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Donor Promiscuity of a Thermostable Transketolase by Directed Evolution: Efficient Complementation of 1-Deoxy-D-xylulose-5-phosphate Synthase Activity

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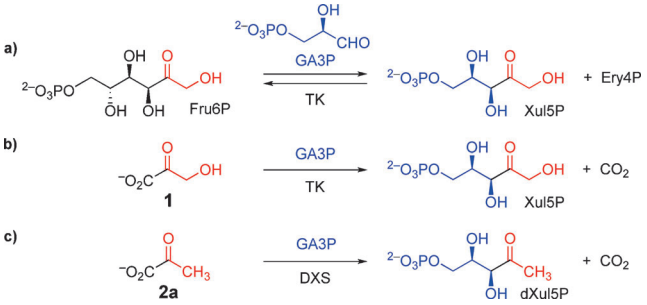
Abstract: *Enzymes catalyzing asymmetric carboligation reactions typically show very high substrate specificity for their nucleophilic donor substrate components. Structure-guided engineering of the thermostable transketolase from Geobacillus stearothermophilus by directed in vitro evolution yielded new enzyme variants that are able to utilize pyruvate and higher aliphatic homologues as nucleophilic components for acyl transfer instead of the natural polyhydroxylated ketose phosphates or hydroxypyruvate. The single mutant H102T proved the best hit toward 3-methyl-2-oxobutyrate as donor, while the double variant H102L/H474S showed highest catalytic efficiency toward pyruvate as donor. The latter variant was able to complement the auxotrophic deficiency of Escherichia coli cells arising from a deletion of the dxs gene, which encodes for activity of the first committed step into the terpenoid biosynthesis, offering the chance to employ a growth selection test for further enzyme optimization.*

Transketolase (TK, EC 2.2.1.1,) is a thiamine diphosphate (ThDP) dependent enzyme that catalyzes in vivo a reversible transfer of a C2 ketol fragment from a phosphorylated ketose to a phosphorylated aldose (Scheme 1).[1] While wild-type TK accepts a large variety of hydroxyaldehydes as the electrophilic acceptor substrates with strict (2*R*)-specificity,[2] we recently engineered variants of the TK from *Geobacillus stearothermophilus* (TK_{gst}) that can also efficiently convert (2*S*)-configured hydroxyaldehydes.[3] In another directed evolution study we succeeded in the discovery of TK_{gst} variants that efficiently convert arylated aldehydes[4] and non-hydroxylated aliphatic aldehydes with high stereocontrol for either native or reversed configuration.[5] Similar studies were also reported for the TK from *E. coli*.[6] On the other hand, TK shows very limited substrate tolerance toward the nucleophilic donor components. Although previous studies showed that substrate phosphorylation is not essential for catalytic activity, apart from its natural (phosphorylated) ketose donors TK accepts only hydroxypyruvate (**1**). This non-natural substrate is particularly attractive for synthetic applications of TK because decarboxylation renders conversions essentially irreversible.[7]



Scheme 1. Comparison of carboligation reactions catalyzed by TK and DXS. a) Native equilibration of sugar phosphates by TK in vivo; b) directed carboligation of TK by using hydroxypyruvate (**1**) in vitro; c) native carboligation by DXS in vivo.

With regard to a broader application scope of TK catalysis, particularly in the development of novel in vitro reaction cascades along the strategic concept of *Systems Biocatalysis*,[8] other 2-oxoacids such as pyruvate (**2a**) and higher homologues would be interesting alternative acyl donor substrates that would open up an access to a range of novel product types. Unfortunately, several authors have reported that TK itself is unable to react with **2a**,[9] in line with the notion that enzymes catalyzing C–C bond formations are generally notorious for having a strict specificity for their nucleophilic substrate.[10]

However, among the superfamily of ThDP-dependent enzymes[11] some enzymes can utilize **2a** as their natural donor substrate for decarboxylative carboligation. This includes the 1-deoxy-D-xylulose-5-phosphate synthase (DXS, EC 2.2.1.7), which specifically catalyzes the transfer of the acetyl group from **2a** to glyceraldehyde-3-phosphate to yield 1-deoxy-D-xylulose 5-phosphate as the first committed step into the non-mevalonate pathway of terpenoid biosynthesis (Scheme 1).[12] Herein we report on structure-guided mutational studies to create variants of TK_{gst} that are able to use **2a** and related 2-oxoacids as donor substrates for a corresponding, highly stereoselective asymmetric C–C bond formation.

