

Discovery and remodeling of *Vibrio natriegens* as a microbial platform for efficient formic acid biorefinery

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Formic acid (FA) has emerged as a promising one-carbon feedstock for biorefinery. However, developing efficient microbial hosts for economically competitive FA utilization remains a grand challenge. Here, we discover that the bacterium *Vibrio natriegens* has exceptional FA tolerance and metabolic capacity natively. This bacterium is remodeled by rewiring the serine cycle and the TCA cycle, resulting in a non-native closed loop (S-TCA) which as a powerful metabolic sink, in combination with laboratory evolution, enables rapid emergence of synthetic strains with significantly improved FA-utilizing ability. Further introduction of a foreign indigoidine-forming pathway into the synthetic *V. natriegens* strain leads to the production of 29.0 g · L⁻¹ indigoidine and consumption of 165.3 g · L⁻¹ formate within 72 h, achieving a formate consumption rate of 2.3 g · L⁻¹ · h⁻¹. This work provides an important microbial chassis as well as design rules to develop industrially viable microorganisms for FA biorefinery.

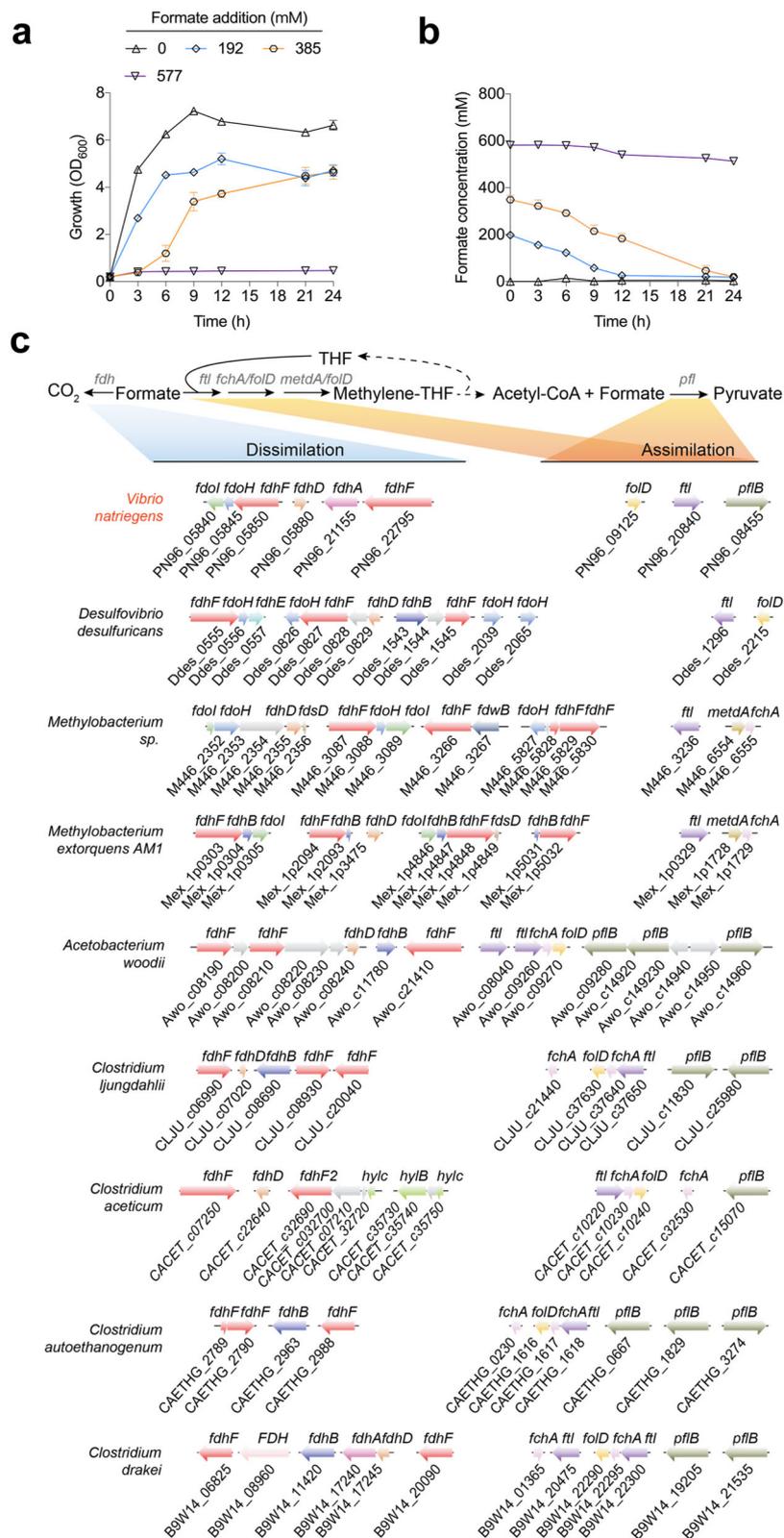
Microbial assimilation and conversion of one-carbon (C1) compounds to produce value-added products have attracted significant attention because it represents an important biological manufacturing route using abundant and highly available carbon sources¹. Formic acid (FA) is a promising C1 feedstock since it can be efficiently derived from CO₂ or syngas (mainly CO and CO₂) with high selectivity via chemical conversions and further utilized by microorganisms². Additionally, the storage of FA as a liquid is more convenient compared to gases³. Thus, FA is a CO₂-equivalent carbon source that is more easily handled than CO₂.

Native FA-utilizing microorganisms mainly employ the Wood-Ljungdahl pathway (also known as the reduced acetyl-CoA pathway), serine cycle, and reductive glycine pathway for FA assimilation⁴. These pathways all depend on a core FA-fixing module called the tetrahydrofolate (THF) cycle which is initiated by formate-tetrahydrofolate ligase (FTL)⁵. Pyruvate formate-lyase (PFL)-catalyzed pyruvate

formation from formate and acetyl-CoA is another important FA-assimilating approach in organisms⁴. However, native FA-utilizing microorganisms generally grow slowly and cannot efficiently utilize this C1 carbon source⁵⁻¹⁷. Hence, efforts have been made to develop synthetic microorganisms such as *Escherichia coli* through rational genetic modifications and adaptive laboratory evolution, wherein FA assimilation was achieved by reconstructing the THF cycle and reverse glycine cleavage¹⁸⁻²⁰. Despite the improved performance of synthetic microorganisms in FA utilization, the efficiency is still much lower than those of traditional carbon sources such as sugars, which is largely due to the native weak FA tolerance and metabolic capacity of these microbial hosts. Thus, the discovery and employment of more suitable microorganisms as a chassis to establish efficient FA bioconversion platforms are needed.

Vibrio natriegens is a rapidly growing bacterium with the shortest doubling time (<10 min) of all known bacteria^{21,22}. It is also regarded as

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a desirable microbial host for bio-manufacturing^{23–25}. *Vibrio natriegens* can grow on diverse substrates including galactose, arabinose, and glycerol^{26,27}, due to its versatile metabolism. Furthermore, genetically modified *V. natriegens* strains are capable of producing multiple chemicals including amino acids, polymers, and polyols^{27–29}. However, its inherent physiological and metabolic characteristics and potential in utilizing C1 resources remain unclear.

