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Supplementary Information for

Accelerating pathway evolution by increasing the gene dosage of chromosomal segments

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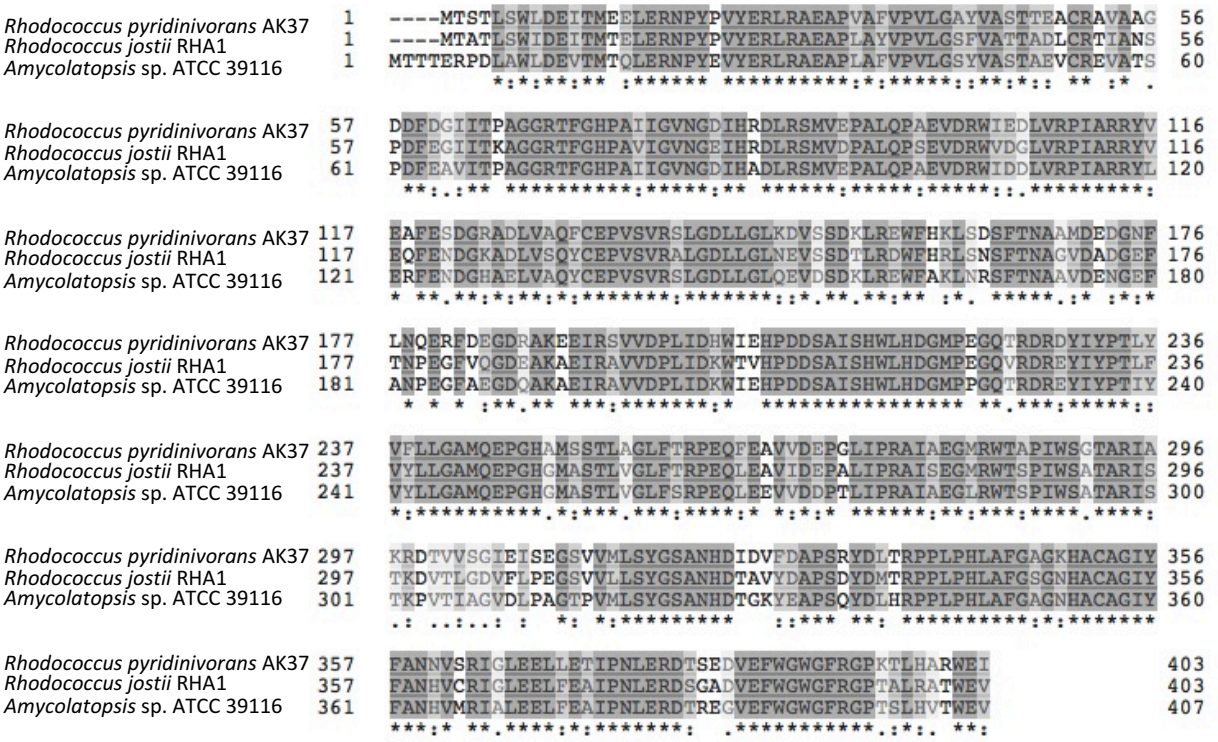

Fig. S1. **Alignment of GcoA Sequences.** Proteins from Table S1 were aligned with Clustal Omega[1] on the UniProt[2] website.

