

# Identification of a mosaic transposable element of *Paracoccus marcusii* composed of insertion sequence *ISPmar4* (ISAs1 family) and an *IS1247a*-driven transposable module (TMO)

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transposition; entrapment vector.

## Introduction

Insertion sequences (ISs) are the simplest transposable elements (TEs), possessing only the genetic information necessary for their own transposition. The majority of ISs only carry a single gene encoding transposase (Tnp), plus short terminal inverted repeats (IRs) that can be recognized by the Tnp. Transposition of these elements usually results in duplication of a short target sequence, which produces direct repeats (DRs) flanking the entire element (Chandler & Mahillon, 2002).

ISs are widespread among bacteria. Because of their recombinational activity, they play a crucial role both in shaping the structure of bacterial genomes and in the mobilization of genomic DNA in horizontal gene transfer. The latter function is realized (1) by the formation of composite transposons, which consist of random segments of genomic DNA bordered by a pair of ISs, or (2) by generation of mobile insertion cassettes, which result from trans-mobilization of genomic DNA by coexisting ISs (e.g. De Palmenaer *et al.*, 2004).

Recently, using entrapment vector pMEC1 (carrying a positive selection cartridge enabling direct identification of functional TEs), we demonstrated that even a single copy of

## Abstract

A *sacB*-based trap plasmid was used to clone a mosaic transposable element (TE) in the carotenoid producer *Paracoccus marcusii* OS22. This element is composed of an insertion sequence (IS) *ISPmar4* (ISAs1 family), which contains an inserted functional transposable module (TMO) generated by a copy of *IS1247a* (of the *IS1380* family). Besides *IS1247a* sequences, the TMO also contains the 3'-end region of a putative  $\alpha/\beta$  hydrolase gene, whose expression might be activated from the  $P_2$  promoter of *IS1247a*. The identification of this novel TE provides evidence that transposition of TMOs may change an IS into a more complex element resembling noncomposite transposons.

an IS (*ISPme1* of the *IS1380* family) can mobilize adjacent segments of genomic DNA, leading to the generation of diverse TEs of various sizes and DNA contents (Bartosik *et al.*, 2008). These *ISPme1*-mediated elements (named transposable modules, TMOs) were identified in *Paracoccus methylutens* DM12 (*Alphaproteobacteria*). We found that transposition of these elements is a frequent phenomenon. We also demonstrated that c. 80% of the sequence of pMTH1 (31 kb), a natural plasmid residing in strain DM12, is formed by TMOs, which indicates the potentially significant role of these elements in shaping the structure of bacterial genomes.

All TMOs have a similar structure. They are composed of a single copy of *ISPme1* (always placed in the same orientation at the 5'-end of the elements) with adjacent DNA fragments of lengths varying from 0.5 to 5 kb (called 3'-end DNA regions). The DNA content of the 3'-end DNA regions depends solely on the location of *ISPme1* in the host genome. A number of copies of *ISPme1* reside in strain DM12 and we identified several types of TMOs with different 3'-end DNA regions. In an attempt to classify these diverse elements, different types (A, B, C, etc.) and classes (1, 2, 3, etc.) of TMOs were distinguished. Within a given type, all

the TMs carry homologous DNA segments adjacent to *ISPme1*, although the length of these segments varies in individual elements (size classes within a type). We also proposed the nomenclature of these elements, for example TMo(*ISPme1*)A1 indicates a class 1 type A TMo generated by the IS *ISPme1* (Bartosik *et al.*, 2008).

In order to determine whether other ISs related to *ISPme1* are able to generate TMs, we searched for such ISs in different strains and species of the genus *Paracoccus*, finally focusing on the carotenoid producer *Paracoccus marcusii* OS22. Using an entrapment vector, we identified a TMo, originally residing within a disrupted (but still functional) novel IS of the ISAs1 family. We found that transposition of TMs may influence the structure of other TEs, and also demonstrated that the acquisition of TMs may convert a simple IS into a noncomposite transposon.

