Styrene oxide isomerase of *Sphingopyxis* sp. Kp5.2

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Styrene oxide isomerase (SOI) catalyses the isomerization of styrene oxide to phenylacetaldehyde. The enzyme is involved in the aerobic styrene catabolism via side-chain oxidation and allows the biotechnological production of flavours. Here, we reported the isolation of new styrene-degrading bacteria that allowed us to identify novel SOIs. Out of an initial pool of 87 strains potentially utilizing styrene as the sole carbon source, just 14 were found to possess SOI activity. Selected strains were classified phylogenetically based on 16S rRNA genes, screened for SOI genes and styrene-catabolic gene clusters, as well as assayed for SOI production and activity. Genome sequencing allowed bioinformatic analysis of several SOI gene clusters. The isolate *Sphingopyxis* sp. Kp5.2 was most interesting in that regard because to our knowledge this is the first time it was shown that a member of the family Sphingomonadaceae utilized styrene as the sole carbon source by side-chain oxidation. The corresponding SOI showed a considerable activity of 3.1 U (mg protein)$^{-1}$. Most importantly, a higher resistance toward product inhibition in comparison with other SOIs was determined. A phylogenetic analysis of SOIs allowed classification of these biocatalysts from various bacteria and showed the exceptional position of SOI from strain Kp5.2.

**INTRODUCTION**

Strains of the genera *Pseudomonas*, *Xanthobacter*, *Corynebacterium*, *Exophiala* and *Rhodococcus* have previously been reported to degrade styrene under aerobic conditions by the pathway of side-chain oxidation (Fig. 1b) (Mooney et al., 2006; Tischler & Kaschabek, 2012). It has been supposed that this pathway represented the major route to mineralize styrene by micro-organisms (Mooney et al., 2006). The upper pathway of styrene degradation commonly comprises a styrene monoxygenase (SMO, encoded by *styA/styB*), a styrene oxide isomerase (SOI, encoded by *styC*) and a phenylacetaldehyde dehydrogenase (PAD, encoded by *styD*). The respective enzymes convert styrene via styrene oxide and phenylacetaldehyde to phenylacetic acid as a central metabolite that can be further degraded (Beltrametti et al., 1997; Bestetti et al., 2004; Hartmans et al., 1990; Itoh et al., 1997; O’Connor et al., 1995; Teufel et al., 2010).

**Abbreviations:** DSMZ, German Collection of Microorganisms and Cell Cultures; PAD, phenylacetaldehyde dehydrogenase; SOI, styrene oxide isomerase; SMO, styrene monoxygenase.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the genes *styC* of 1CP, *styC2* of 1CP, *sty* cluster of 1CP, *styC* of 5.3_2_1, *styC* of Kp5.2, *sty* cluster of Kp5.2, 16S rRNA of 5.3_2_1 and 16S rRNA of Kp5.2 are KF540254–KF540261, respectively.

One supplementary table and three supplementary figures are available with the online Supplementary Material.

Genes encoding the relevant enzymes for the upper pathway are usually clustered and ordered as *sty(SR)ABCD* (Beltrametti et al., 1997; O’Leary et al., 2001; Panke et al., 1998; Toda & Itoh, 2012; Velasco et al., 1998). The genes *styS* and *styR* encode a two-component regulatory element of the *sty* operon (O’Leary et al., 2001), and have so far been found only in pseudomonads (O’Leary et al., 2001; Panke et al., 1998; Tischler & Kaschabek, 2012; Velasco et al., 1998).

Considering the enzymology of styrene degradation, substantial investigations have only been performed with SMOs (Huijbers et al., 2014; Mooney et al., 2006). In contrast, previous studies on SOIs were limited to the actinobacteria *Corynebacterium* sp. AC-5 (Itoh et al., 1997) and *Rhodococcus opacus* 1CP (Oelschlägel et al., 2012), to the alphaproteobacterium *Xanthobacter* sp. 124X (Hartmans et al., 1989), and the gammaproteobacterium *Pseudomonas putida* S12 (Miyamoto et al., 2007).

SOI (EC 5.3.99.7) has been identified as a stable, integral membrane protein that catalyses the isomerization of styrene oxide into phenylacetaldehyde independent of cofactors (Itoh et al., 1997; Oelschlägel et al., 2012). Promising biotechnological applications of SOIs and a rather simple enrichment yielding activities up to 370 U (mg protein)$^{-1}$ have been demonstrated (Itoh et al., 1997; Miyamoto et al., 2007; Oelschlägel et al., 2012, 2014). Phenylacetaldehydes represent important building blocks for the healthcare and pharmaceutical industries, and can serve as flavours in the...
perfume industry (Hölderich & Barsnick, 2001). SOIs can also serve in racemic resolution to produce enantiopure styrene oxides, whilst converting the opposite enantiomer to phenylacetaldehyde (Itoh et al., 1997). A disadvantage of SOIs with respect to their applications is an inhibition by the phenylacetaldehyde formed at higher product concentrations occurring during biotransformation (Oelschlägel et al., 2012). The inhibition is irreversible and can be overcome only to a limited extent by applying immobilized enzyme in two-phase systems (Oelschlägel et al., 2014).