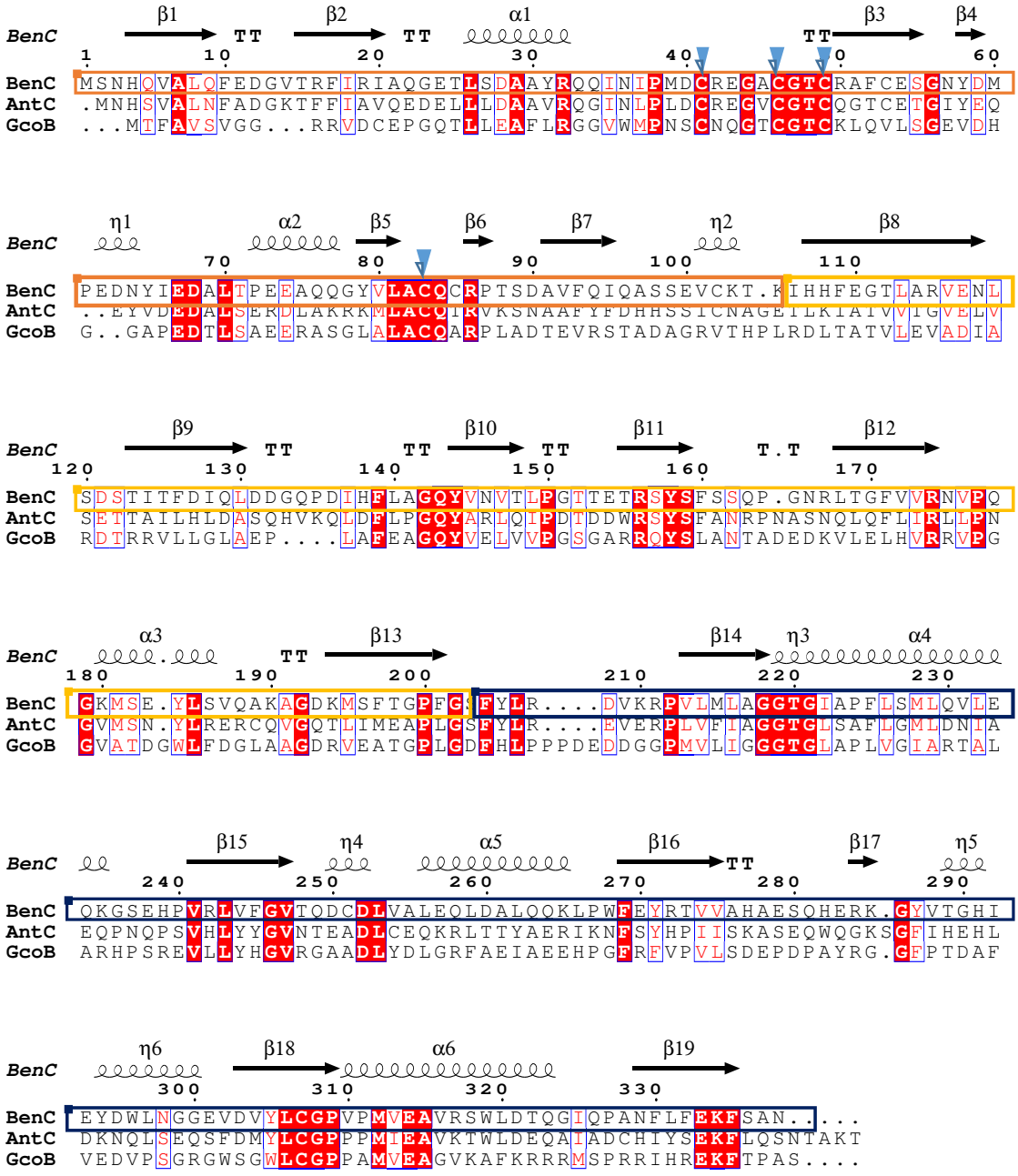

Fig. S2. Alignment of GcoB, BenC, and AntC reductases. Homology searches using the deduced amino acid sequence of GcoB from *Amycolatopsis* sp. ATCC 39116 (WP_020419854) identified significant similarity with BenC and AntC, which are three-domain reductases that partner with dioxygenase components in benzoate 1,2-dioxygenase and anthranilate 1,2-dioxygenase systems, respectively, from *A. baylyi* ADP1.[3-5] Sequences were aligned with Clustal W.[6] Structural elements of BenC[5] are marked by ENDscript/ESPript.[7] In BenC, the N-terminal ferredoxin domain is enclosed in an orange box, and blue triangles mark conserved residues involved in iron-sulfur center binding. The other domains of BenC are indicated by a yellow box, to mark the flavin-binding domain, and a dark blue box, to mark the domain that binds NADH.

