Characterization of Highly Ferulate-Tolerant *Acinetobacter baylyi* ADP1 Isolates by a Rapid Reverse Engineering Method

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**ABSTRACT** Adaptive laboratory evolution (ALE) is a powerful approach for improving phenotypes of microbial hosts. Evolved strains typically contain numerous mutations that can be revealed by whole-genome sequencing. However, determining the contribution of specific mutations to new phenotypes is typically challenging and laborious. This task is complicated by factors such as the mutation type, the genomic context, and the interplay between different mutations. Here, a novel approach was developed to identify the significance of mutations in strains evolved from *Acinetobacter baylyi* ADP1. This method, termed rapid advantageous mutation screening and selection (RAMSES), was used to analyze mutants that emerged from stepwise adaptation to and consumption of high levels of ferulate, a common lignin-derived aromatic compound. After whole-genome sequence analysis, RAMSES allowed rapid determination of effective mutations and seamless introduction of the beneficial mutations into the chromosomes of new strains with different genetic backgrounds. This simple approach to reverse engineering exploits the natural competence and high recombination efficiency of ADP1. Mutated DNA, added directly to growing cells, replaces homologous chromosomal regions to generate transformants that will become enriched if there is a selective benefit. Thus, advantageous mutations can be rapidly identified. Here, the growth advantage of transformants under selective pressure revealed key mutations in genes related to aromatic transport, including *hcaE*, *hcaK*, and *vanK*, and a gene, *ACIAD0482*, which is associated with lipopolysaccharide synthesis. This study provided insights into the enhanced utilization of industrially relevant aromatic substrates and demonstrated the use of *A. baylyi* ADP1 as a convenient platform for strain development and evolution studies.

**IMPORTANCE** Microbial conversion of lignin-enriched streams is a promising approach for lignin valorization. However, the lignin-derived aromatic compounds are toxic to cells at relevant concentrations. Although adaptive laboratory evolution (ALE) is a powerful approach to develop more tolerant strains, it is typically laborious to identify the mechanisms underlying phenotypic improvement. We employed *Acinetobacter baylyi* ADP1, an aromatic-compound-degrading strain that may be useful for biotechnology. The natural competence and high recombination efficiency of this strain can be exploited for critical applications, such as the breakdown of lignin and plastics and abundant polymers composed of aromatic subunits. The natural transformability of this bacterium enabled us to develop a novel approach for rapid screening of advantageous mutations from ALE-derived, aromatic-tolerant, ADP1-derived strains. We clarified the mechanisms and genetic targets for improved tolerance toward common lignin-derived aromatic compounds. This study facilitates metabolic engineering for lignin valorization.

**KEYWORDS** Acinetobacter baylyi ADP1, adaptive laboratory evolution, aromatic acids, reverse engineering, tolerance
The importance of adaptive laboratory evolution (ALE) (1, 2) in generating strains with desired traits is evidenced by the success in improving the tolerance of production hosts toward stresses caused by nonoptimal pH levels (3), high substrate or product concentrations (2, 4, 5), or other growth inhibitors (6, 7). Discovery of the associated genetic change can be accomplished by whole-genome sequencing (1, 2). However, it is challenging to determine the contribution of mutations, alone or in combination, to the evolved phenotype because ALE typically yields multiple mutations (2). In addition, mutations may occur in poorly characterized genes. Some mutations may be neutral while others are important for the evolutionary trajectory but not the final phenotype or may be deleterious hitchhikers (2).

Statistical methods have the potential to predict relevant mutations across many independent ALE experiments (8), but a more profound understanding of the functional relevance of genetic change requires the reconstruction of strains with specific mutations (2). Such reconstruction, also referred to as reverse engineering, can be done by the introduction of selected mutations into reference strains followed by comparative analyses (5, 7, 9–11). However, significant efforts may be required to synthesize alleles and integrate genetic changes in the appropriate location, especially when multiple mutations are tested. The bacterium used in our experiments, *Acinetobacter baylyi* ADP1 (hereafter ADP1), has unique advantages for determining the combinatorial effects of mutations. Its high efficiency of natural transformation and allelic replacement has long been exploited because linear DNA can be added directly to growing cultures. DNA responsible for a new phenotype is readily identified when it confers a growth advantage (12). As described here, we developed a high-throughput method for the simultaneous analysis of multiple ALE-generated mutations.

ADP1 is an ideal model organism for biotechnology and synthetic biology (13) that has been used to study bacterial metabolism, engineering, and evolution (14–17). The potential of ADP1 as a production host for the synthesis of both native (18–22) and non-native products (20, 23, 24) has also been demonstrated. In our previous study, we engineered ADP1 to produce 1-alkenes from ferulate (23), which represents one of the major compounds of alkaline-pretreated lignin (25–27). Engineering aromatic compound catabolism to valorize the lignin fraction of lignocellulose has important industrial potential (28, 29), and ADP1 is among the best bacterial candidates for this purpose (26). However, due to the inhibitory effects of lignin-derived aromatic compounds and the complexity of the associated pathways, natural pathways are not yet optimal for lignin valorization.

In this study, ALE was used to increase the tolerance and utilization of aromatic compounds to improve the suitability of ADP1 for these biotechnology applications. Catabolic pathways for aromatic compound degradation via the β-ketoadipate pathway in ADP1, and other bacteria have been well characterized (30–32). ALE has been shown to be effective in overcoming problematic aspects of complex regulation, transport, and enzyme specificity for aromatic compound degradation (17, 33). Here, we characterized the phenotypes of different ALE lineages followed by whole-genome sequencing. Our new method, termed rapid advantageous mutation screening and selection (RAMSES), facilitated the identification of the relevant mutations and the reverse engineering process. The method relies on the simple recreation of mutated chromosomal alleles by transforming growing cells directly with DNA followed by efficient enrichment of beneficial transformants under suitable selective pressure. Finally, strains with increased tolerance were reconstructed by the new method and then characterized. This study provides insights into enhancing the tolerance of production hosts toward lignin-derived aromatics and highlights the utility of ADP1.