In this work, we discover the previously unreported superior tolerance and metabolic capacity of *Vibrio natriegens* to FA and develop this bacterium into a platform microorganism for FA utilization and conversion. Using a combination of bioinformatic, omics, and experimental validation approaches, multiple potentially crucial genes and metabolic pathways associated with FA metabolism in *Vibrio natriegens* are revealed. Based on these findings, we design and remodel this

Fig. 1 | Comparative analysis of formate metabolic pathways in *V. natriegens*. **a, b** Cell growth (a) and formate consumption (b) profiles with the supplementation of different amounts of sodium formate (HCOONa·2H₂O). Data are presented as the mean ± SD (*n* = 3 biologically independent samples). Error bars show SDs. **c** Putative genes and gene clusters involved in formate assimilation and dissimilation pathways in *V. natriegens* and some other representative formate-utilizing microorganisms. Homologous genes are marked with the same colors. *fdhI* formate dehydrogenase subunit gamma, *fdhB* formate dehydrogenase (NADP⁺) beta subunit, *fdhH* formate dehydrogenase iron-sulfur subunit, *fdhF* formate

dehydrogenase major subunit, *fdsD* formate dehydrogenase subunit delta, *fdhA* formate dehydrogenase (coenzyme F420) alpha subunit, *fdwB* formate dehydrogenase beta subunit, *FdhD* formate dehydrogenase accessory protein, *ftl* formate-tetrahydrofolate ligase, *fchA* methylenetetrahydrofolate cyclohydrolase, *metDA* Methylenetetrahydrofolate dehydrogenase (NADP⁺), *folD* bifunctional methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase, *pflB* pyruvate formate-lyase, *hylB* formate dehydrogenase (NAD⁺, ferredoxin) subunit B, *hylC* formate dehydrogenase (NAD⁺, ferredoxin) subunit C. Source data are provided as a Source Data file.

bacterium, combined with laboratory evolution, achieving efficient FA utilization and indigoidine production.

Results

Superior tolerance and metabolic capacity of native *Vibrio natriegens* to formate

V. natriegens has rapid growth, but its tolerance and metabolic capacity to formate are unknown; thus, we first examined its growth and formate consumption in the presence of different formate concentrations. We observed that *V. natriegens* could grow in the LBv2 medium supplemented with 20 and 40 g·L⁻¹ sodium formate (HCOONa·2H₂O) (equivalent to 192 and 385 mM formate, respectively) (Fig. 1a), and exhausted all the formate within 24 h (Fig. 1b), although the growth rates and highest biomass gradually decreased with increasing formate concentration (Fig. 1a). At a higher sodium formate concentration (60 g·L⁻¹, equivalent to 577 mM), the strain could hardly grow (Fig. 1a), but it still consumed 69 mM formate within 24 h (Fig. 1b). However, when *V. natriegens* was cultivated in the M9 minimal medium (containing 4 g·L⁻¹ glucose), the addition of 288 mM formate (30 g·L⁻¹ HCOONa·2H₂O) could completely inhibit cell growth (Supplementary Fig. 1a), showing poorer formate tolerance relative to that of the LBv2 medium. At a lower formate concentration (192 mM, equivalent to 20 g·L⁻¹ HCOONa·2H₂O) in this minimal medium, *V. natriegens* could grow and consume 95 mM formate within 24 h (3.96 mM·h⁻¹) (Supplementary Fig. 1b, c). Taken together, compared with the reported data from other microorganisms, *V. natriegens* exhibited a superior ability in the consumption of formate (Supplementary Table 1), showcasing great potential in biological FA utilization.

Genes and metabolic pathways for formate metabolism in *V. natriegens*

Natural FA assimilation reactions in organisms are quite scarce, in which two of the best known are FTL-catalyzed formylation of

tetrahydrofolate (THF) and (PFL)-catalyzed pyruvate formation (Supplementary Fig. 2). Additionally, FA can be oxidized to form CO₂ by formate dehydrogenase (FDH), a major FA dissimilation reaction in microorganisms. The *V. natriegens* genome contains six *fdh* genes (two *fdhFs*, one *fdhA*, one *fdhI*, one *fdhH*, and one *fdhD*), two THF cycle genes (*ftl* and *folD*) and one *pfl* gene, enabling the operation of both FA dissimilation and assimilation reactions mentioned above (Fig. 1c); however, the other two THF cycle genes, *fchA* and *metDA*, were missing (Table 1). Furthermore, all the candidate genes responsible for the reactions starting from methylene-THF in the reductive glycine pathway and serine cycle were found in *V. natriegens* (Supplementary Fig. 3a), suggesting that the assimilated formate can be further converted into diverse metabolites. Additionally, energy conservation systems play important roles in microbial growth on organic C1 resources such as formate and methanol²⁰. Of note, diverse energy conservation systems were found in *V. natriegens*, most of which had multiple coding genes and gene clusters in the genome (Supplementary Fig. 3b). This may be crucial for the required energy supply in the growth of *V. natriegens* on formate.

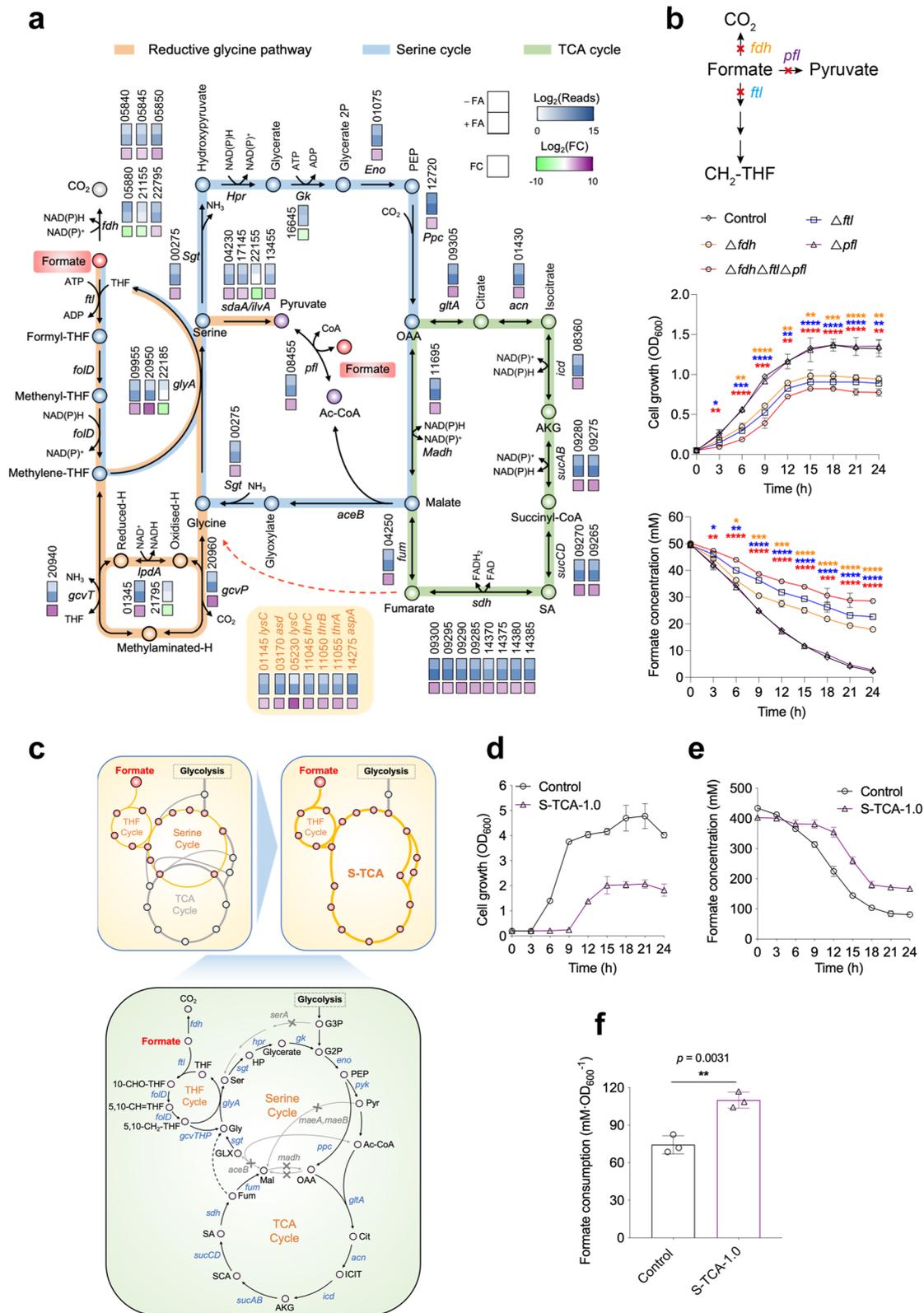
To explore the potential genes and metabolic pathways associated with formate metabolism in *V. natriegens*, we measure the transcriptomic changes with the induction of formate. There were 755 and 606 genes significantly up-regulated and down-regulated, respectively, following formate addition to the culture (Supplementary Fig. 4a). Functional category analysis yielded 28 subsets containing many genes exhibiting significantly altered transcriptional levels (Supplementary Fig. 4b). An obvious positive correlation was detected between qRT-PCR and RNA-seq results for 15 selected genes (Supplementary Fig. 5), suggesting a good quality of the comparative transcriptomic data. We observed that, with formate stress, multiple crucial genes located in the THF cycle, the reductive glycine pathway, and the serine cycle were significantly up-regulated (Fig. 2a). Four (PN96_22795, PN96_05840, PN96_05845, and PN96_05850) out of six FDH-encoding genes were up-regulated with the highest fold change (9.7-fold) occurring for PN96_05850 (Fig. 2a). Furthermore, an 8.9-fold transcriptional increase in the *pfl* gene which encodes pyruvate formate-lyase occurred (Fig. 2a). These findings indicate that the formate assimilation and dissimilation reactions mediated by these genes may contribute to the formate metabolism in *V. natriegens*. Noticeably, the transcriptional levels of nearly all the genes located in the TCA cycle were significantly up-regulated (Fig. 2a). The TCA cycle is the metabolic hub in cells connecting major nutrients, including sugars, lipids, and amino acids^{30,31}. The overall up-regulation of TCA genes indicates that *V. natriegens* enhanced its fundamental metabolism to meet the energy demand in formate assimilation. In addition, multiple genes encoding ionic transporters or associated with unsaturated fatty acid synthesis and DNA repair were significantly upregulated (Supplementary Fig. 6). It is known that the acid tolerance of microorganisms can be affected by the efficiency of ionic transporters and the ratio of unsaturated fatty acids in cell membrane³²⁻³⁴; hence, higher expression of these genes may be crucial for the formate tolerance of *V. natriegens*.

