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Enantioselective oxidation by a cyclohexanone monooxygenase from the xenobiotic-degrading *Polaromonas* sp. strain JS666

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ABSTRACT

A cyclohexanone monooxygenase (CHMO) from the xenobiotic-degrading *Polaromonas* sp. strain JS666 was heterologously expressed in *Escherichia coli*, and its ability to catalyze enantio- and regiodivergent oxidations of prochiral and racemic ketones was investigated. The expression system was also used to evaluate this enzyme's potential role in the oxidation of *cis*-1,2-dichloroethene (cDCE), a groundwater pollutant for which strain JS666 is the only known assimilator. The substrate enantiopreference and -selectivity of the strain JS666 CHMO is similar to that of other CHMO-type enzymes; of note is this enzyme's excellent stereodiscrimination of 2-substituted cyclic ketones. The expression system exhibits no activity with ethene or cDCE as substrates under the tested conditions. Phylogenetic analysis shows that sequence variability among cyclohexanone monooxygenases could be a rich source of new enzyme activities and attributes.

1. Introduction

Cyclohexanone monooxygenases (CHMOs) are a subset of the family of enzymes known as Baeyer-Villiger monooxygenases (BVMOs; E.C. 1.4.13.22), so named because they perform the classical Baeyer-Villiger oxidation reaction. Enzymatic Baeyer-Villiger oxidations have several advantages over their traditional chemical counterparts; namely, they can achieve greater chemo-, regio- and enantioselectivity while avoiding the use of potentially hazardous organic peracids and organometallic catalysts [1,2]. They have been a popular target of study over the last few decades because of their versatility and enantiopure production of chiral lactones, which are valuable building blocks in the pharmaceutical industry.

The CHMO found in *Acinetobacter* sp. NCIMB 9871 is the most extensively researched enzyme in this group, and has exhibited activity on more than 100 ketone substrates [3,4]. Novel CHMOs have been discovered and characterized more recently through genome mining and heterologous expression; this increased enzyme availability has provided opportunities to discover diverse activities and selectivities and to explore the effects of changes in particular regions of the protein sequence [5,6].

Here we report the cloning and heterologous expression of a CHMO from *Polaromonas* sp. strain JS666, a β-proteobacterium capable of degrading a wide variety of xenobiotic compounds, including the recalcitrant groundwater contaminant *cis*-1,2-dichloroethene (cDCE) [7]. Differences in potentially important amino acid residues are noted among the consortium of heterologously expressed CHMOs, and the phylogenetic relationship of $CHMO_{JS666}$ to these other enzymes is illustrated. The acceptance and enantioselective oxidation of 32 ketone substrates is evaluated and compared to reference biotransformations. The new heterologous expression system is also used to test the previously published hypothesis that $CHMO_{JS666}$ is involved in cDCE and ethene oxidation [8].

2. Experimental

2.1. Creation of heterologous expression system

The target CHMO gene (1620 bp, GenBank Accession No. YP_552312) was amplified as a blunt-end PCR product with native start- and stop-codons, using the proofreading Pfx DNA polymerase (Invitrogen). The amplified gene was ligated into the pET 101/D-TOPO vector (Invitrogen) to create the expression vector pAA2, and was then sequenced to check for accuracy against its native 338 kb plasmid sequence (GenBank Accession No. CP000318). *Escherichia coli* BL21 Star (DE3) from Invitrogen was used as the expression strain for all analyses.

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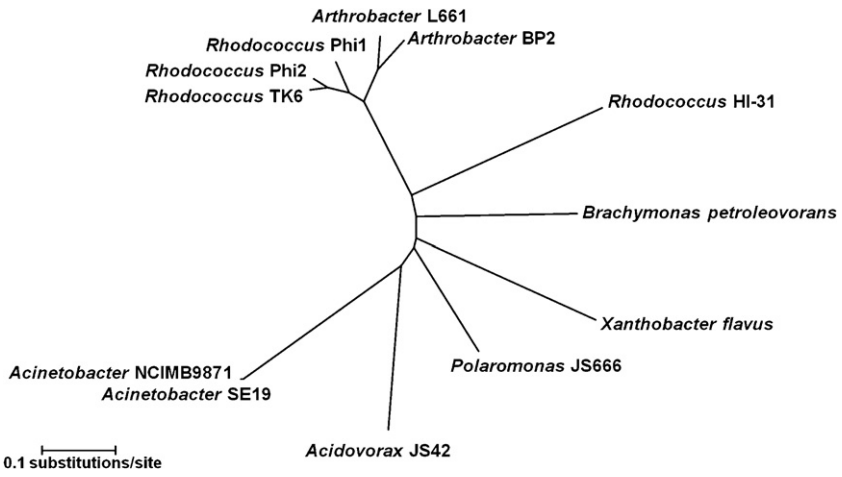