## Materials and methods

### Bacterial strains and plasmids

*Paracoccus marcusii* OS22 (Drewniak *et al.*, 2008) was the host strain of the analysed TEs; *Paracoccus versutus* UW225 (Bartosik *et al.*, 1992) was used for  $\beta$ -galactosidase assays, and *Paracoccus pantotrophus* KL100 (Bartosik *et al.*, 2002) was used to analyse the transposition activities of the TMo. The strains of *Paracoccus* spp. used for hybridization analysis were: *Paracoccus aestuarii* PAT1, *Paracoccus alcaliphilus* JCM 7364R, *Paracoccus alkenifer* DSM 11593R, *Paracoccus aminophilus* JCM 7686R, *Paracoccus aminovorans* JCM 7685R, *Paracoccus halophilus* PHL1, *Paracoccus kondratievae* PKN1, *Paracoccus koreensis* PKR1, *P. marcusii* OS22, *P. methylutens* DM12, *P. pantotrophus* DSM 11072R, *P. pantotrophus* DSM 11073R, *P. pantotrophus* LMD 82.5R, *Paracoccus seriniphilus* PSE1, *Paracoccus solventivorans* DSM 11592R, *Paracoccus sulfuroxidans* PSF1, *Paracoccus thiocyanatus* IAM 12816R, *P. versutus* UW1R, *Paracoccus yeeii* PEI1 and *Paracoccus zeaxantinifaciens* PZX1. *Escherichia coli* TG1 was used for plasmid construction. The plasmids used in this study (pRK415, pBBR1MCS-2, pCM132, and pDS132) have been described previously (Keen *et al.*, 1988; Kovach *et al.*, 1995; Marx & Lindstrom, 2001; Philippe *et al.*, 2004). Entrapment vectors pMAT1 (Km<sup>r</sup>) and pSCM1 (Tc<sup>r</sup> and Cm<sup>r</sup>) were constructed by cloning an XbaI–EcoRV DNA fragment (containing the *sacB* gene) or a PstI–BamHI DNA fragment (containing the *sacB* gene and a chloramphenicol resistance gene), derived from plasmid pDS132, into compatible sites in pBBR1MCS-2 and pRK415, respectively.

### DNA manipulations

The isolation of plasmid DNA and total bacterial DNA as well as common DNA manipulation methods were performed as described by Sambrook & Russell (2001).

### DNA–DNA hybridization

Probes for dot-blot DNA hybridization analysis were amplified by PCR using specific primer pairs: (1) LTNP1TMo/RTNP1TMo (*ISPmar4*), (2) LTNP2TMo/RTNP2TMo (*IS1247a*) and (3) LHYDTMo/RHYDTMo (hydrolase gene) (Table 1). The amplified DNA fragments were gel-purified and labelled with digoxigenin (Roche). Application of total bacterial DNA to the nylon membrane (Roche), hybridization and visualization of bound digoxigenin-labelled probes were carried out as recommended by the supplier.

### Introduction of DNA into bacteria

DNA was introduced into bacterial cells by triparental mating (*Paracoccus* spp.) or transformation (*E. coli*), as described previously (Bartosik *et al.*, 2008).

### Assay for $\beta$ -galactosidase activity

Selected fragments of TMo(*IS1247a*)A1 amplified by PCR using specific primer pairs: (1) TMOMSL1/TMOMSR3 (contains P<sub>1</sub> promoter of *IS1247a*), (2) TMOMSL2/TMOMSR2 (contains P<sub>2</sub> promoter of *IS1247a*) and (3) TMOMSL3/TMOMSR1 (DNA region upstream of the hydrolase gene) (Table 1) were cloned into the promoter probe vector pCM132 carrying a *lacZ* reporter gene.  $\beta$ -Galactosidase activity in *P. versutus* UW225 strains carrying these constructs was measured by the conversion of *O*-nitrophenyl- $\beta$ -D-galactopyranoside into nitrophenol as described by Miller (1972). Assays for  $\beta$ -galactosidase activity were repeated three times.

