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# Promiscuous activity of 3-isopropylmalate dehydrogenase produced at physiological level affords *Escherichia coli* growth on D-malate

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**Promiscuous activities of enzymes may serve as starting points for the evolution of new functions. However, most experimental examples of promiscuity affording an observable phenotype necessitate the artificial overexpression of the target enzyme. Here, we show that 3-isopropylmalate dehydrogenase (IPMDH), an enzyme involved in leucine biosynthesis, has a secondary activity on D-malate, which is sufficient for D-malate assimilation under physiological conditions where the enzyme is upregulated. *In vitro*, the turnover constant ($k_{cat}$) of IPMDH for D-malate is about 30-fold lower than the $k_{cat}$ for 3-isopropylmalate, yet sufficiently high to support the growth on D-malate. From an evolutionary perspective, our results highlight the possibility of phenotype emergence triggered by arbitrary changes in environmental conditions and prior to any mutational event.**

**Keywords:** D-malate dehydrogenase; enzyme; *Escherichia coli*; IPMDH; molecular evolution; promiscuous activity

Enzyme promiscuity plays crucial role in understanding enzyme evolution. Promiscuous activities of enzymes are defined as weak activities that are not physiologically relevant and for which enzymes have not been evolved [1–4]. Enzyme promiscuity is thought to originate from the retention of weak latent secondary activities inherited from primitive ancestors endowed with broad specificities [5–7]. Supporting this hypothesis, the latent activity of one member within an enzyme superfamily is often the native activity of another member [8,9]. Shared activities are not only observed between homologous enzymes. For example, reciprocal promiscuity has been reported also between nonhomologous enzymes, such as *Escherichia coli* alanine racemase (ALR) and cystathionine β-lyase (CBL) [10]. Moreover, enzymes may possess secondary activities that are unrelated to their evolutionary history [1,11,12].

Secondary activities are usually orders of magnitude below the native activity and, as such, should not perturb the cell physiology. In the course of evolution, these activities may be exploited as starting points for neofunctionalization [13–16]. However, if a promiscuous secondary activity is not high enough to be physiologically relevant, the emergence of a new phenotype will require its improvement by the selection of one or more genetic mutations within the coding region of the gene or in the region that controls the level of expression. In agreement with this hypothesis, several studies have shown that the genetic complementation of a functional knockout by a promiscuous enzyme requires its overexpression from a plasmid so that the

**Abbreviations**
ALR, alanine racemase; CBL, cystathionine β-lyase; DmlA, D-malate dehydrogenase; GH5, glycoside hydrolase family 5; HDH, homoisocitrate dehydrogenase; IDH, isocitrate dehydrogenase; IPMDH, isopropylmalate dehydrogenase; 3-IPM, 3-isopropylmalate.

activity can reach a phenotypically relevant level. Nevertheless, a promiscuous activity may directly contribute to a phenotype without the need for any mutagenic event but this scenario has been almost never explored experimentally. In a previous study, we have shown that promiscuity of *E. coli* D-malate dehydrogenase (DmlA) on 3-isopropylmalate (3-IPM) complements isopropylmalate dehydrogenase (IPMDH), an enzyme involved in leucine biosynthesis, in a Δ*ipmdh* knockout strain [17]. With a $k_{cat}$ around 0.1 $s^{-1}$, the IPMDH activity of DmlA is two orders of magnitude lower than its native activity on D-malate but it is sufficient to afford growth on a medium without leucine, even when the enzyme is produced from its chromosomal locus and under the control of its native promoter.

DmlA belongs to the superfamily of NAD(P)-dependent β-decarboxylating dehydrogenases catalyzing the oxidative decarboxylation of substrates having a common R-malate moiety with varying γ-substituents at C-3 position (Fig. S1). Substrate specificity and promiscuity in this superfamily have been extensively studied *in vitro*. For instance, isocitrate dehydrogenase (IDH, ECnonbreakingspace1.1.1.42), a major enzyme in Krebs cycle, is highly specific to negatively charged isocitrate [18] but the promiscuous reduction of α-ketoglutarate into the metabolite α-hydroxyglutarate has been reported for IDH1 of *Mycobacterium tuberculosis* [19] and for oncogenic variants of human IDH1 [20]. Dual specificity for isocitrate and homoisocitrate has been reported for the homoisocitrate dehydrogenase (HDH, ECnonbreakingspace1.1.1.87) of *Thermus thermophilus* and *Pyrococcus horikoshii* [21,22] whereas *Deinococcus radiodurans* has a trifunctional HDH which can recognize isopropylmalate as well [23]. Tartrate dehydrogenase (ECnonbreakingspace1.1.1.93) has native activity on tartrate and promiscuous to 3-isopropylmalate, L- and D-malate *in vitro* [24,25]. *Escherichia coli* DmlA (ECnonbreakingspace1.1.1.83) is a remarkable generalist enzyme of β-decarboxylating dehydrogenase superfamily. Besides its native activity on D-malate [26], it is promiscuous to L-tartrate, isocitrate, D-tartrate and notably to 3-IPM [17]. IPMDHs (ECnonbreakingspace1.1.1.85) are highly active on their native 3-IPM substrate with $k_{cat}/K_M$ in the order of $10^5$ to $10^7$ $M^{-1}s^{-1}$ [27,28], a typical range of efficiency for many metabolic enzymes [4]. Besides, *in vitro* studies showed that IPMDHs have substrate promiscuity. IPMDH from *Thermus thermophilus* HB8 is endowed with broad specificity toward a variety of alkylmalate substrates (methyl, ethyl, isopropyl, isobutyl, tertbutyl, and isoamyl group on C-3) [27]. *Escherichia coli* IPMDH is also reported as promiscuous to ethylmalate and tert-butylmalate [28]. IPMDH from *Thiobacillus ferrooxidans*, an acidophilic chemolithoautotrophic bacterium, has relatively high activity on both D-malate and L-malate *in vitro* [29].