In the absence of an experimental 3D protein structure for TK_{gst} its substrate-binding pocket was modeled[5] based on the close sequence homology with the TK from *Bacillus anthracis*

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(74% identity), for which the X-ray crystal structure of the holoenzyme has recently been solved.[13] In addition, both X-ray protein structures of TK and DXS from *E. coli* are available,[14] as well as a snapshot of TK with covalent cofactor-bound substrate Fru6P.[15] Based on the well-known similarity among the ThDP-dependent enzyme family and the highly conserved sequence motif for the cofactor-binding site,[11] a structure alignment of the two *E. coli* enzymes (Figure 1) confirmed their highly similar active-site construction. The substrate-liganded TK structure indicates that physical contacts of the crucial CH_2OH moiety of the ketol donor exist to Gly116 (hydrogen bond to carbonyl), His68 (hydrophobic contact), His102 and His474 (hydrogen bonds to ring N). However, a major difference is that the His102 residue in TK is changed to phenylalanine in DXS, which plausibly will better accommodate the pyruvate methyl group instead by improving hydrophobic contacts.

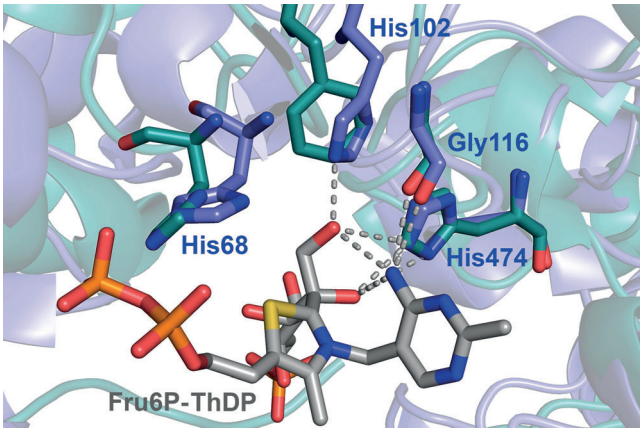


Figure 1. Comparison of the donor binding environments of TK and DXS from *E. coli* by superposition of their active sites based on the X-ray crystal structures (blue = TK, PDB entry 2R8P; green = DXS, PDB entry 2O1S). Graphic was created with PyMOL.[16]

A multiple sequence alignment (MSA) performed for both TK and DXS from a diverse set of organisms confirms their highly conserved active-site organization. Particularly, there is a high sequence homology for the active site residues of TK and DXS enzymes that are directly involved in the donor substrate binding (Figure 2), except that the TK H102 residue is consistently replaced by Phe or Tyr in DXS enzymes.

To test for background activity, standard wild type enzymes from TK_{gst} and the TK from *E. coli* and yeast (TK_{eco} and TK_{sce}, respectively) were assayed for conversion of

	64 72	98 106	112 120	470 478
TK_{gst}	LSAG**H**GSML	KTPG**H**PEYG	EATT**G**PLGQ	DGPT**H**EPIE
TK_{eco}	LSNG**H**GSML	KTPG**H**PEVG	ETTT**G**PLGQ	DGPT**H**QPVE
TK_{sce}	LSNG**H**AVAL	RTPG**H**PEFE	EVTT**G**PLGQ	DGPT**H**QPIE
DXS_{gst}	WDVG**H**QAYV	GLSG**F**PKRS	VWET**G**HSST	DGET**H**QGVF
DXS_{eco}	WDVG**H**QAYP	GLHP**F**PWRG	VLSV**G**HSST	DGQT**H**QGAF

Figure 2. Partial alignment of selected TK and DXS protein sequences. Numbering follows the sequence of TK_{gst}. Residues highlighted in color correspond to those in contact to the ThDP-bound nucleophile (see Figure 1). *Saccharomyces cerevisiae* does not have a gene coding for DXS.