**RESULTS**

Adaptive laboratory evolution of *A. baylyi* ADP1 for high ferulate tolerance. Two parallel evolutions were previously carried out for ADP1 to improve the growth on ferulate as a sole carbon source (23), designated here as the G1 and G2 evolution lines (Fig. S1A). Here, we carried out the ALE experiment for two additional lineages to
improve the tolerance toward ferulate, where 0.2% (wt/vol) Casamino Acids and 10 mM acetate were supplied along with ferulate and designated as the T1 and T2 evolution lines (Fig. S1A). Acetate was added as an additional carbon source because acetate is present in substantial amounts in the commonly used lignin-enriched stream (25, 26). This approach allows the finding of mutations that are potentially advantageous for both tolerance and utilization of aromatic compounds. To develop highly ferulate-tolerant strains, the ferulate concentration was increased stepwise during the evolution experiments (Fig. S1B). A starting concentration of 55 mM was applied to the T1 and T2 evolution lines. The cells were passaged daily to fresh medium for 2 months (370 to 390 generations) with the endpoint ferulate concentration being 135 mM for the T1/T2 evolution line. Individual isolates were obtained from endpoint populations. Four isolates, which were named using ASA strain designations, were used for the subsequent studies (Fig. S1A). ASA500 and ASA501 were from G1 and G2 evolution lines, respectively, and ASA502 and ASA503 were from the T2 evolution line.

Characterization of the evolved strains. The evolved strains, ASA500, ASA501, ASA502, and ASA503, were cultivated at different ferulate concentrations in 96-well plates, and the wild-type (WT) ADP1 was used as the control. It was noted that a biphasic growth pattern was observed in some cases when cells were grown on the aromatic substrates (Fig. S2). Therefore, to evaluate the tolerance of strains consistently and comprehensively at different conditions, we decided to use the time needed for cells to reach an optical density (OD) of 0.8 as an indicator (later tOD 0.8). This indicator is influenced by both the lag phase and growth rate.

All the evolved strains exhibited improved tolerance to ferulate, ASA500 had the best tOD 0.8 value (ASA500 and ASA501 [Fig. 1B]; ASA502 and ASA503 [Fig. S3]). In the presence of 20 mM ferulate, the tOD 0.8 was 42.5 h for WT ADP1, 12 h for ASA500, and 16 h for ASA501 (Fig. 1B). For WT ADP1, a long lag phase accounted for most of tOD 0.8.

When the ferulate concentration was increased to 80 mM, the growth of the WT ADP1 was completely inhibited while the tOD 0.8 value was prolonged to 17 h for ASA500 and 33.5 h for ASA501 (Fig. 1B). A similar experimental set-up was employed to test the growth of ASA500 and ASA501 on p-coumarate and vanillate. p-coumarate, ferulate, and vanillate are all catabolized through the protocatechuate branch of the β-ketoadipate pathway. Vanillate is also an intermediate metabolite in the catabolism of ferulate (34) (Fig. 1A). Improved tolerance toward p-coumarate and vanillate was also observed from ASA500 and ASA501 (Fig. 1B). Vanillate seemed to be less toxic than ferulate and p-coumarate as indicated by the growth of WT ADP1 on this substrate.

Although ASA502 and ASA503 evolved in the presence of 0.2% Casamino Acids and 10 mM acetate, both showed improved growth in ferulate as the sole carbon source (Fig. S3). The two strains were further cultivated in elevated ferurate concentrations while being supplemented with Casamino Acids and acetate. When the ferulate concentration was increased from 40 mM to 80 mM, there was a 5 h increase in the tOD 0.8 for both strains (Fig. S4A), which was shorter than the >10 h increase observed when ferulate was the sole carbon source (Fig. S3). In 40 mM ferulate, the WT ADP1 showed diauxic growth characteristic of the sequential consumption of carbon sources. The aromatic degradative pathway is known to be repressed in the presence of acetate through catabolite repression (31). Diauxic growth was not observed for ASA502 and ASA503. However, HPLC analysis showed that while acetate and ferulate were consumed sequentially when both substrates were present, the ferulate was rapidly consumed after acetate was depleted (data not shown). Interestingly, an increase in the acetate concentration from 10 mM to 50 mM improved the tolerance of the WT ADP1 toward ferulate (Fig. S4B).

The tolerance toward aromatic acids may also be affected by the pH of the medium, which is possibly related to the protonation of the acids. Protonated aromatic acids can passively diffuse across bacterial cell membranes (35). The results in the Supplemental Material demonstrated an improved growth of the WT ADP1 on ferulate in higher pH, which favors deprotonation of weak acids.
FIG 1 Growth of ASA500, ASA501, and WT ADP1 on ferulate, vanillate, and p-coumarate. (A) Possible transport system (porins colored in green and transporters colored in blue) for aromatic acids and the β-ketoadipate pathway in ADP1. The pathway indicated by the dashed arrow (Continued on next page)
Genome sequencing of the evolved strains. Whole-genome sequencing was performed to discover the mutations in the evolved isolates. The sequencing reads from the five sequenced strains (ASA500, ASA501, ASA502, ASA503, and WT ADP1) were mapped to the reference genome of A. baylyi ADP1 (GenBank: CR543861). Because some sequence variants that differed from the reference genome were present in the parent strain, WT ADP1 (Table S1), these variants were subtracted from the mutation pool of the evolved isolates. The mutations in the evolved isolates are summarized in Table 1. The number of mutations in coding and noncoding regions for each strain is summarized in Fig. 2. Because ASA502 and ASA503 were isolated from the same population, they shared several mutations. For all the evolved strains, many of the mutations were found in the genes whose products are membrane proteins or involved in cell envelope modification. Notably, some of these genes are associated with aromatic transport, including hcaE, vanK, and hcaK (34, 36, 37). The gene hcaE encodes an outer membrane porin, and both vanK and hcaK encoded transport proteins belonging to the major facilitator superfamily. The gene hcaE was mutated in all four strains: insertion of the IS (insertion sequence) element, IS1236 for ASA500 and ASA501, and a single nucleotide insertion for ASA502 and ASA503. An 1137 bp deletion extending from the position 732 bp upstream of vanK to its CDS position 405 was identified in ASA500, and a 5 bp deletion in hcaK was identified in ASA501. All the mutations in the three genes would likely result in loss of protein function.

We analyzed the emergence of the IS in hcaE and the 1137 bp deletion in vanK by PCR-amplification of the target regions from the genomes of samples from the evolving populations taken at different times during the experiments. It was found that the hcaE mutation had already emerged on day 3 (∼11 generations; ferulate concentration = 45 mM) for the G1 and G2 evolution lines (Fig. 3A). Considering that the hcaE mutations in the two independently evolved strains, ASA500 and ASA501, were identical, the hcaE mutations were probably from the same origin and had already occurred in the preculture stage where 45 mM ferulate was applied (Fig. S1A). The deletion in the vanK region had already occurred in the G1 evolution line on day 20 (∼119 generations; ferulate concentration = 80 mM) and had been fixed between day 40 (∼236 generations; ferulate concentration = 115 mM) and day 50 (∼299 generations; ferulate concentration = 120 mM) (Fig. 3B).