So far, SOI seems to be the most active enzyme of the upper styrene degradative pathway. Non-enriched SOIs in crude extracts obtained from Corynebacterium sp. AC-5, Pseudomonas fluorescens ST and R. opacus ICP have been reported to have styrene oxide isomerization activities of ~7.6–11.0 U mg⁻¹ (Itoh et al., 1997; Oelschlägel et al., 2012). Therefore, screening procedures for SOI activity should easily provide access to the respective sty gene clusters and to interesting biocatalysts, such as SMOs and SOIs (Tischler & Kaschabek, 2012). Based on these observations, the present study aimed at identifying new SOIs. To achieve this, the distribution and organization of the corresponding catabolic clusters amongst bacteria was highlighted. For micro-organisms harbouring a SOI, phylogeny, activity, substrate specificity and stability of this enzyme were investigated. SOI of the isolate Sphingopyxis sp. Kp5.2 turned out to fulfil criteria which distinguished this enzyme from others reported previously.

**METHODS**

**Chemicals and enzymes.** Standard chemicals, 4-chloro-, 4-fluoro- and non-substituted styrene oxide, styrene oxide enantiomers, phenylacetaldehyde, and substituted phenylacetic acids were purchased from Sigma Aldrich, AppliChem, Merck, Riedel-de Haen, Thermo Fisher Scientific, VWR International, Bio-Rad or Carl Roth at the highest purity available. 4-Methylstyrene oxide was synthesized freshly (Tischler et al., 2009).

**Bacterial strains and culture conditions.** R. opacus 1CP (Gorlatov et al., 1989) was available from the strain collection of the Institute of Biosciences (TU Bergakademie Freiberg, Freiberg, Germany) and represented a positive control for SOI activity (Oelschlägel et al., 2012). Strain 1CP was kept on mineral medium plates (Dorn et al., 1974) in the presence of 5 mM benzoate for preservation. P. fluorescens ST (DSM-6290; Baggi et al., 1983; Beltrametti et al., 1997; Bestetti et al., 2004) and Xanthobacter sp. 124X (DSM-6696; Hartmans et al., 1989) were additionally used as reference strains from the German Collection of Microorganisms and Cell Cultures (DSMZ). These strains were streaked out on solid mineral medium (Dorn et al., 1974) containing 20 g glucose l⁻¹ and incubated at 30 °C. Colonies obtained were used to inoculate liquid media.

Further representatives of the genera Arthrobacter, Gordonia and Rhodococcus (Table S1, available in the online Supplementary Material) from the strain collection of the Institute of Biosciences were investigated for their ability to grow on styrene and cultured as described for the isolates below.

**Isolation of styrene-degrading bacteria.** For isolation of styrene-degrading bacteria, 0.2–0.4 g soil (from meadows, non-contaminated or contaminated with aliphatic and aromatic hydrocarbons, sampled in Freiberg or Hoyerswerda, Saxony, Germany) was suspended in 1 ml water, and incubated at 37 °C and 150 r.p.m. for 2–3 h. Samples were then stored without shaking for 20 min for solids to settle, and
the supernatant was diluted and streaked out onto solid mineral medium without a carbon source. These plates were incubated at room temperature in a 5 l desiccator in which an evaporating aliquot of 50 µl styrene was provided as the sole source of carbon and energy (Oelschlägel et al., 2012). Grown colonies were picked and separated by streaking on solid mineral medium plates with styrene as the sole carbon source supplied via the gas phase. The procedure was repeated until pure isolates were obtained that were able to rapidly metabolize styrene (first visible colony formation after 48–72 h).

**Screening for SOI activity.** Isolates were grown in 100 ml mineral medium in baffled 500 ml Erlenmeyer flasks at 30 °C under constant shaking (120 r.p.m.). Initially, 0.05–0.1 % yeast extract was added in a few cases to accelerate biomass formation and yield, respectively. Growth and SOI induction were stimulated by means of repeated shaking (120 r.p.m.). Initially, 0.05–0.1 % yeast extract was added in a few cases to accelerate biomass formation and yield, respectively. Growth and SOI induction were stimulated by means of repeated shaking (120 r.p.m.).