Fig. 1. Phylogenetic relationships between CHMO protein sequences, where branch length is proportional to inferred evolutionary distance, and GenBank protein accession numbers are as follows: *Acidovorax* sp. JS42: YP_986353; *Acinetobacter* sp. NCIMB 9871: BAA86293; *Acinetobacter* SE19: AAG10021; *Arthrobacter* sp. BP2: AAN37479; *Arthrobacter* sp. L661: ABQ10653; *Brachymonas petroleovorans*: AAR99068; *Polaromonas* sp. JS666: YP_552312; *Rhodococcus* sp. HI-31: BAH56677; *Rhodococcus* sp. Phi1: AAN37494; *Rhodococcus* sp. Phi2: AAN37491; *Rhodococcus* sp. TK6: AAR27824; *Xanthobacter flavus*: CAD10801. Sequences were aligned over 512 positions using MEGA4 [9]; the JS666 CHMO has 539 predicted amino acid residues.

2.2. CHMO expression assays: ketone substrates

Baffled Erlenmeyer flasks were used for preculture and cultivation broth growing. A LB_{amp} preculture (200 mg/L ampicillin) was inoculated with a bacterial single colony from an agar plate and incubated at 37 °C in an orbital shaker overnight. The cultivation medium (LB_{amp}) was then inoculated with 2% (v/v) of the preculture and incubated for 1–2 h under the same conditions until an optical density of 0.2–0.6 was reached. IPTG (0.2 mM) and β-cyclodextrin (4 mM) were added; the mixture was thoroughly mixed and split in 1.0 mL aliquots into multi-well plates. Substrates were added as 0.8 M solutions in 1,4-dioxane to a final concentration of 4 mM. The plates were sealed with adhesive film and incubated at 24 °C in an orbital shaker for 24 h.

2.3. CHMO expression assays: alkene substrates

A freshly transformed overnight *E. coli* BL21 Star (DE3) culture carrying the expression vector pAA2 was inoculated at 1% (v/v) in LB broth with 200 mg/L ampicillin to begin each expression assay. Cultures were grown at 23 ± 1 °C with shaking at 200 rpm until an OD_{600} of 0.5–0.7 was reached, at which point they were induced with 0.1 mM (final concentration) IPTG and transferred to 20 mL glass serum bottles sealed with PTFE-coated rubber septa (4 mL per bottle). Neat cDCE (97%, Sigma) was dissolved in ethanol and added to a final concentration of 2.5 mM (1% ethanol (v/v)) 2 h post-induction. Gaseous ethene (4.1 μmol per bottle) was added to the appropriate cultures through 0.22 μm pore-size filtered syringes 2 h after induction with IPTG.

2.4. Analytical methods

Sequence alignment of heterologously expressed CHMO gene sequences and phylogenetic tree calculation were conducted in MEGA4, using the neighbor-joining method [9].

An adapted protocol for the EPICENTRE EasyLyse Bacterial Protein Extraction Solution [10] was used to separate the soluble and insoluble protein fractions in total *E. coli* cell extracts obtained by French press 2 h after induction with IPTG. These fractions were resuspended in SDS loading buffer (Bio-Rad), heated at 95 °C for 1 min, and then resolved with SDS-PAGE using precast Criterion 12.5% polyacrylamide gels (Bio-Rad) and a constant 200 V for 55 min. The relative intensity of the CHMO protein band in the soluble and insoluble fractions was assessed for a variety of temperatures and IPTG concentrations to determine expression conditions that would maximize the amount of soluble CHMO.

