AEM Accepted Manuscript Posted Online 2 September 2016
Appl. Environ. Microbiol. doi:10.1128/AEM.01907-
Copyright © 2016, American Society for Microbiology. All Rights Reserved.

Accepted Manuscript Posted Online
Applied and Environmental Microbiology
AEM
Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Transposon mutagenesis identifies genes critical for growth of *Pseudomonas nitroreducens* TX1 on octylphenol polyethoxylates

Tuan Ngoc Nguyen,[a] Chen-Wei Yeh,[b] Po-Chun Tsai,[a] Kyoung Lee,[c]# Shir-Ly Huang[a,d]#

Institute of Microbiology and Immunology, National Yang-Ming University, No. 155, Sec. 2, Li-Nong Street, Taipei 112, Taiwan[a]; Department of Life Sciences, National Central University, No.300, Jhongda Rd., Jhongli City, Taoyuan County 32001, Taiwan[b]; Department of Bio Health Science, Changwon National University, Kyongnam 51140, Republic of Korea[c]; Graduate Institute of Environmental Engineering, National Central University, No.300, Jhongda Rd., Jhongli City, Taoyuan County 32001, Taiwan[d].

Running title: *P. nitroreducens* TX1 genes needed for growth on $OPEO_n$

#Co-corresponding authors:

Shir-Ly Huang, Institute of Microbiology and Immunology, National Yang-Ming University, No. 155, Sec. 2, Li-Nong Street, Taipei 112, Taiwan. Tel.: +886-2-28267108; e-mail: sl.huang@ym.edu.tw.

Kyoung Lee, Department of Bio Health Science, Changwon National University, Changwon-si, Kyongnam 51140, Republic of Korea. Tel.: +82 552133486; Fax: +82 552133480; e-mail: klee@changwon.ac.kr

Accepted Manuscript Posted Online

Abstract

Pseudomonas nitroreducens TX1 is of special interest because of its ability to utilize 0.05% to 20% octylphenol polyethoxylates ($OPEO_n$) as sole carbon source. In this study, a library containing 30,000 Tn5-insertion mutants of the wild-type strain TX1 was constructed and screened for the $OPEO_n$ utilization and 93 mutants were found to be unable to grow on $OPEO_n$. In total 42 separate disrupted genes were identified and the proteins encoded by the genes were then classified into various categories, namely information storage and processing (14.3%), cellular processes and signaling (28.6%), metabolism (35.7%) and unknown proteins (21.4%). The individual deletion of genes encoding isocitrate lyase (*aceA*), malate synthase (*aceB*) and glycolate dehydrogenase (*glcE*) was carried out and the requirement for *aceA* and *aceB* but not *glcE* confirmed the role of the glyoxylate cycle in $OPEO_n$ degradation. Furthermore, acetaldehyde dehydrogenase and acetyl-CoA synthetase activity levels were 13.2 and 2.1 fold higher in TX1 cells grown on $OPEO_n$ than in TX1 cells grown on succinate, respectively. Growth of the various mutants on different carbon sources was tested and based on these findings a mechanism involving exo-scission to liberate acetaldehyde from the end of the $OPEO_n$ chain during degradation is proposed for the breakdown of $OPEO_n$.

Importance

Octylphenol polyethoxylates belongs to alkylphenol polyethoxylate ($APEO_n$) nonionic surfactant family. Evidence based on the analysis of intermediate metabolites suggested that the primary biodegradation of $APEO_n$ can be achieved by two possible pathways for the stepwise removal of the C_2 ethoxylate units from the end of the chain. However, direct evidence for these hypotheses is still lacking. In this study, we described the use of transposon mutagenesis to identify genes critical to the catabolism of $OPEO_n$ by *P. nitroreducens* TX1. The exo-scission of

Applied and Environmental Microbiology
AEM
Applied and Environmental Microbiology
Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

46 the ethoxylate chain leading to the liberation of acetaldehyde is proposed. Isocitrate lyase and
47 malate synthase in glyoxylate cycle are required in the catabolism of ethoxylated surfactants. Our
48 findings also provide many gene candidates that may help the elucidation of the mechanisms in
49 stress responses to ethoxylated surfactants by bacteria.

50

51

52

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

53 **Introduction**

54 Octylphenol polyethoxylates ($OPEO_n$, commercial name Triton X-100) is a nonionic
55 surfactant that belongs to the alkylphenol polyethoxylate ($APEO_n$) family. These surfactants are
56 used in a range of industrial and household products (1, 2). $APEO_n$ structures typically consist of
57 hydrophilic polyethoxylate chain bound to a hydrophobic moiety such as alkylphenol or a linear
58 primary/secondary alcohol (3). $APEO_n$ are often discharged into wastewater treatment plants or
59 into the environment, which leads to them ultimately being degraded into shorter exothylate (EO)
60 chains. Sometimes the chains are completely removed to form nonylphenol, octylphenol and
61 alkylphenol monoethoxylates to triethoxylates ($APEO_n$, n = 1~3) (4, 5). Many studies have
62 shown that these $APEO_n$ metabolites, which have increased hydrophobicity, are more toxic than
63 their parent compounds (6). In fact, these metabolites are able to mimic natural hormones thus
64 acting as endocrine disruptors when ingested by wildlife, which in turn can affect the
65 environment and ultimately human health (7).

66 The fate and degradability of $APEO_n$ in the environment have received much attention
67 (8). The biodegradation of $APEO_n$ has been studied using both pure and mixed cultures that grow
68 solely on $APEO_n$ and several bacterial strains have been reported as being able to degrade the EO
69 chains of $APEO_n$ (1, 8-12). Most such isolates are proteobacteria and are often members of the
70 genus *Pseudomonas*. Nguyen and Sigoillot (1997) isolated four *Pseudomonas* strains from
71 coastal seawater that grew on $OPEO_n$ and these generated OPEO with 4–5 units of EO chain as
72 the end products. John & White (1998) reported a strain of *P. putida* that grew on nonylphenol
73 polyethoxylate ($NPEO_n$) as sole carbon and in this case the final accumulating metabolite was
74 identified by gas chromatography-mass spectroscopy as $NPEO_2$. Nishio *et al.* (2002) isolated

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

eleven strains of $OPEO_n$-utilizing bacteria from paddy field soils. One strain, *P. putida* S-5, was shown to transform $OPEO_n$ to form $OPEO_2$ and $OPEO_3$, which were the dominant metabolites accumulating under aerobic conditions. Evidence based on analysis of intermediate metabolites has suggested that the primary biodegradation of $APEO_n$ under aerobic condition is achieved by a stepwise shortening of the EO chains (13-15). Two possible pathways for the stepwise removal of the C_2 ethoxylate units from the end of the chain have been proposed (**Fig. 1**). The first is a non-oxidative hydroxyl shift mechanism using ether scission that yields hemiacetal, which then produces acetaldehyde (13, 16). The second is the cleavage of $APEO_n$ by terminal carboxylation followed by hydrolysis to yield glycolate, which is oxidized to glyoxylate by glycolate dehydrogenase (16, 17). However, clear-cut evidence regarding the operation in organisms of these pathways during $OPEO_n$ degradation is still lacking.

In our previous studies, *P. nitroreducens* TX1, which possesses the ability to grow on $OPEO_n$ at wide range concentrations, was isolated (1, 9-12, 18). The strain is able to use 0.05% to 20% of $OPEO_n$ as a sole carbon source. The LC–MS analysis revealed that the ethoxylate chain was sequentially shortened from the hydroxyl terminal side in this strain (9). To elucidate the biodegradation mechanism of $OPEO_n$, a library containing 30,000 mutants of strain TX1 was prepared using Tn5 transposon insertion mutagenesis during this study. A total of 93 mutants were identified that were unable to grow on $OPEO_n$ and these were found to have disrupted 42 individual genes. In-frame deletion of some the target genes was then performed to confirm the roles of these genes in $OPEO_n$ degradation. The results revealed the important role of the glyoxylate cycle in $OPEO_n$ utilization by strain TX1.