**Identification of SOI genes from selected isolates and reference strains.** A SOI-specific PCR was performed with the newly designed primer pair styb-SOI-fw 5'-CCKTCTMTGTTYTTCAGAGA-TTGAGGTGATCCANCCGCA-3' and screenSOI-rev 5'-TGNGCYTTNGCCCANCCYTCA-3'. These primers allowed the amplification of a 291 bp large genomic fragment of some strains was obtained by PCRs performed with the newly designed primer pair styb-SOI-fw 5'-CCKTCTMTGTTYTTCAGAGA-TTGAGGTGATCCANCCGCA-3' and screenSOI-rev 5'-TGNGCYTTNGCCCANCCYTCA-3'.

**Phylogenetic analysis of the SOI-positive isolates.** If not stated otherwise, all primers, cloning kits and PCR reagents were obtained from Eurofins MWG Operon or Thermo Scientific. Isolates were characterized phylogenetically by amplification and sequencing of a large part of the 16S rRNA gene. A 1495 bp fragment was amplified by using the primers 27F 5'-AGAGTTTGATCCTG-3' (Lane, 1991, modified; Edwards et al., 1989) and 1522R 5'-AAACCTGATACACGGAG-3' (Edwards et al., 1989, modified). Colonies of putative styrene-degrading bacteria were picked from solid mineral medium plates containing 20 g glucose l⁻¹ and 4 °C in a swing mill (MM-200; Retsch). They were then investigated for SOI activity. In addition, 500 µl harvested cells was mixed with 500 µl glycerol and stored at −80 °C as a stock culture.

**Phylogenetic analysis of SOI-related genes.** The styC-encoded protein sequences were aligned by means of clustal_x or clustal_w and phylogenetic trees were reconstructed with the maximum-likelihood, neighbour-joining, minimum evolution, upgma and Fitch–Margoliash algorithms as described previously (Tischler et al., 2009, 2012). Phylib package 3.66 (http://evolution.genetics.washington.edu/phylip.html) and megap4 (Tamura et al., 2011) were utilized.

**SOI induction and production in selected strains for initial characterization.** To determine SOI induction by various inducers, biomass was cultivated for 7 days in 500 ml Erlenmeyer flasks with 100 ml 0.05 % (w/v) yeast-extract containing mineral medium in the presence of one of the following carbon sources: styrene (total 35 µmol, 8.7 µmol aliquots added through an evaporation adaptor), styrene oxide (total 25 µmol, 6.1 µmol aliquots added through an evaporation adaptor), phenylacetaldehyde (total 0.35–0.5 mmol, added in 0.05–0.2 mmol portions directly to the medium) or phenylacetic acid (total 0.8 mmol, added in 0.1–0.5 mmol portions). Cells were then harvested, disrupted and assayed for SOI activity.

To determine SOI substrate specificity and product inhibition, the respective SOIs were produced by a two-step cultivation approach. This procedure for SOI production was performed to improve SOI yields as described previously (Oelschlägel et al., 2012, 2014). It comprised a biomass production step utilizing glucose as the carbon source and a SOI production step applying a suitable inducer compound. In most experiments, a defined amount of yeast extract (0.05–0.1 %, w/v) was added to overcome otherwise prolonged lag phases. First, precultures were cultivated in 1 l baffled Erlenmeyer flasks at 30 °C under constant shaking (120 r.p.m.) in 100 ml mineral medium with 0.05 % yeast extract and in the presence of glucose.
After further addition of glucose (total 8.0 mmol, 1 mmol aliquots) to yield OD<sub>546</sub> > 1. The total volume of mineral medium was then extended to 200 ml. Further addition of glucose (total 2.0–2.6 mmol, 0.3–0.5 mmol aliquots) to yield OD<sub>546</sub> > 1. SOI was induced with overall 182 μmol suitable inducer (8.7–26.1 μmol aliquots added through an evaporation adaptor) over 10 days. Cells were harvested and treated as described above.

During the biomass production step utilizing glucose as the carbon source, doubling times were also determined for the selected strains. Therefore, cultures containing 50 ml mineral medium with 3 mM glucose in 500 ml baffled Erlenmeyer flasks were inoculated with precultures mentioned above to OD<sub>546</sub> 0.4. These flasks were incubated as described for precultures and doubling times were determined by repeated OD<sub>546</sub> measurement.

