xylB* gene product from *S. aromaticivorans* F199 [5] (amino acid identity, 33%). We can, therefore, speculate that *adhC* gene product is involved. This possibility can be tested by employing relevant mutant strain(s), which is not currently available.

Better understanding of the complex interplay of XMO and host-derived factors would lead to controlled oxidation profile and accumulation of desired products such as *p*- and *m*-xylyleneglycol.

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