Isolation of marine xylene-utilizing bacteria and characterization of *Halioxenophilus aromaticivorans* gen. nov., sp. nov. and its xylene degradation gene cluster

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Abstract

Seven xylene-utilizing bacterial strains were isolated from seawater collected off the coast of Japan. Analysis of 16S rRNA gene sequences indicated that six isolates were most closely related to the marine bacterial genera *Alteromonas*, *Marinobacter* or *Aestuariibacter*. The sequence of the remaining strain, KU68F^T, showed low similarity to the 16S rRNA gene sequences of known bacteria with validly published names, the most similar species being *Maricurvus nonylphenolicus* strain KU41E^T (92.6% identity). On the basis of physiological, chemotaxonomic and phylogenetic data, strain KU68F^T is suggested to represent a novel species of a new genus in the family *Cellvibrionaceae* of the order *Cellvibrionales* within the *Gammaproteobacteria*, for which the name *Halioxenophilus aromaticivorans* gen. nov., sp. nov. is proposed. The type strain of *Halioxenophilus aromaticivorans* is KU68F^T (= JCM 19134^T = KCTC 32387^T). PCR and sequence analysis revealed that strain KU68F^T possesses an entire set of genes encoding the enzymes for the upper xylene methyl-monooxygenase pathway, *xylCMABN,* resembling the gene set of the terrestrial *Pseudomonas putida* strain mt-2.

Introduction

Xylenes are toxic pollutants that are widely distributed in the environment and arise from a variety of industries, gasoline, airplane fuel and natural sources (Fay *et al*. 2007). Therefore, the bioremediation of xylene is of great interest, and numerous xylene-utilizing microorganisms, such as members of the genera *Pseudomonas* and *Rhodococcus,* have been isolated, resulting in the reporting of several xylene degradation pathways (Assinder and Williams 1990; Barbieri *et al*. 1993; Jindrová *et al*. 2002; Kim *et al.* 2002; Maruyama *et al.* 2005). The initial steps in these pathways are catalyzed by dioxygenases or monooxygenases that hydroxylate the xylene aromatic ring (ring hydroxylating pathways) and monooxygenases that oxidize the xylene methyl group (methyl monooxygenase pathways). Genetic and/or biochemical data are also available for these pathways (Bertoni *et al*. 1998; Bramucci *et al*. 2002; Harayama *et al*. 1986; Kim and Zylstra 1995, 1999; Kim *et al*. 2004; Maruyama *et al*. 2005). However, these data are derived mainly from terrestrial or freshwater bacteria. While a few studies of marine xylene-utilizing bacteria have been conducted, information on such bacteria from marine environments and their xylene degradation pathways is relatively scarce (Berlendis *et al*. 2010; Jin *et al*., 2013; Wang *et al.* 2008). Indeed, there are no data on the xylene-degrading genes of marine bacteria. Therefore, additional information concerning marine xylene-degrading bacteria and their degradation pathways is required to facilitate the development of effective bioremediation programs.

Here, we report the isolation of novel aerobic xylene-utilizing bacteria. Comparative 16S rRNA gene sequence analysis indicated that one of the isolates, strain KU68F^T, forms an independent branch in the family *Cellvibrionaceae* of the order *Cellvibrionales* within the *Gammaproteobacteria*. Therefore, in this study, we aimed to determine the exact taxonomic position of strain KU68F^T and to analyze the sequence properties of the xylene degradation gene cluster present in strain KU68F^T.

Materials and Methods

Isolation of xylene-utilizing bacteria from seawater

Xylene-utilizing bacteria were isolated from two coastal areas of Japan, Fukui and Tokyo (Ogasawara Islands), according to a previously described procedure with some modifications (Iwaki *et al*. 2012a). Daigo's IMK-SP medium (Nihon Pharmaceutical) was supplemented with *o*-, *m*-, or *p*-xylene as a vapor.

16S rRNA gene sequencing and phylogenetic analysis

Near full-length 16S rRNA genes were amplified by PCR using the bacterial universal primer sets 27f /1492r or 9F/1510R (Table S1; Lane 1991; Nakagawa and Kawasaki 2001). PCR amplification was carried out using BLEND Taq -plus- (Toyobo) according to the manufacturer's instructions. PCR products were sequenced directly using the primers 27f, 357f, 926f, 342r and 1492r or the primers 9F, 785F, 802R and 1510R (Table S1; Lane 1991; Nakagawa and Kawasaki 2001). Alignments were generated using the CLUSTALW tool in MEGA version 6 (Tamura *et al*. 2013). Phylogenetic trees were generated using MEGA version 6 as previously described (Iwaki *et al*. 2013).

Determination of taxonomic characteristics of strain KU68F^{T}

Gram staining was performed using a Favor-G kit (Nissui), and cells were observed under a light microscope (BX50F4, Olympus). Cell morphology was examined under a scanning electron microscope (JSM-6320F, JEOL) at 5 kV by the Hanaichi UltraStructure Research Institute. Physiological and biochemical characterization was performed as described previously (Iwaki *et al*. 2014), except that the cell suspension was adjusted to a 1 McFarland standard for antibiotic susceptibility tests. Contents of DNA G+C, isoprenoid quinones, and fatty acids were analyzed at TechnoSuruga Laboratory Co. Ltd. as described previously (Iwaki *et al*. 2014).

PCR detection of genes encoding initial oxygenase

To screen for possible genes encoding the initial oxygenase, we used the previously described primer sets XYLA-F/XYLA-R and TODC1-F/TODC1-R (Table S1; Hendrickx *et al*. 2006). PCR amplification was carried out using BLEND Taq -plus- (Toyobo) according to the manufacturer's instructions.

Amplification and sequencing of flanking regions of the partial *xylA* gene from strain KU68F^{T}

The flanking regions of a partial *xylA* gene from strain KU68F^{T} were obtained by inverse-PCR (Ochman *et al.* 1988) using the primers invF and invR (Table S1). The inverse-PCR was conducted with step-down cycles using KOD FX Neo DNA polymerase (Toyobo) according to the

manufacturer's instructions. The DNA sequence of the inverse-PCR product was determined by direct sequencing and primer walking.