2a in the presence of glycolaldehyde (**3**) as the best-known acceptor substrate using a recently developed pH-sensitive colorimetric assay.[17] The assay principle is based on the increase of the reaction pH as a result of substrate decarboxylation, which is measured by using a pH indicator. Very low residual activity was observed with all the three wild type TK (Table 1; Supporting Information, Table S4) against a control

Table 1: Kinetic properties of selected TK variants toward pyruvate (**2a**).[a]

Variants	K_M [mM]	k_{cat} [$\times 10^{-3}$ s^{-1}]	k_{cat}/K_M [$\times 10^{-3}$ s^{-1} mM^{-1}]
wt-TK_{gst}	40.2 ± 3.5	39 ± 1	1 ± 0.3
TK_{gst}(H102F)	35.1 ± 5.3	53 ± 3	2 ± 0.5
TK_{gst}(H102L)	29.1 ± 1.6	183 ± 3	6 ± 2.0
TK_{gst}(H474S)	30.0 ± 2.9	101 ± 3	3 ± 1.1
TK_{gst}(H474N)	13.4 ± 2.5	85 ± 4	6 ± 1.5
TK_{gst}(H102L/H474S)	16.6 ± 0.7	170 ± 2	10 ± 2.6
TK_{gst}(H102L/H474N)	6.1 ± 2.1	50 ± 3	8 ± 1.5
DXS_{eco}	3.3 ± 1.1	497 ± 23	150 ± 21.3

[a] Kinetic constants were determined by varying the concentration of substrate **2a** (0–200 mM). The assay solution contained 0.03–0.04 mg purified enzyme, 2.4 mM ThDP, 9 mM $MgCl_2$, 10 mM glycolaldehyde (**3**) and 0.028 mM of phenol red in 200 µL of triethanolamine buffer (2 mM, pH 7.5).

without acceptor. However, the assay is unable to distinguish between plain decarboxylation of **2a** versus true transketolase activity (decarboxylation followed by acyl transfer to acceptor). Therefore, a preparative scale synthesis was attempted with wild type TK_{gst} at 50 °C to check for the carboligation step, from which the product L-1-deoxyerythrulose (**4a**) could indeed be isolated in 9% yield, thereby proving the pH assay results.

Protein engineering to improve the TK activity with **2a** was performed on the thermostable TK_{gst}, which shows mutational robustness.[3,5] First, the rational Phe replacement for His102 was constructed to create a DXS-like situation in the TK_{gst} active site. Notably, the designed H102F variant showed only little improvement in its activity with **2a** as donor (Table 1). Similar low activity with **2a** had earlier been detected with the corresponding H103F variant of TK_{sce}.[18]

Thus, we opted for a semi-rational engineering approach focusing on all residues that are in direct contact to the CH_2OH moiety of **1** (His68, His102, Gly116, His474) with screening for improved reaction rates with **2a**. Iterative saturation mutagenesis (ISM)[19] was chosen to mutate each of the residues individually. This directed evolution approach is a compromise that keeps library sizes small but still allows sampling for cooperative binding effects. His68 and Gly116 were included for reason of comprehensiveness although His68 is involved in cofactor binding, and upon replacement of Gly116 side-chain orientation would be opposite to the substrate. Libraries were screened with **2a** (50 mM) using the pH-dependent assay. Indeed, libraries G116X and H68X did not deliver any positive hits. However, both H102X and H474X libraries yielded 25% of significantly improved variants. The four best unique protein variants from these two single libraries were purified by NTA affinity chromatography for kinetic characterization. All four variants

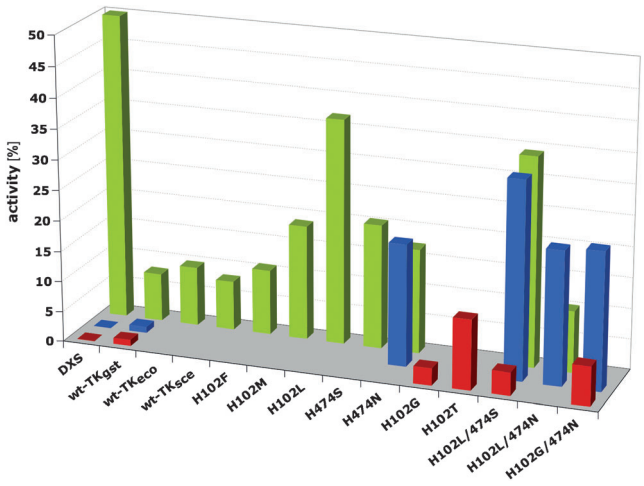


Figure 3. Activity of TK_{gst} variants (k_{cat}) against non-natural nucleophile substrates relative to native DXS_{eco} (set as 100% for reference): ■ pyruvate (**2a**), ■ 2-oxobutanoate (**2b**), ■ 3-methyl-2-oxobutanoate (**2c**).

showed a higher activity than the H102F variant, which corresponds to the DXS situation (Table 1, Figure 3).