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

97 **Materials and Methods**

98 **Bacterial strains and culture conditions**

99 The bacterial strains and plasmids used in this study are listed in **Table 1**. Strain TX1 was
100 routinely grown at 30°C in Luria–Bertani (LB) or minimal salt basal medium (MSB) with an
101 appropriate carbon and energy source (12). In liquid culture, cells were grown in a 50-mL culture
102 volume in 250 mL Erlenmeyer flasks with shaking in an incubator at 180 rpm. For plate culture,
103 agar was added at a final concentration of 1.5% (w/v). *Escherichia coli* was grown in LB
104 medium at 37°C and served as the host organism for plasmid retention. Ampicillin (20 μg mL^{-1}),
105 gentamycin (20 μg mL^{-1}), and kanamycin (20 μg mL^{-1}) were used to select the transformed *E.*
106 *coli* or TX1 cells.

107 **Plasmid and chromosomal DNA isolation**

108 Total DNA and plasmid DNA were extracted using previously described procedures (19).
109 For the polymerase chain reaction (PCR) amplifications, a 50 μl of PCR mixture, consisting of
110 0.2 mM of each of the four dNTPs, 20 pmol of each primer, 10 ng of extracted DNA and 1.25
111 unit of Taq DNA polymerase (TOYOBO Co. Ltd., Japan), with an appropriate amount of
112 reaction buffer, was used. Amplification was performed in a Program Temp. Control System,
113 PC-808 (Astec Co. Ltd.). Other molecular techniques were performed using standard procedures
114 (20) or as recommended by the reagent suppliers. The oligonucleotide primers used in this study
115 are listed in **Table 2**.

116 **Transposon mutagenesis**

117 The plasmid pRL27, which carries the transposon Tn5 with a Km resistance gene, was
118 chosen as the vector for transferring the transposon to *P. nitroreducens* TX1. The introduction

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

and subsequent transposition of the modified mini Tn5 transposon into the *P. nitroreducens* TX1 genome was carried out as previously described by mating TX1 and *E. coli* BW20767, which carries the transposon delivery vector pRL27 (21, 22). The donor BW20767(pRL27), the helper *E. coli* HB101(pRK2013) and the recipient strain TX1 were mixed at a ratio of 1:1:1, spotted on a LB agar plate, and cultured at 30°C for 12 h to allow conjugational transfer of the pRL27 plasmid into the recipient cells. Transconjugants were initially selected on MSB agar medium containing 0.5% succinate, ampicillin, and kanamycin. They were then subjected to $OPEO_n$ utilization screening as follows. First, single colonies were streaked on MSB agar containing 0.5% $OPEO_n$. The plates were then incubated in an inverted position for several days at 30°C. Mutant strains that did not show visible growth on the plate were designated $OPEO_n$-negative mutants and were then subjected to further study.

Plasmid rescue and recovery of interrupted genes

Larsen *et al*. have shown that one-step cloning of the transposon with its associated flanking DNA can be accomplished after mutation by pRL27 (21). The selected $OPEO_n$-negative mutants were grown overnight in LB plus kanamycin and ampicillin, and then 1.5 ml of each culture was transferred to microcentrifuge tubes. Next, the cells were harvested by centrifugation at 10,000 × *g* for 1 minute. Chromosomal DNA was isolated from the pelleted cells as described above and digested with the restriction enzyme *Bam*HI, which does not cut within the transposon sequence of pRL27. The digested DNA was cleaned, which was followed by self-ligation using the T4 ligase at 20°C for 1 hour. Material from ligated mixtures was transformed into *E. coli* DH5α λpir as previous described and plated onto LB agar-kanamycin plates in order to select for cells transformed with the ligated pRL27 plasmid that also contained the flanking *P. nitroreducens* TX1 DNA (23). Next the transposon with its flanking DNA was isolated using the

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

142 Midi plus Ultrapure plasmid extraction system (Viogene, Taipei, Taiwan). Primers tpnRL17-1
143 and tpnRL13-2, which anneal to positions within the transposon sequence in pRL27 and read
144 outwards into flanking DNA regions, were used for sequencing (21). Sequencing of the DNA
145 interrupted by the transposon was carried out on an automatic genetic analyzer (Applied
146 Biosystems). The genes disrupted by the transposon were identified using Bioedit software by
147 local Blast search against the TX1 draft genome sequence and also against the Genbank database.

148 **In-frame deletion mutagenesis**

149 Four genes (*aceA*, *aceB*, *fixC* and *glcE*, which encode isocitrate lyase, malate synthase,
150 dehydrogenase and glycolate dehydrogenase, respectively) were inactivated by in-frame deletion
151 to avoid any polar effect. The in-frame *aceA* deletion mutant of strain TX1 was constructed by
152 allelic replacement as previously described (24). A gene fragment containing about a 98%
153 deletion of the internal region of *aceA* was created by overlap extension PCR as described
154 previously (25). The primers used were Ace_FEco, Ace_RXho, Ace_FXho and Ace_RHind. The
155 internally-deleted gene fragment was cloned into a suicide vector (pK18mobsacB), which was
156 named pKaceA. The *aceA* deletion mutant of TX1 was created by triple mating between strains
157 TX1, *E. coli* DH5α(pKaceA) and *E. coli* HB101(pRK2013). The TX1Δ*aceA* was screened as
158 described previously (24) and confirmed by PCR. The same procedure was applied to construct
159 mutants TX1Δ*aceB*, TX1Δ*fixC* and TX1Δ*glcE* using the primers that are described in **Table 2**.

160 **Complementation of the TX1Δ*aceA*, TX1Δ*aceB* and TX1Δ*glcE* mutants**

161 To determine whether the $OPEO_n$ utilization deficiency was due to an inactivated gene,
162 mutants TX1Δ*aceA*, TX1Δ*aceB* and TX1Δ*glcE* were complemented with appropriate wild type
163 genes expressed in a broad host range vector pBBR1MCS5 (26). For example, the *aceA*

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

164 expression vector was constructed as follows: a PCR fragment (2.6 kb) obtained by amplifying
165 the chromosomal DNA of TX1 with the primers aceA(f)_F and aceA(f)_R was ligated into a
166 pGEM-T easy vector (Promega Co.). The resulting vector (pGaceA) was then amplified in *E.*
167 *coli* DH5α, purified and digested with *Eco*RI and *Hind*III to generate the PCR product. Next, the
168 retrieved PCR-amplified fragment was ligated into pBBR1MCS-5, which had been cut with the
169 same restriction enzymes. The resulting plasmid (pBaceA) was then introduced into TXΔ*aceA*
170 by conjugation using the helper *E. coli* HB101(pRK2013). The presence of the intact *aceA* gene
171 was confirmed by DNA sequencing. The recombinant TX1Δ*aceA*(pBaceA) strain was selected
172 on MSB agar containing 0.5% $OPEO_n$, ampicillin and gentamycin. The same procedure was
173 applied for complementation of mutants TX1Δ*aceB* and TX1Δ*glcE* with pBaceB and pBglcE,
174 respectively, which were constructed using the primers listed in **Table 2**.

175 **Preparation of crude cell extract from strain TX1**

176 0.5L culture grown on either 0.5% OPEOn-MSB or 0.5% succinate-MSB of TX1 was
177 used for preparation of crude cell extract. The cells were collected by centrifugation (11,000 g,
178 10 min, 4°C), washed with 10 mM potassium phosphate buffer (pH 7.0) and suspended in 5 mL
179 of the same buffer. The cell suspension was subjected to sonication for 3 minutes with 50% pulse
180 on ice using the Sonicator 4000 (MISONIX, Farmingdale, NY) to disrupt the cells (27). In the
181 process of cell lysis, 0.15 mM protease inhibitor (phenylmethylsulfonyl fluoride) was added.
182 After the removal of unbroken cells and cell debris by centrifugation (11,000 g, 10 min, 4°C),
183 the supernatant was collected by ultracentrifugation (200,000 g, 1 hour, 4°C) and used as crude
184 cell extract. Protein concentrations were determined using the Bradford protein assay with
185 Bovine Serum Albumin as the standard.