As mentioned above, SOIs were identified as integral membrane proteins in a previous study (Oelschlägel et al., 2012). This allowed a simple enrichment of the membrane-bound enzyme from crude extract, thus avoiding side-reactions of other enzymes during enzyme assays. Partial purification of the SOI from the disrupted cells obtained by two-step cultivation was achieved by an initial centrifugation step at 10 000 g for 20 min at 4 °C to separate whole cells and large cell debris. In a second step at 50 000 g for 24 h at 4 °C, the insoluble SOI was separated from cytosolic proteins as described previously (Oelschlägel et al., 2012). The SOI-containing pellet was suspended in 25 mM phosphate buffer (pH 7.3) and used for activity measurements or stored at −20 °C.

**Enzyme assays and protein quantification.** Activity of SOIs was determined as described previously with GC or reversed-phase HPLC by quantification of the reaction product phenylacetaldehyde formed from the substrate styrene oxide over time (Oelschlägel et al., 2012). HPLC was performed with 50 % (v/v) methanol containing 0.1 % (w/v) phosphoric acid as mobile phase at a flow rate of 0.7 ml min<sup>−1</sup>. The net retention volume (difference of retention times and dead time multiplied by flow rate of the mobile phase) of the analysed product phenylacetaldehyde under these conditions was 3.1 ml. For the determination of the enantioselectivity of SOIs, the assay was used with pure (S)- and (R)-styrene oxide instead of racemic epoxide.

SOI activity toward substituted styrene oxides was determined indirectly by quantification of the corresponding phenylacetic acids that were formed from chemical oxidation from the phenylacetaldehydes (Oelschlägel et al., 2012). The oxidized products were analysed by HPLC. The following net retention volumes were obtained: phenylacetic acid, 2.8 ml; 4-chlorophenylacetic acid, 7.2 ml; 4-fluorophenylacetic acid, 3.4 ml; 4-methylphenylacetic acid, 5.5 ml. Peaks obtained were compared with authentic reference compounds with respect to retention volume and UV spectrum.

For investigation of enzyme stability with respect to product inhibition, 30 μg enzyme preparation was suspended in 400 μl 25 mM phosphate buffer (pH 7.3) containing 5 % (v/v) methanol. Then, 0 or 50 mM phenylacetaldehyde (1 M stock solution in methanol) was added and the batches were incubated for 15 min at room temperature under constant shaking. Samples were diluted by the addition of 1 ml 25 mM phosphate buffer (pH 7.3) and centrifuged (13 000 g, 5 min, 20 °C). The SOI-containing pellet obtained was washed with 25 mM phosphate buffer (pH 7.3), centrifuged (13 000 g, 5 min, 20 °C) and resuspended in 40 μl fresh 25 mM phosphate buffer (pH 7.3). Determination of residual activity was performed as described above.

Determination of protein concentrations was performed by the method of Bradford (Bradford, 1976) as described previously (Oelschlägel et al., 2012).

**RESULTS AND DISCUSSION**

**Isolates with the ability to degrade styrene via side-chain oxidation**

In order to identify new strains that degraded styrene via side-chain oxidation, various actinobacterial strains of the strain collection of the Institute of Biosciences were cultivated initially on solid mineral medium under a styrene atmosphere, thus providing styrene as the sole carbon source. In total, 11 *Gordonia*, two *Arthrobacter* and 18 *Rhodococcus* strains could grow under these conditions, and were further cultivated in liquid cultures with styrene and subsequently screened for SOI activity (Table S1). Of these strains, only *R. opacus* 1CP showed measurable SOI activity. It was thus included in the study as the reference strain (Oelschlägel et al., 2012) beside *P. fluorescens* ST and *Xanthobacter* sp. 124X, which were obtained from the DSMZ and treated similarly.

Additionally, 54 putative styrene-utilizing strains were isolated from soil samples and cultivated as described above. During subsequent assays for SOI activity, 11 of the 54 isolates tested positive (Table S1, Figs S1 and S2). Only these 11 were further investigated to identify possibly novel SOIs. The 16S rDNA amplification and sequencing of these 11 isolates allowed classification into the genera *Rhodooccus* (*n=5*), *Sphingopyxis* (*n=1*) and *Sphingobium* (*n=5*) (data not shown).

Thus, out of 87 styrene-utilizing strains subjected to SOI activity screening only 14 showed SOI activity. It cannot be ruled out completely that in some cases the activity determination of the SOI may have been inhibited by uncommonly high PAD activity, but the low abundance of SOIs amongst the soil bacteria tested indicated that alternative or modified pathways for styrene degradation may have been dominant. A few studies have revealed the possibility of styrene degradation via alternative routes (Patrauchan et al., 2008; Toda & Itoh, 2012; Warhurst et al., 1994).