Biological phenotypes associated with promiscuous activities of IPMDH were never studied experimentally. Here, we identified a physiologically relevant promiscuous activity of *E. coli* IPMDH when produced from its chromosomal locus. We demonstrate that, when IPMDH is induced upon leucine starvation, its secondary activity on D-malate is sufficient to complement *dmlA* deletion (Δ*dmlA*) phenotype in *E. coli* and confirmed this observation by *in vitro* studies. From an evolutionary perspective, our results indicate that a mutagenic event is not necessarily required for trait emergence since it can be associated with an arbitrary change in environmental conditions that fortuitously upregulates a promiscuous activity.

## Results

### Leucine deprivation enables growth on D-malate

D-malate metabolism was studied using the Δ*dmlA* knockout strain from the Keio collection [30]. Serial dilutions of the Δ*dmlA* and parental strain were spotted on M9 minimal agar media supplemented with D-malate (0.15%) and an amino acid mixture. As expected, *dmlA* knockout prevents the utilization of D-malate (Fig. 1A, left). Surprisingly, however, this phenotype was reverted when leucine was omitted in the medium (Fig. 1A, right). In these conditions, the Δ*dmlA* strain grows more slowly than the parental strain and generates very small colonies but with comparable number indicating high viability of individual bacteria. A similar advantage of leucine deprivation on D-malate metabolism by Δ*dmlA* strain was observed in liquid medium (Fig. 1B). When D-glucose was used as a carbon source instead of D-malate, an opposite effect was observed since leucine deprivation leads to a growth disadvantage for both the Δ*dmlA* and the parental strains (Fig. S2). These results suggested that the upregulation of at least one of the leucine biosynthetic enzymes was responsible for D-malate utilization. IPMDH encoded by *leuB* gene was the obvious candidate since it belongs to the same enzyme superfamily as DmlA. To test this hypothesis, the strains were grown on D-glucose with or without leucine and the corresponding cell-free extracts were tested for activity. As expected, leucine deprivation triggers an increase of IPMDH activity (Fig. 2A) in both Δ*dmlA* and wild-type strains. An increase in D-malate dehydrogenase activity independent of the *dmlA* genotype was also