Based on the above analyses, *V. natriegens* is predicted to metabolize formate through FTL and PFL-catalyzed assimilation reactions as well as an FDH-catalyzed dissimilation reaction (Fig. 2b, upper). To

Table 1 | Occurrence of genes involved in formate metabolism in *V. natriegens* and some other representative formate-utilizing microorganisms

Microorganisms	Dissimilation	Assimilation				
	<i>fdh</i>	<i>ftl</i>	<i>fchA</i>	<i>metDA</i>	<i>folD</i>	<i>pfl</i>
<i>Vibrio natriegens</i>	+	+			+	+
<i>Desulfovibrio desulfuricans</i>	+	+			+	
<i>Methylobacterium</i> sp.	+	+	+	+		
<i>Methylobacterium extorquens</i> AM1	+	+	+	+		
<i>Acetobacterium woodii</i>	+	+	+		+	+
<i>Clostridium ljungdahlii</i>	+	+	+		+	+
<i>Clostridium aceticum</i>	+	+	+		+	+
<i>Clostridium autoethanogenum</i>	+	+	+		+	+
<i>Clostridium drakei</i>	+	+	+		+	+

The presence of genes for the corresponding functions is indicated with a "+".



test them, the candidate genes (i.e., *fdh*, *ftl* and *pfl*) responsible for these reactions were separately or simultaneously deleted in *V. natriegens* for phenotypic analysis (Fig. 2b, upper). We observed that the deletion of the *fdh* genes or the *ftl* gene significantly impaired cell growth and formate consumption, indicating that both the formate dissimilation (oxidation) and assimilation pathways mediated by *fdh* and *ftl*, respectively, play roles in the formate metabolism of *V.*

natriegens (Fig. 2b, middle and bottom). However, the deletion of the *pfl* gene had no influence on cell growth and formate consumption (Fig. 2b, middle and bottom), although this gene was up-regulated in the presence of formate (Fig. 2a). This finding indicates the low catalytic activity of PFL towards formate in *V. natriegens*. Furthermore, the simultaneous deletion of all the *fdh*, *ftl*, and *pfl* genes led to less formate consumption compared to the separate deletion of the *fdh* or *ftl*

Fig. 2 | Validation of formate assimilation and dissimilation pathways and reconstitution of formate metabolism in *V. natriegens*. **a** The significantly up-regulated or down-regulated genes involved in the tetrahydrofolate (THF) cycle, TCA cycle, serine cycle, pyruvate formate-lyase (PFL) pathway and formate dehydrogenase (FDH)-mediated formate dissimilation pathways with the supplementation of sodium formate in *V. natriegens*. The bottom box indicates fold change as gene expression with FA over gene expression without FA. **b** Influence of blocking the FTL-mediated THF cycle, pyruvate formate-lyase (PFL) pathway and formate dehydrogenase (FDH)-mediated formate dissimilation pathway (upper) on cell growth (middle) and formate metabolism (bottom) of *V. natriegens*. The deleted *ftl* and *pfl* gene were PN96_20840 and PN96_08455, respectively. The *fdh*-deleted mutant was constructed by simultaneous deletion of the six annotated *fdh* genes (PN96_05840, PN96_05845, PN96_05850, PN96_05880, PN96_21155, and PN96_22795). Sodium formate ($\text{HCOONa}\cdot 2\text{H}_2\text{O}$) and glucose supplemented in the M9 medium was $5.0\text{ g}\cdot\text{L}^{-1}$ and $4.0\text{ g}\cdot\text{L}^{-1}$, respectively. Data are presented as the mean \pm SD ($n = 3$ biologically independent samples). **c** The combined manipulations fused the serine cycle and TCA cycle to create a metabolic sink to promote formate metabolic flux. **d, e** The growth (**d**) and formate consumption (**e**) of the wild type strain and the reconstructed S-TCA-1.0 strain in the LBv2 medium supplemented with $40\text{ g}\cdot\text{L}^{-1}$ sodium formate. **f** Formate consumption per unit biomass of the wild type and S-TCA-1.0 strains in the LBv2 medium supplemented with $40\text{ g}\cdot\text{L}^{-1}$ sodium formate. Data are presented as the mean \pm SD ($n = 3$ biologically

independent samples). *fdh* formate dehydrogenase, *ftl* formate-tetrahydrofolate ligase, *folD* bifunctional methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase, *glyA* glycine hydroxymethyltransferase, *sgt* serine-glyoxylate transaminase, *sdaA* serine dehydratase, *ilvA* threonine dehydratase, *Hpr* glycerate dehydrogenase, *Gk* glycerate 2-kinase, *eno* enolase, *ppc* phosphoenolpyruvate carboxylase, *gltA* citrate synthase, *acn* aconitate hydratase 2, *icd* isocitrate dehydrogenase, *sucA* 2-oxoglutarate dehydrogenase E1 component, *sucB* 2-oxoglutarate dehydrogenase E2 component, *sucC* succinyl-CoA synthetase beta subunit, *sucD* succinyl-CoA synthetase alpha subunit, *pfl* pyruvate formate-lyase, *sdh* succinate dehydrogenase, *fum* fumarate hydratase, *madh* malate dehydrogenase, *aceB* malate synthase, *maeA* malate dehydrogenase (oxaloacetate-decarboxylating), *maeB* malate dehydrogenase (oxaloacetate-decarboxylating) (NADP⁺), *gcvT* glycine cleavage system T protein, *gcvH* glycine cleavage system H protein, *gcvP* glycine cleavage system P protein, *lpdA* dihydrolipoyl dehydrogenase, *pyk* pyruvate kinase, THF tetrahydrofolate, HP hydroxypyruvate, PEP phosphoenolpyruvate, OAA oxaloacetate, AKG alpha-ketoglutaric acid, Cit citrate, ICIT isocitrate, SA succinic acid, SCA Succinyl-CoA, Pyr pyruvate, G2P 2-Phospho-D-glycerate, G3P 3-Phospho-D-glycerate, Mal malate, Fum fumarate, GLX Glyoxylate, Gly glycine, Ser serine. Error bars show SDs. Statistical analysis was performed by a two-tailed Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ versus the control strain. Source data are provided as a Source Data file.