DNA sequencing and sequence analysis methods

DNA fragments were sequenced with the BigDye Terminator Cycle Sequencing Kit version 3.1 and an ABI PRISM 310 or 3130xl genetic analyzer (Life Technologies). DNA sequences were analyzed with GENETYX-Mac software ver. 16 (Genetyx). Nucleotide and protein sequence similarity searches were done using the BLAST program (Altschul *et al*. 1997) via the National Center for Biotechnology Information server.

Results and Discussion

Isolation of xylene-utilizing bacteria and phylogenetic analysis of the 16S rRNA gene sequences of the isolated bacteria

Xylene-utilizing bacteria were isolated from seawater collected in both sampled areas, including three strains from Fukui and four from Tokyo (Ogasawara Islands). Among the isolates, two strains, KU67F and KU67G, were isolated from enrichment cultures with *o*-xylene as the sole carbon source; two strains, KU68F and KU68G, were isolated from enrichment cultures with *m*-xylene as the sole carbon source; and three strains, KU69F, KU69G1 and KU69G4, were isolated from enrichment cultures with *p*-xylene as the sole carbon source (Table 1).

The isolated bacteria were characterized by analysis of their 16S rRNA gene sequences (Table 1, Fig. S1). The determined 16S rRNA gene sequences were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB809162 and LC339512 to LC339517. All isolates were phylogenetically affiliated with the class *Gammaproteobacteria*. Among them, two isolates, KU67F and KU67G, were most closely related to and affiliated with the genus *Marinobacter.* Two strains of the genus *Marinobacter* have been previously shown to degrade *p*-xylene (Berlendis *et al*. 2010), and *Marinobacter* have been suggested to play an important role in the degradation of several organic compounds in the marine environment (Iwaki *et al*. 2012a, 2015). The isolate KU67F was assumed to belong to the same strain as a previously reported phenol degrader, KU17F4, that was isolated from the same seawater sample from Fukui based on its identical 16S rRNA gene sequence (Iwaki *et al*. 2015). Similarly, KU67G and the previously reported phenol degrader KU17G3, which was isolated from the same seawater sample from the Ogasawara Islands, are believed to belong to the same strain.

Among the isolates, three strains were most closely related to and affiliated with the genus *Alteromonas*, and one strain, KU69F, was most closely related to and affiliated with *Aestuariibacter,* a genus that is closely related to *Alteromonas*. Strains KU68G and KU69G1 were also assumed to be the same strain, since they exhibited identical 16S rRNA gene sequences. This is the first time that members of the genera *Alteromonas* and *Aestuariibacter* have been isolated as xylene degraders. Both culture-dependent and -independent methods have indicated that the members of the genus *Alteromonas* play key roles in the degradation of polycyclic aromatic hydrocarbons (Jin *et al*. 2012).

The remaining isolate, strain KU68F^{T}, shared the highest 16S rRNA gene sequence identity with *Maricurvus nonylphenolicus* strain KU41E^{T}, a *p-n*-nonylphenol degrader (Iwaki *et al*. 2012b); however, the identity between these strains was only 92.6% (Table 1). Strain KU68F^{T} was also found to be similar to *Pseudoteredinibacter isoporae* SW-11^{T} (92.5%; Chen *et al*. 2011) and *Simiduia aestuariiviva* J-MY2^{T} (92.0%; Park *et al*. 2014) with lower identities. Levels of identity to other described genera were <92.0%, and strain KU68F^{T} formed a distinct lineage within the family *Cellvibrionaceae* in the order *Cellvibrionales*: the closest neighbor was *Marinagarivorans algicola*

Z1^T (91.6%; Guo *et al*. 2016), as shown in Fig. 1. Therefore, based on the phylogenetic analysis, strain KU68F^T should be classified as a novel genus and species in the family *Cellvibrionaceae* of the order *Cellvibrionales* within the *Gammaproteobacteria*. We therefore selected KU68F^T for further study, including the determination of its precise taxonomic position and characterization of the sequences of its xylene-degrading gene cluster.

Taxonomic characteristics of strain KU68F^T

The cells of strain KU68F^T are gram-negative and were observed to form aggregations on plates containing Marine Agar 2216 (Fig. S2). Cells also formed aggregations or flocs in liquid medium. The formation of aggregates may be due to extracellular polymeric substances (Fig. S3). The strain was capable of utilizing *m*-xylene and *p*-xylene but not *o*-xylene, benzene, toluene, ethylbenzene or cumene as sole carbon and energy sources. Other morphological, phenotypic and chemotaxonomic characteristics are provided in the genus and species descriptions, and those characteristics that differentiate strain KU68F^T from phylogenetically related taxa are listed in Tables 2 and 3.

As demonstrated by the 16S rRNA gene sequence analysis, strain KU68F^T belongs to the family *Cellvibrionaceae* of the order *Cellvibrionales* within the *Gammaproteobacteria* and forms a lineage distinct from related genera. Furthermore, strain KU68F^T can be differentiated from closely related genera by a combination of phenotypic and chemotaxonomic characteristics. Considering the data from the polyphasic study, we suggest that strain KU68F^T represents a novel species of a new genus, for which we propose the name *Halioxenophilus aromaticivorans* gen. nov., sp. nov.