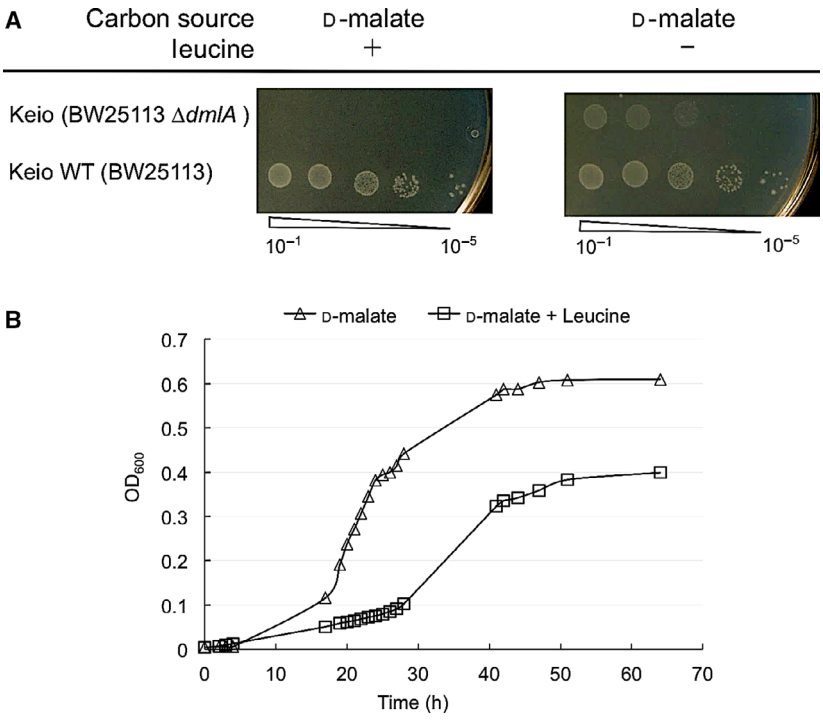


**Fig. 1.** Complementation of Δ*dmlA* phenotype in the context of chromosomal expression. (A) Growth of Keio wild-type (BW25113) and Keio *dmlA* knockout (BW25113 Δ*dmlA*::*kan*(*Km*$^r$) strains on solid M9 minimal media supplemented with D-malate (0.15%), with or without leucine (1 mM); (B) Growth of Keio wild-type (BW25113) and Keio *dmlA* knockout (BW25113 Δ*dmlA*::*kan*(*Km*$^r$) strains in liquid minimal media, containing D-malate as carbon source; the growth of *dmlA* knockout strain was monitored in presence and absence of leucine (1 mM); Keio wild-type was cultivated only in absence of leucine and was used a control. Overnight precultures on minimal media supplemented with D-glucose were washed with M9 buffer and used to inoculate D-malate containing minimal media.

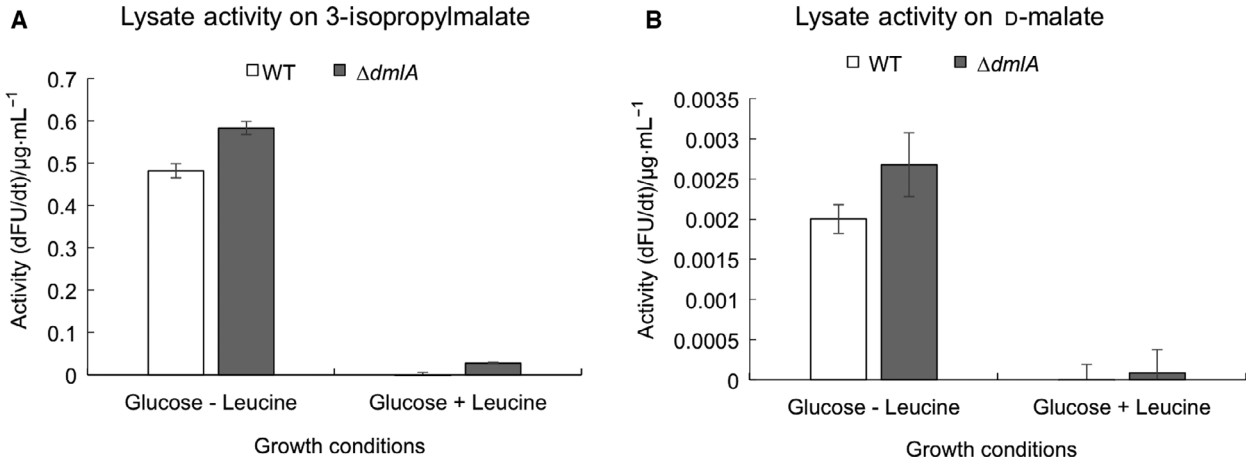


**Fig. 2.** Enzymatic activity in cell-free lysates prepared from Keio wild-type and KeioΔ*dmlA* strains. (A) 3-isopropylmalate dehydrogenase and (B) D-malate dehydrogenase activity in cell lysates. The strains were grown on glucose supplemented without or with leucine in the culture media. All activity measurements were performed at 25 °C in MOPS-KCl buffer (25 mM MOPS, 100 mM KCl, pH 7.5), with 500 µm of substrate (3-IPM or D-malate), NAD (500 µm), and MgCl2 (5 mM). Enzymatic activity is expressed in international units (1 U = 1 µmol·min$^{-1}$) per mg of total protein.

observed upon leucine deprivation although this activity was about 200-fold lower in comparison with IPMDH (Fig. 2B). This result indicates that IPMDH is responsible for the observed activity on D-malate. Both the 3-IPM dehydrogenase and the D-malate dehydrogenase activities are increased by ~ 20–25% in the Δ*dmlA* strain compared to the parental strain. This moderate effect suggests that IPMDH is slightly more concentrated in the Δ*dmlA* strain. These results further support our initial hypothesis that IPMDH has a promiscuous activity on D-malate, which is sufficient for supporting growth.

## IPMDH overexpression affords growth on D-malate

His-tagged version of IPMDH and DmlA was produced from plasmid vectors. The genes were placed

under the control of an IPTG inducible $P_{tac}$ promoter. In order to avoid cross-activity of the endogenous paralogous enzymes in phenotypic experiments, an engineered *E. coli* strain, B3 (HB101 Δ*idh*::*kan*(*Km*$^r$) Δ*leuB* Δ*dmlA*), lacking all beta-decarboxylating dehydrogenases, was built (Fig. S3). Growth of B3 strain transformed with DmlA and IPMDH expression plasmids in liquid minimal media supplemented with D-malate (0.15%) as carbon source and leucine (1 mM) was monitored. Results indicate that, while B3 strain cannot metabolize D-malate, *leuB* overexpression affords growth, even though at a slower rate compared to the strain expressing *dmlA* (Fig. 3A,C). As shown in Fig. 3B,D, very slow growth on D-malate was also observed under leucine deprivation, indicating that DmlA and IPMDH can individually support D-malate metabolism and leucine biosynthesis at the same time, in agreement with the observations from chromosomal expression of *leuB* (Fig. 1) and with our previous report on DmlA promiscuity [17]. In all cases, growths were reduced but still observed without IPTG induction. As shown in Fig. S4, this is due to leaky expression from the plasmids.

