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Enantiocomplementary inverting *sec*-alkylsulfatase activity in cyano- and thio-bacteria *Synechococcus* and *Paracoccus* spp.: selectivity enhancement by medium engineering

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ABSTRACT

Whole resting cells of cyano- and thio-bacteria *Synechococcus* and *Paracoccus* spp. were shown to possess inverting alkylsulfatase activity for a broad spectrum of *sec*-alkylsulfate esters, which furnished either (*R*)- or (*S*)-*sec*-alcohols from the corresponding *rac*-sulfate esters in an enantiocomplementary fashion. Low enantioselectivities (*E*-values 1–4) could be dramatically improved by the addition of lower alcohols (e.g., *t*-BuOH) or by using a biphasic medium containing *t*-BuOMe (*E* >200).

1. Introduction

Sulfatases are a heterogenic group of hydrolytic enzymes,[1] which catalyse the cleavage of the sulfate ester bond to yield the corresponding alcohol (or phenol) and inorganic hydrogen sulfate.[2,3] Depending on the subtype of enzyme, the reaction may proceed via cleavage of the S–O or the C–O bond, which leads to either *retention* or *inversion* of configuration at the stereogenic carbon centre.[4] Whereas the mechanism of action for retaining sulfatases has been studied in great detail mainly on aryl sulfatases, such as human aryl sulfatase A,[5] nothing is known about the catalytic mechanism of inverting sulfatases.[6,7] This latter activity is particularly intriguing, since the S_N2-type base-induced (chemocatalytic) hydrolysis of sulfate esters with concomitant inversion of configuration at carbon is practically impossible,[8] which is in contrast to facile acid-catalysed hydrolysis.[9] Intrigued by the possibility of catalysing a 'chemically impossible' reaction by an enzyme, our search for alkyl sulfatases was driven by the selection of microbial strains possessing unusual sulfur metabolic pathways. In this context, inverting sulfatases were identified in *Rhodococcus*,[7] *Sulfolobus*[10] and *Pseudomonas* spp.,[11] whereas retaining sulfatase activities were identified in marine bacteria.[12]

Prompted by reports on the regulation of the complex (four-component) periplasmic sulfate transport system of unicellular cyanobacteria *Synechococcus* spp.,[13,14] we speculated about the occurrence of (alkyl) sulfatase activities in these obligate photoautotrophs.[15] Based on their unique light-dependent metabolism, these organisms have recently gained increasing interest in biotechnological applications, in particular for the bioremediation of pesticides[16] and the asymmetric reduction of halogenated carbonyl compounds.[17,18] In addition, facultatively lithoautotrophic neutrophilic (thio)bacteria of the genus *Paracoccus* are included in this study due to their ability to oxidise sulfur species in low oxidation states (mainly elemental S_8^0 or hydrogen sulfide) to form sulfuric acid.[19]

2. Results and discussion

Following a previously established protocol,[11] lyophilised whole (resting) cells of *Synechococcus* and *Paracoccus* spp. were used in an initial screening for alkyl sulfatase activity using substrates *rac*-**1a**–**6a**, while the formation of the corresponding *sec*-alcohol **1b**–**6b** was monitored (Scheme 1).

We were able to see that among the range of strains tested,[20] our metabolism-based concept for the strain-selection was successful: For the first time, alkylsulfatase activity was detected in the lithoautotrophic (thio)bacterium *Paracoccus denitrificans* DSM 6392 and in cyanobacteria *Synechococcus* spp. PCC 7942 and RCC 556 (Table 1). In contrast to *Rhodococcus* and *Sulfolobus* spp., which were restricted to linear and non-functionalised *sec*-alkylsulfate esters, the newly detected strains showed a broad substrate tolerance by accepting substrate *rac*-**6a**. Whereas the activities of *Synechococcus* sp. RCC 556 were low, those of *Synechococcus* sp. PCC 7942 and *Paracoccus* DSM 6392 were useful for further studies. For both organisms, the stereochemical course of sulfate ester hydrolysis was shown to proceed via *inversion* of configuration by using (*R*)- and (*S*)-**2a** as substrates, which gave (*S*)- and (*R*)-**2b**, respectively. Despite these encouraging activities, the enantioselectivities were disappointingly low and did not exceed *E*-values of about 4.[33]

In whole-cell transformations of alkylsulfate esters, poor enantioselectivities are often caused by competing (iso)enzymes