Screening for a possible gene encoding initial oxygenase in the first step of xylene degradation in strain KU68F^T

Terrestrial bacteria degrade xylenes via several pathways that include different initial steps, and these can be distinguished based on the xylene methyl-monooxygenase that catalyzes the hydroxylation of the methyl group or the xylene dioxygenase that catalyzes the hydroxylation of the aromatic ring. *m*-Xylene is mainly degraded by terrestrial bacteria via the toluene/xylene methyl-monooxygenase pathway (Gibson *et al*. 1974; Jang *et al*. 2005). To characterize the xylene degradation pathway of marine strain KU68F^T, we screened for a possible gene encoding an initial oxygenase in the first step of the pathway using PCR. As a result, a *xylA* gene encoding a toluene/xylene methyl-monooxygenase was detected with the primers XYLA-F/XYLA-R. In contrast, no *todC1* gene, encoding the largest subunit of a toluene/xylene dioxygenase, was detected with the primers TODC1-F/TODC1-R. Sequence analysis showed that the deduced amino acid sequence based on the nucleotide sequence of the *xylA* PCR fragment shared 80.7% identity with that of XylA of *Pseudomonas putida* strain mt-2 (Suzuki *et al*. 1991). This result suggests that strain KU68F^T has a two-component diiron xylene

methyl monooxygense system (XylMA) and degrades xylene via the so-called xylene methyl-monooxygenase pathway, which is initiated by the hydroxylation of a methyl group to form methylbenzyl alcohol, followed by the formation of methylbenzealdehyde and methylbenzoate.

Isolation and characterization of a possible gene cluster encoding xylene methyl-monooxygenase pathway genes from strain KU68F^T

To further characterize the xylene degradation pathway of strain KU68F^T, we amplified the flanking regions of the partial *xylA* gene and determined the sequence of a contiguous segment of 9869 bp; this was deposited in the DDBJ under the accession number LC339836. Within this sequence region, seven open reading flames (ORFs) were deduced (Fig. 2). The deduced amino acid sequences of these ORFs were used for BLASTP searches, and five ORFs were assigned to *xylCMABN*$_{KU68F}$. The deduced amino acid sequences of *xylCMABN*$_{KU68F}$ exhibit 77–87% identity with the corresponding proteins of the well characterized xylene methyl-monooxygenase pathway of *Pseudomonas putida* strains mt-2 and MT53 (Table S2). These high identity values are sufficient for determination of the functional identities of the proteins, confirming the presence of a xylene methyl-monooxygenase pathway in strain KU68F and allowing for the assignment of the *xyl* gene products to the upper part of the xylene methyl-monooxygenase pathway (Fig. 2). The gene order of *xylCMABN*$_{KU68F}$ is identical to that of terrestrial *Pseudomonas* spp. A notable difference in the *xyl* locus between KU68F and terrestrial *Pseudomonas* involves the fact that two genes with unknown functions in xylene degradation are not present in strain KU68F (Fig. 2): *xylU,* which is not related to any functionally identified protein in any protein databases, and *xylW,* which encodes a probable long-chain zinc-dependent alcohol dehydrogenase (Harayama *et al*. 1986; Williams *et al*. 1997). The presence of genes in the xylene methyl-monooxygenase pathway represents a major feature of the species, and the analysis of the *xylCMABN* genes should aid in species identification.

Detailed sequence features

The predicted coding region of *xylC*$_{KU68F}$, encoding a methylbenzaldehyde dehydrogenase, is preceded by a putative σ^{70} –35 sequence, TTGACT, and –10 sequence, TTTGAT (Hertz and Stormo 1996). This coding sequence consists of 1467 nucleotides with appropriately positioned consensus Shine-Dalgarno (SD) sequences, GGAG and GAGG, 2 bp and 7 bp from the putative ATG start site, respectively. The deduced amino acid sequence of XylC$_{KU68F}$ exhibits 84.0% identity with the sequence of the (methyl)benzaldehyde dehydrogenase, XylC, of *P. putida* strain mt-2 (Inoue *et al.* 1995). This is a member of the superfamily of NAD(P)$^+$-dependent aldehyde dehydrogenases, which act on a broad variety of aldehyde and semialdehyde substrates by transforming them into carboxylic acids (Hempel *et al*. 1993). Based on what is generally known about aldehyde dehydrogenases, Cys289 of XylC$_{KU68F}$

is the predicted catalytic amino acid residue, and the GxTxxG sequence at position 233–238 is a putative NAD^+ binding site (Yeung *et al*. 2008). Glu255, which is important for the catalytic mechanism of class 2 aldehyde dehydrogenases, is also conserved (Yeung *et al*. 2008).

The predicted coding region of $xylM_{KU68F}$, encoding the hydroxylase component of the xylene methyl-monooxygenase, is 87 bp downstream of *xylC* in the same direction. This coding sequence consists of 1092 nucleotides with an appropriately positioned consensus SD sequence, AGGAGG, 5 bp from the putative ATG start site. The deduced amino acid sequence of $XylM_{KU68F}$ exhibits 81.3% identity with the sequence of the membrane-integrated hydroxylase component of the xylene methyl-monooxygenase, XylM, of *P. putida* strain mt-2 (Suzuki *et al*. 1991). Nine histidine residues, which are potential ligands for the diiron atoms, are contained within four conserved motifs, HxxxH, HxxxHH, HxxHH and NYxEHYG, in $XylM_{KU68F}$. The presence of the histidine residues in these motifs is characteristic of the bacterial integral-membrane hydroxylase family (Shanklin *et al*. 1994; Morikawa 2010; Ratajczak *et al*. 1998).

The predicted coding region of $xylA_{KU68F}$ is 298 bp downstream of *xylM* in the same direction. This coding sequence consists of 1053 nucleotides with an appropriately positioned consensus SD sequence, GGA, 9 bp from the putative ATG start site. The deduced amino acid sequence of the complete $XylA_{KU68F}$ protein is 77.1% identical to that of the NADH:acceptor reductase component of the xylene methyl-monooxygenase, XylA, of *P. putida* strain mt-2 (Suzuki *et al*. 1991). $XylA_{KU68F}$ possesses consensus sequences characteristic of plant-type iron sulfur proteins for the binding of a [2Fe-2S] cluster (Cys-X_4-Cys-X_2-Cys-X_n-Cys), which are conserved among various ferredoxin reductases such as the reductases of phthalate dioxygenase (Correll *et al*. 1993) and naphthalene dioxygenase (Simon *et al*. 1993). Consensus sequences involved in a possible FAD binding domain (RxYS; Dym and Eisenberg 2001) and NAD(P)H binding domain (GGxGxxP; Correll *et al*. 1992) were also observed in the deduced amino acid sequence of $XylA_{KU68F}$.