genes (Fig. 2b, bottom); but interestingly, the mutated strain (with the deletion of all the *fdh*, *ftl*, and *pfl* genes) still maintained partial consumption ability of formate (Fig. 2b, bottom), indicating that these pathways were not completely blocked or there are other unknown approaches contributing to the formate metabolism in *V. natriegens*.

Designing and creating a metabolic sink to promote formate metabolic flux

The above findings prompted us to reprogram *V. natriegens* to further enhance its formate utilization. To this end, the following basic rationale was adopted: construction of a powerful metabolic sink that is linked to formate assimilation by integrating the TCA cycle and the serine cycle, aiming at increasing the flux of the serine cycle and its upstream THF cycle by the strong pull from the TCA cycle (Fig. 2c, upper). This was achieved by manipulating the following pathways and genes (Fig. 2c, bottom): (i) disruption of three *madh* genes (PN96_06470, PN96_19465, and PN96_11695), two *maeAB* genes (PN96_07295 and PN96_14755), and the *aceB* gene (PN96_10585) to fuse the serine cycle and the TCA cycle, thus forcing the metabolic flux of the serine cycle to go through the TCA cycle; (ii) disruption of the *serA* gene (PN96_00930) to enable the generation of more serine from formate assimilation rather than glycolysis.

The resulting strain S-TCA-1.0 was cultivated with the supplementation of $40\text{ g}\cdot\text{L}^{-1}$ sodium formate ($\text{HCOONa}\cdot 2\text{H}_2\text{O}$) (equivalent to 385 mM formate). Compared with the wild type strain, S-TCA-1.0 exhibited impaired growth and less total formate consumption (Fig. 2d, e); but interestingly, its formate consumption per unit biomass was much higher than that of the wild type strain (Fig. 2f), indicating the utility of our metabolic engineering strategy in enhancing the formate metabolic flux of individual *V. natriegens* cells. To confirm that the S-TCA cycle truly contributed to the enhanced formate consumption, we examined the transcriptional differences of all the S-TCA genes between the S-TCA-1.0 and the wild-type strains. As expected, all these genes could be properly expressed in S-TCA-1.0, and moreover, most of them exhibited higher transcription levels in S-TCA-1.0 relative to the wild-type strain (Supplementary Fig. 7). We also assayed multiple metabolites (glycine, serine, hydroxypyruvate, glycerate, phosphoenolpyruvate, α -Ketoglutarate, succinic acid, and fumarate) throughout the S-TCA cycle in the S-TCA-1.0 strain. We found that all these eight compounds could be detected, in which five showed higher concentration in S-TCA-1.0 relative to the wild-type strain, while the other three had no significant differences between the two strains (Supplementary Fig. 8). Our analysis suggests that the S-TCA cycle

exerts its function in the cells of S-TCA-1.0, leading to enhanced formate metabolic flux. If the growth deficiency of S-TCA-1.0 can be remedied, this strain will exhibit advantages over the wild type strain in formate utilization.

Adaptive laboratory evolution of S-TCA-1.0 leads to a high-performance strain grown on formate

To efficiently overcome the growth limitation of S-TCA-1.0, this strain was subjected to adaptive laboratory evolution (ALE) with gradually increasing sodium formate concentrations to improve its growth under formate stress. The strain was initially cultivated in the LBv2 medium supplemented with approximately $20\text{ g}\cdot\text{L}^{-1}$ sodium formate ($\text{HCOONa}\cdot 2\text{H}_2\text{O}$) (equivalent to 192 mM formate) and subcultured once every 12 h (Fig. 3a). Several rounds of subculturing resulted in the biomass of grown cells reaching $\text{OD}_{600} \sim 1.0$. At this point, the formate concentration was increased and a new evolutionary period began. This approach gradually improved the tolerance of S-TCA-1.0 to formate as well as its growth. Finally, an evolved strain (named S-TCA-2.0) capable of growing in the presence $140\text{ g}\cdot\text{L}^{-1}$ sodium formate (equivalent to 1,346 mM formate) was obtained after serial subculturing (Fig. 3b). More importantly, S-TCA-2.0 exhibited exceedingly high formate utilization ability, capable of growing to $\text{OD}_{600} \sim 1.6$ with the consumption of $78.9\text{ g}\cdot\text{L}^{-1}$ sodium formate (equivalent to 759 mM formate) within 24 h following initial supplementation of $85\text{ g}\cdot\text{L}^{-1}$ sodium formate (equivalent to 817 mM formate) in the medium (Fig. 3c), reaching a formate consumption rate of $1.42\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ($31.6\text{ mM}\cdot\text{h}^{-1}$) which was several times higher than the highest level reported in microorganisms (Supplementary Table 1).

An independent ALE experiment was performed in parallel to improve the formate utilization of the wild-type strain of *V. natriegens*; however, the cellular adaptation of this strain under formate stress was much slower than that of S-TCA-1.0, and enhancement was difficult when the concentration of sodium formate reached $70\text{ g}\cdot\text{L}^{-1}$ (equivalent to 673 mM formate) (Supplementary Fig. 9). These results suggest that the reconstituted formate metabolic pathways in *V. natriegens* (S-TCA-1.0) facilitated the strain evolution to adapt to high formate stress, increasing its formate consumption accordingly.

To better elucidate the genetic basis for the performance of S-TCA-2.0 in using formate, we chose three isolates to sequence their genome. A total of nine mutated genes were found in all the three isolates of S-TCA-2.0 relative to the S-TCA-1.0 strain (Fig. 3d and Table 2). Among them, *rpoS*, *ZapC*, and *actP*, encoding a general stress responsible regulator³⁵, a division protein³⁶, and an acetate permease³⁷,