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Enzyme assays

The acetaldehyde dehydrogenase activity assay was performed by measuring the rate of appearance of NADH at 340 nm in l cm path cuvette at 25°C with a Beckman DU640 spectrophotometer (Beckman–Coulter, Krefeld, Germany). The incubation mixtures contained the following constituents in a final volume of 1 ml: potassium phosphate buffer, pH 7.0, 10 mM; 2-mercaptoethanol, 10 mM; NAD^+, 2 mM; cell crude extract, 0.5 mg; and acetaldehyde, 1 mM. The reaction was started by the addition of acetaldehyde. For isocitrate lyase activity, a spectrophotometric assay was used to measure isocitrate-dependent formation of glyoxylate. The standard reaction mixture (0.5 ml) contained 10 mM potassium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 2 mM dithioerythritol, 3.5 mM phenylhydrazine, and 0.5 mg cell crude extract. The reactions were started by the addition of 2 mM isocitrate and the formation of the glyoxylate phenylhydrazone derivative was monitored at 324 nm. Malate synthase activity in the crude cell extracts was measured by the method of Srere *et al*. (1963) by following the glyoxylate- and acetyl-CoA-dependent release of CoA (28). Acetyl-CoA synthetase activity in the crude cell extracts was measured in the presence of ATP, acetate, and CoA as described in a previous study (29).

Accepted Manuscript Posted Online

Results and discussion

Transposon mutagenesis of *P. nitroreducens* TX1

The availability of the draft genome sequence of *P. nitroreducens* TX1 provides an opportunity for investigating genes that play significant roles in $OPEO_n$ utilization (18). In order to identify genes involved in $OPEO_n$ catabolism, the transposon Tn5 vector pRL27 (21), which has been used widely in *Pseudomonas* genetics, was used to construct a mutant library of strain TX1 as described in the Materials and Methods. In total, 30,000 Tn5 insertion mutants of TX1 were successfully obtained. After 48h of incubation at 30°C, these mutants were screened and 93 (0.31%) of them failed to grow on 0.5% $OPEO_n$-MSB but still grew on 0.5% succinate-MSB.

A total of 6,341 ORFs have been putatively identified in the TX1 draft genome (18) and therefore the Tn5 mutant library would seem to have 4.7 × ORF coverage of the TX1 genome. The stability of the Tn5 transposon in the transformants was also tested using two of the mutants (C94-4 and A16-7). After ten generations of the single colony propagation by subculture on LB plates, the initial and final colonies were found to still retain the kanamycin resistance and, in addition, the Tn5 transposon had remained in the same genomic position on the genome with this being determined by PCR. These findings confirm that the transposon insertion events present in the transformants from this library are stable.

The transposon insertion points of the 93 mutants defective in $OPEO_n$ utilization were identified by the plasmid rescue followed by nucleotide sequencing using a transposon-derived primer set (21). The insertion loci harboring Tn5 were then mapped to forty-two independent coding genes, three non-coding sequences and one 23S ribosomal RNA. These results are summarized in **Table 3**. Of these forty two genes, eleven had been mutated multiple times to give a number of different mutant strains for a given specific gene. These were the *dedA* gene,

Applied and Environmental Microbiology
AEM
Applied and Environmental Microbiology
Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

which had twelve mutants; the *hit* gene with eleven mutants; the *aceB* gene with seven mutants; the *aceA* and *rfaG* gene with six mutants; the *rfe* gene with four mutants; the *coq7* gene and a gene encoding a hypothetical protein with three mutants; the *yfgC*, *fixC* gene and a gene encoding a transcriptional regulator with two mutants (**Table 3**). These genes with multiple mutations were used for further investigation. Among these forty-two disrupted genes, thirty-nine genes could be located within the draft genome of TX1 (accession no. AMZB01000000), while the other three genes, namely mutants K11-8, C2-42 and T43-26, were found to be similar to genes present in the genomes of other strains including *Mycobacterium abscessus*, *Comamonas testosteroni* and *Pseudomonas knackmussii* (accession numbers WP_049232543, WP_003060809 and WP_043252236.1, respectively), but not within the draft genome of TX1 (18).

Functional characterization of the mutant genes

The proteins encoded by the forty-two unique genes identified in this study ($OPEO_n$ growth-associated proteins) were grouped into functional classes using the Cluster of Orthologous Groups (COG) as shown in **Table 3**. When this was done, six proteins (14.3%) were classified into the information storage and processing related categories (K and L). Furthermore, the cellular processes and signaling related categories (O, M, N, T and V) consisted of twelve proteins (28.6%). Fifteen proteins (35.7%) were present in the metabolism related categories (C, G, E, F, H and I). Two poorly characterized COG groups (R and S) were found to contain six proteins (14.3%). In addition, for the category ‘‘No related COGs’’ (the protein is not predicted to belong to any of the currently defined COGs) there were found to be three proteins (7.1%).

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

252 Overall, twelve (28.5%) genes were predicted to encode catabolic enzymes. Three
253 proteins (isocitrate lyase, malate synthase and FixC dehydrogenase) are known to be involved in
254 energy production and conversion, whereas nine proteins (diadenosine tetraphosphate hydrolase,
255 NAD^+-specific glutamate dehydrogenase, thiamine pyrophosphate-requiring enzyme, β-
256 glucosidase-related glycosidase, glucose-6-phosphate 1-dehydrogenase, ɣ-glutamylcysteine
257 synthetase, UbiE methylase involved in ubiquinone biosynthesis, demethoxyubiquinone
258 hydroxylase and 3-hydroxyacyl-CoA dehydrogenase) are known to be involved in metabolism of
259 amino acids, carbohydrates, coenzymes and lipids. Of these, diadenosine tetraphosphate
260 hydrolase (*hit*) is a key enzyme controlling the *in vivo* concentration of the dinucleotide
261 diadenosine tetraphosphate that has been proposed to play a range of roles in processes such as
262 control of DNA replication and repair, signaling in stress response and apoptosis (30).
263 $OPEO_n$ is one of the most widely used nonionic surfactants; it is used in biology to lyse
264 cells to allow the extraction of protein and other cellular organelles. Previous studies have
265 suggested that $OPEO_n$ affects the cell membrane by disrupting its structural integrity and
266 functioning, which results in increased membrane fluidity and permeabilization (31). In our
267 previous work, we found that *P. nitroreducens* TX1 is able to tolerate up to 20% of $OPEO_n$ in
268 MSB medium. Therefore, the membrane of TX1 might have special structure features that allow
269 $OPEO_n$ to be tolerated. In this context it should be noted that seven (16.6%) out of the forty-two
270 identified proteins, namely three glycosyltransferases (*rfaG, gt2* and *wcaA*), a UDP-*N*-
271 acetylglucosamine-1-phosphate transferase (*rfe*), a membrane protein related to
272 metalloendopeptidase (*nlpD*), an UTP-glucose-1-phosphate uridylytransferase (*galU*) and a
273 nucleoside-diphosphate-sugar epimerase (*wcaG*), are known to be linked to cell
274 wall/membrane/envelope biogenesis. For example, the deletion of *ssg*, which encodes a

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

glycosyltransferase in *P. alkylphenolia* KL28, has been shown to cause the loss of lipopolysaccharide O antigen, which alters the composition of the exopolysaccharide. Furthermore, this mutant strain was found to have reduced surface spreading, reduced pellicle formation and reduced biofilm formation; these were probably due to the cumulative effects of lipopolysaccharide truncation and alterations to the cell's exopolysaccharide composition (23). In some bacterial species, UDP-N-acetylglucosamine-1-phosphate transferase has been shown to play an important role in the biosynthesis of various polymers within the bacterial cell wall such as common antigen and lipopolysaccharide O-antigen (32). UTP-glucose-1-phosphate uridylytransferase is an enzyme associated with glycogenesis. This enzyme synthesizes UDP-glucose from glucose-1-phosphate. UDP-glucose has been reported to be involved in galactose utilization, in glycogen synthesis, and in the synthesis of various carbohydrate moieties such as glycolipids, glycoproteins, and proteoglycans (33). Interestingly, *yfgC* codes for a putative Zn-dependent protease and although its function is unknown, the chemical and genetic data suggest that it also plays a role in outer membrane protein biogenesis (34). Furthermore, five (11.9%) out of the forty-two genes were predicted to encode transport proteins among which two ABC transporters were identified. Such transport proteins were likely to play important roles in the transport of $OPEO_n$ into the cell. For the functions of the DedA membrane protein, the recent genetic approaches have revealed important roles in membrane homeostasis. Bacterial DedA family mutants display such intriguing phenotypes as cell division defects, temperature sensitivity, altered membrane lipid composition, elevated envelope-related stress responses, and loss of proton motive force (35). Finally, there are a significant number of mutants that fall within the hypothetical, unknown and unclassified gene categories, which suggests that there is still a large number of unknown genes across the *P. nitroreducens* genome potentially involved

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

298 in $OPEO_n$ metabolism that remain to be explored, including the three not found in the TX1 draft
299 genome. Further studies on these genes using complementation and other strategies will improve
300 our understanding of the strain TX1 and its tolerance and degradation of $OPEO_n$.