In addition to representatives of the genus *Rhodococcus*, five *Sphingobium* isolates and one strain related to *Sphingopyxis* showed SOI activity (Tables 1 and S1). To our knowledge, styrene degradation via side-chain oxygenation has not been shown previously for members of the family *Sphingomonadaceae* and corresponding SOIs have so far not been characterized. Therefore, genetic and biochemical studies were focused on the isolates *Rhodococcus* sp. 5.3_2_1, *Sphingobium* sp. Kp5.1_1 and Sp8.3_1b, and *Sphingopyxis* sp. Kp5.2.

**Identification and characterization of styC genes and sty gene clusters**

Based on conserved regions of available SOI sequences of *Rhodococcus* sp. ST-5 (Toda & Itoh, 2012) and various *Pseudomonas* strains (Beltrametti et al., 1997; Lin et al., 2010; Panke et al., 1998; Park et al., 2006; Velasco et al., 1998), the primers screenSOI-fw and screenSOI-rev were used for SOI screening.
designed. This SOI-specific PCR was applied to the reference strains *P. fluorescens* ST, *R. opacus* 1CP and *Xanthobacter* sp. 124X as well as to *Rhodococcus* sp. 5.3_2_1, *Sphingobium* sp. Sp8.3_1b and *Sphingopyxis* sp. Kp5.2. To our knowledge, of all strains tested, only the *styC* sequence of *P. fluorescens* ST had been known previously.

PCR products of the expected size of ~140 bp were obtained for almost all strains (Fig. S3) encoding sequences related to SOI genes (sequence data not shown). In the case of *Xanthobacter* sp. 124X, amplification of such a fragment was not possible, indicating larger variations in the primer binding regions.

**Table 1. SOI activity of strains after growth on various substrates**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Highest specific SOI activity [U (mg protein)^{-1}]</th>
<th>Relative specific SOI activity (%) in crude cell extract after growth on</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Styrene</td>
<td>Styrene oxide</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ST</td>
<td>20.7 ± 1.4</td>
<td>85.9 ± 8.5</td>
<td>100 ± 7*</td>
</tr>
<tr>
<td><em>Rhodococcus</em> sp. 5.3_2_1</td>
<td>1.76 ± 0.18</td>
<td>53.0 ± 16.8</td>
<td>100 ± 10*</td>
</tr>
<tr>
<td><em>R. opacus</em> 1CP</td>
<td>10.0 ± 4.5</td>
<td>100 ± 45*</td>
<td>17.9 ± 3.0</td>
</tr>
<tr>
<td><em>Sphingobium</em> sp. Kp5.1_1</td>
<td>4.66 ± 0.44</td>
<td>32.3 ± 3.5</td>
<td>100 ± 9*</td>
</tr>
<tr>
<td><em>Sphingobium</em> sp. Sp8.3_1b</td>
<td>7.58 ± 0.57</td>
<td>72.3 ± 7.2</td>
<td>100 ± 8*</td>
</tr>
<tr>
<td><em>Sphingopyxis</em> sp. Kp5.2</td>
<td>3.09 ± 0.39</td>
<td>100 ± 13*</td>
<td>70.8 ± 7.4</td>
</tr>
<tr>
<td><em>Xanthobacter</em> sp. 124X</td>
<td>8.65 ± 0.23</td>
<td>50.4 ± 6.2</td>
<td>33.1 ± 7.6</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD of four independent measurements.
*The highest specific activity found on one of the substrates is given and was set as 100 %.

**Fig. 2.** Localization and organization of *sty* genes. The organization of *sty* catabolic gene clusters is illustrated for *R. opacus* 1CP (GenBank accession number KF540256), *Rhodococcus* sp. ST-5 and ST-10 (Toda & Itoh, 2012), *Pseudomonas* sp. Y2 (Velasco et al., 1998), and *Sphingopyxis* sp. Kp5.2 (GenBank accession number KF540259). Genes involved in styrene metabolism were: *styA/styB* of SMO, *styC* of SOI, *styC2* of a *StyC*-similar protein (59 % identical positions on protein level), *styD/feaB* of PAD with different sizes depending on two potential start codons (indicated by an asterisk), and *styS* and *styR* of sensor and regulator proteins, respectively. *paaK* encoded a phenylacetyl-CoA ligase.
The SOI amino acid sequences of strain 1CP and of isolate and styR ST-5 (Toda & Itoh, 2012). Regulatory genes, such as et al. pseudomonads (Velasco styA/styB number KF540256) (Fig. 2). The styrene catabolic genes sty classified as between both strains. Strain 1CP had previously been on PCR products obtained by SOI-specific PCR. Later obtained after further PCRs as described in Methods based 1999) and isolate 5.3_2_1 can be assigned to (genome sequence GCA_000325625) harboured a further [Rhodococcus anisopliae ARSEF 23, Methylibium petroleiphilum PM1, R. opacus 1CP [this study, StyC_1CP (GenBank accession number KF540254) and StyC2_1CP (GenBank accession number KF540255)], Rhodococcus sp. 5.3_2_1 [this study, StyC_5.3_2_1 (GenBank accession number KF540257)], Rhodococcus sp. ST-5, Rhodococcus wratislavienesis IFP 2016, Pseudomonas chlororaphis subsp. aureofaciens, P. fluorescens ST, P. putida SN1, Pseudomonas sp. 19-rim, Pseudomonas sp. LO26, Pseudomonas sp. VLB120, Pseudomonas sp. Y2, Sphingomonas melonis and Sphingopyxis sp. Kp5.2 [this study, StyC_Kp5.2 (GenBank accession number KF540258)].