The predicted coding region of $xylB_{KU68F}$, encoding a methylbenzyl alcohol dehydrogenase, is 45 bp downstream of *xylA*. This coding sequence consists of 1101 nucleotides with an appropriately positioned consensus SD sequence, GGAG, 6 bp from the putative ATG start site. The deduced amino acid sequence of $XylB_{KU68F}$ exhibits 86.3% identity with the sequence of XylB of *P. putida* strain mt-2, a (methyl)benzyl alcohol dehydrogenase belonging to the zinc-dependent long-chain alcohol dehydrogenase family. Notable sequence features present in $XylB_{KU68F}$ including a catalytic zinc-binding motif (GHExxGxxxxxGxxV), structural zinc-binding motif (CxxCxxCxxxxxxxC), and coenzyme-binding motif (GxGxxG) (Reid and Fewson 1994). An Asp residue at position 218, which is expected to be essential for the binding of NAD^+, is also conserved.

The predicted coding region of $xylN_{KU68F}$ is 178 bp downstream of *xylB*. This coding sequence consists of 1395 nucleotides with an appropriately positioned consensus SD sequence, AGG, 5 bp

from the putative ATG start site. The deduced amino acid sequence of $XylN_{KU68F}$ exhibits 77.8% identity with XylN from *P. putida* strain mt-2, an outer membrane protein involved in *m*-xylene uptake (Kasai *et al*. 2001). PSORTb v3.0 (http://www.psort.org/psortb; Yu *et al*. 2010) and PRED-TMBB (http://biophysics.biol.uoa.gr//PRED-TMBB; Bagos *et al*. 2004) predict that $XylN_{KU68F}$ is also an outer membrane protein, like XylN of *P. putida* strain mt-2. Furthermore, the signal sequence at the N-terminal end of $XylN_{KU68F}$ was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP; Petersen *et al*. 2011), with a predicted cleavage site at position 25.

The remaining two ORFs, designated *orf1* and *orf2*, encode putative regulators. The predicted *orf2* coding region is located upstream of *xylC* with 665 bp of intervening intergenic sequence, and it is in the opposite orientation from the *xylCMABN* genes (Fig. 2). A BLAST search revealed that the deduced amino acid sequence of Orf2 shows homology with putative regulators classified in the IclR family. An IclR family helix-turn-helix domain was found in the N-terminal region of the putative Orf2 protein using an InterPro search (Apweiler *et al*. 2000).

The predicted *orf1* coding region is located 58 bp upstream of *orf2* in the opposite orientation from *orf2* and the same direction as *xylCMABN.* A BLAST search revealed that the deduced amino acid sequence of Orf1 shows homology with putative regulators classified in the AraC family, including XylS, which is the toluene/xylene catabolic *meta*-cleavage pathway operon regulator from *P. putida* (40.0% identity) (Ramos *et al*. 1997). An AraC family helix-turn-helix domain was found in the C-terminal region of Orf1 according to both Conserved Domain Database (CDD) (Marchler-Bauer *et al*. 2017) and InterPro searches (Finn *et al*. 2017). The expression of the *Pseudomonas xyl* upper pathway operon is under the control of a σ^{54} promoter and the NtrC family regulator XylR. In strain $KU68F^T$, Orf1 and/or Orf2 are probable regulators of the *xylCMABN* genes, rather than a σ^{54}-dependent NtrC family regulator. This is supported by the presence of a σ^{70} promoter sequence (TTGACT-N_{16}-TTTGAT) 43 bp upstream of the putative ATG start codon of *xylC,* which is the first of the *xylCMABN* genes. A detailed biochemical and genetic study of the *xylCMABN* operon and its regulation is outside the scope of this study.

Description of *Halioxenophilus* gen. nov.

Halioxenophilus (Ha.li.o.xe.no'phi.lus. Gr. adj. *halios,* of the sea; Gr. adj. *xenos,* foreign; Gr. masc. n. *philos,* friend; N.L. masc. n. *Halioxenophilus,* friend of foreign compounds, referring to the isolation of the type species by enrichment on *m*-xylene from the sea)

Cells are Gram-negative, aerobic pleomorphic rods, and non motile. Sodium ions are required for their growth. The predominant fatty acids are Summed Feature 3 ($C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7c), $C_{18:1}$

ω7c, $C_{16:0,}$ unknown fatty acid (equivalent chain-length 11.799), $C_{10:0}$ 3-OH. The predominant respiratory quinone is Q-8. The type species is *Halioxenophilus aromaticivorans.*

Description of *Halioxenophilus aromaticivorans* sp. nov.

Halioxenophilus aromaticivorans (a.ro.ma.ti.ci.vo'rans. N.L. adj. *aromaticus,* aromatic, fragrant; L. part. adj. *vorans*, devouring; N.L. part. adj. *aromaticivorans*, devouring aromatic (compounds))