Other mutations that were likely to cause the loss of function were found in the genes ACIAD2265, iscR, gacA, and ACIAD0602 (Table 1). ACIAD2265, which was mutated in ASA501, is predicted to encode a lytic transglycosylase that is involved in cell wall organization. The other genes, iscR, gacA, and ACIAD0602, were mutated in ASA502 and ASA503. iscR potentially encodes a repressor of the iscRSUA operon, which is involved in the assembly of Fe-S clusters. Fe-S clusters are important in enzymes for aromatic compound degradation. For example, they act as cofactors of a two-component vanillate dehydrogenase (VanAB) for the conversion of vanillate into protocatechuate (38). The gene gacA encodes a response regulator whose deletion has been characterized in A. baumannii and would lead to up/downregulation of many genes (39). Interestingly, further analysis of the up/downregulated genes showed that some genes are related to aromatic catabolism and uptake. ACIAD0602 encoded a putative glycosyltransferase that shares >80% amino acid identity with GtrOC4 in A. baumannii by NCBI protein BLAST. GtrOC4 was proposed to be involved in the outer core synthesis of lipo-oligosaccharides (40).

Besides the IS1236 insertion in hcaE in ASA500 and ASA501, IS1236 was also identified in two noncoding regions in ASA502 and ASA503 (Table 1): one is 21 bp upstream of ACIAD2867 and another one is 135 bp upstream of ACIAD0481. Consistent with the previous report (41), all the IS1236 insertions generated a small duplication that resulted in
| Gene locus ID (name) | Position | Description | Mutation type | DNA change | Protein effect | ASA500 | ASA501 | ASA502 | ASA503 |
|---------------------|----------|-------------|---------------|------------|---------------|--------|--------|--------|--------|
| ACIAD1702 (pcaU)    | 1708197  | Regulatory protein for pca operon (activator) | Substitution (transition) | G → A | P250L (CCA→CTA) | +      |        |        |        |
| ACIAD1722 (fcaE)    | 1730279-1730280 | Porin | Insertion (tandem repeat) | (G3 → (G)4 | Frameshift | +      |        |        |        |
| ACIAD1722 (fcaE)    | 1730384-1730385 | Porin | Insertion(IS element) | +IS +AGG | Frameshift | +      |        |        |        |
| ACIAD1727 (fcaK)    | 1736544-1736548 or 1736548-1736552 | Transporter | Deletion | -TGCTG or -GTGCT | Frameshift | +      |        |        |        |
| ACIAD0982 (vanK)    | 967651-96787 | Porin | Insertion (tandem repeat) | (G)3 → (G)4 | Frameshift | +      |        |        |        |
| ACIAD0982 (vanK)    | 280753   | Porin | Insertion(IS element) | 1              | Frameshift | +      |        |        |        |
| ACIAD0982 (vanK)    | 2236575-2236576 | Transduction | Insertion (tandem repeat) | (T)5 → (T)4 | Frameshift | +      |        |        |        |
| ACIAD0982 (vanK)    | 2322575  | Lipid transport protein | Substitution (transition) | G → A | A486V (GCG→GTG) | +      |        |        |        |
| ACIAD0481 (secA)    | 639202   | Preprotein translocase | Substitution (transition) | C → T | Δ166-169 | +      |        |        |        |
| ACIAD1405 (iscR)    | 1399615  | Repressor of the iscRSUA operon | Substitution (transition) | G → A | Truncation | +      |        |        |        |
| ACIAD0504 (gacA)    | 261189   | Response regulator | Deletion | -A | Frameshift | +      |        |        |        |
| ACIAD3465 (rpoC)    | 3392835  | Putative two-component sensor | Substitution (transversion) | G → T | G881C (GGT→GTT) | +      |        |        |        |
| ACIAD2274 (sthA)    | 2243241  | Soluble pyridine nucleotide transhydrogenase | Substitution (transition) | G → A | A462V (GCT→GTT) | +      |        |        |        |
| ACIAD0438 (rne)     | 436298   | Ribonuclease E | Deletion | -A | Frameshift | +      |        |        |        |
| ACIAD3194 (rpoA)    | 3122059  | RNA polymerase alpha subunit | Substitution (transversion) | G → T | P291Q (CCA→CAA) | +      |        |        |        |
| ACIAD0481 (rpoC)    | 307439   | RNA polymerase beta subunit | Substitution (transition) | A → G | D285G (GAT→GCT) | +      |        |        |        |
| ACIAD1220           | 1223930  | Conserved hypothetical protein | Substitution (transversion) | C → G | G84A (GGA→GCA) | +      |        |        |        |
| ACIAD3457 to ACIAD3481 | 3380313-3408297 | Noncoding region 21 bp upstream of ACIAD2867 | Duplication (IS element) | N/A | Duplication | N/A |        |        |        |
| ACIAD3459 to ACIAD3486 | 3380938-3413938 | Noncoding region 135 bp upstream of ACIAD0487 | Duplication (IS element) | N/A | Duplication | N/A |        |        |        |
| N/A                 | 2808313-2808314 | Noncoding region 21 bp upstream of ACIAD2867 | Insertion (IS element) | +IS +AAC | N/A |        |        |        |        |
| N/A                 | 474157-474158 | Noncoding region 135 bp upstream of ACIAD0487 | Insertion (IS element) | +IS +TGT | N/A |        |        |        |        |
| N/A                 | 2766926-2766927 | Noncoding region between ACIAD2829 and ACIAD2832 | Insertion (tandem repeat) | (T)6 → (T)7 | N/A |        |        |        |        |
| Gene locus ID (name) | Position | Description | Mutation type | DNA change | Protein effect | ASA500 | ASA501 | ASA502 | ASA503 |
|---------------------|----------|-------------|---------------|------------|----------------|--------|--------|--------|--------|
| N/A                 | 2236147  | Noncoding region between ACIAD2264 (mleB) and ACIAD2265 | Deletion (tandem repeat) | (T)7 → (T)6 | N/A            | +      | +      |        |        |
| N/A                 | 967592   | Noncoding region 17 bp upstream of ACIAD0980 (vanA) | Substitution (transition) | C → T     | N/A            | +      |        |        |        |

*a Locus IDs, mutation positions, and descriptions were assigned according to the reference genome CR543861 (GenBank entry).

*b For HcaK, the UniProt entry Q7X0E0 (with 410 residues) was used as a reference to evaluate the protein effect.
3 bp repeats flanking the inserted IS element, which is known to occur for the mechanism of its transposition.