The sequences of the complete SOI genes from R. opacus 1CP (GenBank accession number KF540254) and strain 5.3_2_1 (GenBank accession number KF540257) were obtained after further PCRs as described in Methods based on PCR products obtained by SOI-specific PCR. Later genome sequencing of strain 1CP (unpublished) verified the styC sequence obtained and provided insights into the clustering of sty genes in strain 1CP (GenBank accession number KF540256) (Fig. 2). The styrene catabolic genes styA/styB, styC and styD were clustered similarly to those of pseudomonads (Velasco et al., 1998) and Rhodococcus sp. ST-5 (Toda & Itoh, 2012). Regulatory genes, such as styS and styR, could neither be identified in nor neighboured to the sty cluster of strain 1CP.

The SOI amino acid sequences of strain 1CP and of isolate 5.3_2_1 (both 168 aa) showed 100 % identity, whereas the 16S rDNA results obtained allowed us to distinguish between both strains. Strain 1CP had previously been classified as R. opacus (Gorlatov et al., 1989; Tsitko et al., 1999) and isolate 5.3_2_1 can be assigned to Rhodococcus jostii. The SOI match could be a consequence of horizontal gene transfer, which has often been reported for rhodococci (Larkin et al., 1998). The SOIs of 1CP and 5.3_2_1 were 64 and 86 % identical on the amino acid level to SOIs of Pseudomonas sp. Y2 [GenBank accession number CAA04002 (Velasco et al., 1998)] and Rhodococcus sp. ST-5 [GenBank accession number AB594507 (Toda & Itoh, 2012)], respectively (Figs 3 and 4).

Interestingly, Rhodococcus strains 1CP and IFP 2016 (genome sequence GCA_000325625) harboured a further styC-like gene in addition to styC. Whilst styC was part of a sty gene cluster, the other styC-like gene is localized somewhere else in the genomes (Fig. 2). The second hypothetical SOI shares ~60 % identical amino acids (166 aa) with those encoded by the sty clusters (Figs 3 and 4). These findings raise questions about the function of these SOI-like proteins which might be solved in future investigations.

For the Sphingobium and Sphingopyxis isolates, we attempted to amplify complete styC genes by additional PCRs as described in Methods. However, no PCR products were obtained thus indicating a different organization of the sty clusters in both strains. Finally, the styC gene (GenBank accession number KF540258) of Sphingopyxis sp. Kp5.2 was obtained from a genome-sequencing project with the CeBiTec (Bielefeld, Germany; unpublished). The rest of the relevant styrene-catabolic cluster was also predicted (GenBank accession number KF540259) (Fig. 2). Indeed, clustering of the genes styA/styB, styC and styD in isolate Kp5.2 differed from known sty operons of pseudomonads and Rhodococcus strains (Toda & Itoh, 2012; Velasco et al., 1998). The styD (seaB) in Sphingopyxis was not directly adjacent to the sty genes, but was located ~1100 bp upstream of styABC. This fact explained the unsuccessful attempts to obtain the complete styC gene by PCRs mentioned above. The regulatory elements styS and styR known from pseudomonads were not identified in the respective cluster of Sphingopyxis. However, a hypothetical outer membrane protein and a hypothetical histidine kinase sensor protein were found to be encoded downstream and a LysR transcriptional regulator upstream of styABC. The presence of a StyS-like histidine kinase sensor protein and a LysR regulator may indicate a regulatory function of the respective genes for styrene metabolism in strain Kp5.2. In contrast to 1CP, no further SOI-related gene was identified on the genome of strain Kp5.2.