The description is identical to that for the genus, with the following additions. Cells are 1.0–1.3 μm in length and 0.4–0.5 μm in width. Colonies are yellow, circular, smooth, pulvinate, 1.0 mm in diameter, and with an entire margin after 2 days incubation on Marine Agar 2216. Oxidase- and catalase-positive. Growth occurs at temperatures of 10–30^{o}C, at pH 6 from 9.5, and at NaCl concentrations of 1–4%. The cells are susceptible to ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), penicillin G (10 U), polymyxin B (300 U), rifampicin (5 μg), streptomycin (10 μg), and tetracycline (30 μg), but not to lincomycin (2 μg). In API ZYM system, cells are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase, and weakly positive for cystine arylamidase, but negative for all other enzymes. Hydrolysis of Tween 40, Tween 80 and esculin are positive. In API20NE system, hydrolysis of esculin, β-galactosidase, and utilization of D-glucose and caprate are positive, but negative for all other tests. The cells utilize the following compounds as sole carbon and energy sources: *m*-xylene, *p*-xylene, D-glucose, L-rhamnose, *myo*-inositol, cellobiose, lactose, sucrose, acetate, caprate, *n*-hexanoate, propionate, pyruvate, succinate, L-alanine, L-glutamate, but not the following compounds: *o*-xylene, benzene, toluene, ethylbenzene, cumene, phenol, L-arabinose, D-arabitol, *N*-acetyl-glucosamine, D-galactose, D-fructose, D-mannitol, D-mannose, D-ribose, D-sorbitol, D-xylose, glycerol, D-maltose, trehalose, adipate, citrate, gluconate, formate, DL-malate, L-asparagine, L-asparate, L-histidine, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine. The DNA G + C content is 51.4 mol%.

The type strain, KU68F^{T} (= JCM 19134^{T} = KCTC 32387^{T}), was isolated from seawater obtained from the coastal region of Fukui, Japan.

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Table 1. Xylene-degrading isolates from seawater

Enriched substrate	Source	Strain	16S rRNA gene sequence analysis	
			Closest species in database (Acc. No.)	Identity (%)
o-xylene	Fukui	KU67F	*Marinobacter hydrocarbonoclasticus* (X67022)	97.6
o-xylene	Tokyo (Ogasawara islands)	KU67G	*Marinobacter shengliensis* (KF307780)	99.2
m-xylene	Fukui	KU68F	*Maricurvus nonylphenolicus* (AB626730)	92.6
m-xylene	Tokyo (Ogasawara islands)	KU68G	*Alteromonas macleodii* (Y18228)	99.6
p-xylene	Fukui	KU69F	*Aestuariibacter aggregatus* (FJ847832)	99.1
p-xylene	Tokyo (Ogasawara islands)	KU69G1	*Alteromonas macleodii* (Y18228)	99.6
p-xylene	Tokyo (Ogasawara islands)	KU69G4	*Alteromonas macleodii* (Y18228)	97.8

Table 2. Differential characteristics of strain KU68F^T and closely related genera

Characteristic	1	2	3	4	5
Motility	–	+	+	+	+
NaCl range for growth (%, w/v)	1–4	1–4	2–4	0.5–7.0	0.5–6.0
pH range for growth	6–9.5	7–8	7–8	5.5–8.0	6.5–8.5
Temperature range for growth (°C)	10–30	20–35	10–45	15–40	10–33
Catalase	+	+	+	+	–
Nitrate reduction	–	+	+	+	–
Hydrolysis of:					
Starch	–	–	w	+	+
Gelatin	–	+	+	+	+
Enzyme activities (API ZYM tests)					
Lipase (C14)	+	+	+	–	–
Cystine arylamidase	W	–	+	–	+
Trypsin	–	–	+	–	–
Chymotrypsin	–	–	+	+	–
Acid phosphatase	+	–	+	+	–
Naphthol-AS-BI-phosphohydrolase	+	+	+	–	+
Carbon utilization of:					
D-Arabitol	–	–	+	NR	–
D-Mannose	–	–	+	–	–
L-Rhamnose	+	–	–	NR	–
D-Xylose	–	–	–	+	–
Lactose	+	–	–	NR	–
Cellobiose	+	–	+	+	+
D-Maltose	–	–	–	–	+
Sucrose	+	–	–	+	–
Trehalose	–	+	–	+	–
Glycerol	–	–	+	NR	–
myo-Inositol	+	–	–	NR	–
Acetate	+	+	+	–	NR
Citrate	–	–	–	–	+
Pyruvate	+	+	–	–	NR
Succinate	+	–	+	+	NR
L-Glutamate	+	–	+	–	NR
Susceptibility to:					
Ampicillin	+	+	–	+	+
Kanamycin	+	+	+	–	NR
Polymyxin B	+	+	NR	–	+
Tetracycline	+	+	+	+	–
Isoprenoid quinone(s)	Q-8(97.7%) Q-7(1.7%) Q-9(0.6%)	Q-8	Q-9 (79%), Q-8 (21%)	Q-8	Q-8
DNA G+C content (mol%)	51.4	48.6	51.6	54.8	45.1

Genera: 1, strain KU68F^T; 2, *Maricurvus nonylphenolicus* KU41E^T (data from Iwaki *et al*., 2012); 3, *Pseudoteredinibacter isoporae* SW-11^T (Chen *et al*., 2011); 4, *Simiduia aestuariiviva* J-MY2^T (Park *et al*. 2014); 5, *Marinagarivorans algicola* Z1^T (Guo *et al*. 2016). +, positive reaction; –, negative reaction; w, weakly positive reaction; NR, not reported.

Table 3. Cellular fatty acid compositions (%) of strain KU68F^T and closely related genera

Fatty acid	1	2	3	4	5
$C_{9:0}$	–	1.0	–	–	–
$C_{10:0}$	–	6.4	3.5	–	5.1
$C_{11:0}$	–	–	2.5	–	–
$C_{12:0}$	1.2	–	3.0	–	–
$C_{14:0}$	1.6	1.3	1.1	2.5	–
$C_{15:0}$	–	1.8	–	–	–
$C_{16:0}$	18.1	17.0	10.3	20.7	14.3
$C_{17:0}$	–	1.8	5.1	4.5	–
$C_{18:0}$	–	1.6	–	1.5	1.1
$C_{10:0}$ 3-OH	5.9	9.4	3.2	2.7	8.3
$C_{11:0}$ 3-OH	–	–	4.0	–	–
$C_{12:0}$ 3-OH		–	2.4	–	–
$C_{12:1}$ 3-OH	–	–	–	2.5	–
$C_{17:1}$ ω6c	–	–	1.2	1.1	–
$C_{17:1}$ ω8c	–	5.6	13.4	12.0	–
$C_{17:1}$ anteiso ω9c	–	–	10.0	–	–
$C_{18:1}$ ω6c	–	3.0	13.1[a]	–	49.1
$C_{18:1}$ ω7c	28.8	19.8		12.3	14.3
Summed Feature 3[b]	34.5	28.4	22.3[c]	36.7[c]	17.4
Summed Feature 7[b]	–	–	–	–	1.2
unknown ECL 11.799[d]	8.5	–	–	–	–