The best variants H102L and H474N showed practically identical catalytic efficiency but differed significantly in their K_M values. The variant H102L was found to be the most active, showing a k_{cat} of about one third of native DXS_{eco} under saturating conditions, while the variant H474N showed the best substrate affinity with a K_M value about four-fold compared to that of DXS_{eco}.

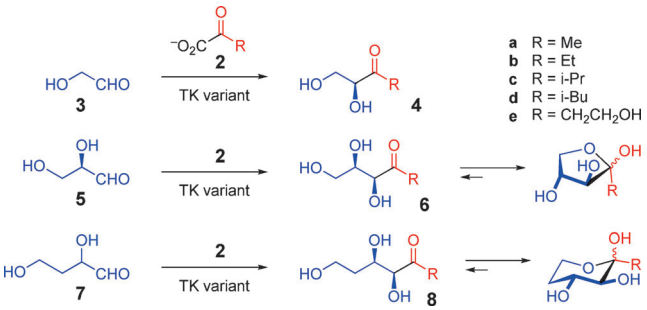
In a subsequent round, the positive hits were used as templates to generate four secondary saturation libraries (H102L/H474X, H102M/H474X, H474N/H102X and H474S/H102X). Screening furnished eight positive hits, from which sequencing identified H102L/H474N and H102L/H474S as unique (Table 1; Supporting Information, Table S4). Probably, more stringent assay conditions (< 50 mM of **2a**) might have been more efficient to identify variants with improved substrate affinity. The double variant H102L/H474N showed the best K_M value for **2a** of all variants, approaching the affinity of the native DXS_{eco} (Table 1, Figure 3). The variant H102L/H474S displayed a ten times higher catalytic efficiency than wild type TK_{gst} and five-fold better than the H102F variant. Interestingly, all of these engineered TK variants retained high stability against thermal unfolding (T_m = 75.3–78.5 °C; Supporting Information, Table S2) and were still able to convert **1** at various degree (Supporting Information, Table S3).

In an effort to study the donor substrate scope of the variants all single- and double-site libraries were screened toward activity with the sterically more demanding branched-chain oxoacids 3-methyl-2-oxobutanoate (**2c**) and 4-methyl-2-oxopentanoate (**2d**). Interestingly, in screenings with **2c** (10 mM) two single variants (H102G and H102T) and two double variants (H102L/H474S and H102G/H474N) were found to be active, while no positive hits revealed activity toward the more bulky higher homologous **2d**. After purification, kinetic data for these variants were determined (Figure 3; Supporting Information, Table S6). Clearly, variant H102T showed the highest rates and efficiency. Finally, top candidates identified from both previous screenings were also kinetically analyzed against 2-oxobutanoate (**2b**) and 4-hydroxy-2-oxobutanoate (**2e**)[20] (each at 10 mM) as donor substrates of intermediate size (Figure 3; Supporting Information, Table S5). The best candidate (highest k_{cat}) found with **2b** was the double variant H102L/H474S that showed a catalytic rate very similar to that with **2a** while profiting from significantly enhanced substrate affinity. Notably, the polar substrate analogue **2e** was not accepted.

Apparently, an exchange of the two histidine residues by smaller side chains is required to make room for the larger nucleophilic substrates (**2b**, **2c**). In position His474 it seems to be essential that a hydrogen bond donating capacity to the ketol carbonyl moiety is preserved, which is attained by replacements for serine or asparagine that both are also of similar, slightly reduced size. A replacement of His102 by a non-polar leucine residue not only will better accommodate the extra space requirements but also improve the binding of a hydrophobic alkyl chain of the substrate in the otherwise highly polar active site of TK as reflected in the reduced K_M values. Curiously enough, His474 stabilizing the acyl oxygen by hydrogen bonding is invariant among all species except for mammalian TKs where it is replaced by glutamine.[21]

Although the overall catalytic efficiency of the variants (k_{cat}/K_M) with substrates **2a** and **2c** are relatively low in comparison to that of the native DXS_{eco} enzyme (with **2a**), with **2b** some variants interestingly reach up to 28–39% of its catalytic efficiency, probably reflecting a compromise of good steric fit and positive hydrophobic interactions.