At selected time points during ketone oxidation assays, 0.5 mL of culture medium was extracted with ethyl acetate plus methyl benzoate as internal standard. The organic phase was removed and dried over anhydrous $MgSO_4$, then analyzed by chiral GC with an Agilent 6897N gas chromatographer fitted with a Cyclodextrin E column (Solutions and Tools GmbH, 25 m × 0.25 mm × 0.125 μm), FID, and H_2 carrier gas. *Normal* lactone peaks (see Section 3.5) were identified through *meta*-chloroperbenzoic acid (*m*CPBA) oxidation of racemic ketones, using a biphasic reaction protocol adapted from Mohan and Whalen [11]. Ketone substrates were also oxidized by *E. coli* cultures expressing the *Acinetobacter* sp. NCIMB 9871 CHMO gene from vector pMM4 [12], using the same transformation and culture conditions as described for pAA2. As the enantioselectivity of the *Acinetobacter* CHMO is known for these substrates, these oxidations provided identification controls for R- and S-enantiomers in the chiral GC analysis. Enantioselectivity (*E*) values were calculated using the *Selectivity* software developed by Faber et al. [13].

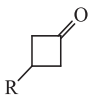
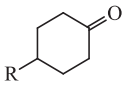
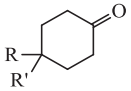
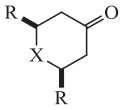
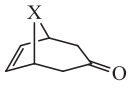
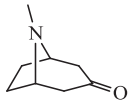
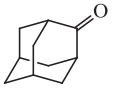
Headspace samples (100 μL) were taken from the cDCE- and ethene-containing cultures at selected time points, and analyzed by GC; cDCE on a J&W Scientific GSQ capillary column (30 m × 0.25 mm × 0.53 μm), and ethene and epoxyethane on a Supelco 1% SP-1000 60/80 Carbopack B packed column, both with FID and N_2 carrier gas.

3. Results and discussion

3.1. Phylogenetic analysis

Investigations into sequence relationships between CHMO-type enzymes can reveal both conserved domains and regions of variation that may account for differences in substrate acceptance and enantioselectivity, and it can be expected that in general, closely related sequences will exhibit similar activities. The phylogenetic relationships of sequenced CHMOs whose activities have been verified through heterologous expression are shown in Fig. 1. $CHMO_{JS666}$ is most similar to CHMO from *Brachymonas petroleovorans* (66% amino acid identity), and its sequence also clusters with the CHMOs from *Xanthobacter flavus* and *Acidovorax* sp. JS42. The $CHMO_{JS666}$ has 62% amino acid identity with the well-studied *Acinetobacter* sp. NCIMB 9871 CHMO. These amino acid similarities

Table 1
Desymmetrization of prochiral cycloketones (substrates **1a**–**15a**).

Substrate	R, R′, X	Compound no.	JS666		Reference biotransformation			
			% Conv.[a]	% *ee*[b]	% Yield/% Conv.[a]	% *ee*[b]	Biocatalyst	Reference
	Bn	**1**	+	78 (−)	+++	88 (−)	$CHMO_{Xantho}$	[19]
	3,4,5-triMeO-Bn	**2**	+	96 (−)	+++	95 (−)	$CHMO_{Xantho}$	[19]
					72	79 (+)	$CHMO_{Brevi1}$	[20]
	3,4-(OCH_2O)-Bn	**3**	+++	36 (+)	+++	99 (−)	$CHMO_{Xantho}$	[19]
					61	75 (+)	$CHMO_{Brevi1}$	[20]
	Ph	**4**	+	97 (−)	+++	98 (−)	$CHMO_{Xantho}$	[19]
	CO_2Et	**5**	+	98 (−)	+++	98 (−)	$CHMO_{Xantho}$	[19]
					83	64 (+)	$CPMO_{Coma}$	[21]
	Me, Me	**6**	++	n.a.	61	n.a.	$CHMO_{Acineto}$	[22]
					45	n.a.	$CPMO_{Coma}$	[23]
	—$(CH_2)_5$—	**7**	n.c.	n.a.	n.a.	n.a.	n.a.	n.a.
	X = CH_2 R = Me	**8**	+	>99 (−)	+++ ++	97 (−) 99 (+)	$CHMO_{Xantho}$ $CPMO_{Coma}$	[19]
	X = O R = H	**9**	++	n.a.	+++ ++	n.a. n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[19]
	X = N-Bn R = H	**10**	n.c.	n.a.	n.a.	n.a.	n.a.	n.a.
	X = CH_2	**11**	n.c.	n.a.	71	>99 (−)	$CHMO_{Xantho}$	[19]
	X = O	**12**	n.c.	n.a.	n.c.	n.a.	$CHMO_{Acineto}$	[24]
					53	95 (+)	$CPMO_{Coma}$	
	X = N-CO_2Me	**13**	n.c.	n.a.	n.c.	n.a.	n.a.	n.a.
		**14**	n.c.	n.a.	n.a.	n.a.	n.a.	n.a.
		**15**	+	n.a.	+++	n.a.	$CHMO_{Acineto}$	[25]