Genera: 1, strain KU68F^T; 2, *Maricurvus nonylphenolicus* KU41E^T (data from Iwaki *et al*., 2012); 3, *Pseudoteredinibacter isoporae* SW-11^T (Chen *et al*., 2011); 4, *Simiduia aestuariiviva* J-MY2^T (Park *et al*. 2014); 5, *Marinagarivorans algicola* Z1^T (Guo *et al*. 2016). Values are percentages of the total fatty acids; fatty acids that make up <1% of the total are not shown or indicated by "–". [a] $C_{18:1}$ ω6c and/or $C_{18:1}$ ω7c; [b] Summed features are groups of two fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH or [c] $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c; Summed feature 7 comprises $C_{19:1}$ ω6c and/or unknown ECL 18.846; [d]ECL, Equivalent chain-length.

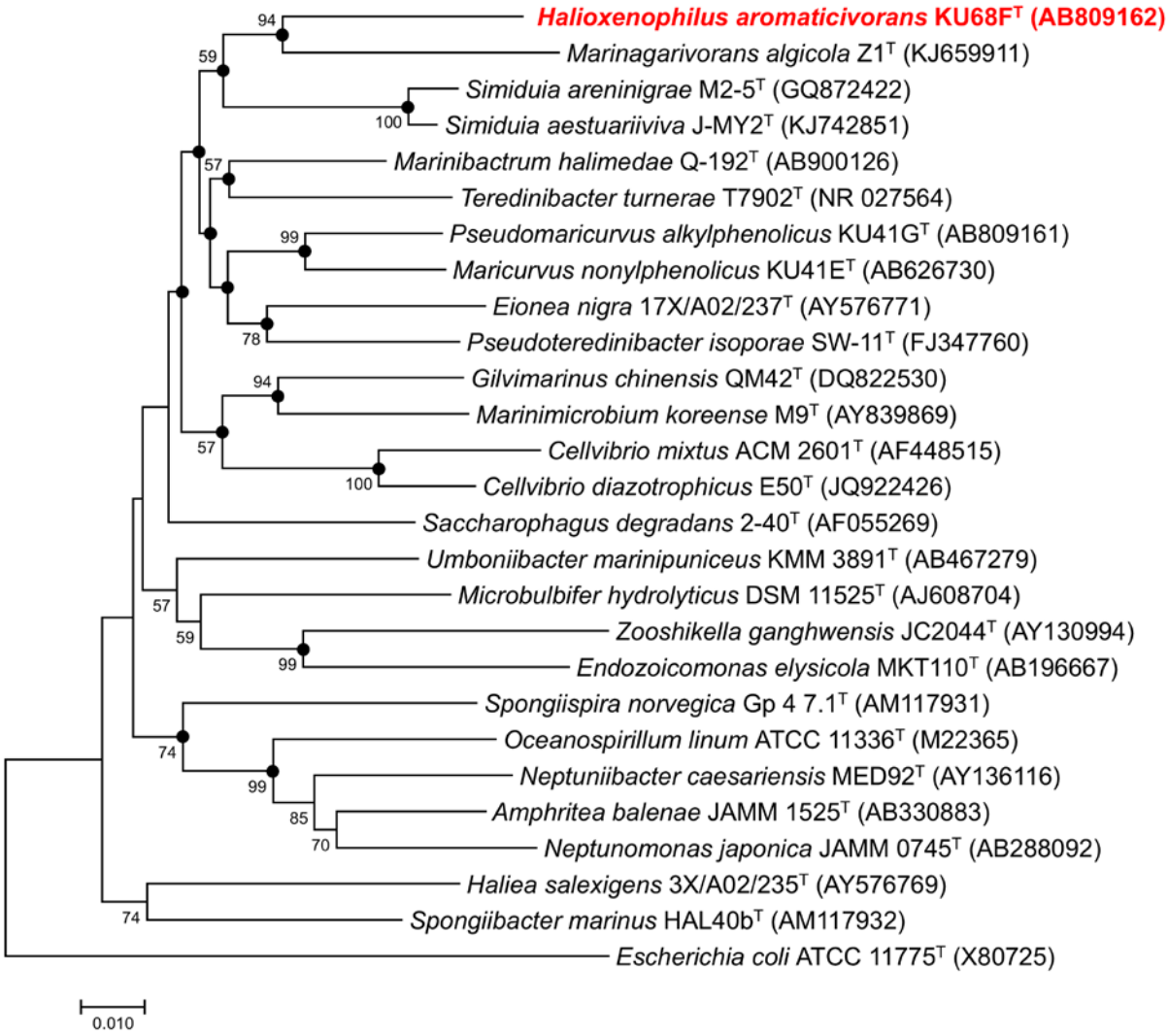

Fig. 1. Phylogenetic relationships between strain KU68F^T and other members of the class *Gammaproteobacteria*. The tree was constructed using the neighbor-joining algorithm. Numbers at nodes are bootstrap percentages based on 1000 replications; only values > 50% are shown. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum likelihood algorithm. Bar, 0.01 substitutions per nucleotide position.