301 **Role of the glyoxylate cycle in $OPEO_n$ degradation by TX1**

302 Isocitrate lyase and malate synthase, which are both key enzymes in the glyoxylate cycle,
303 were frequently detected during screening of mutants unable to grow on 0.5% $OPEO_n$. In the
304 glyoxylate cycle, two molecules of acetyl-CoA are converted into oxaloacetate, thus bypassing
305 the reactions of the TCA cycle in which CO_2 is released. The glyoxylate cycle is essential when
306 cells are growing on C_2 compounds because this pathway allows the synthesis of all cellular
307 compounds with three or more carbon atoms; examples include the biosynthesis of amino acids
308 and nucleotides. To confirm the role of these two enzymes in $OPEO_n$ utilization, in-frame
309 deletion mutants lacking the *aceA* and *aceB* genes were created from strain TX1. As expected
310 from the transposon mutagenesis results, the mutants TX1Δ*aceA* and TX1Δ*aceB* were unable to
311 grow on $OPEO_n$. The in-frame deletion mutants were then used for complementation analysis.
312 Two plasmids, pBaceA and pBaceB, which carry the respective *aceA* and *aceB* genes, were
313 constructed and transferred independently from *E. coli* into the TX1Δ*aceA* and TX1Δ*aceB*
314 mutant strains by conjugation. In both cases the introduction of the wild-type copy of the gene
315 resulted in a return to the wild-type growth pattern, confirming the role of the *aceA* and *aceB* in
316 $OPEO_n$ utilization. In addition, isocitrate lyase and malate synthase activities in cell extract of
317 wild-type cells grown on $OPEO_n$ were 277.6±5 and 15.1±5 nmol min^{-1} mg^{-1}, respectively. These
318 activities were downregulated 8.8- and 1.7-fold in cells grown on succinate (31.3±6.5 and 8.7±2
319 nmol min^{-1} mg^{-1}, respectively). These results clearly reveal that the glyoxylate cycle plays a
320 critical role in OPEOn degradation by strain TX1.

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Growth of the mutants on different carbon sources

Eleven mutants (TX1Δ*aceA*, TX1Δ*aceB*, TX1Δ*fixC*, C48-18, C73-29, C82-3, C85-47, B2-48, C3-45, B3-46 and C11-3), which are representatives of the eleven genes present in the Tn5 library as multiple events, TX1Δ*glcE* and the three complemented strains (TX1Δ*aceA*(pBaceA), TX1Δ*aceB*(pBaceB) and TX1Δ*glcE*(pBglcE)) were tested for their growth on 0.1% $OPEO_n$, 0.1% $NPEO_n$, 0.1% dodecyl octaethoxylate (AEO_8), 0.1% ethanol, 0.1% acetate, 0.1% glycolate or 0.1% pyruvate as sole carbon source (**Table 4**). The *glcE* gene was deleted to test the cleavage of $OPEO_n$ by terminal carboxylation followed by hydrolysis to yield glycolate in strain TX1 (**Fig. 1**, oxidation). Wild-type TX1 is unable to grow on polyethylene glycol 400 (PEG_{400}), polyethylene glycol 1000 (PEG_{100}), acetaldehyde or glyoxylate, therefore these compounds were not used for growth testing of the mutants. The generation time of wild-type TX1 was much longer when grown on glycolate (10.8 to 11.8 h) than on $OPEO_n$, $NPEO_n$, AEO_8, ethanol, acetate or pyruvate (1.5-4.8 h) (**Table 4**). All mutants were able to grow on pyruvate with generation times ranging from 3.6 to 4 h. When $NPEO_n$ or AEO_8 was used as sole carbon source, five mutants TX1Δ*fixC*, TX1Δ*glcE*, TX1Δ*aceA*(pBaceA), TX1Δ*aceB*(pBaceB) and TX1Δ*glcE*(pBglcE) showed growth on these compounds with generation times ranging from 1.5 to 3.7 h, whereas the rest of mutants failed to grow at all. When ethanol or acetate was used as sole carbon source, the two glyoxylate cycle mutants (TX1Δ*aceA*, TX1Δ*aceB*) failed to grow, but others were able to grow on ethanol or acetate with generation times ranging from 2.7 to 4.8 h. In addition, the two mutants TX1Δ*aceB* and TX1Δ*glcE* failed to grow on 0.1% glycolate, but the other mutants were able to grow on it with generation times ranging from 10.8 to 11.8 h.

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

343 **Proposed mechanism for the degradation of $OPEO_n$ by *P. nitroreducens* TX1**

344 The biodegradation of long-chain $APEO_n$ has been studied using isolated bacterial strains
345 that grow solely on $OPEO_n$ or $NPEO_n$. Most isolates are from the genus *Pseudomonas*, which
346 belongs to the *gamma*-Proteobacteria (9, 13, 15). Based on the results of metabolite analyses, the
347 initial degradation reaction of $APEO_n$ has been found to be a shortening of the ethoxylate chain
348 by exo-scission of the EO chains from the hydroxyl terminal side (13-15) (**Fig. 1**). Our group
349 isolated *Pseudomonas* strain TX1, which has the ability to grow on $OPEO_n$ at wide range
350 concentrations (9). LC–MS analysis of the intermediate metabolites revealed the presence of a
351 gradual shortening process that affected the EO chains during $OPEO_n$ utilization. The results
352 suggested that the same exo-type biodegradation process is occurring in TX1 as in other isolates.
353 The terminal oxidation model for the biodegradation of PEG under aerobic conditions has
354 been reviewed previously (36, 37). In this model, the biodegradation of the PEG molecules is
355 initiated through the oxidation of the terminal EO unit to a carboxyl group, which is followed by
356 the liberation of glycolate. The latter product is converted to glyoxylate by glycolate
357 dehydrogenase (GlcE). Tasaki *et al.* isolated the *adh1* gene from *P. putida* S-5 and expressed this
358 gene in *E. coli*. By measuring the reduction of 2,6-dichlorophenolindophenol (DCPIP)
359 spectrophotometrically at 600 nm using a cell crude extract, alcohol dehydrogenase (Adh1) was
360 shown to have activity against $OPEO_n$ of varying EO chain lengths (38). However, its relevance
361 to the ether cleavage step in $OPEO_n$ biodegradation remains unknown. The use of redox dye-
362 based assay with crude extracts can be misleading because crude cell extract may contain
363 multiple activities that are able to utilize DCPIP as an electron acceptor (16). Furthermore, the
364 detection of glycolate and an increase in GlcE activity have not been reported; such findings
365 would support the terminal oxidation model. In our study, a *fixC* gene encoding a dehydrogenase

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

366 was found among the mutants. However, an in-frame deletion mutant of the *fixC* gene retained
367 the ability to grow on $OPEO_n$ in a manner similar to the wild-type. These results suggest that the
368 transposon insertion into *fixC* has a polar effect and the *fixC* gene product itself may not play an
369 important role in $OPEO_n$ utilization. In addition, TX1 grows only slowly on glycolate but does
370 not grow at all on glyoxyate, PEG_{400} or PEG_{1000} as sole carbon source. In order to determine
371 whether glycolic acid is involved in the metabolism of $OPEO_n$ by strain TX1, a *glcE* in-frame
372 deletion mutant was constructed. As shown in **Table 4**, TX1Δ*glcE* grew on $OPEO_n$ to a level
373 comparable to wild-type. Interestingly, TX1Δ*glcE* and TX1Δ*aceB* were unable to grow on
374 glycolate, whereas TX1Δ*aceA* was able to grow. Interestingly, one isocitrate lyase gene was
375 found in TX1 genome (18). In addition, the isocitrate lyase activity in cell extract of TX1Δ*aceA*
376 cells grown on glycolate or succinate was not detectable. These results suggest that the
377 degradation of glycolate does not involve the glyoxylate cycle because the isocitrate lyase
378 mutant was able to grow on glycolate in a manner similar to the wild-type TX1 (**Fig. 2**).
379 Considering the results, we conclude that the degradation of $OPEO_n$ by strain TX1 does not
380 follow an oxidative pathway involving the liberation of glycolate.