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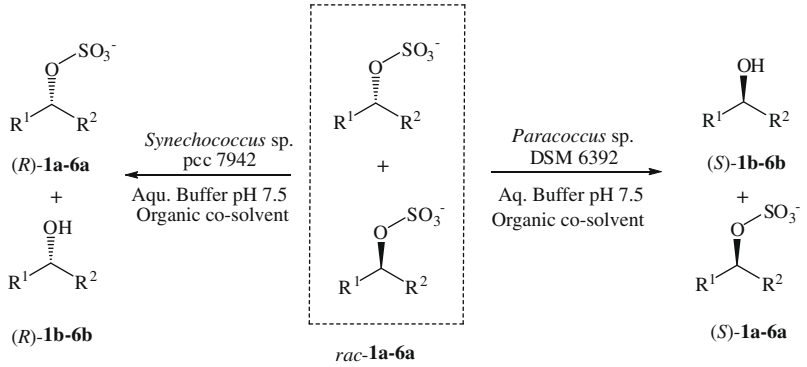

R^1 = large group, R^2 = small group

Substrate	1a,b	2a,b	3a,b	4a,b	5a,b	6a,b
R^1	n-C_5H_{11}	n-C_6H_{13}	n-C_7H_{15}	n-C_5H_{11}	n-C_4H_9	$(CH_2)_2$-CH=C$(CH_3)_2$
R^2	CH_3	CH_3	CH_3	C_2H_5	n-C_3H_7	CH_3

Scheme 1. Enantio- and stereoselective microbial hydrolysis of *sec*-alkylsulfate esters *rac*-**1a**–**6a** (cf. Table 2).

Table 1
Substrate tolerance of *Synechococcus* spp. PCC 7942, RCC 556 and *Paracoccus denitrificans* DSM 6392

Substrate	*Synechococcus* sp. RCC 556			*Synechococcus* sp. PCC 7942			*Paracoccus denitrificans* DSM 6392		
	c (%)	ee_P (%)	E[a]	c (%)	ee_P (%)	E[a]	c (%)	ee_P (%)	E[a]
rac-**1a**	<1	—	—	4	53 (*R*)	3	5	50 (*S*)	3
rac-**2a**	1	n.d.	n.d.	11	29 (*R*)	2	4	28 (*S*)	2
rac-**3a**	1	n.d.	n.d.	24	3 (*S*)	~1	8	<1	~1
rac-**4a**	1	n.d.	n.d.	11	16 (*S*)	~1	7	<1	~1
rac-**5a**	8	<1	~1	11	12 (*S*)	~1	1	7 (*S*)	~1
rac-**6a**	n.c.	—	—	9	49 (*R*)	4	6	22 (*S*)	2

n.c. = no conversion; n.d. = not determined due to low conversion; c = conversion; ee_P = enantiomeric excess of product **1b**–**6b**.
[a] E-value;[33] time: RCC 556 = 96 h; PCC 7942 = 72 h; DSM 6392 = 24 h.

possessing lower (or even) opposite stereo- and/or enantiopreferences[6] by acting through retention or inversion of configuration, or by preferring opposite substrate enantiomers. This assumption was supported by the fact that depending on the substrate, *Synechococcus* sp. PCC 7942 produced the opposite enantiomeric products (*R*)-**1b**, (*R*)-**2b**, (*R*)-**6b** and (*S*)-**3b**–**5b** in low to moderate enantiomeric excesses (Table 1). Since the overall activities of *Synechococcus* sp. PCC 7942 and *P. denitrificans* DSM 6392 were satisfactory, we considered improving their enantioselectivities by medium engineering (Table 2).

In order to improve insufficient regio- or stereoselectivities of biocatalysts, various techniques have been developed, which the aim of (i) the modification of the substrate[21] or cosubstrate structure[22] (substrate engineering); (ii) changing the enzyme by chemical[23] or genetic methods[24] (enzyme engineering); and (iii) tuning of the reaction medium (medium engineering). The latter technique makes use of various components, such as carbohydrates (e.g., cyclodextrins), PEG, detergents[25] and metal ions[26] which are added in low amounts and are believed to act as enantioselective inhibitors.[27] Alternatively, the bulk-solvent as a whole may be

Table 2
Selectivity enhancement for *Paracoccus denitrificans* DSM 6392 and *Synechococcus* sp. PCC 7942