**Table 1.** Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
A489SB	CAGACCCTAACACAGTACA
A869SB	TTAGGATCTCCGGCTAATGC
B824SB	ACTATCACGGCTACCACATC
B1253SB	TTGTCGCTGAGCTGTAGTT
C1225SB	GATGAAGGCAACTACAGCTC
C1639SB	ACGTAATGCCGTCAATCGTC
D1619SB	TGACGATTGACGGCATTACG
D1928SB	CCTTGTTCAAGGATGCTGTC
LTNP1TMo	GAACACGCGACATGACTTGG
RTNP1TMo	AGGACAGCGCGAAGTACCTC
LTNP2TMo	GTGATGCGCGAGCTTGATGA
RTNP2TMo	GAAGAACCCTTGACCTTGG
LHYDTMo	TTCTGGCCGAAGGTTACCGC
RHYDTMo	GTTACCACATCAGGATGCG
TMOMSL1	GC <b>GAATC</b> CCCCACATTTCCCAAGTAAGG
TMOMSR3	GT <b>GGATCC</b> CTGTCCATGTCCAGAACGAT
TMOMSL2	AC <b>GAATTC</b> TCGCCGTATCCGCCGATTG
TMOMSR2	AT <b>GGATCC</b> GACACGTCTCTGCATTCC
TMOMSL3	AC <b>GAATTC</b> GGAATGCAGGAGGACGTGTC
TMOMSR1	AT <b>GGATCC</b> GGTGGAAACATGACAGGCTGC

Introduced EcoRI and BamHI restriction sites are shown in bold.

## Sequence analyses and annotation

Nucleotide sequences were determined using a dye terminator sequencing kit and an automatic sequencer (ABI 377 Perkin Elmer). DNA sequence similarity searches were performed using ISFINDER (<http://www-is.biotoul.fr/is.html>) and the BLAST programs (Altschul *et al.*, 1997) provided by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## Nucleotide sequence accession numbers

The nucleotide sequences of *ISPmar4*[TMo(IS1247a)A1] reported here have been deposited in the GenBank/EMBL database under accession number FJ422380. The nucleotide sequences of *ISPmar4* and *IS1247a* have also been submitted to the ISFINDER database.

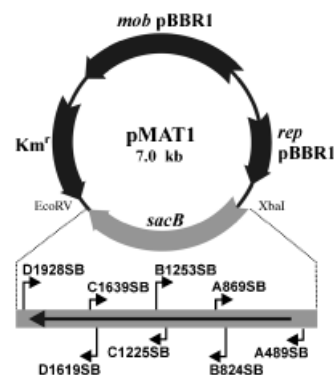
## Results

### Identification of relatives of *ISPme1* in *Paracoccus* spp.

The distribution of *ISPme1* homologues in different strains of *Paracoccus* spp. was examined by dot-blot DNA–DNA hybridization analysis. The full nucleotide sequence of *ISPme1*, amplified by PCR, was labelled with digoxigenin and used as a probe against total DNA extracted from the strains of *Paracoccus* spp. listed in Materials and methods. We observed strong hybridization signals with the DNAs of three environmental strains: (1) *P. methylutens* DM12 (positive control), (2) *P. marcusii* OS22 and (3) *P. halophilus* PHL1. A weaker, but still distinct, signal was detected with the DNA of a clinical isolate of *P. yeiii* PE11 (data not shown). In this study, we focused our interest on the carotenoid producer *P. marcusii* OS22.

### Identification and analysis of a pool of insertion mutants

To analyse transposition of the *ISPme1* relatives and the possible formation of TMs in the strain OS22, we used the trap plasmid pMAT1, which enables direct *in vivo* capture of functional TEs (Fig. 1). This plasmid is composed of a mobilizable, broad-host-range vector pBBR1MCS-2 ( $Km^r$ ) and the *sacB* gene of *Bacillus subtilis*. The expression of *sacB* is lethal for the bacterial host in the presence of sucrose (Gay *et al.*, 1985; Solyga & Bartosik, 2004). This allows direct selection of *sacB* mutants (e.g. carrying inserted TEs), whose growth is not affected under these conditions. The *sacB* gene therefore serves as a cassette enabling positive selection of transposition mutants. Plasmid pMAT1 was introduced into *P. marcusii* OS22 by triparental mating and transposition mutants were selected on Luria–Bertani medium supplemented with sucrose and kanamycin.  $Km^r$  and sucrose<sup>r</sup>



**Fig. 1.** The genetic organization of the entrapment vector pMAT1 containing the *sacB* gene. The four pairs of nested primers used to localize the insertion site of TEs by PCR (listed in Table 1) are indicated.