Preparative-scale synthesis with non-natural nucleophiles was performed at 50 °C to profit from the higher activity of the TK_{gst} variants at elevated temperatures (Scheme 2, Table 2). In a combinatorial manner, nucleophiles **2a**-**2c** were reacted with **3**, D-glyceraldehyde (**5**) and *rac*-2,4-



Scheme 2. Preparative syntheses catalyzed by best identified TK_{gst} variants for the conversion of oxoacids **2a–c** using aldehydes **3**, **5** and **7**.

dihydroxybutanal (**7**) as electrophiles, respectively, using the appropriate best TK_{gst} variants (H102L/474S for **2a**/**2b** and H102T for **2c**). The corresponding products **4a–4c**, **6a–6c** and **8a–8c** were obtained in good overall yields, somewhat lower only for the sterically more bulky series **c**. Reactions proceeded with high enantio- and diastereoselectivity for the natural configuration, as determined by $[\alpha]_D$ comparison, chiral-phase GC and NMR analysis.

Table 2: Synthetic reactions performed with non-natural nucleophiles using best TK_{gst} variants.[a]

Aldehyde	Oxo-acid	TK_{gst} variant	Product	Configuration	*ee*/*de* [%]	Yield [%]
3	**2a**	H102L/H474S	**4a**	(*S*)[b]	>95	88
3	**2b**	H102L/H474S	**4b**	(*S*)	–[c]	72
3	**2c**	H102T	**4c**	(*S*)	>99	69
5	**2a**	H102L/H474S	**6a**	(2*R*,3*S*)	>97[d]	72
5	**2b**	H102L/H474S	**6b**	(2*R*,3*S*)	>97[d]	60
5	**2c**	H102T	**6c**	(2*R*,3*S*)	>97[d]	52
7[e]	**2a**	H102L/H474S	**8a**	(3*R*,4*S*)	>97[d]	62
7[e]	**2b**	H102L/H474S	**8b**	(3*R*,4*S*)	>97[d]	60
7[e]	**2c**	H102T	**8c**	(3*R*,4*S*)	>97[d]	48[f]

[a] Reaction solution contained 12 mg purified enzyme (0.5 mol% relative to oxoacid), 2.4 mM ThDP, 9.4 mM $MgCl_2$, 50 mM aldehyde (**3** or **5**) and 50 mM oxoacid in 25 mL H_2O (adjusted to pH 7.5 by pH stat using 0.1 N HCl; 50°C, 12 h). [b] Configuration by comparison of $[\alpha]_D$ to authentic material. [c] Enantiomers could not be resolved by GC/HPLC. [d] de, determined by NMR analysis. [e] 100 mM *rac*-**7**. [f] Separation from starting material difficult.

Complete kinetic resolution of *rac*-**7** yielded the pure (2*R*)-derived products, analogous to the wild-type ability,[22] as evident by ^{1}H NMR analysis using the deoxy methylene group as stereochemical reporter group in the cyclic isomers of **8a–c**.[17,23] Identification of stereoisomers was facilitated via direct correlation with products obtainable by catalysis from fructose-6-phosphate aldolase (FSA), which gives products **4a**,**b** (from methanal),[24] **6a**,**b** (from **3**),[25,26] and **8a**,**b** (from 3-hydroxypropanal) with identical constitution and stereoconfiguration.[23,25] Thus, TK and FSA offer a directly complementary approach for the series **a** and **b**, while products **4c**, **6c** and **8c** are uniquely available only by TK catalysis because FSA is unable to convert nucleophiles carrying a branching point directly next to the carbonyl group.[23] DXS_{eco}, which is highly specific for its native substrate (Figure 3), has been shown to catalyze the synthesis of **4a** and **6a** only.[27] Another advantage of TK catalysis is that syntheses are driven by substrate decarboxylation, while the success of FSA synthesis depends on the magnitude of the aldol equilibrium constants.

To verify the functionality of wild-type TK_{gst} and its evolved variants for activity against **2a** in vivo, a complementation test was performed using the engineered auxotrophic *E. coli* strain EcAB4-2 (*dxs:CAT*),[28] in which the *dxs* gene had been disrupted by insertion of a selection marker. Because *E. coli* cells depend on the *dxs* gene for terpenoids synthesis,[12] its disruption leads to an auxotrophic strain that will only be able to grow if suitable metabolites can be taken up from the medium, or if the strain is complemented by a plasmid based gene replacement. This strain requires either supplementation by mevalonic acid (MVA) from the medium for growth or complementation by a non-chromosomal *dxs*-type gene to support the reaction depicted in Scheme 1c. For that purpose, strain EcAB4-2 was transformed with plasmids carrying the mutant gene for TK_{gst}(H102L/H474S), the wild-type TK_{gst}, the *dxs* gene for expression of native DXS_{eco} (positive control), or the empty vector (negative control). As shown in Figure 4, in the absence of MVA the transformants of strain EcAB4-2 were unable to grow when carrying only the empty vector, but grew successfully when complemented by the native *dxs* gene or the mutant gene for TK_{gst} with very similar growth rates. Surprisingly, even the wild-type gene for TK_{gst} could sustain growth, albeit at a significantly retarded rate. Presumably, the latter observation must be explained by the fact that larger concentrations of the inefficient TK_{gst} enzyme, which become available by efficient intracellular overexpression from a multi-copy vector, can also compensate for the loss of the *dxs* gene.