**Evidence for a novel SOI from Sphingopyxis sp. Kp5.2**

In strain Kp5.2, styC was found to have a size of 561 bp encoding a product of 186 aa. Compared with known SOIs, SOI from Sphingopyxis sp. Kp5.2 seems to be 26–27 aa longer at the N-terminal side, but 10 aa shorter at the C terminus (Fig. 3). One additional isoleucine was also incorporated in the first transmembrane helix of the protein (Fig. 3, position 64). Like the other membrane-bound SOIs it also contained four predicted transmembrane helices (Fig. 3; Krogh et al., 2001). At the protein level, the SOI from strain Kp5.2 shared 62 % identical positions with SOIs from Pseudomonas sp. Y2 (GenBank accession number CAA04002 (Velasco et al., 1998)] and Rhodococcus sp. ST-5 [GenBank accession number AB594507 (Toda & Itoh, 2012)].

Conclusively, the SOI of the alphaproteobacterium Sphingopyxis sp. Kp5.2 (SOI-4; GenBank accession number
KF540258) in the dendrogram (Fig. 4) represented a novel branch amongst styrene oxide isomerases that was more related to the SOI-inactive outgroup proteins [GenBank accession numbers AF012555 (Dutta et al., 2010), EAW31154, EAQ96818, ABE29428 and AEO27340] than other functional SOIs.

**Induction and production of the WT SOIs for initial characterization**

With the aim of revealing conditions under which SOI was expressed, induction of SOI with the potential inducers styrene, styrene oxide, phenylacetaldehyde and phenylacetic acid as the sole carbon source was investigated (Table 1). Only these substrates were investigated initially because previous investigations had shown a high specificity of the induction of styC in *R. opacus* 1CP by substrates and intermediates of styrene side-chain oxidation, and no or only a minor effect on SOI expression by other structurally similar compounds (Oelschlägel et al., 2014). Further studies (Hartmans et al., 1989; O’Connor et al., 1995) had also shown the highest enzyme activities of proteins encoded by *sty* genes after growth on substrates involved in the side-chain oxidation.

For *P. fluorescens* ST, styrene oxide was proven to be the most efficient inducer (Table 1). Amongst the rhodococci, the highest SOI activity in strain 5.2_3_1 was achieved with styrene oxide and in strain 1CP with styrene. With a relative SOI activity of 1.5% compared with styrene-induced biomass, phenylacetaldehyde does not seem to be an efficient inducer for strain 1CP based on this study, whilst a relative SOI activity of 16% had been determined during previous investigations (Oelschlägel et al., 2012). In contrast to the present investigation, biomass in the previous study had been cultivated with 0.8 instead of
0.35 mmol substrate and for 14 instead of 7 days. This suggested that the induction of the SOI is also dependent on incubation time and inducer concentration.

For Xanthobacter sp. 124X, phenylacetaldehyde was identified to be the best inducer (Table 1). The two Sphingobium strains showed similar expression patterns after induction with various substrates, of which styrene oxide yielded the highest isomerase activities. For Sphingopyxis sp. Kp5.2, styrene was the best substrate for efficient styC expression. Interestingly, Sphingopyxis sp. Kp5.2 showed styC expression also after growth on glucose alone (1.1 ± 0.3 U mg⁻¹). This again indicated a different regulation compared with pseudomonads and rhodococci because in the case of P. putida CA-3, glucose had been reported to be a sty operon repressor (O’Leary et al., 2001).

With respect to total SOI activities, the highest activities were reached by P. fluorescens ST (20.7 U mg⁻¹), R. opacus 1CP (10 U mg⁻¹) and Xanthobacter sp. 124X (8.7 U mg⁻¹), whereas Sphingopyxis sp. Kp5.2 yielded a SOI activity of only 3.1 U mg⁻¹ (Table 1). Similar activities had been determined previously for Corynebacterium sp. AC-5 (7.6 U mg⁻¹) after induction with styrene (O’Leary et al., 2001).

As it had been found previously that a two-step cultivation may be beneficial for the production of SOI, such a cultivation with glucose for biomass production and a suitable inducer such as styrene for SOI expression was investigated for the new isolates and reference strains (Table 2). Styrene was chosen for the second step because it induced SOI activity in all strains investigated (Table 1) and its toxic effect on the biomass was lower than that of styrene oxide (Oelschlägel et al., 2014). The SOI enrichment procedure, which is described in Methods, worked well for R. opacus 1CP, P. fluorescens ST and also for isolate 5.3_2_1, but not for the alphaproteobacteria. This was also indicated by a comparison of SOI activity in crude extracts of biomass induced with a total of 350 μmol styrene l⁻¹ (assuming complete dissolution in aqueous phase) over 7 days (Table 1) with the activity in the membrane fraction from biomass obtained after two-step cultivation with glucose and subsequent induction with 910 μmol styrene l⁻¹ over 10 days (Table 2). In the latter membrane fraction, a drastic loss of specific activity was observed for the alphaproteobacteria, which might have been due to a different form of anchoring in the membrane(s) and which might make an adjustment of the enrichment protocol necessary. This feature clearly distinguishes SOIs of alphaproteobacteria including strain Kp5.2 from known epoxide isomerases.