381 In the non-oxidative biodegradation model, biodegradation of the EO chain proceeds via
382 the shift of a hydroxyl group followed by the liberation of acetaldehyde, which is then quickly
383 transformed to acetate by dehydrogenases that are ubiquitously distributed throughout the cell
384 (39). The pathway for acetate degradation is well known and involves the conversion of acetate
385 into acetyl-CoA, which then enters in the central carbon metabolism of the cell (39). In the
386 glyoxylate cycle, the input of two molecules of acetyl-CoA results in a net synthesis of one
387 molecule of succinate, which is then available for biosynthetic purposes (40). As shown in **Table**
388 **4**, TX1 was able to grow on either ethanol or acetate as sole carbon source with generation times

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

ranging from 2.7 to 4.8 h but TX1Δ*aceA* and TX1Δ*aceB* was unable to grow on these carbon sources. These results suggest that the glyoxylate cycle plays a critical role in ethanol or acetate catabolism. The ethanol oxidation product acetate must first be activated to acetyl-CoA in order that it can be used as a carbon source and for the replenishment of intermediates within the TCA cycle; this would occur via the glyoxylate bypass in strain TX1. Our results suggest that non-oxidative biodegradation with the liberation of acetaldehyde is the most likely pathway for $OPEO_n$ utilization in strain TX1 (**Fig. 2**). In addition, acetaldehyde dehydrogenase and acetyl-CoA synthetase activities were upregulated 13.1- and 2.1-fold in cells grown on $OPEO_n$ (211±14 and 68.8±1.1 nmol min^{-1} mg^{-1}) than in TX1 grown on succinate (16±12 and 32.6±1.2 nmol min^{-1} mg^{-1}), respectively. Further studies on the mechanism used for liberation of acetaldehyde will be needed in order to improve our understanding of the degradation of $OPEO_n$ by strain TX1.

Acknowledgements

This work was supported by Ministry of Science and Technology, Taiwan (NSC102-2628-B-008-001-MY3) and NRF-NSC cooperation program by National Research Foundation of Korea (NRF-2013K2A1B8053138) and Ministry of Science and Technology, Taiwan (NSC 102-2923-B-008-001-MY2).

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

References

1. **Chen HJ, Tseng DH, Huang SL.** 2005. Biodegradation of octylphenol polyethoxylate surfactant Triton X-100 by selected microorganisms. Bioresour Technol **96:**1483-1491.
2. **Saito I, Onuki A, Seto H.** 2004. Indoor air pollution by alkylphenols in Tokyo. Indoor Air **14:**325-332.
3. **Pagano M, Lopez A, Volpe A, Mascolo G, Ciannarella R.** 2008. Oxidation of nonionic surfactants by Fenton and H_2O_2/UV processes. Environ Technol **29:**423-433.
4. **Giger W, Brunner PH, Schaffner C.** 1984. 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. Science **225:**623-625.
5. **Huang SL, Tuan NN, Lee K.** 2016. Occurrence, human intake and biodegradation of estrogen-like nonylphenols and octylphenols. Curr Drug Metab **17:**293-302.
6. **Tanenbaum DM, Wang Y, Williams SP, Sigler PB.** 1998. Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. Proc Natl Acad Sci U S A **95:**5998-6003.
7. **Sahambi SK, Pelland A, Cooke GM, Schrader T, Tardif R, Charbonneau M, Krishnan K, Haddad S, Cyr DG, Devine PJ.** 2010. Oral *p*-tert-octylphenol exposures induce minimal toxic or estrogenic effects in adult female Sprague-Dawley rats. J Toxicol Environ Health A **73:**607-622.
8. **Ying GG, Williams B, Kookana R.** 2002. Environmental fate of alkylphenols and alkylphenol ethoxylates--a review. Environ Int **28:**215-226.
9. **Lin YW, Guo GL, Hsieh HC, Huang SL.** 2010. Growth of *Pseudomonas* sp. TX1 on a wide range of octylphenol polyethoxylate concentrations and the formation of dicarboxylated metabolites. Bioresour Technol **101:**2853-2859.
10. **Chen HJ, Guo GL, Tseng DH, Cheng CL, Huang SL.** 2006. Growth factors, kinetics and biodegradation mechanism associated with *Pseudomonas nitroreducens* TX1 grown on octylphenol polyethoxylates. J Environ Manage **80:**279-286.
11. **Chen HJ, Huang SL, Tseng DH.** 2004. Aerobic biotransformation of octylphenol polyethoxylate surfactant in soil microcosms. Environ Technol **25:**201-210.
12. **Tuan NN, Hsieh HC, Lin YW, Huang SL.** 2011. Analysis of bacterial degradation pathways for long-chain alkylphenols involving phenol hydroxylase, alkylphenol monooxygenase and catechol dioxygenase genes. Bioresour Technol **102:**4232-4240.

Accepted Manuscript Posted Online

13. **John DM, White GF.** 1998. Mechanism for biotransformation of nonylphenol polyethoxylates to Xenoestrogens in *Pseudomonas putida*. J Bacteriol **180:**4332-4338.

14. **Maki H, Masuda N, Fujiwara Y, Ike M, Fujita M.** 1994. Degradation of alkylphenol ethoxylates by *Pseudomonas* sp. strain TR01. Appl Environ Microbiol **60:**2265-2271.

15. **Nguyen MH, Sigoillot JC.** 1996. Isolation from coastal sea water and characterization of bacterial strains involved in non-ionic surfactant degradation. Biodegradation **7:**369-375.

16. **White GF, Russell NJ, Tidswell EC.** 1996. Bacterial scission of ether bonds. Microbiol Rev **60:**216-232.

17. **Nishio E, Ichiki Y, H. T, S. M, K. W, H. Y.** 2002. Isolation of bacterial strains that produce the endocrine disruptor, octylphenol diethoxylates, in paddy fields. Biosci Biotechnol Biochem **66:**1792-1798.

18. **Huang SL, Chen H, Hu A, Tuan NN, Yu CP.** 2014. Draft genome sequence of pseudomonas nitroreducens strain tx1, which degrades nonionic surfactants and estrogen-like alkylphenols. Genome Announc **2**.

19. **Ausubel FM, Roger. B, Kingston RE, Moore DD, Seidman JG, Smith JA, Kevin S.** 2003. Current protocols in molecular biology, ringbou ed, John Wiley & Sons Inc.

20. **Sambrook J, Fristsch EF, Maniatis T.** 1989. Molecular cloning: A laboratory manual, vol 1,2,3. Cold Spring Harbor Laboratory Press, NY.

21. **Larsen RA, Wilson MM, Guss AM, Metcalf WW.** 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Arch Microbiol **178:**193-201.

22. **Figurski DH, Helinski DR.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A **76:**1648-1652.

23. **Veeranagouda Y, Lee K, Cho AR, Cho K, Anderson EM, Lam JS.** 2011. Ssg, a putative glycosyltransferase, functions in lipo- and exopolysaccharide biosynthesis and cell surface-related properties in *Pseudomonas alkylphenolia*. FEMS Microbiol Lett **315:**38-45.

24. **Schafer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69-73.

25. **Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51-59.

26. **Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, 2nd, Peterson KM.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175-176.

27. **Tuan NN, Lin YW, Huang SL.** 2013. Catabolism of 4-alkylphenols by *Acinetobacter* sp. OP5: genetic organization of the oph gene cluster and characterization of alkylcatechol 2, 3-dioxygenase. Bioresour Technol **131:**420-428.

28. **Srere PA, Brazil H, Gonen L.** 1963. The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. Acta Chemica Scandinavica **17:**129-134.

29. **Gardner JG, Grundy FJ, Henkin TM, Escalante-Semerena JC.** 2006. Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD(+) involvement in *Bacillus subtilis*. J Bacteriol **188:**5460-5468.

30. **Szurmak B, Wyslouch-Cieszynska A, Wszelaka-Rylik M, Bal W, Dobrzanska M.** 2008. A diadenosine 5',5"-P1P4 tetraphosphate (Ap4A) hydrolase from Arabidopsis thaliana that is activated preferentially by Mn2+ ions. Acta Biochim Pol **55:**151-160.