## IPMDH is active on D-malate in vitro

His-tagged IPMDH was purified and kinetic parameters were estimated for 3-IPM and D-malate substrates

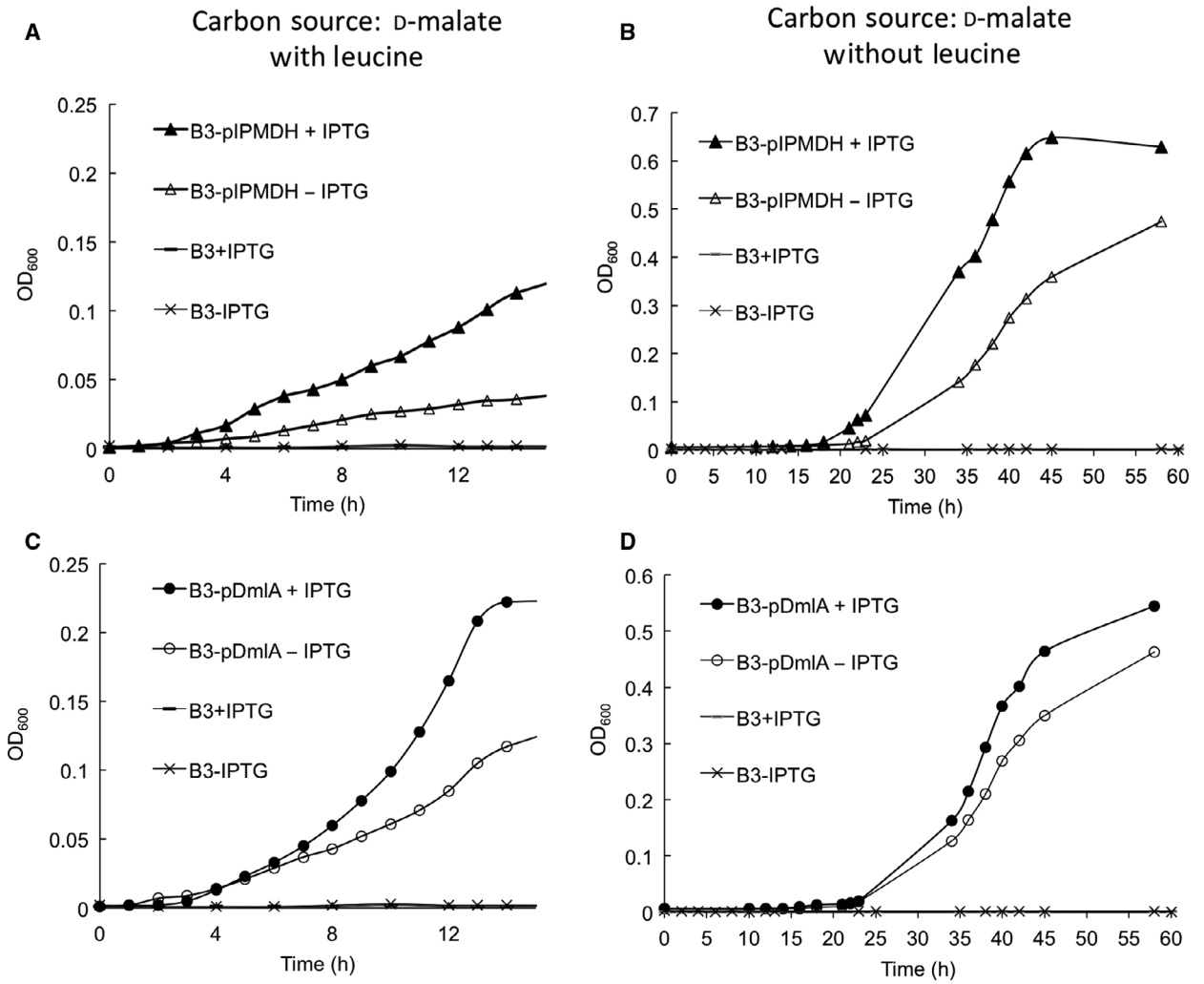


**Fig. 3.** Expression of IPMDH from a plasmid affords D-malate utilization. Growth curves of the B3 (HB101Δ*idh*::*kan*(*Km*$^r$) Δ*ipmdh* Δ*dmlA*) strain transformed with pIPMDH (A and B) or pDmlA (C and D) expression vectors without or with IPTG (1 mM) induction in EZ MOPS defined media supplemented with D-malate (0.15%) as a sole carbon source, amino acids (0.25 mM each except Glu, Gln and Leu, and 2 mM of glutamate/glutamine), (A, C) with leucine supplement (1 mM); (B, D) without leucine supplement.