### Table 2. SOI activity of strains after growth on glucose and styrene induction

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time on glucose (h)</th>
<th>Specific SOI activity from enriched membrane fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Racemic styrene oxide (S)-styrene oxide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. fluorescens ST</td>
<td>1.62 ± 0.01</td>
<td>10.1 ± 2.3</td>
</tr>
<tr>
<td>Rhodococcus sp. 5.3_2_1</td>
<td>1.69 ± 0.28</td>
<td>ND</td>
</tr>
<tr>
<td>R. opacus 1CP</td>
<td>2.68 ± 0.03</td>
<td>6.99 ± 0.95</td>
</tr>
<tr>
<td>Sphingobium sp. Kp5.1_1</td>
<td>3.27 ± 0.26</td>
<td>0.056 ± 0.012</td>
</tr>
<tr>
<td>Sphingobium sp. Sp8.3_1b</td>
<td>3.51 ± 0.03</td>
<td>0.0015 ± 0.0004</td>
</tr>
<tr>
<td>Sphingopyxis sp. Kp5.2</td>
<td>3.86 ± 0.01</td>
<td>0.063 ± 0.014</td>
</tr>
<tr>
<td>Xanthobacter sp. 124X</td>
<td>10.61 ± 0.11</td>
<td>0.14 ± 0.04</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD of three to four independent measurements.
ND, Not determined.

Substrate specificity and stability of SOIs

The enzymes enriched in the membrane fractions by centrifugation as described above were used for biotransformation studies (Table 2, Fig. 5). Owing to identical styC genes of Rhodococcus strains 1CP and 5.3_2_1 (Figs 3 and 4), these studies were only performed with the enzyme of strain 1CP.

All enzymes tested showed the highest activity with styrene oxide as substrate (Fig. 5). Sphingobium sp. Sp8.3_1b showed remarkably high relative SOI activities for 4-chlorostyrene oxide and 4-fluorostyrene oxide of 16.1 ± 2.2 and 36.1 ± 12.7 % compared with styrene oxide, respectively. The lowest activity with 4-fluorostyrene oxide was observed for Sphingopyxis sp. Kp5.2 at 8.52 ± 4.40 %. For the SOIs obtained from the two Sphingobium strains and from Sphingopyxis sp. Kp5.2, the relative activity for 4-methyl-styrene oxide was only 17–28 % compared with styrene oxide, whilst the other strains tested showed relative values of 66–81 %.

With respect to enantioselectivity, all enzymes investigated showed a similar preference for (S)-styrene oxide and only ~36–64 % relative activity toward the (R)-enantiomer (Table 2, Fig. 5). Amongst all enzymes investigated so far, SOI of Sphingopyxis sp. Kp5.2 was found to possess the highest selectivity for the (S)-epoxide. It therefore may be a valuable candidate for racemic resolution of styrene oxides as previously investigated for other SOIs by Itoh et al. (1997).

The stability of 30 μg enriched protein toward the previously described irreversible product inhibition
(Oelschlägel et al., 2012) was investigated. A 15 min incubation of the enzyme preparation in 50 mM phenylcetataldehyde resulted in considerable inactivation of almost all SOIs investigated. However, the SOIs of Sphingopyxis sp. Kp5.2 and Xanthobacter sp. 124X showed remarkable residual activities of 11–12 %, indicating a higher stability.

The results obtained by the initial enzymic characterization presented here indicate that SOIs from Sphingopyxis and Sphingobium strains behave significantly differently compared with the isomerases known from pseudomonads and rhodococci. In particular, the more stable SOI of strain Kp5.2 seemed to have a higher suitability for (S)-styrene oxide conversion despite a narrower substrate acceptance in general. This also indicated an outstanding position of the enzyme – a conclusion also derived from phylogenetic calculations (Fig. 4). These facts lead us to conclude that SOI of strain Kp5.2 is a novel SOI representative possibly useful for biotechnological applications.

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REFERENCES


**Fig. 5.** Substrate specificity of investigated SOIs. Activity measurements were performed as described in Methods. Data represent the mean ± SD of four independent measurements. aRelative activities presented refer to specific SOI activity toward racemic styrene oxide (Table 2). bRelative specific SOI activity in the presence of (R)-styrene oxide refers to the activity toward the (S)-enantiomer (Table 2).
Styrene oxide isomerases of soil bacteria