Entry	Substrate	Strain	Additive[a]	c (%)	ee_P (%)	E
1	*rac*-**6a**	*Synechococcus* PCC 7942	None	2	71 (*R*)	6
2	*rac*-**6a**	*Synechococcus* PCC 7942	EtOH 10%	<1	n.d.	—
3	*rac*-**6a**	*Synechococcus* PCC 7942	*i*-PrOH 10%	<1	n.d.	—
4	*rac*-**6a**	*Paracoccus denitrificans* DSM 6392	None	6	22 (*S*)	2
5	*rac*-**6a**	*Paracoccus denitrificans* DSM 6392	$FeCl_3$ 5 mM	11	48 (*S*)	3
6	*rac*-**6a**	*Paracoccus denitrificans* DSM 6392	MeOH 10%	9	85 (*S*)	13
7	*rac*-**6a**	*Paracoccus denitrificans* DSM 6392	EtOH 10%	9	93 (*S*)	31
8	*rac*-**6a**	*Paracoccus denitrificans* DSM 6392	EtOH 20%	3	>99 (*S*)	>200
9	*rac*-**6a**	*Paracoccus denitrificans* DSM 6392	*i*-PrOH 10%	5	92 (*S*)	25
10	*rac*-**6a**	*Paracoccus denitrificans* DSM 6392	*t*-BuOH 10%	7	>99 (*S*)	>200
11	*rac*-**2a**	*Synechococcus* PCC 7942	None	3	42 (*R*)	3
12	*rac*-**2a**	*Synechococcus* PCC 7942	*t*-BuOMe 50%	3	89 (*R*)	17
13	*rac*-**6a**	*Paracoccus denitrificans* DSM 6392	*t*-BuOMe 50%	3	>99 (*S*)	>200
14	*rac*-**6a**	*Synechococcus* PCC 7942	*t*-BuOMe 50%	3	>99 (*R*)	>200

[a] Concentrations are denoted as v:v; time: 24 h.

altered by the addition of water-miscible or -immiscible organic cosolvents, in order to furnish mono- or biphasic reaction media, respectively. Although the molecular reasons of the selectivity enhancement by medium engineering are still poorly understood, they provide a powerful tool.[28] As a rule of thumb, an increase in stereoselectivity usually results in a loss of catalytic activity. In general, water-miscible cosolvents, such as acetone, DMSO,[29] THF,[30] acetonitrile and lower alcohols,[31] are often employed with hydrolytic enzymes acting on substrates of medium polarity, such as esterases and proteases, lipophilic organic (co)solvents to yield biphasic media that are popular for enzymes acting on an interface, such as lipases.

In our previous study on *sec*-alkylsulfatase RS2 from *Rhodococcus ruber* DSM 44541, $FeCl_3$ and hexadecyltrimethyl-ammonium bromide turned out to be powerful selectivity enhancers.[32] Although $FeCl_3$ did not exhibit any effect on the enantioselectivity of *Paracoccus* DSM 6392 (entry 5), lower alcohols such as MeOH, EtOH, *i*-PrOH and *t*-BuOH had a strong impact. Although initial tests showed that *Synechococcus* sp. PCC 7942 was very sensitive towards these solvents (entries 1–3), they were very effective with *P. denitrificans* DSM 6392 (entries 4–10). Variation of the nature and concentration of alcohol revealed two trends:

(i) The selectivity enhancing effect increased with the steric requirements of the alcohol, that is, *t*-BuOH was more effective than MeOH (entries 6 and 10), with EtOH and *i*-PrOH as intermediates (entries 7 and 9); and (ii) increasing the amount of (ethyl) alcohol led to enhanced selectivities with concomitant enzyme deactivation (entries 7 and 8). No selectivity enhancement was detected for *Alcaligenes* sp. DSM 2625, *R. ruber* DSM 44540, *Norcadia nova* DSM 43843 or *Ralstonia* sp. DSM 6428 (data not shown).

Since water-immiscible organic cosolvents are generally better tolerated by enzymes, a range of biphasic aqueous–organic solvent systems were tested.[34] Among them, *t*-BuOMe proved to be the best. Substrate *rac*-**2a**, whose selectivity enhancement using alcohols failed with *P. denitrificans* DSM 6392, showed acceptable results (*E*-values from 3 to 17, entries 11 and 12), and *rac*-**6a** could be hydrolysed with perfect enantioselectivity (*E* >200, entries 13 and 14) with both organisms in an enantiocomplementary fashion to yield (*R*)- or (*S*)-**6b** in >99% ee using *Synechococcus* PCC 7942 or *P. denitrificans* DSM 6392, respectively. The modest conversion may be caused by solvent toxicity or by enzyme inhibition.