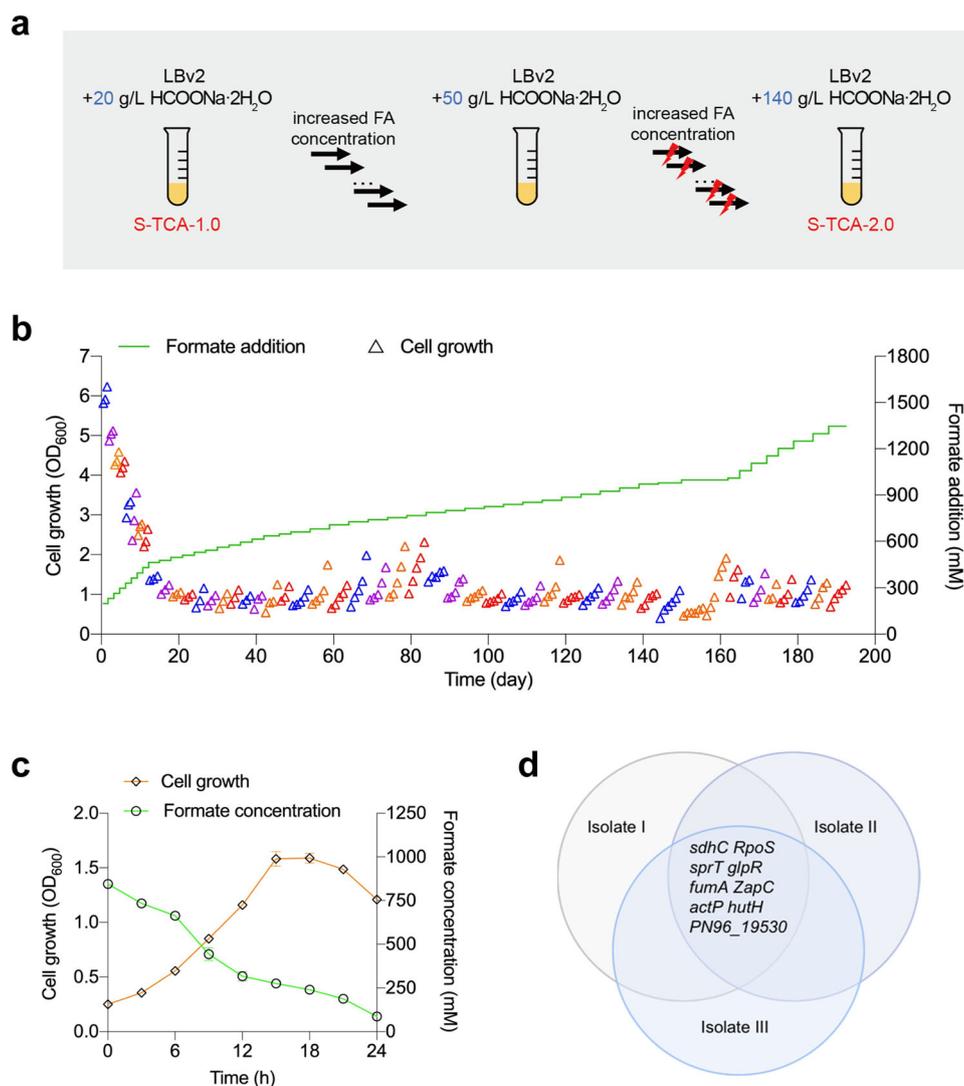


Fig. 3 | Adaptive laboratory evolution of the *V. natriegens* strains further enhances formate tolerance and metabolism. **a** Flowchart for the adaptive laboratory evolution of the *V. natriegens* S-TCA-1.0. S-TCA-2.0 was the final evolved strain with significantly enhanced formate metabolism ability in LBv2 medium. The lightning symbols represent UV mutagenesis, which was carried out every three passages, aiming to accelerate the strain mutation and evolutionary efficiency. **b** Evolution trajectory of S-TCA-1.0 to obtain the S-TCA-2.0 strain with gradually enhanced formate utilization. LBv2 medium was used with an increasing sodium

formate ($\text{HCOONa}\cdot 2\text{H}_2\text{O}$) concentration from 20 to 140 $\text{g}\cdot\text{L}^{-1}$ (192 to 1346 mM equivalent formate). Triangles indicate the bacterial biomass of each passage; the same colors represent passages with the same formate concentration. **c** Validation of the growth and formate consumption of S-TCA-2.0 within 24 h using the LBv2 medium initially supplemented with 85 $\text{g}\cdot\text{L}^{-1}$ sodium formate (817 mM equivalent formate). Data are presented as the mean \pm SD ($n = 3$ biologically independent samples). **d** The nine mutated genes occurred in all the three isolates of the S-TCA-2.0 strain. Source data are provided as a Source Data file.

respectively, are commonly observed to be mutated in ALE experiments³⁸. Thus, the mutations of these three genes are unlikely to be specifically linked to the phenotypes (high formate tolerance and consumption) of S-TCA-2.0. Of note, we found a mutation (A44E) in *fumA*, the gene encoding fumarate hydratase that converts fumarate toward malate (a main flux branch point of the S-TCA cycle). The mutation of this enzyme may reduce the diversion of metabolic flux from the S-TCA cycle. Another potentially key mutation is in *sdhC*, encoding a cytochrome subunit of succinate dehydrogenase (catalyzing the conversion of succinate to fumarate in the TCA cycle), whose inactivation may change the metabolic flux of TCA cycle. For the remaining four mutated genes, there is currently no clue linked to formate consumption.

Next, we sought to evaluate how much formate can be assimilated in S-TCA-2.0. For this we cultivated this strain in the M9 minimal medium supplemented with ^{13}C -labeled sodium formate and then collected cells for isotopomer analysis. Based on the specific

consumption rates of formate, glucose, and glycine, the specific formate assimilation rate was calculated to be 43.3 $\text{mg}\cdot\text{gDCW}^{-1}\cdot\text{h}^{-1}$, accounting for approximate 12.1% of the total consumed formate (Supplementary Table 2). Considering that some formate could be converted to the metabolites that are secreted outside cells, the exact amount of formate assimilated by this strain should be higher.

Engineering S-TCA-2.0 for efficient synthesis of indigoidine from formate

Considering the excellent formate utilization ability of S-TCA-2.0, we attempted to evaluate if it could be reprogrammed to efficiently convert formate into valuable compounds. Here, indigoidine was chosen as a target product because it is an important pigment widely used in food, medicine, and dyeing industries, and moreover, can be synthesized using α -ketoglutarate in the TCA cycle as the precursor.

The indigoidine-producing pathway consisting of the *Streptomyces lividans* Idgs and *Bacillus subtilis* Sfp, which catalyze the

Table 2 | The nine mutated genes occurred in all the three isolates of the S-TCA-2.0 strain

Name	Gene ID	Gene product	Mutation type
<i>RpoS</i>	PN96_01115	RNA polymerase sigma factor RpoS	SNP
<i>sprT</i>	PN96_00870	SprT family zinc-dependent metalloprotease	SNP
<i>ZapC</i>	PN96_05610	Cell division protein ZapC	SNP
<i>actP</i>	PN96_14245	Cation acetate symporter	SNP
<i>glpR</i>	PN96_01950	DeoR/GlpR family transcriptional regulator	SNP
<i>fumA</i>	PN96_04250	Fumarate hydratase	SNP
<i>hutH</i>	PN96_17630	Histidine ammonia-lyase	SNP
<i>sdhC</i>	PN96_09300	Succinate dehydrogenase cytochrome b556 subunit	Indel
<i>PN96_19530</i>	PN96_19530	ATP-binding protein	SNP

conversion of glutamine to indigoidine, was introduced into S-TCA-2.0 via an expression plasmid, yielding the strain S-TCA-2.0-IE (Fig. 4a). Next, the S-TCA-2.0-IE strain was cultivated in the LBv2 medium supplemented with 85 g·L⁻¹ sodium formate (HCOONa·2H₂O). As expected, the culture of S-TCA-2.0-IE appeared blue color, which was caused by indigoidine, after 24 h of fermentation, whereas no blue occurred for the control (the strain S-TCA-2.0 harboring a blank plasmid) (Fig. 4b), indicating the production of indigoidine by S-TCA-2.0-IE in the presence of high concentration of formate. To further validate the formate assimilation in S-TCA-2.0-IE, the amino acids formed from the artificial S-TCA cycle (glycine, serine, isoleucine, lysine, methionine, aspartic acid, glutamate, and phenylalanine) or pyruvate (alanine, valine, and leucine) were analyzed through the isotopic labeling experiment using ¹³C-labelled formate. The result showed that all the detected amino acids were labeled by ¹³C (mostly, *m*+1), in which serine and methionine contained higher proportions of ¹³C-labelled amino acids (45.7% and 39.4%, respectively), probably due to that these two amino acids relative to the other amino acids are more closely linked to formate assimilation (THF cycle) (Fig. 4c).