**Table 1.** Activity of purified IPMDH on 3-IPM and D-malate. Kinetic assays were performed in MOPS-KCl buffer (25 mM MOPS, 100 mM KCl, pH 7.5) with fixed concentration of $NAD^+$ (500 μm), $MgCl_2$ (5 mM), and varying the 3-isopropylmalate or D-malate substrate concentrations.

| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($M^{-1}{\cdot}s^{-1}$) |
|---|---|---|---|
| 3-Isopropylmalate | 12 ± 1 | 11 ± 4 | (1.15 ± 0.3) × $10^6$ |
| D-malate | 0.41 ± 0.02 | 301 ± 15 | (1.36 ± 0.3) × $10^3$ |

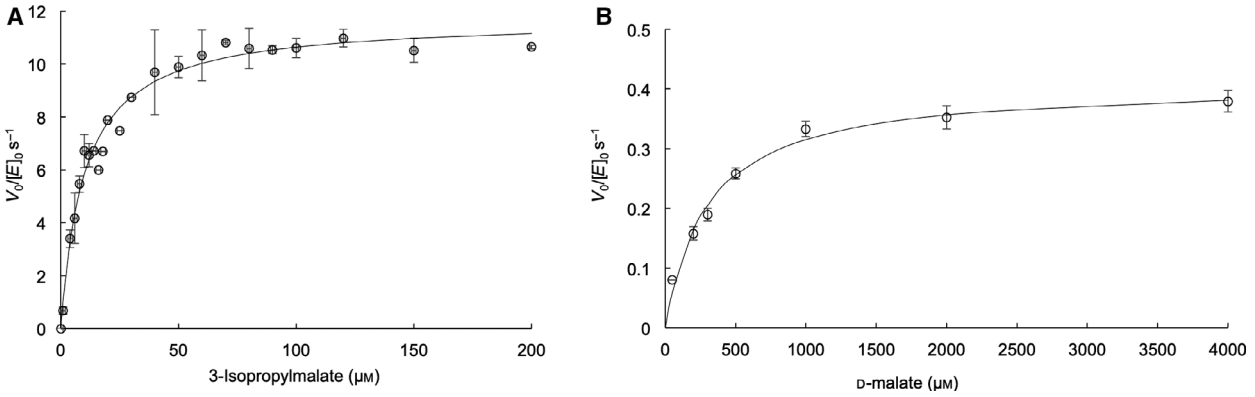


**Fig. 4.** *In vitro* activity of purified IPMDH on (A) 3-isopropylmalate and (B) D-malate. All kinetic measurements were performed in MOPS-KCl buffer (25 mM MOPS, 100 mM KCl, pH 7.5), NAD (500 μm), MgCl2 (5 mM), and varying the substrate (3-isopropylmalate or D-malate) concentrations.

at 25 °C (Table 1). Kinetic profiles on both substrates follow classical Michaelis–Menten hyperbolic behavior (Fig. 4). The catalytic efficiency constant ($k_{cat}/K_m$) on D-malate is about 1000-fold lower than the constant for 3-isopropylmalate while the turnover number ($k_{cat}$) is only reduced by about 30-fold. These values are in semiquantitative agreement with the 200-fold decrease in activity observed in lysates (Fig. 2) since those assays were performed under substrate saturation conditions for 3-IPM but not for D-malate. In culture experiments, D-malate is at 11 mM concentration in the medium (0.15%). This is about 300 times higher than the $K_m$ (0.3 mM). Even though the intracellular concentration of D-malate is not known, a decrease by more than two orders of magnitude seems unlikely so D-malate is probably at saturating concentration *in vivo*.

## Discussion

Leucine biosynthesis genes in *E. coli* are clustered in *leuABCD* operon, *leuB* encoding the IPMDH enzyme. The operon expression is regulated by a transcription attenuation mechanism controlled by the presence of leucine [31]. Our results show that, upon leucine starvation, the physiological concentration of IPMDH raises to a level that is sufficient for metabolizing D-malate thanks to substrate promiscuity. Moreover, under these conditions, IPMDH must also play its native role in leucine biosynthesis so the enzyme is phenotypically active in two core metabolic pathways at the same time. This observation has important implications from an evolutionary perspective. While it is generally accepted that promiscuous activities may serve as starting points for the neofunctionalization of proteins, the requirement of a mutagenic event boosting this promiscuous activity at the birth of a new phenotype can be questioned. Our results show that, for a new phenotypic trait to appear, a genetic mutation is not strictly needed, whereas environmental conditions may play an arbitrary role in enabling or not promiscuous phenotypes. In our case, D-malate metabolism is made possible by leucine deprivation, which is a priori unrelated. We have previously shown that the promiscuous activity of DmlA on 3-IPM could complement a *leuB* knockout phenotype in *E. coli* [17] in a very similar and symmetric way as the one we report here. Indeed, in this case, leucine prototrophy is only observed when D-malate is the carbon source, that is, when *dmlA* expression is induced. Both DmlA and IPMDH possess functions that overlap each other and the physiological relevance of IPMDH promiscuity on

D-malate is the reciprocal property of the DmlA promiscuity on 3-isopropylmalate. Thus, this study exemplifies the retention of physiological significant reciprocal promiscuous activity between two members of β-decarboxylating dehydrogenase superfamily. From a more general perspective, we infer that such conditional promiscuous phenotypes may not be exceptional and that evolutionary trajectories may start with fortuitous environmental conditions that enable a promiscuous phenotype. Subsequently, a mutagenic event could then occur so that the robustness of the emerging phenotype enhances, for example by disrupting the transcriptional regulation responsible for the conditional function and/or by duplicating the gene.