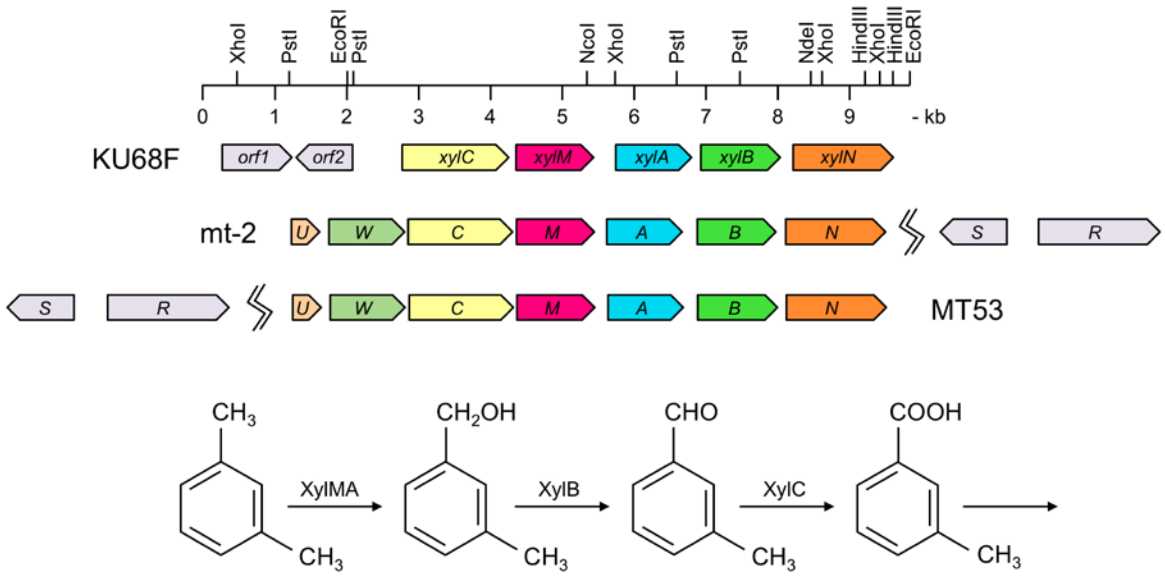

Fig. 2. Genetic structure of the *m*-xylene degradation upper pathway gene (*xyl*) cluster isolated from *Halioxenophilus aromaticivorans* strain KU68F^{T} and assignment of the *xyl* gene products to the xylene degradation upper pathway. Genetic organization of the *xyl* gene clusters in *Pseudomonas putida* strains mt-2 and MT53 are also shown.

Supporting Information

Isolation of marine xylene-utilizing bacteria and characterization of *Halioxenophilus aromaticivorans* gen. nov., sp. nov. and its xylene degradation gene cluster

Hiroaki Iwaki, Taisei Yamamoto, Yoshie Hasegawa

Table S1. Sequences of primers used in this study.

Primer	Sequence (5' to 3')
27f	AGAGTTTGATC(A/C)TGGCTCAG
1492r	TACGG(C/T)TACCTTGTTACGACTT
342r	CTGCTGC(G/C)(C/T)CCCGTAG
357f	CTCCTACGGGAGGCAGCAG
926f	AAACT(C/T)AAA(G/T)GAATTGACGG
9F	GAGTTTGATCCTGGCTCAG
1510R	GGCTACCTTGTTACGA
785F	GGATTAGATACCCTGGTAGTC
802R	TACCAGGGTATCTAATCC
inv-F	TGCGGCAGCAGCGTGATTTATATTGCCTTG
inv-R	GTTGGGTTCCGGTCATGCTCTCCAAAATGC
XYLA-F	CCAGGTGGAATTTTCAGTGGTTGG
XYLA-R	AATTAACTCGAAGCGCCCACCCCA
TODC1-F	CAGTGCCGCCA(C/T)CGTGG(C/T)ATG
TODC1-R	GCCACTTCCATG(C/T)CCRCCCCA

Table S2. Xylene degradation pathway gene products of strain KU68F and identities with the corresponding *xylCMABN* gene products from *Pseudomonas putida* strains mt-2 and MT53.

gene	deduced function	aa	% identity (aa) [accession no.]	
			P. putida mt-2	*P. putida* MT53
$xylC_{KU68F}$	benzaldehyde dehydrogenase	488	84 (408/486) [BAA09661]	84 (409/486) [BAF02444]
$xylM_{KU68F}$	xylene monooxygenase hydroxylase subunit	363	81 (291/358) [BAA09662]	81 (291/358) [BAF02445]
$xylA_{KU68F}$	xylene monooxygenase electron transfer component	350	77 (266/344) [BAA09663]	77 (265/344) [BAF02446]
$xylB_{KU68F}$	benzyl alcohol dehydrogenase	366	86 (315/365) [BAA09664]	87 (318/365) [BAF02447]
$xylN_{KU68F}$	outer membrane protein involved in xylene uptake	464	78 (362/465) [BAA09665]	79 (366/461) [BAF02448]