We next attempted to explore the real potential of S-TCA-2.0-IE in using formate for indigoidine production in a fed-batch mode. This strain was grown in the LBv2 medium containing approximately 60 g·L⁻¹ initial sodium formate (HCOONa·2H₂O) (equivalent to 577 mM formate), with additional sodium formate (60 g·L⁻¹) added into the medium every 12 h (repeated five times). We observed that S-TCA-2.0-IE continued to grow with the supplementation of formate, achieving the highest biomass (OD₆₀₀ ~ 3.1) at 60 h (Fig. 4d, left); in contrast, the biomass of S-TCA-2.0-IE stopped to increase after 18 h in the medium without formate addition, reaching to the highest biomass of OD₆₀₀ ~ 1.5 (Fig. 4d, left). Concurrently, S-TCA-2.0-IE consumed all the formate (approximately 165.3 g·L⁻¹ formate) added into the medium and produced 29.0 g·L⁻¹ of indigoidine within 72 h (Fig. 4d, right), achieving a specific formate consumption rate of 2.3 g·L⁻¹·h⁻¹. Such an indigoidine production level, to our knowledge, is higher than the reported highest indigoidine production through microbial synthesis although under different cultivation conditions (Supplementary Table 3). In contrast, the indigoidine produced by S-TCA-2.0-IE was only 4.7 g·L⁻¹ without the supplementation of formate (Fig. 4d, right). Altogether, these results confirmed the great potential of S-TCA-2.0-IE in indigoidine production using formate as the co-substrate.

Discussion

FA chemically converted from CO₂ is an attractive feedstock for biological production of various value-added chemicals. In this study, we have two important findings. First, the exceptional tolerance and

metabolic capacity of *V. natriegens* to formate represent a superior FA-utilizing platform strain. Second, a combination of rational metabolic remodeling that generated the non-native S-TCA loop and laboratory evolution represents a successful strategy with potential universality for enhancing microbial FA utilization.

A large number of pathways and genes were found to be active during the formate metabolism in *V. natriegens*. Therefore, it seems that this organism has metabolic flexibility and versatility in the presence of formate. Multiple formate dehydrogenase genes were significantly up-regulated under formate stress (Fig. 2a), indicating that these FDHs were activated to oxidize the available formate to CO₂ with the generation of reducing power. Although the key genes (*ftl* and *fold*) responsible for the THF cycle exhibited similar expression levels with and without formate (Fig. 2a), the majority of the genes associated with the downstream reductive glycine pathway, the serine cycle, and the TCA cycle were significantly up-regulated following formate addition (Fig. 2a), which would pull the flux of the THF cycle and promote formate conversion into various metabolites. Noticeably, *V. natriegens* still remained partial formate consumption ability even after the deletion of all the predicted *fdh*, *ftl*, and *pfl* genes (Fig. 2b, bottom), suggesting the existence of potential alternative pathways for formate assimilation. One possibility is the pathways starting with the reduction of formate to formaldehyde⁴: (i) formate can be ligated with CoA by an acetyl-CoA synthetase to form formyl-CoA followed by the reduction to formaldehyde by an acyl-CoA reductase. (ii) formate can be activated by a kinase to produce formyl phosphate followed by the reduction to formaldehyde by a formyl phosphate reductase. These two pathways have been constructed and tested in vivo recently^{39–41}; however, whether they occur in nature in microorganisms and contribute to formic assimilation remain unclear.

Formate assimilation has been previously used to relieve the serine-auxotrophy of *E. coli*^{2,42}, suggesting that formate assimilation fluxes could be directed toward serine synthesis. In our design, serine production from glycolysis was blocked by deleting the *serA* gene, which would enhance the THF cycle flux to form serine. Additionally, the artificial S-TCA cycle created by integrating the serine cycle and the TCA cycle enabled the high-flux TCA to pull the upstream carbon supply and consequently enhance formate assimilation and conversion. This metabolic design is crucial for the following rapid evolution of *V. natriegens* into the strains with exceptional formate metabolic capacity since the wild-type strain did not achieve the goal using the same adaptive laboratory evolution strategy. Although the growth of *V. natriegens* was impaired after multiple gene deletions for constructing the S-TCA module (Fig. 2d), the following adaptive laboratory evolution rapidly recovered cellular fitness. This finding indicates that *V. natriegens* has excellent plasticity in metabolic remodeling. Therefore, the rapid generation of the evolved strain S-TCA-2.0 with strikingly enhanced formate metabolism should be attributed to the combination of metabolic engineering and adaptive laboratory evolution. This metabolic design may also work in other microbial chassis to create diverse cell factories for formate utilization. However, even though the S-TCA-2.0 strain exhibited efficient formate consumption, potential bottlenecks may still exist in the artificial S-TCA cycle. For example, we found that the overexpression of some genes in the glycine cleavage system (GCS) and the serine cycle could affect the formate consumption of S-TCA-2.0 (Supplementary Fig. 10), indicating ample room for further improvement of this metabolic pathway.

It should be noted that formate assimilation to acetyl-CoA via the THF cycle in microorganisms is an energy consuming process. The energy required for formate assimilation should depend on other approaches, such as the metabolism of other organic carbon sources or FDH-catalysed formate oxidation. Moreover, all the reported native and artificial bacteria showed poor growth using formate as the sole organic carbon source^{5–7,9,10,19,20}. Therefore, the LBv2 medium which contains yeast extract and peptone was used here to evaluate the real