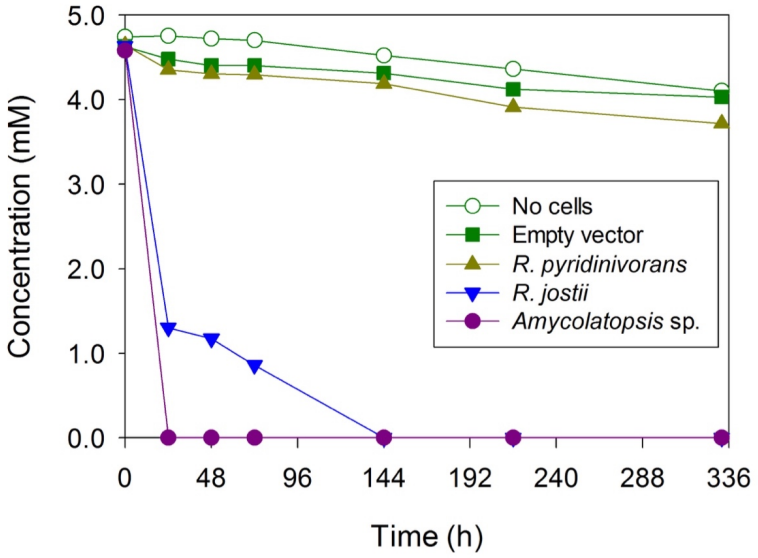

Fig. S3. Guaiacol metabolism by *P. putida* KT2440 carrying plasmids with *gcoAB* genes. The guaiacol remaining in culture supernatants (Y axis) is shown for *P. putida* grown in minimal medium with glucose and guaiacol. Cells carried a plasmid vector (pBLT-2) with predicted *gcoAB* genes from *R. pyridinivorans* (pCJ120), *R. jostii* (pCJ114), or *Amycolatopsis* sp. (pCJ021). The concentration of guaiacol in the culture supernatant was analyzed by HPLC. Representative data from two independent experiments is shown. Plasmids are described in Table S3.

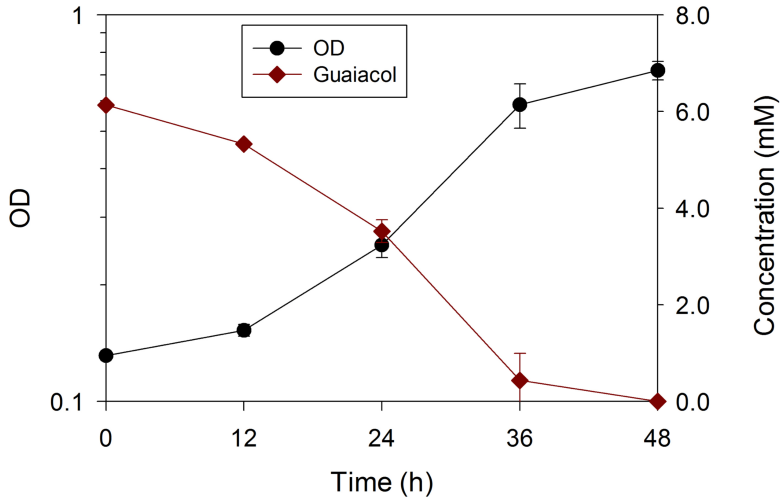

Fig. S4. **Introduction of foreign DNA enables growth on guaiacol**. With plasmid-expressed *Amycolatopsis gcoAB* in *P. putida*, strain KT2440(pCJ021) completely consumed 6 mM guaiacol provided as the sole carbon source in minimal medium. The concentration of guaiacol in the culture supernatant was analyzed using HPLC. Growth was assessed by OD at 600 nm. Data points represent averages of 3 biological replicates, and error bars indicate the standard deviations of the measurements.