In this study the semi-rational evolution of the thermostable TK_{gst} led to the discovery of several new variants that are able to convert pyruvate (**2a**) as a non-natural nucleophilic substrate to imitate the native reaction catalyzed by DXS. Notably, the mutagenesis strategy revealed several protein variations that were far superior over the rationally designed H102F variant, which mimics the native DXS sequence. Even sterically more demanding oxoacids 2-oxobutanoate (**2b**) and branched-chain 3-methyl-2-oxobutanoate (**2c**) could be converted with good rates, and products were obtained with high enantioselectivity and diastereoselectivity that match the ability of the wild-type TK_{gst} with hydroxypyruvate (**1**). While TK reactions with substrates **2a** and **2b** offer a synthetic access complementary to using FSA, TK products derived from **2c** are unique because branching is inaccessible via FSA catalysis. We are confident that the promiscuity of TK for nucleophilic substrates could be further expanded by suitable mutagenesis to address novel product structures that are valuable as chiral building blocks.

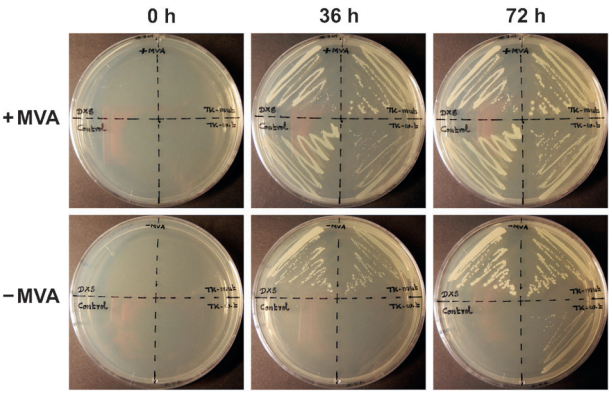


Figure 4. Complementation study of *E. coli dxs* auxotrophic strain by plasmid based genes coding for DXS_{eco} (positive control), TK_{gst}(H102S/H474L) and wild-type TK_{gst} against empty vector as negative control (from top left quadrant in clockwise order, respectively). Recombinant *E. coli* strains were plated on LB medium containing as selection markers 100 μg mL^{-1} ampicillin, 17 μg mL^{-1} chloramphenicol, 25 μg mL^{-1} kanamycin, and 0.5 mM IPTG. The medium was supplemented with *rac*-MVA (1 mM) as indicated. Plates were incubated at 37°C for the times indicated.

The ability of TK_{gst} mutants to complement the *dxs*$^-$ phenotype was proven by transformation of the auxotrophic *E. coli* strain EcAB4-2. We note that this observation offers an opportunity for further evolution of optimized DXS-like activity for TK_{gst} variants by random mutagenesis followed by growth selection under suitable limiting conditions.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: acyloins · asymmetric synthesis · biocatalysis · carboligation · protein engineering

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Communications

Protein Engineering

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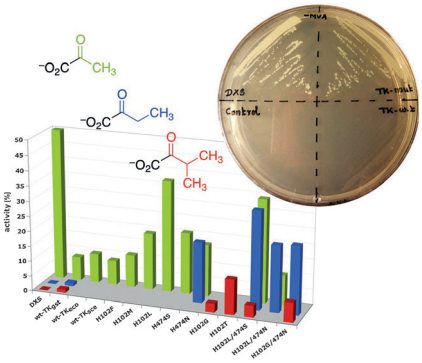
Donor Promiscuity of a Thermostable Transketolase by Directed Evolution: Efficient Complementation of 1-Deoxy-D-xylulose-5-phosphate Synthase Activity



Structure-guided engineering of a bacterial transketolase yielded enzyme variants capable of using aliphatic oxoacids as non-natural nucleophilic components for stereospecific acyloin synthesis. The double variant H102L/H474S showed high catalytic efficiency toward pyruvate and was able to complement a deletion of the *dxs* gene coding for 1-deoxyxylulose-5-phosphate synthase, which catalyzes the first step of the terpenoid biosynthesis.