31. **Koley D, Bard AJ.** 2010. Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). Proc Natl Acad Sci U S A **107:**16783-16787.

32. **Al-Dabbagh B, Mengin-Lecreulx D, Bouhss A.** 2008. Purification and characterization of the bacterial UDP-GlcNAc:undecaprenyl-phosphate GlcNAc-1-phosphate transferase WecA. J Bacteriol **190:**7141-7146.

33. **Thoden JB, Holden HM.** 2007. The molecular architecture of glucose-1-phosphate uridylyltransferase. Protein Sci **16:**432-440.

34. **Oh E, Becker AH, Sandikci A, Huber D, Chaba R, Gloge F, Nichols RJ, Typas A, Gross CA, Kramer G, Weissman JS, Bukau B.** 2011. Selective ribosome profiling reveals the cotranslational chaperone action of trigger factor in vivo. Cell **147:**1295-1308.

35. **Doerrler WT, Sikdar R, Kumar S, Boughner LA.** 2013. New functions for the ancient DedA membrane protein family. J Bacteriol **195:**3-11.

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

36. **Kawai F, Kimura T, Fukaya M, Tani Y, Ogata K, Ueno T, Fukami H.** 1978. Bacterial oxidation of polyethylene glycol. Appl Environ Microbiol **35:**679-684.

37. **Kawai F.** 2002. Microbial degradation of polyethers. Appl Microbiol Biotechnol **58:**30-38.

38. **Tasaki Y, Yoshikawa H, Tamura H.** 2006. Isolation and characterization of an alcohol dehydrogenase gene from the octylphenol polyethoxylate degrader *Pseudomonas putida* S-5. Biosci Biotechnol Biochem **70:**1855-1863.

39. **Alber BE, Spanheimer R, Ebenau-Jehle C, Fuchs G.** 2006. Study of an alternate glyoxylate cycle for acetate assimilation by *Rhodobacter sphaeroides*. Mol Microbiol **61:**297-309.

40. **Kornberg HL, Krebs HA.** 1957. Synthesis of cell constituents from C_2-units by a modified tricarboxylic acid cycle. Nature **179:**988-991.

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

528 **Figure Legends**

529 **Figure 1.** Proposed pathways for the biodegradation of alkylphenol polyethoxylates from the

530 literatures (13, 16, 17).

531 **Figure 2.** Proposed pathway and mechanism (black lines) for the degradation of $OPEO_n$ by *P.*

532 *nitroreducens* TX1.

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

533 **Table 1.** Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	References
Strain		
Pseudomonas nitroreducens strain		
TX1	Wild-type strain TX1, Amp^R	Laboratory collection
Δ*aceA*	Strain TX1 with in-frame deletion of *aceA* gene, Amp^R	This study
Δ*aceB*	Strain TX1 with in-frame deletion of *aceB* gene, Amp^R	This study
Δ*fixC*	Strain TX1 with in-frame deletion of *fixC* gene, Amp^R	This study
Δ*glcE*	Strain TX1 with in-frame deletion of *glcE* gene, Amp^R	This study
Δ*aceA*(pBaceA)	Mutant Δ*aceA* harboring plasmid pBaceA, Amp^R, Gm^R	This study
Δ*aceB*(pBaceB)	Mutant Δ*aceB* harboring plasmid pBaceB, Amp^R, Gm^R	This study
E. coli		
DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR*17(r_k^-,m_k^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96	Laboratory collection
DH5α/λpir	λpir lysogen of DH5α	Laboratory collection
BW20767	Containing pRL27; Used as donor for pRL27 conjugations	Larsen et al., 2002
HB101	Containing pRK2013; Used as helper for pRL27 conjugations	Figurski & Helinski, 1979
Plasmid		
pGEM-Teasy	PCR cloning vector, Amp^R	Promega
pGaceA	Amp^R , pGEM-Teasy with 2.6-kb fragment including *aceA*	This study
pGaceB	Amp^R , pGEM-Teasy with 3.2-kb fragment including *aceB*	This study
pRL27	Km^R; mini-Tn*5* transposon (*ori*R6K) delivery vector	Larsen et al., 2002
pRK2013	Km^R; encoding for RK2 transfer genes	Figurski & Helinski, 1979
pK18mobsacB	Km^R, oriT(RP4), *sacB*, *lacZ*alpha, Plac, Pmbi, mobilization and counter selection	Schafer et al., 1994
pKaceA	pK18mob*sacB* containing a gene fragment with 98% deletion of the internal region of *aceA*	This study
pKaceB	pK18mob*sacB* containing a gene fragment with 98% deletion of the internal region of *aceB*	This study
pKfixC	pK18mob*sacB* containing a gene fragment with 98% deletion of the internal region of *fixC*	This study
pKglcE	pK18mob*sacB* containing a gene fragment with 98% deletion of the internal region of *glcE* (WP_017520229.1)	This study
pBBR1MCS-5	Broad host range cloning vector, *lacZ*, Gm^R	Kovach et al., 1995
pBaceA	Gm^R , pBBR1-MCS5 with 2.6-kb fragment including *aceA*	This study
pBaceB	Gm^R , pBBR1-MCS5 with 3.2-kb fragment including *aceB*	This study
pBglcE	Gm^R , pBBR1-MCS5 with 1.5-kb fragment including *glcE*	This study

534 [a] Km^R, Gm^R and Amp^R indicate resistance to kanamycin, gentamicin and ampicillin, respectively.

535

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

536 **Table 2.** Oligonucleotide primers used in this study

Name	Sequence (5'-3')[a]	Note	Restriction site	References
tpnRL17-1	AACAAGCCAGGGATGTAACG	For sequencing of transposon insertion		Larsen et al., 2002
tpnRL13-2	CAGCAACACCTTCTTCACGA			Larsen et al., 2002
AceA_FEco	GCGAATTCACGTGCTCGACTAAGCCTTC	For in-frame deletion of the *aceA* in TX1	*Eco*RI	This study
AceA_RXho	GCCTCGAGTGCGGACATGGCCAATCCTTC		*Xho*I	This study
AceA_FXho	GCCTCGAGTTCCACTAAGAACTGGCTCAC		*Xho*I	This study
AceA_RHind	GCAAGCTTCTCTTGATCAGCGGCAGTTT		*Hind*III	This study
AceB_FEco	GCGAATTCGAAGCGAAGAACGGGTACAG	For in-frame deletion of the *aceB* in TX1	*Eco*RI	This study
AceB_RXho	GCCTCGAGTTCAGTCATTGCTTGCCTCAC		*Xho*I	This study
AceB_FXho	GCCTCGAGGGTCTGTAAGCACCTCGCGCC		*Xho*I	This study
AceB_RHind	GCAAGCTTAACCGTCTGCAGTCTTTCTGA		*Hind*III	This study
FixC_FEco	GCGAATTCCAGTGATCCGTCGAAGGTG	For in-frame deletion of the *fixC* in TX1	*Eco*RI	This study
FixC_RSma	GCCCCGGGGGCGGGCATCAGAAGGGCTCC		*Sma*I	This study
FixC_FSma	GCCCCGGGGCCAGCTGAATAGGACAAGG		*Sma*I	This study
FixC_RHind	GCAAGCTTCAGTTCAGCTCCACCTCGAT		*Hind*III	This study
GlcE_FEco	GCGAATTCAGTTTCGATTCGGTGGAAAA	For in-frame deletion of the *glcE* in TX1	*Eco*RI	This study
GlcE_RXho	GCCTCGAGATCGGCCATCAGAAACGCTCC		*Sma*I	This study
GlcE_FXho	GCCTCGAGGAGCTCTGATGCAAACCAACC		*Sma*I	This study
GlcE_RHind	GCAAGCTTCGTCTGCACTATGGCTTCG		*Hind*III	This study
aceA(f)_F	GCGAATTCACGTGCTCGACTAAGCCTTC	For construction of pBaceA	*Eco*RI	This study
aceA(f)_R	GCAAGCTTCTCTTGATCAGCGGCAGTTT		*Hind*III	This study
aceB(f)_F	GCGAATTCGAAGCGAAGAACGGGTACAG	For construction of pBaceB	*Eco*RI	This study
aceB(f)_R	GCAAGCTTAACCGTCTGCAGTCTTTCTGA		*Hind*III	This study
glcE(f)_F	TGCCCGGGGAGCTGGAACGCGCCG	For construction of pBglcE	*Sma*I	This study
glcE(f)_R	GCACAAGCTTGATCAGGTAGATGCGCC		*Hind*III	This study