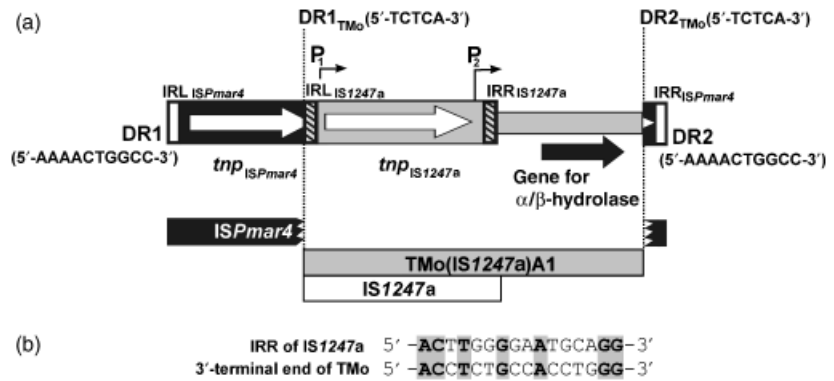
clones appeared at a frequency of  $7 \times 10^{-5}$ . We analysed the plasmid pattern of 100 clones. Detailed restriction analysis revealed the presence of three classes of elements ‘captured’ in pMAT1, of approximate sizes 1.2 kb (94% of clones), 1.6 kb (3%) and 4.1 kb (1%). The remaining 2% of clones carried plasmids of the original size representing putative point mutations or small deletions.

Hybridization analysis of the pMAT1 insertion derivatives performed with the *ISPme1*-specific digoxigenin-labelled probe revealed that all 1.6 and 4.1 kb inserts carried sequences homologous to *ISPme1* (data not shown). Plasmids containing these inserts were used as the template DNA for PCR with four sets of nested cartridge-specific primers (Fig. 1, Table 1). This confirmed that the transposition had occurred within the *sacB* gene and allowed rough localization of the insertion site (data not shown).

### Bioinformatic analyses of the captured TEs

The nucleotide sequences of the ‘captured’ TEs of 1.6 kb (randomly selected plasmid pMAT1/2) and 4.1 kb (plasmid pMAT1/6) were determined. These were identified as (1) an IS and (2) an IS-driven element of mosaic structure, respectively.

The pMAT1/2 IS (1671 bp) carries one large ORF (comprising 81% of the element) encoding a putative transposase composed of 451 amino acids (aa). Similarity searches of the ISFINDER database showed a very high level of identity of this IS (99% at the nucleotide sequence level) to *IS1247* (the *IS1380* family), previously identified by van der Ploeg *et al.* (1995) in *Xanthobacter autotrophicus* GJ10. The ‘captured’ IS therefore represents an isoform of *IS1247* and was designated *IS1247a*. The only difference between these two elements is the transition C → T at position 1293 (of the *IS1247a* nucleotide sequence), which results in the substitution of threonine (*IS1247*) by isoleucine (*IS1247a*) at position 400 in the amino acids sequence of the transposase. The



**Fig. 2.** (a) Genetic structure of the mosaic TE *ISPmar4*[*TMo*(*IS1247a*)*A1*] identified with entrapment vector *pMAT1* in *Paracoccus marcusii* OS22. Promoters *P*<sub>1</sub> and *P*<sub>2</sub> identified within *IS1247a* are indicated. See text for details. (b) Comparison of the nucleotide sequences of the 3' termini of *IS1247a* (*IRR*) and of the *TMo*.

deposited nucleotide sequence of *IS1247* is 1 bp longer than *IS1247a* (additional C residue adjacent to the 5'-end of the *IRL*), which may have been due to the incorrect definition of the *IS1247* terminus. *IS1247a* also shows a substantial similarity to *ISPme1* (90% identity at the DNA level), which explains the positive hybridization signal observed with the *ISPme1*-derived probe.

The second element 'captured' in *pMAT1/6* (4087 bp) encodes three ORFs placed in the same orientation (Fig. 2a). Sequence analysis revealed a mosaic structure composed of (1) a novel IS with overall similarity to members of the *ISAs1* family (designated *ISPmar4*), which has been disrupted (but remains functional in transposition) by (2) a *TMo* generated by *IS1247a* (Fig. 2a). The predicted *TMo* has been inserted into the terminal part of a gene encoding the transposase of *ISPmar4* (*in silico* predictions suggest that the *Tnp* is lacking 4 aa at the C-terminus; data not shown). Consequently, the complete mosaic element contains identical 9 bp IRs of *ISPmar4* at both ends, and is bordered (within the *sacB* cassette) by DRs of 9 bp – a size characteristic for members of the *ISAs1* family (Fig. 2a).