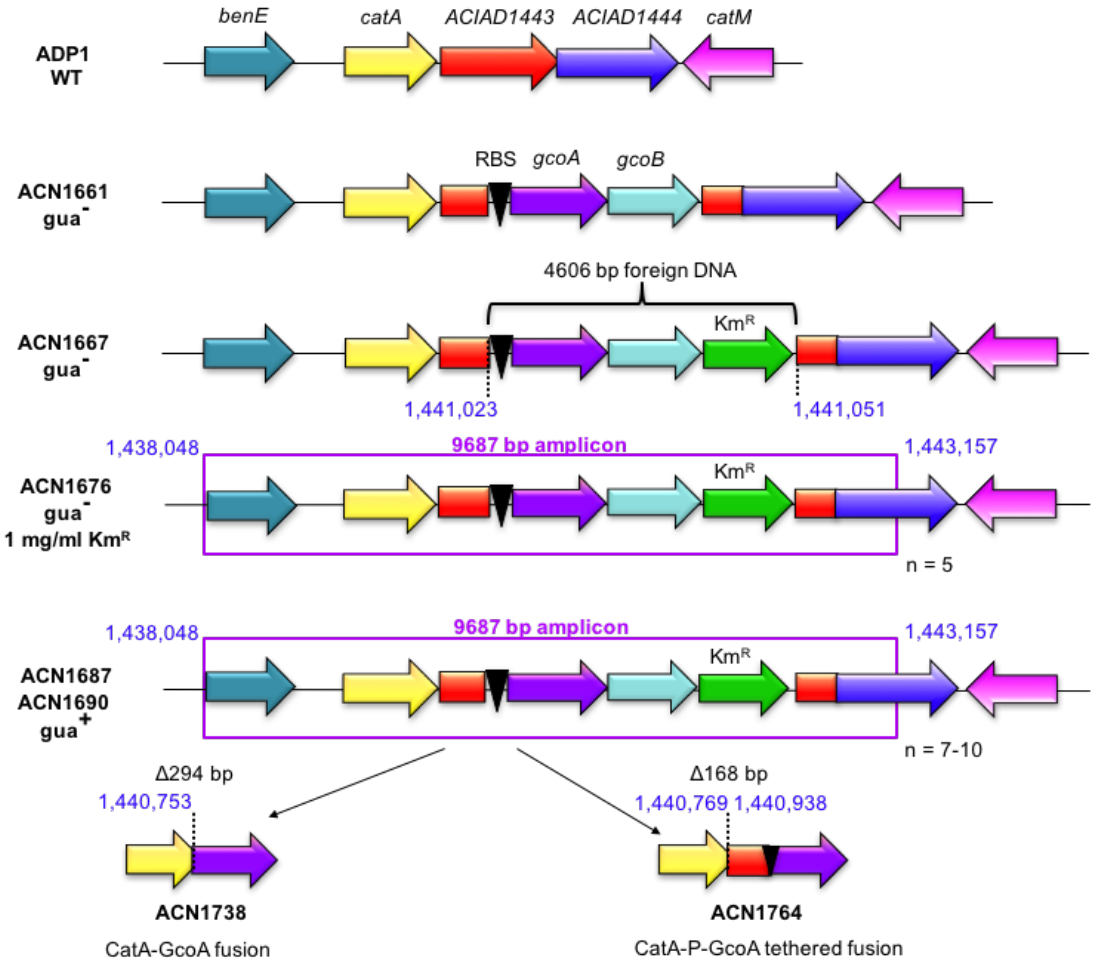

Fig. S5. ***A. baylyi* genetic arrangements.** Foreign DNA was inserted in the ADP1 chromosome (not drawn to scale). Blue numbers mark genomic positions in ADP1 (NCBI reference NC_005966). The insertion of 15 bp, GAGAGGAGGACAGCT (black triangle), includes a ribosome binding site (RBS). Since this DNA can create an in-frame coding sequence with *gcoA,* the loss of DNA spanning the ends of *catA* and ACIAD1443 can result in gene fusions that encode one protein with a functional portion of CatA connected to GcoA (in its entirety) by a peptide linker (P) consisting of some ACIAD1443 residues and 5 amino acids encoded by the sequence shown in black (ERRTA). In ACN1764, this type of tethered fusion includes a 34-amino acid peptide linker. In ACN1738, regions of CatA and GcoA are directly connected. The purple box indicates the amplified region (amplicon). A copy number of 5 to 10 was detected in different amplification mutants. The 4606 bp region of foreign DNA has a GC content of 63%. ADP1 DNA from *benA* to *catM* has a GC content of 43%.