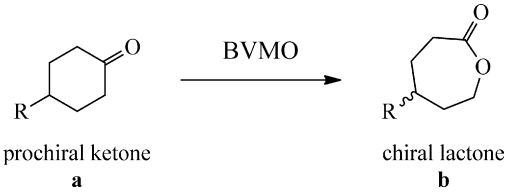
n.a.: not available, n.c.: no conversion, rac.: racemic.
[a] Relative conversion (Conv.) of starting material determined by chiral phase GC after 24 h: +++ >90%, ++ 50–90%, + <50%.
[b] Enantiomeric excess values determined by chiral phase GC; sign of optical rotation or absolute configuration is given in parentheses and assigned on the basis of reference biotransformations.

still leave substantial room for sequence variation, which is a likely source of novel activities, selectivities, and variations in enzyme stability [6,14], and a compelling reason to continue to expand and investigate the collection of known CHMOs.

All of the heterologously expressed CHMOs share the consensus $NADP^+$-binding motif and conserved substrate binding pocket residues identified by Mirza et al. [1], but there are differences in the "mobile loop" sequence hypothesized to flexibly close around the substrate in the binding pocket (Fig. S1). These steric differences may affect substrate acceptance and enantioselectivity, so variation in these residues is a logical target of future research into CHMO substrate range and enzyme engineering.

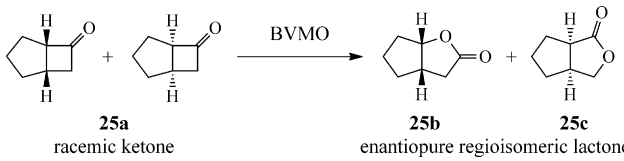
3.2. Improvement of the CHMO expression system

Initial expression assays with *E. coli* BL21 Star (DE3) and plasmid pAA2 using the vector manufacturer's recommended conditions (37 °C, 1.0 mM IPTG) exhibited no oxidation of cyclohexanone, even though SDS-PAGE with total cell extracts showed high production of a protein of the CHMO's expected size. Given that insoluble protein aggregates (or inclusion bodies) are often responsible for reductions in heterologous protein activity [15], soluble/insoluble protein fraction analysis was performed as described above, and it was discovered that essentially all of the heterologous protein was present in the insoluble phase (Fig. S2). Temperatures from



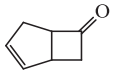
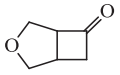
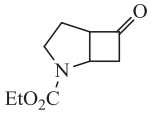
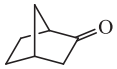
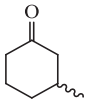
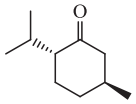
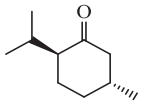
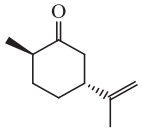
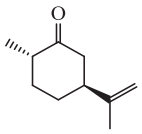
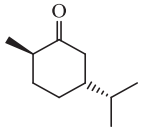
Scheme 1. Desymmetrization of prochiral cycloketones with BVMOs.