The predicted 'passenger' *TMo* (2874 bp) contains *IS1247a* (placed at the 5'-end) and a 3'-end DNA region of length 1203 bp (Fig. 2a). This 3'-end region contains only one ORF, whose putative product shows high similarity to a number of proteins containing the  $\alpha/\beta$  hydrolase fold (Remington *et al.*, 1992). The highest similarity (73%) was observed with the  $\alpha/\beta$  hydrolase of *Roseovarius* sp. TM1035. This presumptive *TMo* is bordered by 5 bp DRs – a remnant of the ancient transposition event that led to insertion of this element into *ISPmar4*.

The *TMo* (according to the previously proposed nomenclature) was designated *TMo*(*IS1247a*)*A1* and the complete mosaic element was named *ISPmar4*[*TMo*(*IS1247a*)*A1*].

To definitely prove the presence of *ISPmar4*[*TMo*(*IS1247a*)*A1*] in the OS22 genome and exclude the possibility that this mosaic element is a result of two successive transposition events that occurred in the entrapment vector *pMAT1*, we performed PCR analyses. For the analysis, we

used total DNA of the wild-type OS22 strain (as a template DNA) and two primers, *LTNP1TMo* and *RHYDTMo* (Table 1), which anneal close to both ends of the *ISPmar4*[*TMo*(*IS1247a*)*A1*]. As a result, we obtained a PCR amplification product of the expected size, whose nucleotide sequence showed an identity to the *ISPmar4*[*TMo*(*IS1247a*)*A1*] (data not shown). This confirmed the existence of the composite structure in the OS22 genome.

#### Analyses of the transposition activity of *TMo*(*IS1247a*)*A1*

To prove that the *in silico*-predicted *TMo* is a functional TE, we attempted to demonstrate its transposition from one entrapment vector to another. For this purpose, two plasmids were used: (1) *pMAT1/6* containing *ISPmar4*[*TMo*(*IS1247a*)*A1*] and (2) a compatible *Tc*<sup>r</sup> plasmid *pSCM1* with an 'empty' *sacB* trap cassette. Both replicons were introduced into *P. pantotrophus* strain KL100 and a pool of *Tc*<sup>r</sup> clones grown in the presence of sucrose (sucrose<sup>r</sup>) was analysed as described previously. The strain KL100 was used for two reasons: (1) it does not harbour any plasmids (unlike *P. marcusii* OS22, which carries approximately eight plasmids of sizes ranging from 5 to 100 kb) and (2) it does not contain ISs homologous to *IS1247a* (as inferred by DNA hybridization analysis; data not shown). Analysis of 100 *Tc*<sup>r</sup> and sucrose<sup>r</sup> clones revealed that the predicted *TMo* of *ISPmar4*[*TMo*(*IS1247a*)*A1*] as well as *IS1247a* itself could transpose into *pSCM1* at frequencies of 1% and 38%, respectively. In both cases, transposition resulted in the duplication of 5 bp target sequences (Table 2). The length of the transposed *TMo* was identical to our previous *in silico* prediction.

#### Promoters within the *TMo*

We next investigated whether transcription of the putative  $\alpha/\beta$  hydrolase gene of *TMo*(*IS1247a*)*A1* is driven by its own promoter or by a promoter(s) derived from *IS1247a*. For this purpose, DNA sequences located (1) between the right

**Table 2.** Characteristic features of TEs of *Paracoccus marcusii* OS22

TE	Length (bp)	G+C (%)	IR (bp)	DR (bp)	IS family/group
IS1247a	1671	61.6	16/13	5	IS1380
ISPmar4*	1218	65	19/19	10	ISAs1
TMo(IS1247a)A1	2847	62	–	5	–
ISPmar4[TMo(IS1247a)A1]	4087	62.8	19/19	10	–

\**In silico* prediction after removing TMo(IS1247a)A1 and one of the DRs.