3. Conclusion

In conclusion, enantiocomplementary inverting *sec*-alkylsulfatase activity has been detected for the first time in cyano- and thio-bacteria *Synechococcus* and *Paracoccus* spp., which were preselected for potential sulfatase activities due to their special sulfur-metabolism. Low initial enantioselectivities (*E*-values up to 4) could be improved by the addition of water-miscible organic cosolvents (such as *t*-BuOH) or by using a biphasic medium containing *t*-BuOMe (*E* >200). Isolation, characterisation and cloning of inverting *sec*-alkylsulfatases are currently being undertaken to provide sufficient amounts of proteins for the preparative-scale deracemisation *sec*-alcohols via enantioconvergent chemoenzymatic hydrolysis of their corresponding sulfate esters, either by employing a single inverting sulfatase in combination with retaining chemical hydrolysis, or by using a matching pair of an inverting and retaining enzyme.[35]

4. Experimental

4.1. General

Substrates *rac*-**1a**–**6a** and non-racemic reference compounds **1b**–**6b** were synthesised as previously described.[36] The absolute configuration of products **1b**–**6b** was determined by coinjection with an authentic reference material on GC using a chiral stationary phase. Achiral and chiral GC-analyses were performed as previsouly reported.[11]

Strains: *Alcaligenes* sp. DSM 2625, *R. ruber* DSM 44540, *N. nova* DSM 43843, *Ralstonia* sp. DSM 6428 and *P. denitrificans* DSM 6392 were obtained from DSMZ (http://www.dsmz.de/) and were grown for 72 h in media containing yeast extract (10 g/L), bacteriological peptone (10 g/L), glucose (10 g/L), NaCl (2 g/L), $MgSO_4{\cdot}7H_2O$ (0.15 g/L), K_2HPO_4 (4.4 g/L) and NaH_2PO_4 (1.3 g/L) at 30 °C and 120 rpm in shaking flasks. *Synechococcus* sp. PCC 7942 and RCC 556 were provided by the Pasteur Culture Collection of Cyanobacteria (http://www.pasteur.fr/recherche/banques/PCC/) and Roscoff Culture Collection (http://www.sb-roscoff.fr), respectively. Both cyanobacterial strains were cultivated according to the method reported by Franco-Lara et al.[37] on a 20 L-scale. For the limnic strain *Synechococcus* sp. PCC 7942, BG-11 medium[38] was used, whereas the marine *Synechococcus* sp. RCC 556 was grown in PCR-Tu_2[39] with artificial seawater as the base. After 264 h, cells were harvested by centrifugation (4528*g*, 30 min), they were washed with Tris–HCl buffer (100 mM, pH 7.5) and lyophilised.

4.2. General screening-procedure for *sec*-alkylsulfatase activity

Lyophilised whole cells (50 mg) were rehydrated in 700 μL of Tris–HCl buffer (100 mM, pH 7.5) for 1 h at 30 °C and shaking at 120 rpm. Next, 200 μL of substrates **1a**–**6a** from a stock solution [30 mg/mL, in Tris-buffer (100 mM, pH 7.5) was added and the reaction mixture was incubated at 30 °C with shaking at 120 rpm for 24 and 72 h; in the case of *Synechococcus* sp., 96 h was required. RCC 556. Work-up was performed by adding 600 μL of EtOAc. After centrifugation (13,000 rpm, 2 min, rt), the organic layer was dried over Na_2SO_4 and 100 μL of 1-decanol from a stock solution (10 mg/mL in EtOAc) was added as the internal standard for the determination of conversion. Samples were centrifuged again and subjected to GC analysis on an achiral CP1301 or DB1701 column. Positive hits were derivatised overnight using acetic anhydride (60 μL) and catalytic 4-(dimethylamino)-pyridine (DMAP). After quenching with water, the organic layer was treated as described above without addition of internal standard. Derivatised samples were measured on a chiral DEX-CB column, *E*-values were calculated from ee_P and conversion.[33]

4.3. General procedure for selectivity enhancement of *P. denitrificans* DSM 6392 and *Synechococcus* sp. PCC 7942

Lyophilised whole cells (50 mg) were rehydrated in 700 μL of Tris–HCl buffer (100 mM, pH 7.5) for 1 h at 30 °C and shaken at 120 rpm. Water-miscible (10% v/v) or -immiscible organic cosolvents (50%) or $FeCl_3$ (5 mM) or hexadecyltrimethyl-ammoniumbromide (5 mM) was added. Then 200 μL of substrate from a stock solution [30 mg/mL, *rac*-sulcatylsulfate **6a** in Tris-buffer (100 mM, pH 7.5) was added and the reaction mixture was incubated at 30 °C with shaking at 120 rpm for 24 h. Work-up was performed by adding 600 μL of EtOAc (in the case of water-miscible cosolvents, $FeCl_3$ and hexadecyltrimethyl-ammoniumbromide) or the corresponding water-immiscible solvent, respectively After centrifugation, work-up and analysis were carried out as described above.

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