**IS-involved gene duplication was identified in the evolved strains.** Gene duplication was found in the evolved strains ASA500 and ASA503, which were probably mediated by IS1236. Sequencing analysis showed that the strains ASA500 and ASA503 had DNA regions at similar genomic positions with sequencing coverages 2-fold higher than those of the genomes (Fig. S5 and Table S2), suggesting a duplication event. The region in ASA500 had a size of approximately 28 kb and covered the whole coding sequences of the genes from ACIAD3457 to ACIAD3481, while the region in ASA503 had a size of about 33 kb extending from ACIAD3459 to ACIAD3486 (Table S2). Most of the involved genes were shared by the duplicated regions in ASA500 and ASA503. However, these genes were either not explicitly related to aromatic metabolism or not well characterized. The sequence of IS1236 was found at each junction of the region (Fig. S6), suggesting that the duplicated region was flanked by IS1236. In addition, the original DNA sequences were present at the junction sites (Fig. S6), indicating that the genomic context of the original copy of the duplicated region was not changed. Junctions between the duplicated region and other locations of the genome were not found. Thus, one possibility is that the region could be duplicated in the form of a composite transposon that might be inserted in one of the original IS1236 sites. The duplications in the two strains were not identical, indicating that they resulted from independent events. However, the duplication was absent in ASA502, which was isolated from the same population as ASA503.

**Rapid selection of advantageous mutations and reverse engineering.** To select and reverse engineer the mutations that confer significantly improved tolerance toward ferulate, a novel approach (RAMSES; Fig. 4) was implemented. This method is based on ADP1’s natural transformation, an active homologous recombination machinery, and efficient enrichment of advantageous mutants under selective conditions. As illustrated in Fig. 4, the transformation is done by simply adding the amplified mutated alleles containing flanking regions of sequence identity to the chromosome to the cell culture (for liquid medium transformation) or the colony (for solid medium transformation). The cultures after transformation were then used to inoculate different selective media with incremental selective pressures (here 20 mM, 40 mM, 60 mM, and 80 mM ferulate; 40 mM, 60 mM, 80 mM, and 100 mM vanillate). The use of a range of selective pressures enabled finding a suitable condition for selection. The advantageous mutations were selected if the cells transformed with the corresponding alleles showed significantly improved growth under the conditions used for growth.

We focused on the evolved isolates from the G evolution lines, ASA500 and ASA501, and performed RAMSES with seven mutated alleles from them, including the pcaU500 (P250L),

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**FIG 2 Number of mutations in the genomes of each evolved strain.**

![Number of mutations in the genomes of each evolved strain](image-url)
hcaE500 (frameshift by IS insertion), vanK500 (loss of function by deletion), hcaK501 (frameshift), ACIAD2867_500 (A247V), ACIAD2265_501 (frameshift), and ACIAD0482_500 (Δ166-169 in amino acid sequence). These mutations were expected to have effects on ferulate tolerance. The transposon-free *A. baylyi* ADP1 (42), designated ISx, was used as the background strain for the RAMSES. The strain in which all the six copies of IS1236 were deleted has been shown to exhibit a more stable phenotype and increased transformability (42). The transformation was performed in a liquid medium. The transformed cells were transferred to the selective medium containing 20 mM, 40 mM, 60 mM, and 80 mM ferulate. At 60 mM ferulate, the cells transformed with hcaE500 and hcaK501 showed evident benefit, and their growth curves could be clearly distinguished from those of the controls (Fig. 5), indicating the significance of the hcaE and hcaK mutations. The benefit from the mutation in hcaE is consistent with the observation that hcaE was mutated in all four evolved isolates. The other mutated alleles, such as the pcaU500 and ACIAD0482_500 mutations, showed detectable but less prominent effects or large variances between replicates.

Although the mutation-transformed cells could be directly isolated from the initial screening experiment, we confirmed the reproducibility of the method by reintroducing
the mutations to the ISx strain by solid medium transformation. Because the *hcaE* mutation found in ASA500/ASA501 occurred in the early stage of the G evolution lines (Fig. 3A), it was chosen as the first mutation to be introduced into ISx. ISx showed improved growth at the elevated ferulate concentration (20 mM) after being transformed with *hcaE*500 (Fig. 6A). An additional round of cultivation under the selection condition was performed to further enrich the *hcaE* mutant. PCR analysis from the genome of the enriched population showed the existence of both wild-type and the mutated *hcaE* genotypes (Fig. 6A), indicating the enrichment of the *hcaE* mutant. The pure strain containing the mutant *hcaE*500 was further isolated and designated ASA504.

The mutated allele *vanK*500 was also chosen to transform ISx given its propagation in the G1 evolution population over time (Fig. 3B) and the role of the gene related to aromatic transport (36). However, only one of the two replicate populations that were transformed with *vanK*500 showed improved growth and enrichment of the *vanK* mutant (Fig. S7). The pure strain containing *vanK*500 was further isolated and designated ASA505.

We next used ASA504 (reconstructed mutant *hcaE*) as the parent strain for the introduction of other mutated alleles, including *vanK*500, *hcaK*501, *pcaU*500, and ACIAD0482_500.

**FIG 4** Schematic of RAMSES. The mutated allele amplified from the evolved strain is used to transform the background strain by direct addition of the purified PCR product to the exponentially growing cells (in small volume) or newly emerging colony. The linear DNA is incorporated into the chromosome by allelic replacement. The cultures after transformation are used to inoculate different mediums with incremental selective pressures (here aromatic concentration) in a multiwell plate. The growth is monitored based on the measurement of optical density. The mutants (colored in red) containing the advantageous mutation can grow robustly and get enriched at the level of selective pressure such that it outcompetes the background strain, resulting in a different growth profile compared to the control. The mutant can be recovered from the culture by an additional enrichment step under the same (or higher) selective pressure and subsequent isolation, and the corresponding mutation(s) can be confirmed by sequencing (or PCR if possible).
The ASA504 populations transformed with *vanK* and *pcaU*, respectively, did not show significantly improved growth at the ferulate concentrations tested (20 to 80 mM) (data not shown). However, it was found that ASA504 had poor growth on vanillate while ASA505 (reconstructed mutant *vanK*) had improved growth in the same condition (Fig. S8A). Therefore, we hypothesized that *vanK* would restore the growth of ASA504 on vanillate. Next, we transformed ASA504 with *vanK* and used vanillate as the selective pressure for mutant enrichment. As expected, the population of ASA504 showed improved growth on vanillate after being provided with the *vanK* (Fig. 6B).

![Image of Figure 5 showing initial screening of advantageous mutations by RAMSES.](image)

**FIG 5** Initial screening of advantageous mutations by RAMSES. Growth of ISx in 60 mM ferulate after being transformed with the selected mutated alleles is shown. Control 1: ISx without any treatment. Control 2: ISx treated with water. Control 3: ISx treated with a nonmutated allele. The OD values were calculated from two replicates. The error bars indicate the standard deviations. The y axis is shown in the log10 scale.