Interestingly, in directed evolution experiments, selection for promiscuous activity does not necessarily result in dramatic loss of native activity of the enzymes. Most of the mutations imparting selection advantages or improving promiscuity are shown to alter the specificity of the substrates rather than changing active site residues [32]. It was proposed that such mutational robustness of native activity is linked with the weak trade-offs between new and native activity [33–35]. For example, substantial improved promiscuous activity of serum paraoxonase (PON1), bacterial phosphotriesterase (PTE), and carbonic anhydrase II (CAII) in directed evolution experiments was reported to have small impact (if any) on the native functions of the enzymes [35]. However, molecular evolution experiments with stringent selection and absence of purifying selection against the ancestral activity may artificially favor multifunctional mutants [36]. Given these two contrasting ideas regarding native activity robustness, however, they do not rule out the possible coexistence of high-level promiscuity together with specialist behavior in an enzyme. Supporting this, dual functionality was achieved by only a single mutation in glutamylphosphate reductase (in proline biosynthesis) that improved the promiscuous N-acetylglutamylphosphate reductase activity (in arginine biosynthesis) and retained low native activity [37]. The loss of native activity was compensated with overexpression required for the survival of the organism in the absence of proline and arginine. Interestingly, the strong reduction of native activity may have been advantageous for lowering the competition by the native substrate. The coexistence of dual function is also reported in other enzyme families. For example, a conserved substrate recognition motif enabled the coexistence of high endoglucanase and mannanase activity among glycoside hydrolase family 5 (GH5) enzymes [38]. In our case, the bifunctionality of IPMDH is endogenous but the dual phenotype is conditional. IPMDH promiscuity is most likely inherited from the common ancestor of β-decarboxylating dehydrogenase superfamily. Phylogenetic analysis showed that DmlA/TDH belongs to a monophyletic cluster and diverge from the same point as the NAD-dependent IDHs and IPMDHs [17]. Natural selection of IPMDH for 3-IPM activity probably reached at a level sufficient to meet the native physiological needs. With an isopropyl group at the C3 position, 3-IPM is bulkier than D-malate (Fig. S1). Smaller substrates compared to native ones cannot be easily discriminated in enzymes, and, without proof editing, it is impossible for IPMDH not to accommodate D-malate in its active site. Hence, a residual 3% activity on D-malate with reduced affinity is not surprising at all. The specificity constant ($k_{cat}/K_m$) for D-malate is 1000-fold lower compared to 3-IPM. This is comparable to discrimination against a substrate smaller by a methyl in some highly selective enzymes. For example, isoleucyl-tRNA synthetase discriminates against Val by ~ 200-fold [39].

Competition by the native substrate may reduce the evolutionary advantage of promiscuous activity [36,40]. In our experiments, under leucine starvation, 3-IPM is present and therefore competes with D-malate (Fig. 1. and 3B.). Although the intracellular concentration of 3-IPM is unknown, competition is not sufficient to prevent growth on D-malate. In the environment, if D-malate is the carbon source, then substrate competition will potentially slow down the leucine biosynthesis, so there could be a selection pressure on IPMDH against D-malate activity; however, decreasing this activity by mutations is probably not straightforward and the pressure on IPMDH will be applied only if IPMDH activity on 3-IPM is rate limiting in leucine biosynthesis and if D-malate is present at high concentration. And even under these circumstances, one can easily imagine that the selection of a regulatory mechanism increasing IPMDH expression in the presence of D-malate will be easier than a reduction of its intrinsic promiscuous activity.

## Experimental procedures

### Escherichia coli strains

Keio wild-type (BW25113) and Keio Δ*dmlA* (BW25113Δ*dmlA*::*kan*(*Km*$^r$)) strains were provided by Jean-François Collet, Institut de Duve, UCLouvain, Belgium. A triple gene deletion strain of *E. coli*; B3 (HB101Δ*idh*::*kan*(*Km*$^r$) Δ*ipmdh* Δ*dmlA*) was constructed from HB101 using P1 phage transduction [41] (Fig. S3). Three paralogous genes, *leuB*-encoding

isopropylmalate dehydrogenase (IPMDH), *icd*-encoding isocitrate dehydrogenase (IDH), and *dmlA*-encoding D-malate dehydrogenase (DmlA), were successively deleted and replaced by Keio construct possessing kanamycin-resistant cassette from donor strains of Keio collection [30]. The Keio construct was integrated in the targeted gene locus of acceptor strain by homologous recombination using the RecA recombinase encoded from pFL352 vector (donated by Pr. Bernard Hallet, Louvain Institute of Biomolecular Science and Technology, UCL, Belgium), whereas the flap (FLP) recombinase (pCP20) [42] was used to remove the kanamycin cassette in transduction process. The final engineered B3 (HB101 Δ*idh*::*kan*(*Km*$^r$) Δ*ipmdh* Δ*dmlA*) strain was cured from the two vectors. The deletion of the three genes was confirmed by PCR (Fig. S3) using loci-specific primers and sequencing. Oligonucleotide sequences are provided in Supporting Information. The triple gene deletion was also phenotypically characterized (Fig. S4).