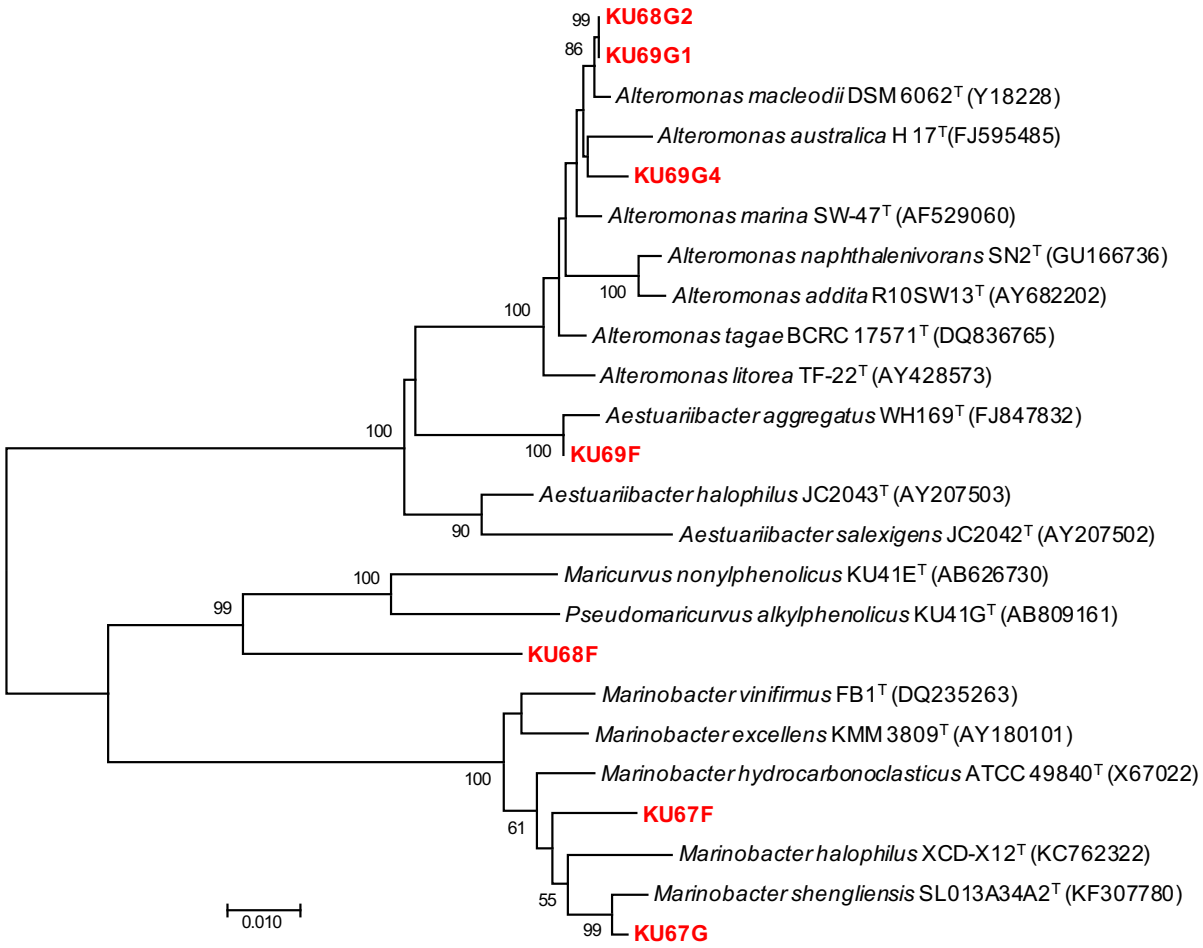

Fig. S1. Phylogenetic relationships among xylene-degrading isolates and closely related genera. The tree was constructed using the neighbor-joining algorithm. Numbers at nodes are bootstrap percentages based on 1000 replications; only values > 50% are shown. Bar, 0.01 substitutions per nucleotide position.

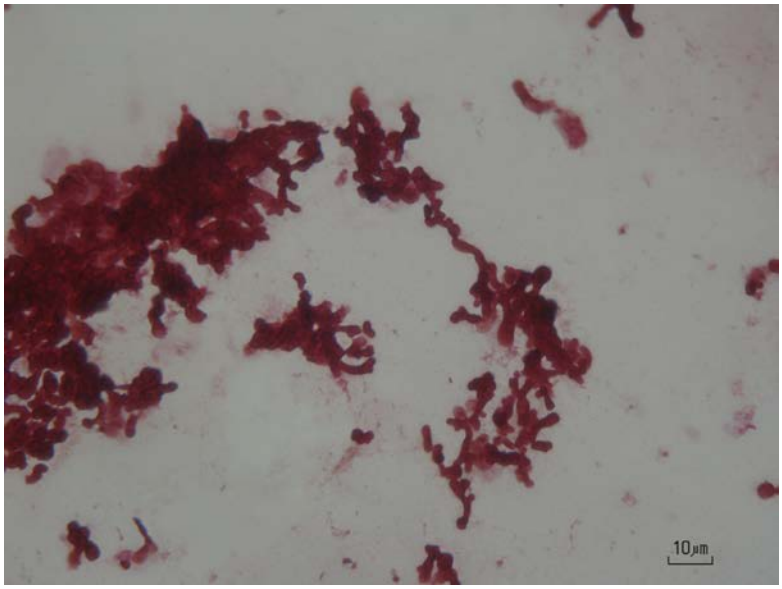

Fig. S2. Light micrograph of Gram-strained KU68F^T cells.

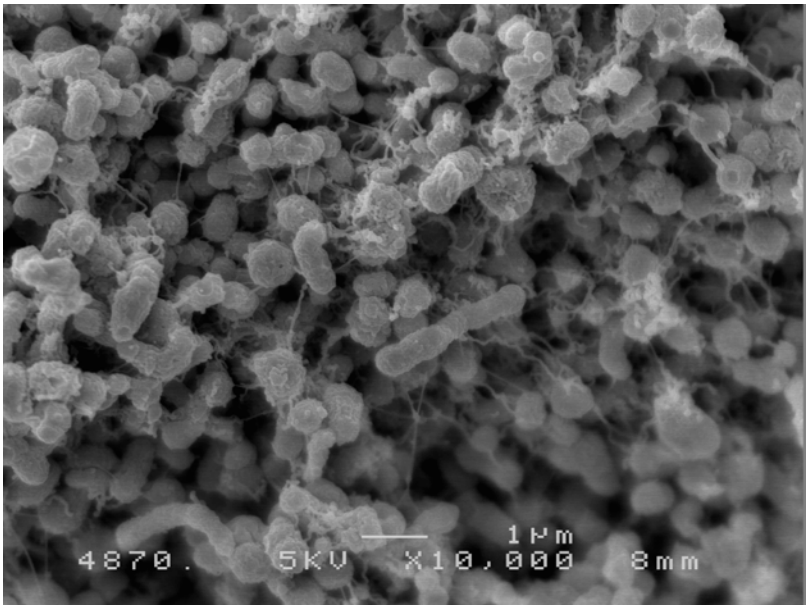

Fig. S3. Scanning electron microscope image of KU68F^T cells grown on Marine Agar 2216. Extracellular polymeric substances can be seen.