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![Image of Figure 6 showing reverse engineering of the selected mutations by RAMSES.](image)

**FIG 6** Reverse engineering of the selected mutations by RAMSES. (A) Reverse engineering of *hcaE* into ISx. Growth of ISx in 20 mM ferulate after transformation is shown. Control: ISx without transformation. To analyze *hcaE*, PCR analysis was performed from the genome of the transformed population after further enrichment. Lane 1: the transformed population. (B) Reverse engineering of *vanK* into ASA504 (reconstructed mutant *hcaE*). Growth of ASA504 in 40 mM vanillate after transformation is shown. Control: ASA504 without transformation. To analyze *vanK*, PCR analysis was performed from the genome of the transformed population after further enrichment. Lane 1: the transformed population. Lane 2: ASA504 containing wild-type *vanK*.

(C) Reverse engineering of *hcaK* and ACIAD0482 into ASA504 (reconstructed mutant *hcaE*). Growth of ASA504 in 60 mM ferulate after transformation is shown. Control: ASA504 without transformation. The genes *hcaK* and ACIAD0482 of the single isolates from the enriched populations were analyzed by sequencing. Pure mutant strains were recovered by further isolation. (D) The reconstructed strains were derived from ISx. The OD values were calculated from two replicates. The error bars indicate the standard deviations. The y axis is shown in the log10 scale.
from the genomic DNA extracted from the enriched population showed only the band of vanK500 (Fig. 6B), indicating the predominance of this allele. A streak-purified isolate was designated ASA506. Transforming ASA504 with hcaK501 and ACIAD0482_500 led to improved growth at 60 mM ferulate (Fig. 6C). After further enrichment with the same selective pressure, pure isolates were obtained from each of the populations. Six isolates from the hcaK501 transformed population and five isolates from the ACIAD0482_500 transformed population was analyzed by Sanger sequencing for hcaK and ACIAD0482, respectively. All these isolates were shown to contain the corresponding mutated alleles (Fig. 6C). The mutation in hcaK501 resulted in a frameshift (based on the HcaK sequence from UniProt entry Q7X0E0) and likely caused the loss of protein function, while the mutation in ACIAD0482_500 led to the deletion of 4 amino acids. The resulting mutants were designated ASA507 and ASA508, respectively. All the reconstructed strains were summarized in Fig. 6D.

**Characterization of the reconstructed strains.** To compare the growth on ferulate between the reconstructed strains, they were cultivated at different ferulate concentrations. The WT ADP1, ISx, ASA500 (evolved isolate), and ASA501 (evolved isolate) were also cultivated for comparison. Although the WT ADP1 seemed to differ from ISx in only the copy number of the IS1236 element, it had better growth than ISx at 20 mM ferulate (Fig. 7). Compared to the reference strain ISx, the single mutants, ASA504 (reconstructed mutant hcaE) and ASA505 (reconstructed mutant vanK), showed improved growth at 20 mM ferulate (Fig. 7). Both strains exhibited improved tolerance also toward p-coumarate (Fig. S8B). However, the growth of the two single mutants was strongly inhibited at 40 mM ferulate. The introduction of vanK500 only slightly improved the growth of ASA504 as indicated by the growth of ASA506 (reconstructed mutant hcaE and vanK) (Fig. 7). This result was consistent with the previous failed attempt to enrich the hcaE and vanK double mutant using ferulate. However, the growth of ASA504 on vanillate was significantly improved by introducing vanK500 (Fig. S8A). The genes vanK and vanP may be under the control of the same promoter due to their proximity. The mutation in vanK500 would likely cause the loss of the vanK promoter region, which may negatively affect the expression of the downstream gene vanP. It was further explored (see Supplemental Material) whether deletion of both vanK and vanP alone had the same effect as the mutation in vanK500 in terms of improving the tolerance of ASA504 to vanillate. The hcaK and ACIAD0482 mutations further improved the tolerance of ASA504 to ferulate as indicated by the robust growth of ASA507 (reconstructed mutant hcaE and hcaK) and ASA508 (reconstructed mutant hcaE and ACIAD0482) at 40 mM (Fig. 7). ASA507 had the best growth on ferulate among the reconstructed strains because it was the only reconstructed strain that grew robustly at 60 mM ferulate. Although a direct comparison between the evolved strain ASA500/ASA501 and ASA507 is complicated by their derivations from different parent strains, it was clear that there is potential to further recapitulate the evolved tolerance patterns by introducing other mutations.

**DISCUSSION**

Lignocellulose biorefining has been demonstrated as a sustainable method to produce fuels, chemicals, and materials (43). To date, the polysaccharide fraction of lignocellulose is of primary interest for the downstream conversion, whereas the lignin fraction is usually regarded as a waste, a low value-added product, or a source for process heat. Recent analysis has indicated that lignin valorization is essential to increase the sustainability and economic viability of lignocellulose-based industries (44, 45). The success of lignin valorization largely relies on lignin depolymerization and subsequent upgrading of the heterogeneous lignin-derived aromatics (44). However, due to the heterogeneity and impurity of lignin, it is a very challenging feedstock for chemical processes (29). The microbial valorization of lignin has been suggested (25, 27, 29). The aromatic catabolic pathways in some microbes allow the “funneling” of various aromatic species into two key aromatic ring-cleavage substrates, commonly protocatechuate and catechol, which can be further channeled
to central carbon metabolism. Salvachúa et al. (26) examined 14 bacteria for their ability to utilize a lignin-enriched substrate. *A. baylyi* ADP1 was the best among the tested bacteria both to degrade high molecular weight lignin and to consume low molecular weight lignin-derived compounds. The use of *A. baylyi* ADP1 as a host of engineering for biological lignin valorization is further warranted by its straightforward genome editing (14, 46) and the rapidly increasing number of available genetic tools (46).

Apart from the availability of lignin-derived aromatics for use as a substrate, their toxicity must be considered, which is regarded as a major challenge in the biological upgrading of lignin-enriched streams (47, 48). All the aromatic compounds tested in the current study showed various degrees of toxicity when used as sole carbon sources. The growth of the wild-type ADP1 on ferulate was significantly impaired when the concentration was increased from 20 mM to 40 mM. Moreover, 80 mM was lethal.
to the cells, and cell growth was not observed in 20 mM \( p \)-coumarate (Fig. 1B). In a previous study, a 33% reduction in the growth rate was observed in glucose-grown \( P. \) \textit{putida} KT2440 and \( E. \) \textit{coli} MG1655 in the presence of 61 mM and 30 mM \( p \)-coumarate, respectively (49). Consequently, the use of batch fermentation is greatly limited, and suitable fed-batch strategies need to be developed for substrate feeding without reaching toxicity limits. Some aromatics can cause severe growth impairment at much lower concentrations. For example, benzoate and catechol have been reported to completely inhibit glucose-grown \( P. \) \textit{putida} KT2440 at 50 mM and 8 mM, respectively (47, 50). In addition, prolonged contact with toxic aromatic compounds, even at low concentrations, may lead to other cellular malfunctions, such as cellular energy shortage as demonstrated by Kohlstedt et al. (47). Biotransformation can become more challenging when the lignin-derived aromatics serve as the sole carbon source, especially if the products of interest require high levels of a carbon substrate to sustain the synthesis of desired products.