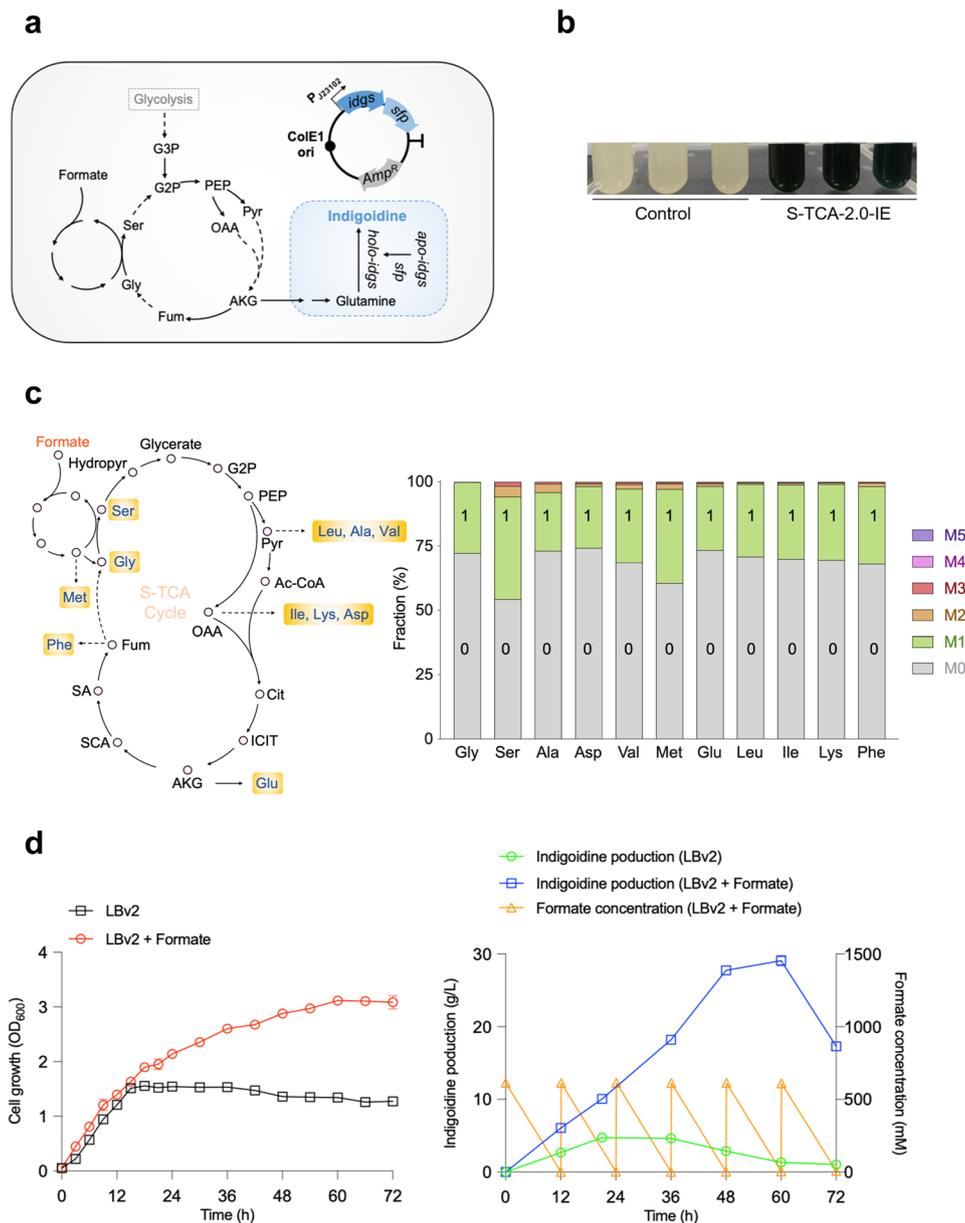


Fig. 4 | Engineering the evolved *V. natriegens* strain for efficient production of indigoidine from formate. **a** The synthetic pathway and plasmid for indigoidine production. Idgs: indigoidine synthetase. Sfp: phosphoryltransferase. **b** Indigoidine production by S-TCA-2.0-IE. The S-TCA-2.0 strain harboring a blank shuttle vector was used as the control. Strains were grown in LBv2 medium for 24 h, with initial supplementation of 85 g · L⁻¹ sodium formate (HCOONa·2H₂O). **c** The average ¹³C fraction of eleven analyzed amino acids of the S-TCA-2.0-IE strain grown in the LBv2 media supplemented with both unlabeled (40 g · L⁻¹) and ¹³C-labeled (60 g · L⁻¹) sodium formate (HCOONa·2H₂O). The left panel represented the metabolic pathways for the formation of amino acids, in which dashed lines represent the multiple-step pathways. Data are presented as the mean ± SD (*n* = 3 biologically independent

samples). **d** Growth (left), indigoidine production (right), and formate consumption (right) of S-TCA-2.0-IE in a fed-batch mode. Initial sodium formate (HCOONa·2H₂O) concentration in medium was 60 g · L⁻¹. Extra sodium formate (60 g · L⁻¹) was added into the medium every 12 h for five times. Hydroxy-pyruvate, PEP phosphoenolpyruvate, OAA oxaloacetate, AKG alpha-ketoglutaric acid, Cit citrate, ICIT isocitrate, SA succinic acid, SCA Succinyl-CoA, Pyr pyruvate, G2P 2-Phospho-D-glycerate, G3P 3-Phospho-D-glycerate, Mal malate, Fum fumarate, Gly glycine, Ser serine, Met methionine, Leu leucine, Ala alanine, Val valine, Ile isoleucine, Asp aspartic acid, Glu glutamic acid, Phe phenylalanine, Lys lysine. Data are presented as the mean ± SD (*n* = 3 biologically independent samples). Source data are provided as a Source Data file.

potential of the strain S-TCA-2.0-IE in formate consumption and indigoidine production, rather than using the minimal medium containing formate as the sole carbon source. Although yeast extract and peptone in the LBv2 medium enabled growth and indigoidine synthesis of S-TCA-2.0-IE, further enhanced biomass and indigoidine production were observed with the supplementation of formate (Fig. 4d), strongly indicating the contribution of formate consumption to indigoidine production. We analyzed the proportion of ¹³C-labelled glutamine (the precursor of indigoidine) to total glutamine in the S-TCA-2.0-IE strain and found that the ¹³C-glutamine (M1) accounted for 12.9% of total

glutamine (Supplementary Fig. 11). This analysis confirmed that the carbon atom in formate could be incorporated into indigoidine but the ratio was not high. Therefore, we deduce that a large portion of the consumed formate was oxidized to form CO₂ (generating reducing power) and assimilated into biomass components in S-TCA-2.0-IE, which would facilitate cell growth or other co-substrates (e.g., tryptone and yeast extract) to be more used for indigoidine synthesis. This may also partially explain why the addition of formate could significantly enhance the production of indigoidine by S-TCA-2.0-IE grown in the LBv2 medium.

treated with methanol, isopropanol, water, ethanol, and hexane for three rounds, aiming to lyse cells and remove contaminating proteins and metabolites. The pellet was dried overnight and then resuspended in dimethylsulfoxide (DMSO) at a concentration of $1\text{ mg}\cdot\text{mL}^{-1}$. The purity of indigoidine was determined by HPLC and mass spectrum (Supplementary Fig. 12). The purified indigoidine reagent was used to establish a standard curve correlating the concentration of indigoidine to the absorbance at OD_{600} (Supplementary Fig. 13). To quantify indigoidine produced by *V. natriegens*, 1 mL of the fermentation broth was collected and treated by the same procedure described above. The concentration of indigoidine was determined by measuring the optical absorbance at 600 nm using a spectrophotometer (DU730, Beckman Coulter, Inc., USA).

The measurement of glycine was carried out based on the method described previously with a few modifications⁴⁶. In brief, glycine was measured by HPLC with *o*-phthalaldehyde (OPA) (500 mg OPA, 5 ml ethanol, and 500 μl mercaptopropionic acid in 50 ml of 0.4 M borate buffer) as a precolumn derivatization reagent. Mobile phase A and B were sodium acetate solution (50 mM) and methanol, respectively. The flow rate was $1\text{ mL}\cdot\text{min}^{-1}$ and the detection wavelength was 334 nm. The Zorbax Eclipse XDB-C8 ($4.6\times 150\text{ mm}$, $3.5\text{ }\mu\text{m}$) column was used with a matched guard column. The gradient program for HPLC was as follows: 0–7 min, 30% solution B; 7–14 min, 45% solution B; 14.5–18 min, 30% solution B. The sample loading volume was 5 μl . The column temperature was kept at 35 °C.

RNA-seq analysis

V. natriegens strains were cultured in the LBv2 medium with or without the supplementation of sodium formate ($\text{HCOONa}\cdot 2\text{H}_2\text{O}$) ($40\text{ g}\cdot\text{L}^{-1}$). The grown cells (6 h of cultivation) were harvested by centrifugation ($6000\times g$, 4 °C, 20 min) and then frozen in liquid nitrogen. The RNA-seq assays were finished by the Majorbio Co. Ltd. in China. In brief, total RNA was extracted using the TRIzol® reagent (R0016, Beyotime, China) followed by the removal of genomic DNA using DNase I (Takara, Japan). The 16S and 23S ribosomal RNA (rRNA) in total RNA were eliminated using a Ribo-Zero Magnetic kit (Epicenter Biotechnologies, WI, USA). Thereafter, mRNA was broken into short fragments (~200 bp) with the fragmentation buffer. Next, cDNA was synthesized via reverse transcriptase using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina, USA). The second strand of cDNA was generated by incorporating deoxyuridine triphosphate (dUTP) into the place of deoxythymidine triphosphate (dTTP), aiming to create blunt-ended cDNAs. The yielding double-stranded cDNAs were subjected to end repair, phosphorylation, 3' adenylation, and adapter ligation in sequential. Next, the second strand of cDNA with dUTP was degraded using UNG enzyme (Uracil-N-Glycosylase). cDNA fragments were separated on a 2% agarose gel. The DNA fragments of ~200 bp were extracted from the gel and used as the template for the construction of cDNA libraries by PCR amplification using the Phusion DNA polymerase (NEB, USA) with 15 reaction cycles. After quantification with a micro fluorometer (TBS-380, TurnerBioSystems, USA), the libraries were sequenced on the Illumina HiSeq × Ten sequencer using paired-end sequencing.