23 °C to 37 °C and IPTG concentrations from 0.01 mM to1.0 mM were assessed with both soluble/insoluble protein fraction analysis and cyclohexanone oxidation assays to find improved conditions for CHMO expression and activity. Out of the tested combinations, the best conditions for strain JS666's CHMO activity in this heterologous expression system were found to be 23 °C (room temperature) and 0.1–0.2 mM IPTG. No CHMO activity in the *E. coli* strain



Scheme 2. Regiodivergent Baeyer-Villiger oxidations of fused bicyclic cyclobutanones.

Table 2
Regiodivergent bio-oxidations of cycloketones (substrates **16a**–**25a**).

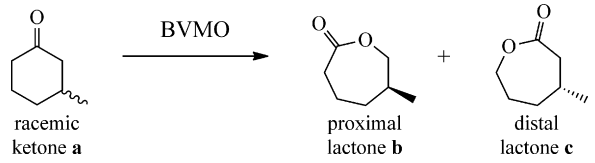
Substrate	Compound no.	JS666		Reference Biotransformation			
		% Conv.[a]/ratio[b]	% *ee*[c]	% Conv.[a]/ratio[b]	% *ee*[c]	Biocatalyst	Reference
	**16**	++[d] 38:62	*8 (−)* 62 (−)	85 51:49	96 (−) *>99 (−)*	$CHMO_{Brevi1}$	[26]
	**17**	n.c.	n.a.	70 50:50	97 (−) *98 (−)*	$CHMO_{Acineto}$	[27]
	**18**	n.c.	n.a.	78 48:52	98 (+) *89 (−)*	CPDMO	[28]
	**19**	n.c.	n.a.	+++ 97:3	12 (+) *88 (n.d.)*	$CHMO_{Acineto}$	[29]
	**20**	+[d] 63:36	*>99 (−)* 36 (−)	+++/50:50 +++/100:0	*95 (−)*, 94 (−) rac.	$CHMO_{Acineto}$ $CPMO_{Coma}$	[30,31] [28]
	**21** (+)-menthone	Complex product mixture	n.a.	+++ 100:0	[e]	$CHMO_{Acineto}$	[32,33]
	**22** (−)-menthone	n.c.	n.a.	+++ 100:0	[e]	CPDMO	[28]
	**23** (+)-*trans*-dihydrocarvone	n.c.	n.a.	+++ 0:100	[e]	$CHMO_{Acineto}$	[32,33]
	**24** (−)-*trans*-dihydrocarvone	Complex product mixture	n.a.	+++ 100:0	[e]	$CHMO_{Acineto}$	[32,33]
	**25** (+)-*trans*-carvomenthone	+ 0:100		+++ 0:100	[e]	$CHMO_{Xantho}$	[34]

n.a.: not available, n.c.: no conversion, rac.: racemic.
[a] Relative conversion (Conv.) of starting material determined by chiral phase GC after 24 h: +++ > 90%, ++ 50–90%, + < 50%.
[b] Ratio of regioisomers (normal:abnormal).
[c] Enantiomeric excess values determined by chiral phase GC; sign of optical rotation or absolute configuration is given in parentheses and assigned on the basis of reference biotransformations; ee_{normal} in italics, $ee_{abnormal}$ in normal font.
[d] Starting material was racemic within measurement error.
[e] Starting material was optically pure; no epimerization observed.

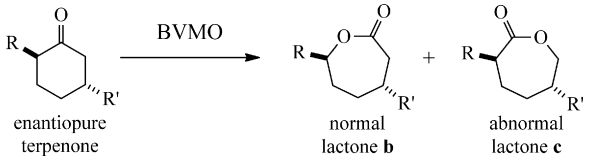
carrying pAA2 was observed at 37 °C (the manufacturer's recommended temperature), and only minimal activity was detected at 30 °C. Finding the optimal activity at a low temperature is logical for a heterologously expressed protein from strain JS666, which has a growth optimum between 20 and 25 °C [16].