## DNA techniques

A synthetic *leuB* gene (GenScript, USA) of HB101 was fused with a sequence encoding a C terminus GSSG linker followed by a 6-histidine tag and cloned in a pACYC-derived plasmid [42] under the control of isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible lacI$^q$/Ptac expression system. The dmlA was amplified from HB101 genome by PCR (Phusion, New England Biolabs) and cloned between the *NdeI* and *XhoI* sites of the same type of expression vector with a C terminus fusion of GSSG linker and 6xHistidine tag. The constructs were verified by sequencing. Primary sequences of recombinant DmlA and IPMDH are provided in Supporting Information.

## Phenotypic test and culture media

For the chromosomal expression test, Keio wild-type and KeioΔ*dmlA* growth analysis were performed on M9 minimal media [43]. The M9 media was supplemented with D-glucose/D-malate (0.1% or 0.15%), $MgSO_4$ (5 mM), $CaCl_2$ (0.1 mM), thiamine (0.01%), amino acid mixture (0.25 mM each except glutamate, glutamine, and leucine), glutamate/glutamine (2 mM each) and without or with leucine (1 mM). Keio wild-type and KeioΔ*dmlA* overnight cultures at 37 °C in LB and LB with kanamycin (25 μg/mL), respectively, were pelleted and washed twice in M9 buffer to remove residual LB and used to inoculate the M9 minimal media (100ml). The culture was incubated at 37 °C with 180 rpm of agitation and growth was followed by optical density at 600 nm ($OD_{600}$) at 2 h intervals. Solid M9 minimal plates were prepared by adding 2.6% of agar (Difco™ Bacto Agar, BD). From the same starter culture, 10-fold serial dilutions prepared in M9 buffer to spot 10 μL from each dilution on solid M9 plates and incubated at 37 °C for 18 h. To perform expression assays, B3 strain transformed with the *dmlA* (pDmlA) and *ipmdh* (pIPMDH) expression vectors were cultured (5 mL) in LB with tetracycline (5 μg·mL$^{-1}$) at 37 °C and at 180 rpm of agitation. The bacteria were pelleted by centrifugation at 3000 ***g*** for 5 min and washed twice in 5 mL of M9 buffer to remove any residual LB. EZ-rich MOPS (3-morpholinopropane-1-sulfonic acid) based defined media [44] (100 mL) was inoculated and incubated at 37 °C and 180 rpm of agitation. The media was supplemented with D-malate (0.15% w/v), amino acid mixture (0.25 mM each except glutamate/glutamine and leucine), glutamate/glutamine (2 mM) and without or with leucine (1 mM). Growth of the bacteria in each case was followed by optical density at 600 nm ($OD_{600}$) at 2-hour intervals for both basal level of expression (without IPTG induction) and for overexpression mode induced by IPTG (1 mM).

## Activity assay in extracts

Cultures (50 mL) of Keio wild-type and Keio Δ*dmlA* strain at 37 °C in M9 media supplemented with D-glucose (0.1%) and with leucine (1 mM) as appropriate were harvested by centrifugation at 3000 ***g*** for 10 min when $OD_{600}$ was between 0.4 and 0.5. Pellets were suspended in 5 mL of MOPS-KCl buffer (25 mM MOPS, 100 mM KCl, pH 7.5) supplemented with lysozyme (1 mg·mL$^{-1}$), DNase I (50 μg·mL$^{-1}$), and protease inhibitors (1 complete mini EDTA-free tablet for 10 mL buffer, Sigma-Aldrich, St, Louis, MO, USA) and incubated on ice for 1 h. The cells were lysed on ice by sonicating the pellets for 5 min. The lysates were obtained upon centrifugation at 15 000 ***g*** for 20 min to remove cell debris. The lysates were cleared by filtration (0.45 μm) before being used for activity assay. For testing the leakiness of $P_{tac}$ promoter, 10 mL LB cultures of B3 strain transformed with pDmlA or pIPMDH vector and nontransformed strain were used (Fig. S5). The cultures were supplemented with or without IPTG (1 mM) and incubated at 37 °C for 4 h to reach absorbance ($OD_{600}$) of 0.4. The lysates were prepared as above. All activity measurements were performed in triplicates with MOPS-KCl buffer (pH 7.5) at 25 °C using 96-well plates (Black wall flat transparent bottom) in Tecan plate reader (Tecan Infinite 200 pro). The 125 μL assay reaction mixture was