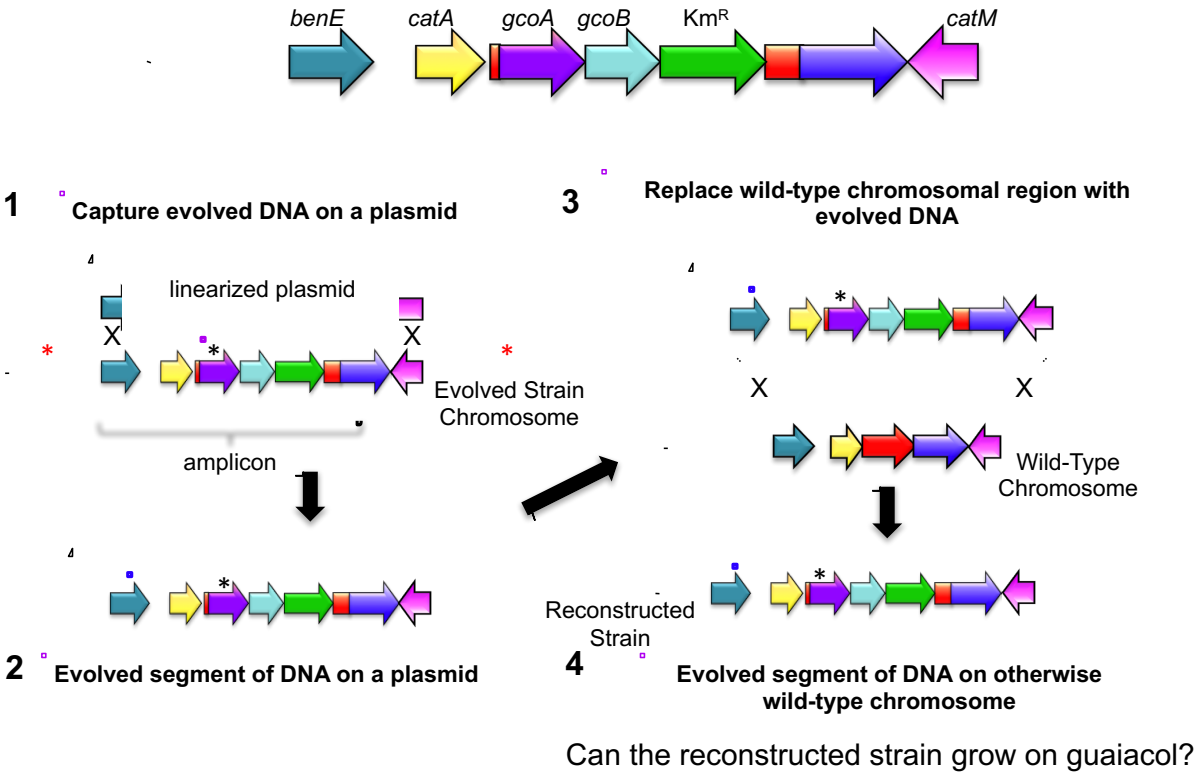

Fig. S6. Replacement of wild-type genomic DNA with DNA from an evolved Gua$^+$ strain. Mutants derived from parent strain ACN1667 (top) may have mutations (*) in the foreign DNA (purple box), and/or elsewhere in the chromosome (*). 1) The gap-repair method enables plasmid-based capture of a target region via homologous recombination (X) in vivo.[8] 2) Plasmids thus generated carry DNA from the mutant (blue box). 3) This gap-repaired plasmid can then be linearized and used to transform a wild-type recipient to generate reconstructed strains by allelic replacement (selected by Km^R). 4) The reconstructed strain can be tested to determine whether a single copy of the evolved DNA is sufficient for Gua$^+$ growth.