In our attempts to discover the tolerance mechanisms behind the evolved strains, aromatic-specific transport was found to play an important role. The loss-of-function mutations in the genes \( hcaE, hcaK, \) and \( vanK \) were identified to be advantageous by RAMSES. Reconstruction of the \( hcaE \) mutation improved the tolerance toward both ferulate and \( p \)-coumarate. This gene encodes an outer membrane porin and clusters with other genes responsible for ferulate and \( p \)-coumarate catabolism (34), implying that the porin may act on hydroxycinnamates. Interestingly, the \( hcaE \) mutation resulted in decreased tolerance of vanillate. \( HcaE \) might have low specificity for vanillate. The tolerance toward vanillate was improved by the reconstruction of the \( vanK \) mutation. The gene \( vanK \) encodes a transporter belonging to the major facilitator superfamily. Its location near the \( vanAB \) operon, which is responsible for vanillate catabolism, implies that vanillate may be transported by VanK, as proposed previously (51). VanK has been also reported to mediate the uptake of two other intermediates in the aromatic catabolic pathway, protocatechuate, and \( p \)-hydroxybenzoate (36). The combination of the \( hcaE \) and \( hcaK \) mutations further improved the tolerance to ferulate. The gene \( hcaK \), transcribed in the opposite direction of the \( hca \) operon by a bidirectional promoter (52), encodes a transporter which also belongs to the major facilitator superfamily. It is possible that ferulate and \( p \)-coumarate are both transported by \( HcaK \). Loss of function mutations in these genes related to aromatic acid transport suggests a mechanism for tolerance/growth improvement by reducing the entry of aromatics. This is in line with a previous study in \( P. \) \textit{putida} (9), which showed that deletion of an outer membrane porin PP_3350 in a wild-type strain decreased the lag phase in 20 g/L \( p \)-coumarate (\(-123\) mM) by \( >30 \) h. In a recent study, Kusumawardhani et al. (53) elucidated that several genes associated with porins and with transport proteins were downregulated in an ALE-derived toluene-tolerant \( P. \) \textit{putida} S12. Besides the machinery associated with molecule uptake, efflux pumps have also been shown to contribute to the tolerance toward aromatic compounds (9, 49, 53). It is commonly known that aromatic compounds can disrupt cell membrane integrity due to their lipophilic (or partially lipophilic) nature (54, 55) and are suggested to exert toxic effects intracellularly through different modes of actions (55, 56). Therefore, this mechanism of tolerance against aromatic compounds may result from their toxic effects in the periplasm or cytoplasm. In nature, the aromatic transport systems can be important for nutrient uptake but, in concentrations relevant to applications, their role becomes less important because aromatic acids in their protonated form can diffuse down the concentration gradient across cell membranes (35, 56). This may also explain our observation that the wild-type ADP1 showed improved growth on ferulate at a higher pH, which can promote deprotonation and decrease the proportion of permeable aromatic acids.

The mutation in \( ACIAD0482 \) was surprisingly found to be advantageous in ferulate tolerance. The product of \( ACIAD0482 \) has not been reported in \( A. \) \textit{baylyi} but has homology to LpsB of \( A. \) \textit{cinetobacter baumannii} with \( >80\% \) identity. LpsB was reported to be a
glycosyltransferase of the lipopolysaccharide (LPS) core (57). The 12 bp deletion in ACIAD0482 would lead to the deletion of four amino acids from position 166 to position 169 in the protein sequence. However, the effect of the deletion on the protein function has yet to be explored. Interestingly, another gene that is associated with lipooligosaccharide (LOS) was found to be mutated in ASA502 and ASA503: a single nucleotide deletion in ACIAD0602, which may lead to loss of protein function. ACIAD0602 encodes a putative glycosyltransferase sharing 80% identity with GtrOC4 in A. baumannii, and GtrOC4 was proposed to be involved in the synthesis of the outer core of LOS (40). LPS/LOS is known to provide a barrier protecting Gram-negative bacteria from hydrophobic substances (58, 59), but the mechanism of the tolerance improvement by the glycosyltransferase mutation remains unclear.

The IS1236 element played an important role in the mutation development of the evolution experiment presented here. In addition to its insertion in hcaE in ASA500/ASA501, IS1236 was found to be inserted in two noncoding regions in the strain ASA502 and ASA503. Interestingly, the gene duplications observed in ASA500 and ASA503 were found to be related to IS1236. The duplicated region was flanked by IS1236, but the genomic context of the original copy of the region seemed not to change. In addition, junctions between the duplicated region and other locations of the genome were not found. One possibility is that the duplication may result from the formation of a new composite transposon by IS1236 flanking the duplicated region followed by integration of the composite transposon into one of the IS1236 sites. Although the duplications in the two strains originated from independent evolution events and most of the duplicated genes were shared by the two strains, the roles of the duplications are not obvious. Because the strain ASA502, which was from the same population and shared many common mutations with ASA503, only showed a slight difference from ASA503 in the growth on ferulate but did not carry the duplication. In addition, the genes in the duplicated region are not obviously related to aromatic metabolism. Nevertheless, it cannot be excluded that they may have underlying effects on the tolerance or metabolism of aromatics. For example, some of the genes may have regulatory effects or encode membrane protein. A. baylyi ADP1 contains 6 copies of a single type of IS element, IS1236, 5 of which are identical (60). In a previous evolution study, it was reported that IS1236 was responsible for 41% of mutations in ADP1 after propagation for 1000 generations in rich nutrient broth (61). Although IS elements may play a role in fitness improvement during evolution, they can also contribute to undesired genetic instability in engineered strains (42). This prompted us to use the transposon-free A. baylyi ADP1 (42), ISx, as the background strain for reverse engineering, although it seemed to have a decreased tolerance toward ferulate compared to the wild-type ADP1.