537 [a]Restriction enzyme sites are underlined

538

539

540

541

Accepted Manuscript Posted Online

542
543 **Table 3.** Characterization of the $OPEO_n$-negative mutants of *P. nitroreducens* TX1 obtained by Tn5 insertional mutagenesis

Mutants[a]	Gene	Function	Protein accession no.	Function group (COGs)[b]
C94-4; C100-12; N8-29; N10-35; N36-50; P30-12	*aceA*	Isocitrate lyase	WP_017517106.1	COG2224C
A16-7; C87-31; C90-8; K1-02; N38-2; P10-33; T16-15	*aceB*	Malate synthase	WP_017520204.1	COG2225C
B1-31; B1-33	*fixC*	Dehydrogenase (flavoprotein)	WP_017520493.1	COG0644C
A2-42	*hisP*	ABC-type histidine transport system	WP_017517948.1	COG4598E
C39-17	*gdh2*	NAD-specific glutamate dehydrogenase	WP_017519332.1	COG2902E
C96-34	*rhtB*	Putative threonine efflux protein	WP_017521173.1	COG1280E
T8-50	*ilvB*	Thiamine pyrophosphate-requiring enzyme	WP_017516325.1	COG0028EH
N43-34	*putP*	Na^+/proline symporter	WP_017518917.1	COG0591ER
C48-18; C77-4; C95-33; P19-3; P19-32; P38-47; P42-36; T10-24; T15-48; T57-1; T58-5	*hit*	Diadenosine tetraphosphate hydrolase and other HIT family hydrolases	WP_017517344.1	COG0537FGR
B3-49	*bglX*	Beta-glucosidase-related glycosidase	WP_017517232.1	COG1472G
P40-18	*zwf*	Glucose-6-phosphate 1-dehydrogenase	WP_017520335.1	COG0364G
K32-8	*gshA*	Gamma-glutamylcysteine synthetase	WP_017520855.1	COG2918H
A21-39	*ubiE*	Methylase involved in ubiquinone biosynthesis	WP_017521165.1	COG2226H
C73-29; C97-46; P24-25	*coq7*	Demethoxyubiquinone hydroxylase	WP_017517343.1	COG2941H
B3-29	*fadB*	3-Hydroxyacyl-CoA dehydrogenase	WP_017519287.1	COG1250I
A11-1	*yafY*	Predicted transcriptional regulator	WP_017518996.1	COG2378K
C4-30	*araC*	AraC-type DNA-binding domain-containing protein	WP_017521913.1	COG2207K
B5-50	*aRO8*	Transcriptional regulator	WP_017518869.1	COG1167KE
C15-19	*hepA*	Superfamily II DNA/RNA helicase	WP_017522209.1	COG0553KL
K11-8	*dob10*	DEAD/DEAH box helicase	WP_049232543	COG4581L
C2-42	*Tn3*	Transposase	WP_003060809	COG1961L
C82-3; T44-10; T45-21; T67-19; T78-50; T77-39	*rfaG*	Glycosyltransferase	WP_017520449.1	COG0438M
C85-47; T41-40;T66-4; T66-19	*rfe*	UDP-*N*-acetylglucosamine-1-phosphate transferase	WP_017520347.1	COG0472M
N52-26	*gt2*	Glycosyltransferases	WP_017520448.1	COG1216M
P33-4	*nlpD*	Membrane protein related to metalloendopeptidase	WP_017517352.1	COG0739M
P55-13	*wcaA*	Glycosyltransferase involved in cell wall biogenesis	WP_017517134.1	COG0463M
T43-26	*galU*	UTP-glucose-1-phosphate uridylytransferase	WP_043252236.1	COG1210M
B7-14	*wcaG*	Nucleoside-diphosphate-sugar epimerase	WP_017520346.1	COG0451MG
B6-16	*tar*	Methyl-accepting chemotaxis protein	WP_017516259.1	COG0840NT
N24-8	*anmK*	Predicted molecular chaperone	WP_017517351.1	COG2377O
A21-12	COG4590	ABC-type uncharacterized transport system	WP_017520681.1	COG4590R
A7-47	*ybcL*	Membrane-bound metal-dependent hydrolase	WP_017520412.1	COG1988R
B2-48; B7-43	*yfgC*	Putative Zn-dependent protease	WP_017519734.1	COG4783R
K17-10	*roxA*	Ribosomal protein L16 Arg81 hydroxylase	WP_017517102.1	COG2850S
K21-12	*tauE*	Cytochrome biogenesis protein	WP_017516824.1	COG2836S
C3-45; C5-33; C9-50; C61-29; C63-28; C64-38; P32-21; P33-22; T11-1; T15-44; T17-21; T46-48	*dedA*	Membrane protein	WP_017521718.1	COG0586S
N30-8	*atoC*	Response regulator	WP_017518918.1	COG2204T
P36-32	*GGDEF*	GGDEF domain, diguanylate cyclase	WP_017517199.1	COG2199T

Applied and Environmental Microbiology
AEM
Applied and Environmental Microbiology
Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

C12-38	*norM*	Na^+-driven multidrug efflux pump	WP_017518643.1	COG0534V
B3-46; B4-25		Transcriptional regulator	WP_017516487.1	-
C11-3; T56-25; T57-21		Hypothetical protein	WP_017520115.1	-
A28-42		Hypothetical protein	WP_017520049.1	-
T12-28		23S ribosomal RNA		-
A6-49		Non-coding sequence		-
B2-47		Non-coding sequence		-
P34-24		Non-coding sequence		-

[a]The representative strains (in bold) were selected for further investigation as described in the text.

[b]The proteins were classified into functional categories according to the Cluster of Orthologous Groups (COG). The functional categories are information storage and processing, including COG categories K (transcription) and L (replication, recombination and repair); cellular processes and signaling, including O (posttranslational modification, protein turnover, chaperones), M (cell wall/membrane/envelope biogenesis), N (cell motility), T (signal transduction mechanisms) and V (defense mechanisms); metabolism, including C (energy production and conversion), G (carbohydrate transport and metabolism), E (amino acid transport and metabolism), F (nucleotide transport and metabolism), H (coenzyme transport and metabolism) and I (lipid transport and metabolism); poorly characterized including R (general function prediction only) and S (function unknown); and '-', which refers proteins not classified into a COG.

Applied and Environmental Microbiology
AEM
Applied and Environmental Microbiology
Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

557 **Table 4.** Growth of TX1 mutants on various different carbon sources

Strains[a]	Description	Generation time (h)						
		$OPEO_n$	$NPEO_n$	AEO_8	Ethanol	Acetate	Pyruvate	Glycolate
TX1	Wildtype	1.5±0.1	3.2±0.1	3.7±0.2	4.8±0.2	2.7±0.2	3.6±0.4	11.3±3.1
TX1Δ*aceA*	Deletion of isocitrate lyase gene	-	-	-	-	-	3.6±0.4	11.5±2.5
TX1Δ*aceB*	Deletion of malate synthase gene	-	-	-	-	-	3.6±0.4	-
TX1Δ*fixC*	Deletion of dehydrogenase gene	1.5±0.1	3.1±0.1	3.7±0.1	4.8±0.2	2.7±0.2	3.6±0.4	10.8±2.2
TX1Δ*glcE*	Deletion of glycolate dehydrogenase gene	1.5±0.1	3.2±0.1	3.7±0.1	4.7±0.1	2.9±0.3	4±0.2	-
C48-18	Mutation at diadenosine tetraphosphate hydrolase gene	-	-	-	4.7±0.2	2.7±0.2	4±0.2	11.2±4.2
C73-29	Mutation at demethoxyubiquinone hydroxylase gene	-	-	-	4.7±0.2	2.7±0.2	4±0.2	11.3±3.3
C82-3	Mutation at glycosyltransferase gene	-	-	-	4.7±0.2	2.9±0.3	4±0.2	11.5±2.8
C85-47	Mutation at UDP-*N*-acetylglucosamine-1-phosphate transferase gene	-	-	-	4.7±0.1	2.7±0.2	4±0.2	11.7±2.5
B2-48	Mutation at putative Zn-dependent protease gene	-	-	-	4.8±0.2	2.9±0.3	4±0.2	10.9±3.2
C3-45	Mutation at gene encoding membrane protein	-	-	-	4.7±0.2	2.9±0.3	4±0.2	11.6±2.7
B3-46	Mutation at gene encoding transcriptional regulator	-	-	-	4.8±0.1	2.9±0.3	4±0.2	11.8±2.8
C11-3	Mutation at gene encoding unknown protein	-	-	-	4.7±0.1	2.9±0.3	4±0.2	11.7±3.1
TX1Δ*aceA*(pBaceA)	Complementation of isocitrate lyase gene	1.5±0.1	3.2±0.1	3.6±0.2	4.7±0.1	2.9±0.3	4±0.2	11.1±3.2
TX1Δ*aceB*(pBaceB)	Complementation of malate synthase gene	1.5±0.1	3.1±0.1	3.7±0.1	4.7±0.2	2.9±0.3	4±0.2	10.8±2.6
TX1Δ*glcE*(pBglcE)	Complementation of glycolate dehydrogenase gene	1.5±0.1	3.1±0.1	3.7±0.1	4.8±0.2	2.9±0.3	4±0.2	11.2±2.8