prepared with fixed concentration of 3-isopropylmalate (IPMDH activity) or D-malate (DmlA activity) (500 μm), $NAD^+$ (500 μm), $MgCl_2$ (5 mM). The reaction started by adding 25 μL of cleared lysate and the production of NADH was monitored by fluorescence (excitation at 340 nm and emission at 460 nm) over time. Activity (dFU/dt, change in fluorescence per time unit) was normalized by the total protein content [45] (Bio-Rad Protein Assay, Hercules, CA, USA). For IDH activity measurement of B3 and wild-type strain, HB101, LB cultures ($OD_{600}$ of 0.5) were harvested and lysates were prepared using glass beads (Fast prep protocol) (Fig. S5). In brief, sterile 200-μL glass beads suspension in MOPS-KCl butter was mixed with 800 μL of culture and vigorously agitated for 30 min and then the lysates were clarified by centrifuging at 15 000 ***g*** for 15 min. Finally, a 25 μL lysate was used in the kinetic reaction mixture (125 μL) containing fixed concentration of isocitrate (500 μm), $NAD^+$ (500 μm), $MgCl_2$ (5 mM). Production of NADH was followed at 340 nm.

### Purification and characterization of IPMDH

IPMDH was produced in B3 strain transformed with pIPMDH. Terrific broth (500 mL) [46] was inoculated from a starter culture in LB with tetracycline (5 $\mu g \cdot mL^{-1}$). Terrific broth culture was supplemented with D-glucose (1 mM) and tetracycline (5 $\mu g \cdot mL^{-1}$) and culture incubated at 37 °C at 180 rpm. The culture was harvested when $OD_{600}$ reached to 0.4, the cells were centrifuged (4000 ***g***, 10 min) and suspended in fresh terrific broth without glucose supplement and with IPTG (1 mM) for induction. The *ipmdh* gene encoding the protein was overexpressed for 4 h at 25 °C and at 180 rpm. Bacteria were harvested by centrifuging at 4000 rpm for 10 min and lysate was prepared according to the protocol stated in activity assay section. Lysate was dialyzed (10 kDa cutoff membrane) against 2 L wash buffer (50 mM Tris pH 7.5, 300 mM NaCl, 20 mM imidazole) at 4 °C for 1 h. One column volume (2 mL) Ni-NTA resin (Novex, Carlsbad, CA, USA) equilibrated with wash buffer was mixed with 5 mL of lysate and incubated at 4 °C with gentle shaking for 1 h. The column was washed six times with 1 column volume of wash buffer and enzyme was eluted with elution buffer (50 mM Tris pH 7.5, 300 mM NaCl, 250 mM Imidazole). The enzyme was desalted (10 mM Tris, pH8) and was concentrated using Amicon (10 kDa cutoff) concentrator (Millipore, Burlington, MA, USA). Protein concentration was measured by following absorbance at 280 nm in Nanodrop and using the predicted extinction coefficient ($\varepsilon_{280}$ = 25820 $M^{-1} \cdot cm^{-1}$) of the protein in CLC Main Workbench 7 tool. Kinetic assay was performed in MOPS-KCl buffer (25 mM MOPS, pH 7.5, 100 mM KCl) with fixed concentration of $NAD^+$ (500 μm), $MgCl_2$ (5 mM) and varying concentrations of 3-isopropylmalate (0 to 200 μm) or D-malate (0 to 1.2 mM) in 96-well flat-bottom black plates. Fluorescence was monitored over time (excitation at 340 nm and emission at 460 nm). Rates of NADH production ($V_0$) were deduced according to a standard NADH fluorescence curve. Apparent kinetic constant ($k_{obs}$) was calculated according to equation; $k_{obs} = V_0/[E]_0$, where $V_0$ is initial velocity of the reaction and $[E]_0$ is the IPMDH concentration. From the observed kinetic parameter ($k_{obs}$) and known 3-isopropylmalate concentrations, nonlinear theoretical Michaelis–Menten curve fitting was performed according to the equation, $k_{obs} = (k_{cat}*[S])/(K_m+[S])$. In case of D-malate, $k_{obs}$ was plotted against substrate concentrations to have $k_{cat}/K_m$ estimates.

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## Author contributions

MSK and PS designed the experiments. SG performed the kinetic analysis of IPMDH on D-malate with purified enzyme. MSK performed all the other experiments. MSK, SG and PS wrote the manuscript.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** The members of the β-decarboxylating dehydrogenase family catalyze several reactions on D-malate based substrates

**Fig. S2.** Keio wild type (BW25113) and Keio *dmlA* knockout (BW25113*ΔdmlA::kan* (*Kmr*) strains growth curve on D-glucose.

**Fig. S3.** Genotype validation of engineered strain, B3 (HB101*Δidh::kan(Kmr)ΔipmdhΔdmlA*).

**Fig. S4.** Auxotrophic and D-malate assimilation test of B3 (HB101*Δidh::kan(Kmr)*Δ*ipmdhΔdmlA*).

**Fig. S5.** B3 lysate activity assay with or without IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction grown on LB.

**Fig. S6.** Isocitrate dehydrogenase activity measurement in *E. coli* lysates.

**Fig. S7.** D-malate dehydrogenase activity complementation in DmlA negative strains.

**Fig. S8.** SDS-PAGE analysis of the purified IPMDH.