From the point of view of rational engineering, it is desirable to find the “minimal set” of mutations resulting in significant improvement of a phenotype. Here, by employing the RAMSES methodology, we were able to rapidly identify and reintroduce two key mutations (the hcaE and hcaK mutations) that alone significantly improved the tolerance of A. baylyi ADP1 toward ferulate. Such a method would be particularly useful when screening a large number of mutations (and their combinations), which is in contrast to the individual construction of knock-in cassettes. The high capability of screening would also make it possible to expand the subset of mutations to be tested beyond the mutations that are either intuitive or convergent between ALE replicates, increasing the potential to discover novel mechanisms behind the improved phenotypes. Here, for example, the ACIAD0482 mutation, which may not be considered beneficial intuitively, was found to be advantageous. Moreover, the RAMSES approach can be easily automated with the use of a liquid handling robot, owing to the possibility of transforming A. baylyi directly in liquid culture. In principle, the RAMSES approach can be used to study most types of evolved alleles that can be introduced using conventional allelic replacement methods. An adaptation of the type of donor DNA used for transformation also allows the study of duplication, a type of genetic change that is
traditionally difficult to analyze. For such a mutation type, transformation with a linear synthetic bridging fragment (SBF) can recapitulate a specific duplication by providing a platform for homologous recombination between sequences downstream and upstream of a target region (17). SBFs can be used for the amplification of desired genes or genomic target regions and, as for other types of mutations, the enrichment of duplicated transformants under selection can be informative (17).

**Conclusion.** We exploited the natural competence and high recombination efficiency of *A. baylyi* ADP1 in developing a simple and rapid method for screening, identifying, and reverse engineering advantageous mutations that arose during ALE. The method was applied on strains that were evolved for high ferulate tolerance and then subjected to whole-genome sequencing. Among numerous mutations, we were able to determine that mutations in *hcaE* and *hcaK* played a major role in the improved tolerance. By simply introducing the combination of these two mutations in a parent strain, the high tolerance against ferulate could be restored. This study highlighted the potential of applying the naturally competent *A. baylyi* ADP1 for evolution studies and strain development and facilitated the construction of more robust cell factories for aromatic substrate valorization.

**MATERIALS AND METHODS**

**Strains and medium.** Wild-type *Acinetobacter baylyi* ADP1 (DSM 24193, DSMZ, Germany) was used as a starting strain for ALE and was designated as WT ADP1. The transposon-free *A. baylyi* ADP1 (42) (a kind gift from the Barrick lab), designated ISx, was used as the parent strain for reverse engineering. *Escherichia coli* XL1-Blue (Stratagene, USA) was used as the host in cloning steps. All the strains used in this study are listed in Table 2.

Mineral salts medium (MSM) was used for ALE, growth study, and reverse engineering. The carbon sources, including ferulate, vanillate, *p*-coumarate, Casamino Acids, and acetate, were added when appropriate. The composition of MSM was 3.88 g/L K$_2$HPO$_4$, 1.63 g/L Na$_2$PO$_4$, 2.00 g/L (NH$_4$)$_2$SO$_4$, 0.1 g/L MgCl$_2$, 6H$_2$O, 10 mg/L Ethylenediaminetetraacetic acid (EDTA), 2 mg/L ZnSO$_4$.7H$_2$O, 1 mg/L CaCl$_2$.2H$_2$O, 5 mg/L FeSO$_4$.7H$_2$O, 0.2 mg/L Na$_2$MoO$_4$.2H$_2$O, 0.2 mg/L CuSO$_4$.5H$_2$O, 0.4 mg/L CoCl$_2$.6H$_2$O, 1 mg/L MnCl$_2$.2H$_2$O. The stock solutions of ferulate, vanillate, and coumarate were prepared with a concentration of 200 mM. Briefly, the proper amount of aromatic acid (all purchased from Sigma-Aldrich, USA) was added in deionized water and dissolved by slowly adding NaOH while stirring. The final pH of the stock solutions was 8.2 to 8.3. The stock solutions were further sterilized by filtration with sterile filters (pore size 0.2 μm; Whatman). The stock solutions were freshly prepared before each experiment. E. coli strains were maintained on a modified LB medium (10 g/L tryptone, 5 g/L yeast extract, 1 g/L NaCl) supplemented with 1% glucose. For solid medium, 15 g/L agar was added. Spectinomycin (50 μg/mL) was added when appropriate.

**Adaptive laboratory evolution of Acinetobacter baylyi** ADP1. Two parallel evolutions with ferulate as a sole carbon source, designated G1 and G2 evolution lines here, have been described previously (23). Here, two additional parallel evolutions were carried out to improve the tolerance on ferulate, designated T1 and T2 evolution lines, in which acetate and Casamino Acids were supplemented

| Table 2: Bacterial strains used in the study |
| --- |
| Name | Description | Source/reference |
| E. coli XL1-Blue | Wild-type E. coli XL1-Blue | Stratagene, USA |
| WT ADP1 | Wild-type *A. baylyi* ADP1 | DSM 24193, DSMZ |
| ASA500 | Isolate from G1 evolution line | (23) |
| ASA501 | Isolate from G2 evolution line | (23) |
| ASA502 | Isolate from T2 evolution line | This study |
| ASA503 | Isolate from T2 evolution line | This study |
| ISx | Transposon-free *A. baylyi* ADP1 | (42) |
| ASA504 | Reconstructed mutant containing hcaE500, descended from ISx, constructed by RAMSES with ferulate | This study |
| ASA505 | Reconstructed mutant containing vanK500, descended from ISx, constructed by RAMSES with ferulate | This study |
| ASA506 | Reconstructed mutant containing hcaE500 and vanK500, descended from ASA504, constructed by RAMSES with vanillate | This study |
| ASA507 | Reconstructed mutant containing hcaE500 and hcaK501, descended from ASA504, constructed by RAMSES with ferulate | This study |
| ASA508 | Reconstructed mutant containing hcaE500 and ACIAD0482_500, descended from ASA504, constructed by RAMSES with ferulate | This study |
| ASA509 | ∆vanKP::spec’ mutant descended from ASA504, 732 bp region upstream of vanK was also deleted | This study |
| ASA510 | ∆vanKP::spec’ mutant descended from ASA504 | This study |
in addition to ferulate. The ALE cultivation was performed in Erlenmeyer flasks (100 mL) containing 10 mL medium at 30°C and 300 rpm. The wild-type ADP1 was first plated on solid MSM, and 25 mM ferulate, 10 mM acetate, and 0.2% (wt/vol) Casamino Acids were supplemented. The single colony from the plate was precultivated in MSM supplemented with 55 mM ferulate, 10 mM acetate, and 0.2% (wt/vol) Casamino Acids. The preculture was transferred to two Erlenmeyer flasks containing the same medium, resulting in the two parallel evolution populations. The cells were transferred to fresh medium before reaching the stationary phase daily. The optical density at 600 nm (OD_{600}) was measured before each transfer. The amount of inoculum for each transfer was adjusted so that the initial OD after each transfer was between 0.03 and 0.1. The cells were cryopreserved at −80°C every two transfers. The concentration of ferulate was gradually increased during the evolution (Fig. S1B): a 5 mM or 10 mM concentration increase was applied if the ODs could be maintained at a high level (>3) before passaging. Individual isolates were streak-purified twice on LB-agar plates from the end population of each evolution line.