Bioinformatics analysis was performed using a cloud platform (Shanghai Majorbio Bio-Pharm Technology Co., Ltd., China) based on the data generated by the Illumina platform. The main methods are as follows: RSEM was used to quantify gene and isoform abundances. The TPM method was used to calculate expression levels. Differentially expressed genes were identified by using the DESeq2 packages (<http://bioconductor.org/packages/release/bioc/html/DESeq2.html>).

Quantitative real-time-PCR

Quantitative real-time-PCR (qRT-PCR) was performed to verify the RNA-seq data. Briefly, cultures (cultivation in the LBv2 medium with or

without the supplementation of sodium formate) were taken after 6 h of cultivation, frozen quickly in liquid nitrogen, and ground into powders. RNA samples were then extracted using a Ultrapure RNA Kit (SparkJade, China), followed by the removal of genomic DNA with DNase I (SparkJade, China). Next, qRT-PCR was carried out in a MyiQ2 thermal cycler (Bio-Rad, USA) using a SYBR Green PCR premix kit (SparkJade, China). The transcript levels of the tested genes were normalized to that of the 16S rRNA gene (PN96_00205, an internal control) of *V. natriegens*. The primers used are listed in Supplementary Data 1.

¹³C isotopomer analysis

¹³C isotopomer analysis of amino acids in the *V. natriegens* S-TCA-2.0-IE strain was carried out as follows. Briefly, the *V. natriegens* cells were grown in 4 mL of the LBv2 medium ($40\text{ g}\cdot\text{L}^{-1}$ of $\text{HCOONa}\cdot 2\text{H}_2\text{O}$) at 30 °C. When cells grew to the plateau stage, $60\text{ g}\cdot\text{L}^{-1}$ of ¹³C-labeled sodium formate ($\text{HCOONa}\cdot 2\text{H}_2\text{O}$) was added into the medium. After 24 h of cultivation, 0.01 mL of the grown cells were inoculated into the same medium ($40\text{ g}\cdot\text{L}^{-1}$ of $\text{HCOONa}\cdot 2\text{H}_2\text{O}$) for cultivation again with the supplementation of $60\text{ g}\cdot\text{L}^{-1}$ of ¹³C-labeled sodium formate. Such a subculturing was repeated three times. The grown cells of the last passage were harvested by centrifugation ($6000\times g$, 4 °C, 20 min) and incubated with 200 μl 6 M HCl at 105 °C for 24 h. The samples were dried in a vacuum centrifuge and then derivatized with 100 μl of pyridine and 50 μl of *N*-methyl-*N*-[*tert*-butyldimethylsilyl] trifluoroacetamide at 85 °C for 1 h. After filtration (0.45 μm pore size, Millipore), 1 μl of samples were injected into a GC-MS system equipped with a DB-5HT column ($30\times 0.25\text{ mm}$, 0.1 μm), which was operated in an electron shock (EI) mode at 70 eV. Amino acids were determined by matching masses and retention times to authenticated standards library.

Isotopomer analysis of glutamine in the S-TCA-2.0-IE strain was performed as follows. The cells were grown in 5 mL of the LBv2 medium supplemented with $60\text{ g}\cdot\text{L}^{-1}$ of ¹³C-labelled sodium formate ($\text{HCOONa}\cdot 2\text{H}_2\text{O}$). After 24 h of cultivation, 0.25 mL of the grown cells were inoculated into the same medium for cultivation again. Such a subculturing was repeated three times. The grown cells of the last passage were collected by centrifugation ($6000\times g$, 4 °C, 20 min). Then, cells were mixed with acetonitrile/methanol/water (40:40:20), incubated at -20 °C for 20 min, and centrifuged ($12,000\times g$, 4 °C, 20 min). The supernatant was collected to analyze glutamine by ultrahigh-performance liquid chromatograph (UHPLC; Acquity; Waters) coupled to a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific, Inc., USA). Metabolites were separated with a Waters Corp Acquity UPLC HSS T3 column ($100\times 2\text{ mm}$, 1.8 μm). The mass spectrometer was run in both electrospray ionization positive (ESI+) and negative (ESI-) modes. Data were acquired using full scan over 70–1000 *m/z* at 70,000 resolution. MS/MS spectra were acquired with 30-eV collision energy. Data processing was performed by Compound Discoverer 2.0 (Thermo Fisher Scientific, Inc., USA), with a mass tolerance of 5 ppm for both noise filtering and database search.

Metabolomics analysis

The grown cells (OD_{600} ~1.0) were harvested by centrifugation ($6000\times g$, 4 °C, 20 min). Then, cells were mixed with acetonitrile/methanol/water (40:40:20) and incubated at -20 °C for 20 min. Next, the samples were centrifuged ($12,000\times g$, 4 °C, 20 min), and the supernatant was collected. The metabolites were analyzed through the ultrahigh performance liquid chromatograph (Acquity, Waters) with a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific, Inc., USA). 10 μl of the samples were injected into the LC-MS system. Metabolites were separated by a Luna NH2 column ($100\text{ mm}\times 2\text{ mm}$, 3 μm particle size, Phenomenex) with a solvent flow rate of $0.3\text{ mL}\cdot\text{min}^{-1}$. The analysis was carried out with the column

temperature maintained at 15 °C. Solvent A (20 mM ammonium acetate, adjusted to pH 9.0 with ammonium hydroxide) and solvent B (acetonitrile) served as the mobile phases with a gradient as follows: 0 min, 85% solvent B; 10 min, 45% solvent B; 15 min, 2% solvent B; 18 min, 2% solvent B; 18.1 min, 85% solvent B; 24 min 85% solvent B. The mass spectrometer with a heated electrospray ionization source was operated in a positive mode. Data were generated through full scan over 70–1000 *m/z* at 70,000 resolution. MS/MS spectra were acquired with 30-eV collision energy. The compound identities were verified by mass and retention-time match to authenticated standards.

Analysis of ¹³C ratio in biomass

Formate assimilation ratio was performed by analyzing the proportion of ¹³C in the total carbon of biomass as previously described¹⁸. In brief, the *V. natriegens* S-TCA-2.0 cells were grown in the modified M9 medium (supplemented with 2.0 g·L⁻¹ glucose, 0.25 g·L⁻¹ glycine, and 10 g·L⁻¹ ¹³C-labeled HCOONa·2H₂O) at 30 °C. After 48 h of cultivation, 1 mL of the grown cells were harvested by centrifugation (6000 × *g*, 4 °C, for 10 min) and then washed twice with the double distilled water. Next, the samples were freeze-dried and used for determining ¹³C contents by an isotope ratio mass spectrometer coupled with an elemental analyzer (Thermo Fisher Scientific, Inc., USA).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The RNA-seq data and genome resequencing data generated in this study have been deposited in the NCBI SRA database under Bioproject accession [PRJNA843433](#) and [PRJNA1022572](#), respectively. Source data are provided with this paper.

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Author contributions

J.T. conceived and performed strain modifications, adaptive laboratory evolution and isotopic labeling experiments; W.D. and Z.Z. constructed plasmids; J.T. and W.D. analyzed the phenotypic changes of the mutants; W.D. performed bioinformatics analyses and isotopic labeling experiments; Y.G. and W.J. supervised and directed the study. Y.G., W.J., J.T. and W.D. wrote the manuscript. G.Y., G.Z., S.Y., and J.X. discussed results and offered advices.

Competing interests

The authors declare no competing interests.

Additional information

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