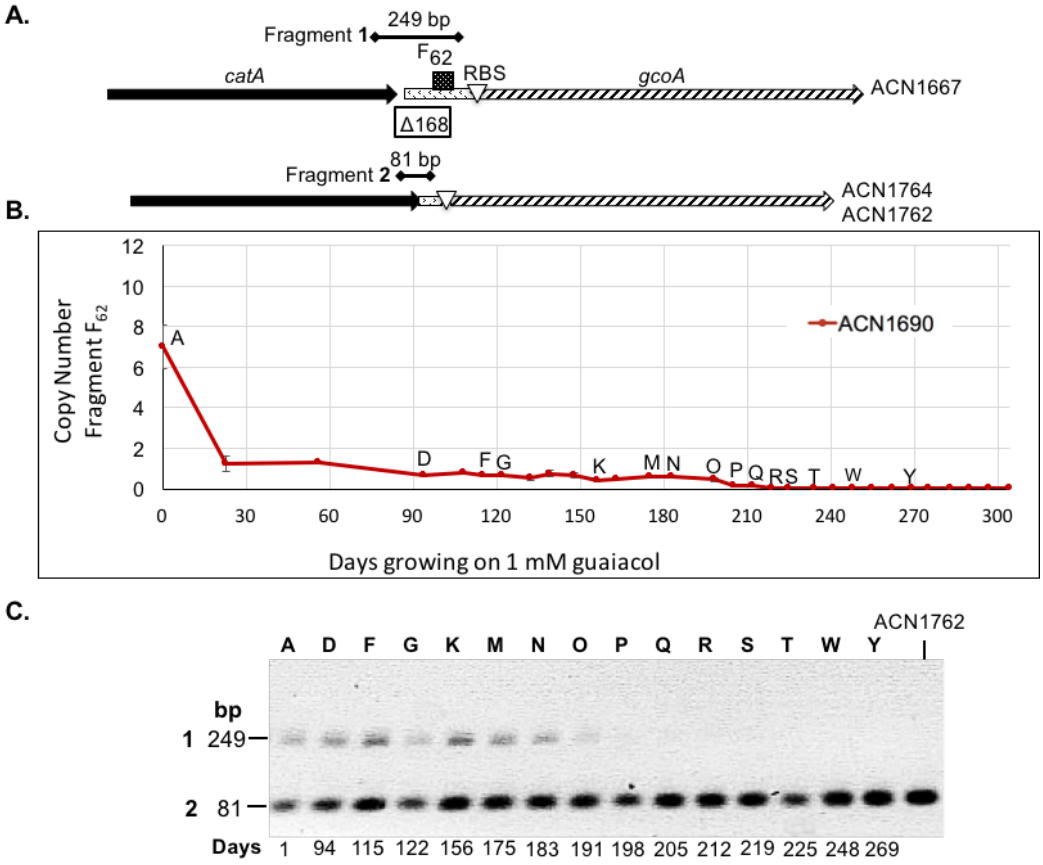

Fig. S7. Selection of a gene fusion. A. A 249-bp PCR product (Fragment 1) is generated with primers MTV706 and MTV707 using ACN1667 template DNA. With template DNA from ACN1764 or ACN1762, a smaller, 81-bp product forms (Fragment 2), which corresponds to a 168-bp deletion in this region. ACN1764 is a Gua^{+} derivative of ACN1690 after EASy evolution, and ACN1762 is a reconstructed strain. **B**. Chromosomal copy number of F_{62}, was monitored by qPCR for samples taken during continuous culturing of ACN1690. Because F_{62} cannot be detected in strains with the 168 bp deletion, copy number drops to zero when most of the cells in the population have been selected to encode the fused protein. **C**. PCR was used to analyze genomic DNA from samples of the evolving population of ACN1690. Letters marking the lanes in panel C correspond to the samples analyzed by qPCR for F_{62} in panel B. In samples A through O, two PCR products were evident: Fragment 1 (249-bp), the parent strain configuration, and Fragment 2 (81-bp), carrying the 168 bp deletion. Fragment 2 predominated at later time points. In the right-most lane, template DNA from ACN1762 was used.