The number of generations (n) per flask was calculated with the following equation:

\[ n = \frac{\log \left( \frac{N}{N_0} \right)}{\log(2)} \]

where \( N \) is the final OD_{600} of the culture and \( N_0 \) is the initial OD_{600}.

**Phenotype characterization.** The growth of different strains on different aromatic substrates was tested by cultivations in 96-well plates (Greiner Bio-One CellStar μClear). The cells were precultivated in MSM supplemented with 5 mM aromatic substrate (ferulate/vanillate/p-coumarate) at which both the evolved strains and the reference strains can grow. After overnight cultivation, the cultures were inoculated (initial OD 0.05) into the medium supplemented with the corresponding aromatic substrate at higher concentrations (as indicated in the result section). For strains from the evolution line T2, appropriate amounts of acetate and Casamino Acids were added when needed. The culture (200 μL) was transferred to the 96-well plate and incubated in Spark multimode microplate reader (Tecan, Switzerland) at 30°C. Double orbital shaking was performed for 5 min twice an hour with an amplitude of 6 mm and a frequency of 54 rpm. The OD was measured every hour. The cultivations were performed in duplicate. To study the effect of increased pH on cell growth, the pH of the medium was adjusted by adding concentrated NaOH. The medium was further sterilized by filtration. The cultivation was performed with the same procedure as mentioned above. For precultivation, the medium without pH adjustment was used.

**Whole-genome sequencing of the evolved strains.** Approximately 1 μg of genomic DNA from each strain was isolated using the Nucleospin gDNA cleanup kit (Macherey-Nigel), then fragmented by sonication to an average size of 300 to 500 bp. End repair, A-tailing, and adapter ligation reactions were performed on the fragmented DNA using the NEBNext Ultra II kit (New England Biolabs). Illumina paired-end sequencing was performed on a NextSeq500 device at the Georgia Genomics Facility (University of Georgia).

The sequences were analyzed using both Geneious prime version 8.1 with default settings (62) and the Bresseq (version 0.35.4) computational pipeline (63). Version 2.4.1 of bowtie2 and version 4.0.0 of R (University of Georgia).

**Reverse engineering of key mutations.** The selected mutated alleles were PCR-amplified from the evolved strains with Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Finland), using the primers listed in Table 3. The amplified DNA fragments contained at least 500 bp of homology on each side of the mutated region. The PCR products were then loaded onto the agarose gel for electrophoresis. To avoid cross-contamination between the PCR products of the different mutated alleles, it is important to leave one well empty between the samples and not to overload the PCR products. The amplified DNA was purified with GeneJET Gel Extraction kit (Thermo Fisher Scientific) and eluted with prewarmed water. The concentrations of the purified PCR products ranged from 30 ng/μL to 100 ng/μL.

For natural transformation, ISx was first precultivated in LB medium supplemented with 0.4% glucose. When the cells were in the early exponential phase, 20 μL of the purified DNA was directly added to 180 μL of the culture, and then the mixture was incubated in a 14 mL cultivation tube at 30°C and 300 rpm for 3 to 4 h. The cells were treated with water, an unmutated allele (gene entry: ACIAD3383) amplified from the evolved strain, and without any treatment were used as the controls. To adapt the cells to the medium used for the downstream process, 5 mL of MSM supplemented with 5 mM ferulate was added to the tube, and the culture was incubated overnight. After the incubation, 10 μL of the cells were transferred to different wells of a 96-well plate (Greiner Bio-One CellStar μClear) containing 140 μL of MSM with elevated ferulate concentrations (20 mM, 40 mM, 60 mM, and 80 mM). The plate was incubated in a Spark multimode microplate reader (Tecan, Switzerland) at 30°C, and the OD was measured every hour.

The ground strain, ISx, was streaked on LB-agar, and the plate was incubated at 30°C overnight. The purified DNA (0.5 μL) was added onto single colonies and mixed well by pipetting up and down. After overnight incubation at 30°C, the colony treated with the DNA was scraped and suspended in MSM supplemented 5 mM the corresponding aromatic substrate (ferulate or vanillate). As the control, a colony without DNA treatment was subjected to the same process. The suspension was further incubated.
at 30°C and 300 rpm for 0.5 to 10 h. After incubation, the suspension was used to inoculate 200 μL of MSM supplemented with elevated concentrations of the aromatic substrate (ferulate: 20 mM, 40 mM, 60 mM, and 80 mM; vanillate: 40 mM, 60 mM, 80 mM, and 100 mM) in different wells of the 96-well plate. The plate was incubated in Spark multimode microplate reader (Tecan, Switzerland) at 30°C, and the OD was monitored every hour. If the cells treated with the mutated allele showed improved growth over the control at the elevated aromatic concentration, 50 μL of the cells were taken from the well and used to inoculate 5 mL of MSM containing the same (or higher) concentration of the corresponding aromatic substrate for further mutant enrichment. The culture was further streaked on LB-agar. The clones carrying the mutated allele were identified by picking single colonies for PCR analysis or Sanger sequencing.

**Genetic engineering.** ASA509 was constructed by transforming ASA504 with a linear integration cassette containing the spectinomycin resistance gene flanked by the sequences homologous to the sequences surrounding the vanKP region. The cassette was constructed by overlap extension PCR with the left flanking sequence (amplified with primers P1-F and P2-R), the spectinomycin resistance gene (amplified with primers spec-F and spec-R), and the right flanking sequence (amplified with primers P3-F and P4-R). The linear cassette was later cloned to a previously described plasmid (18), and the left flanking sequence was replaced with another flanking sequence amplified with primers P5-F and P6-R. The resulting plasmid (nonreplicating plasmid in ADP1) was used to transform ASA504 to obtain ASA510.

**Analysis of substrate consumption.** The concentrations of ferulate, vanillate, and acetate were analyzed using Agilent Technology 1100 Series HPLC (UV/VIS system) equipped with G1313A autosampler, G1322A degasser, G1311A pump, and G1315A DAD. Rezex RFQ-Fast Acid H1 (8%) (Phenomenex) was used as the column and placed at 80°C. Sulfuric acid (0.005 N) was used as the eluent with a pumping rate of 0.8 mL/min.

**Data availability.** Next-generation sequencing data generated in this study are available in NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA761218.

### Supplemental material

Supplemental material is available online only.

**Supplemental File 1, PDF file, 1.5 MB.**
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JL, SS, and VS designed the study. JL and EM carried out the research work. JL, EM, and SB analyzed the data. SS, EN, and VS supervised the study. All authors participated in writing the manuscript.

We declare no conflict of interest.

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