558 [a]Each strain was cultured in 5 mL MSB + 0.1% of the relevant carbon source. -, no growth. $OPEO_n$, Triton X-100; $NPEO_n$, Triton N-

559 101; AEO_8, dodecyl octaethoxylate. Mean values and standard deviations are shown for three independent experiments.

560

561

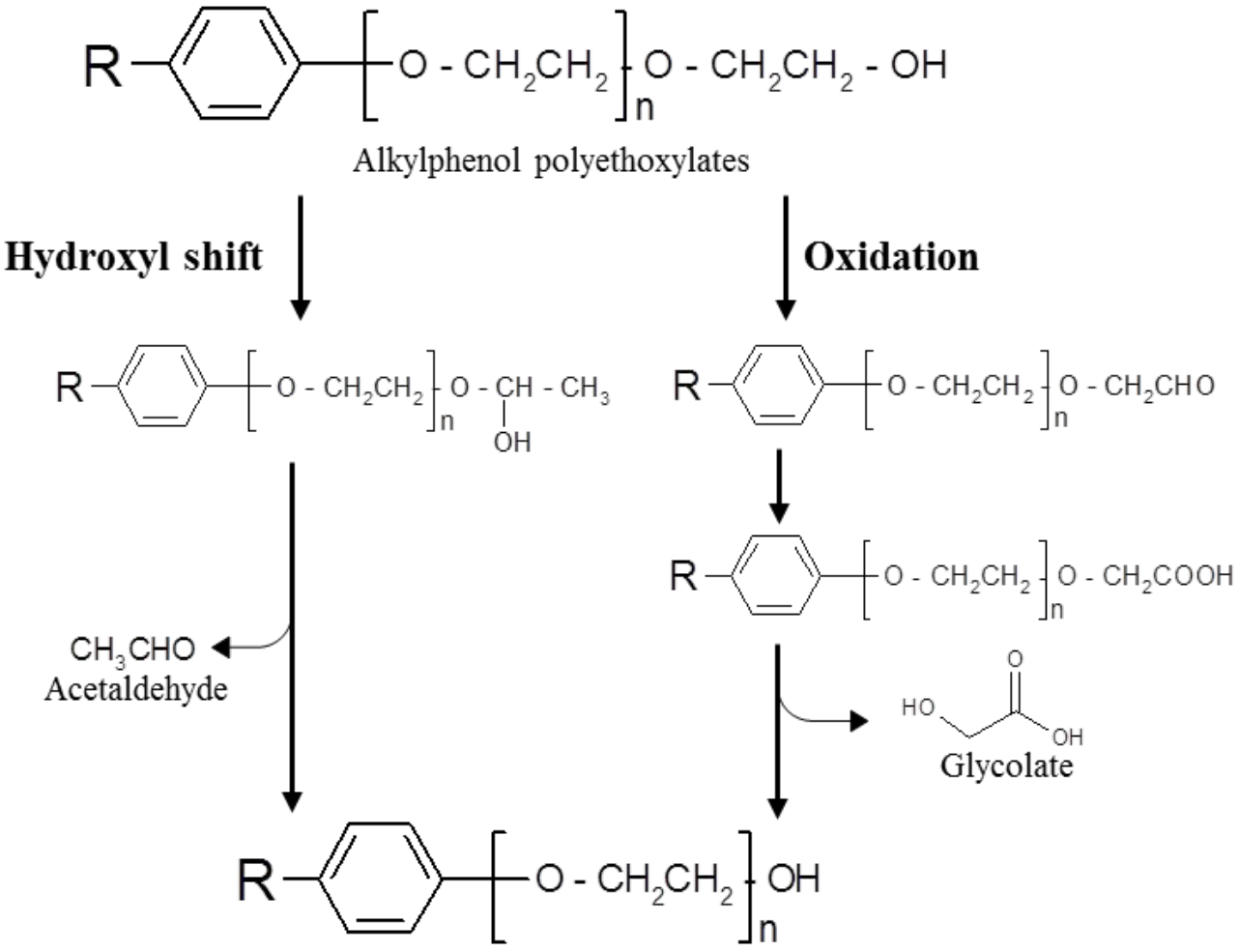
562

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology



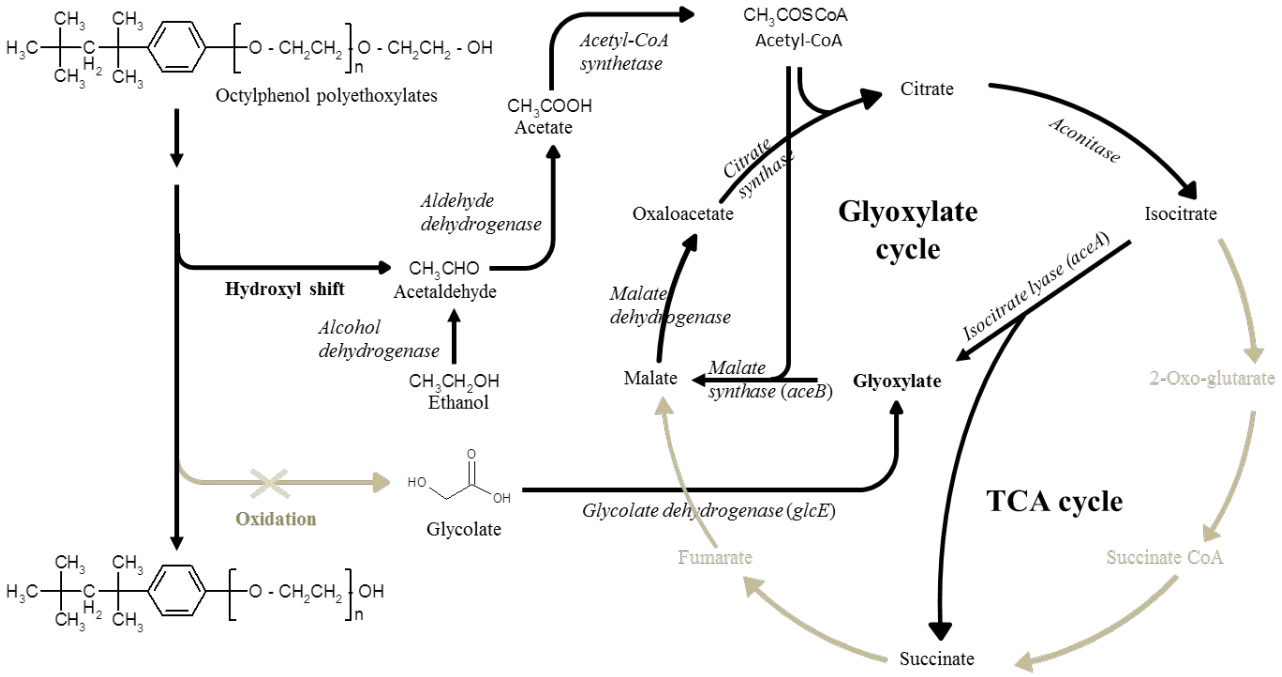
Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology



Downloaded from http://aem.asm.org/ on September 3, 2016 by CORNELL UNIVERSITY