3.3. Ethene and cDCE oxidation

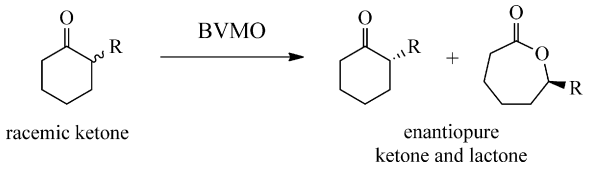
Polaromonas sp. strain JS666 is unique in its ability to couple growth to aerobic oxidation of the toxic groundwater contaminant cDCE [16]. The CHMO gene in strain JS666 is encoded on the smaller of two plasmids [7], and is upregulated during growth on cDCE as a sole carbon source [17]. Strain JS666's degradation of cDCE is observed to be of two distinct types: one relatively rapid and maintainable over successive batch cultures (growth-coupled), and one slow and limited in duration (cometabolic). It has been hypothesized that $CHMO_{JS666}$ is responsible for cometabolic-type cDCE degradation by catalyzing the production and eventual accumulation of the toxic cDCE epoxide (2,3-dichlorooxirane) [8]. This CHMO alkene-oxidation explanation is consistent with the



Scheme 3. Regiodivergent oxidation of β-substituted cycloketones to proximal and distal lactones.



Scheme 4. Regiodivergent transformations of optically pure terpenones **28a**–**31a**.



Scheme 5. Schematic view of a kinetic resolution of 2-substituted cyclic ketones catalyzed by BVMOs.

observed increase in ethene epoxidation rate (and accumulation of epoxyethane with no culture growth) in JS666 cultures following exposure to cDCE [16] or cyclohexanol [unpublished results].

Under the conditions described (in which heterologously expressed $CHMO_{JS666}$ readily converted cyclohexanone to ε-caprolactone), no activity was observed with either ethene or cDCE. Specifically, no degradation of cDCE or ethene or production of epoxyethane (detection limit < 0.1 μmol/bottle) was measured during the 24 h period following introduction of the substrate to induced cultures. This result suggests that $CHMO_{JS666}$ is not involved in either growth-coupled or cometabolic cDCE or ethene oxidation, and implies the need to reevaluate past hypotheses about the cDCE degradation pathway(s) in strain JS666. One important caveat is that the CHMO may behave differently in *E. coli* than in its native host strain; in fact, post-translational modification of this enzyme was proposed by Jennings et al. [17].

3.4. Desymmetrization of prochiral cycloketones

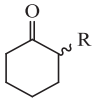
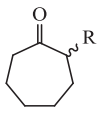
The *Polaromonas* strain JS666 CHMO shows moderate acceptance of symmetric cycloketones (substrates **1a**–**14a**, Table 1, Scheme 1). All three tested 3-substituted cyclobutanones were converted, but only piperonyl compound **3a** reached >90% conversion within 24 h, however with poor enantioselectivity (36% *ee*). 4-Substituted cyclohexanones **4a** and **5a** were both oxidized to the corresponding (−)-lactones with high optical purity (97 and 98% *ee*), but again at a slow rate (<50% conversion). The same behavior was observed for *cis*-3,5-dimethylcyclohexanone **8a**. Bridged bicyclic ketones **11a**–**14a** were not accepted for Baeyer-Villiger oxidation. A known side reaction, epoxidation of the double bond, was also not detected. Only polycyclic substrate **15a**, adamantanone, was slowly converted to the lactone. Generally, in this substrate class $CHMO_{JS666}$ has the same enantiopreference and similar selectivity as other CHMO-type enzymes.

3.5. Regiodivergent biooxidations of cycloketones

Substrates **16a**–**25a** were tested to assess the biocatalyst's ability to perform regiodivergent biooxidations (Table 2). In contrast to chemical Baeyer-Villiger oxidations, several BVMOs catalyze a parallel kinetic resolution: one enantiomer is converted to the expected migration product (rearrangement of the more nucleophilic residue to the *normal* lactone) while the other is transformed to the regioisomeric product (*abnormal* lactone). In general, starting material remains almost racemic during the course of the reaction, as the difference in reaction rates is rather small. Regiodivergent oxidations of fused bicyclic cyclobutanones, β-substituted cycloketones, and optically pure terpenones are illustrated in Schemes 2–4, respectively.

$CHMO_{JS666}$ does not show a specific trend in these substrate classes. Of three fused bicyclic cyclobutanones, only **16a** is accepted and transformed with low enantioselectivity (ee_{normal} = 8%, $ee_{abnormal}$ = 62%). 3-Methylcyclohexanone **20a** was preferentially transformed to the normal lactone **20b** with excellent stereoselectivity (>99% *ee*), however at a slow rate (conversion

Table 3
Kinetic resolutions of 2-substituted cycloketones (substrates **26a–31a**).