IR (IRR) of IS1247a and the ATG start codon of the  $\alpha/\beta$  hydrolase gene and (2) between the TAA stop codon of the IS1247a transposase gene and the IRR of this element were separately amplified by PCR (see Materials and methods for details) and inserted into the broad-host-range promoter probe vector pCM132 to generate transcriptional fusions with a promoterless *lacZ* reporter gene. The resulting plasmid constructs were introduced into *P. versutus* UW225 (a strain routinely used by us for such analyzes) and  $\beta$ -galactosidase activity assays were used to examine promoter strength. The absence of  $\beta$ -galactosidase activity indicated that the  $\alpha/\beta$  hydrolase gene does not contain its own promoter. In contrast, we found that IS1247a carries an outwardly oriented promoter ( $P_2$ ) located in the terminal part of the element (Fig. 2a;  $\beta$ -galactosidase activity:  $124.7 \pm 5.04$  Miller units), which can drive the transcription of downstream genes. We also confirmed the presence of the  $P_1$  promoter for the transposase gene (Fig. 2a;  $\beta$ -galactosidase activity:  $40.8 \pm 5.57$  Miller units).

### Localization of TMs in the *P. marcusii* genome

To examine the localization and copy number of ISPmar4[TMo(IS1247a)A1] (and its component parts distinguished *in silico*) in *P. marcusii* OS22, genome hybridization analysis was performed. For this purpose, three probes (PCR-amplified digoxigenin-labelled DNA fragments), specific for (1) ISPmar4, (2) IS1247a and (3) the  $\alpha/\beta$  hydrolase gene, were hybridized with appropriately digested total DNA of the OS22 strain as well as the DNA of plasmids harboured by this strain. The results obtained revealed that (1) IS1247a and the hydrolase gene (i.e. the TMo) are present in one copy and (2) ISPmar4 is present in two copies in the OS22 genome. All the elements are also harboured by a large (c. 60 kb) natural plasmid in this strain (data not shown).

### Discussion

The results of this study provide further evidence that ISs of the IS1380 family are able to mobilize adjacent DNA regions to generate TMs. The TMo described here is composed of (1) a copy of IS1247a and (2) the 3'-end DNA 'tail' carrying a putative  $\alpha/\beta$  hydrolase gene. This element, designated

TMo(IS1247a)A1, resides within the IS ISPmar4. *In silico* sequence analysis demonstrated that insertion of the TMo caused disruption of the terminal part of the ISPmar4-encoded transposase gene. Despite this, the transposition machinery of ISPmar4 is still functional, and it can mobilize the whole mosaic element, named ISPmar4[TMo(IS1247a)A1]. Because of the transposition of the TMo, the IRs of ISPmar4 are widely separated, which closely resemble noncomposite transposons such as those of the Tn3 family. It is believed that Tn3 and its relatives also originated from an IS (ancestral element to IS1071), which evolved by capturing a site-specific recombination system for cointegrate resolution and other accessory genes (Nakatsu *et al.*, 1991). The element analysed in the present study is, therefore, a further example of how the acquisition of additional genetic information may change a simple IS into a more complex element. Our findings also provide evidence that TMs may significantly influence the structure of other TEs.

It may be speculated that TMs might be generated by a process called one-ended transposition, which produces random end points at one end of the transposed elements. Alternatively, it is possible that the IS-encoded transposase might mistakenly recognize genomic sequences, which act as functional analogues of the IS IRR (Bartosik *et al.*, 2008). Here, we found that the TMo that transposed into the entrapment vector pSCM1 was identical to one already residing in ISPmar4[TMo(IS1247a)A1], which suggests that the 3' terminus of the TMo might be preferentially selected for transposition. Comparative analysis of the nucleotide sequences at the termini of IS1247a (IRR) and TMo(IS1247a)A1 showed apparent similarities (7/16 identical nucleotides) (Fig. 2b), which supports the above suggestion; however, further experiments are required to verify these observations.

The 'passenger' gene in the TMo, coding for a putative  $\alpha/\beta$  hydrolase, does not carry its own promoter (or its promoter is nonfunctional in the analysed strain), but its transcription might be activated from the  $P_2$  promoter of IS1247a. Proteins with the  $\alpha/\beta$  hydrolase fold constitute one of the largest known superfamilies, comprising many hydrolytic enzymes with diverse catalytic functions (e.g. proteases, lipases, esterases, dehalogenases, peroxidases and epoxide hydrolases) (Nardini & Dijkstra, 1999). It is likely that that acquisition of this gene might confer some advantage to the host strain, although this needs to be verified by determination of the activity and substrate specificity of the predicted protein.

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