Substrate	R	Compound no.	JS666			Reference Biotransformation				
			% Conv.[a]	% ee_S, % ee_P[a]	*E*	% Conv.[a]	% ee_S, % ee_P[a]	*E*	Biocatalyst	Reference
	Me	**26**	35	*18 (+)* 70 (−)	7	35	*35 (+)* 61 (−)	6	$CHMO_{Acineto}$	[35]
	Et	**27**	92[b]	*>99 (R)* 30 (S)	>200	79	*>98 (R)* *95 (S)*	>200	$CHMO_{Acineto}$	[36]
	CH_2—CH=CH_2	**28**	75[b]	*81 (S)* >99 (R)	>200	59	*>98 (S)* *>98 (R)*	>200	$CHMO_{Acineto}$	[36]
	Ph	**29**	41	*71 (−)* >99 (+)	>200	40	*76 (−)* >99 (+)	>200	$CHMO_{Xantho}$	[34]
	CH_2—CH_2—CN	**30**	32	*61 (S)* 99 (*R*)	>200	42	*95 (S)* 97 (*R*)	>200	$CHMO_{Acineto}$	[18]
	Bn	**31**	n.c.	*n.a.* n.a.	n.a.	20	*13* 45	3	$CHMO_{Xantho}$	[28]

n.a.: not available, n.c.: no conversion, rac.: racemic.

[a] Relative conversion (Conv.) of starting material and enantiomeric excess values determined by chiral phase GC; sign of optical rotation or absolute configuration is given in parentheses and assigned on the basis of reference biotransformations; ee_S (substrate) in italics, ee_P (product) in normal font.

[b] Expression assays for these substrates were conducted with the conditions described for alkene substrates (Section 2.3). **27a** and **28a** were dissolved in ethanol and added to a final concentration of 2.5 mM (1% ethanol (v/v)) 2 h post-induction.

<50% after 24 h). Biotransformations with CHMO$_{JS666}$ of terpenones **21a**–**25a** yielded mostly either untouched starting material (**22a** and **23a**) or complex product mixtures (**21a** and **24a**). The composition of these mixtures and identity of byproducts was not further examined within this study. Only (+)-*trans*-dihydrocarvone **25a** was cleanly converted to abnormal lactone **25c** at a low rate.

3.6. Kinetic resolution of 2-substituted cyclohexanones

CHMO$_{JS666}$ catalyzes kinetic resolutions of 2-substituted cyclohexanones with excellent stereodiscrimination (Table 3, Scheme 5). Racemic compounds **27a–30a** are converted to the corresponding lactones with >99% *ee* ($E > 200$). Lactone product **30b** could even be obtained with higher optical purity than has been previously reported [18]. 2-Methylycylohexanone **26a** represents a difficult substrate for BVMOs, as the best result published so far reached $E = 6$ with CHMO$_{Acineto}$. CHMO$_{JS666}$ could not significantly improve this value ($E = 7$). The larger 2-benzylcycloheptanone **31a** was not converted by the biocatalyst.

Although it reached 70% conversion within 24 h, the CHMO$_{JS666}$ did not proficiently catalyze the kinetic resolution of β-acetoxy ketone **32a** ($E = 10$). Detailed results and preparative information for this linear ketone substrate can be found in Table S1 [37].

4. Conclusions

The newly expressed cyclohexanone monooxygenase from the xenobiotic-degrading *Polaromonas* sp. strain JS666 exhibits similar substrate enantiopreference and -selectivity to other CHMO-type enzymes for the 32 tested ketone substrates. This new expression system adds to the toolbox of heterologously characterized CHMOs, aiding future exploration of the effects of sequence variation on activity and enantioselectivity. The results of this work appear to contradict one of the current theories of cDCE degradation in strain JS666, illustrating that more research is necessary to uncover the mechanisms at work in the dichotomous growth-coupled/cometabolic cDCE degradation behavior of this strain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2012.03.002.

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