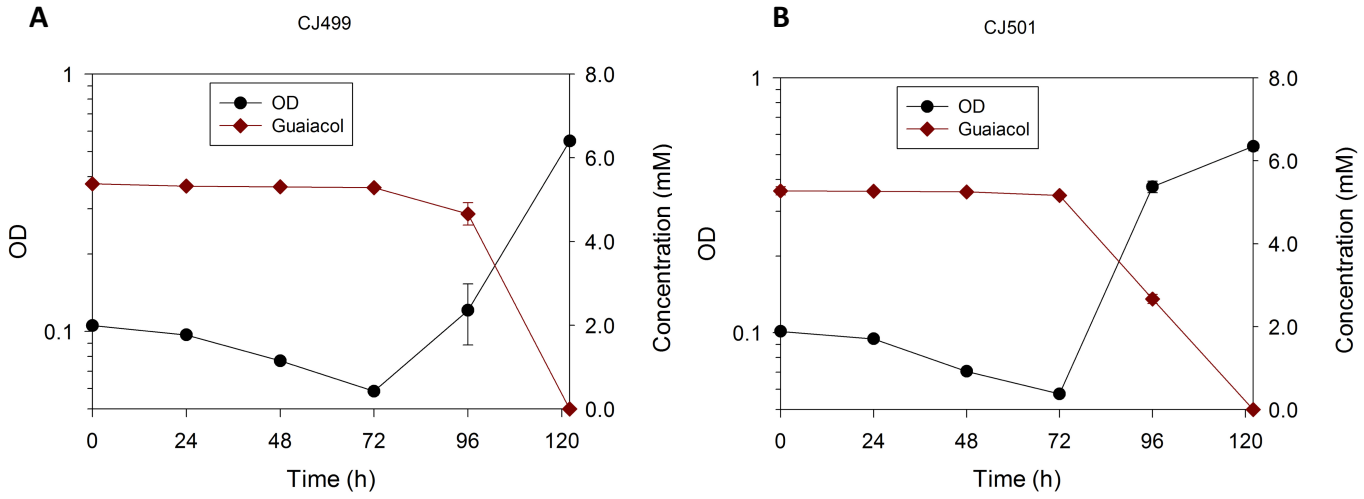

Fig. S8. Growth on guaiacol (6 mM) as the sole carbon source by *P. putida* strains with *catA-gcoA* and *gcoB* genes integrated in the chromosome. A. Unlike the results when *gcoA* and *gcoB* were chromosomally located as separate genes, the fused gene, *catA-gcoA,* and *gcoB* enabled a KT2440-derived strain, CJ499, to consume guaiacol as the sole carbon source, after a lag. **B**. Another KT2440-derived strain, CJ501, differs from CJ499 because it carries a deletion of a second *catA2*. In *P. putida* there are two *catA* genes. In CJ501 the fused *catA-gcoA* is the only gene encoding catechol 1,2-dioxygenase. The concentration of guaiacol in the culture supernatant was analyzed using HPLC. Growth was assessed by OD at 600 nm. Data points represent averages of 3 biological replicates, and error bars indicate the standard deviations of the measurements.

Table S1. **Sequences with homology to N-terminal residues from a guaiacol *O*-demethylating cytochrome P450**

Bacterium	**Amino acids in protein**	**NCBI identifier**	**% identity to the N-terminal query sequence**[a]	**% identity to WP_006553158.1**	**Protein classification**
Rhodococcus pyridinivorans AK37[a]	403	EHK82401.1 WP_006553158.1	100	100	CYP255A4
Rhodococcus jostii RHA1	403	WP_011595125.1	85.7	75.9	CYP255A2
Amycolatopsis sp. ATCC 39116[b]	407	WP_020419855.1	61.9	76.4	CYP255A6

[a] The query sequence used for the NCBI BLAST program[9] was MTSTLSWLDEITMEELERNPY, which was determined for a guaiacol *O*-demethylating cytochrome P450 from *Rhodococcus rhodochrous* strain 116, $P450_{RR1}$.[10]

[b] Previously known as *Streptomyces setonii* or *Streptomyces griseus* strain 75iv2

Table S2. *Acinetobacter baylyi* [a] and *Pseudomonas putida* strains Relevant Characteristics		Source
***A. baylyi* strains**		
ADP1	Wild type (BD413)	11,12
ACN1643	ACIAD1443::*sacB*-Km^R*51643;* counter-selectable marker inserted downstream of *catA* to facilitate subsequent strain construction pBAC1215/AatII X ADP1[b] selected by Km^R	This study
ACN1661	ACIAD1443::*gcoAB51661;* pCJ021 served as the *gcoAB* gene source pBAC1255/EcoRI X ACN1643[b] selected by growth in the presence of sucrose	This study
ACN1667	ACIAD1443::*gcoAB51661;* Km^R*51667* (ΩK[d] cassette inserted downstream of *gcoB*), Gua$^-$ parent strain single copy of *gcoAB* region pBAC1261/EcoRI X ACN1661[b] selected by Km^R	This study
ACN1676	ACIAD1443::*gcoAB51661;* Km^R*51667;* multiple chromosomal copies of a 9.7 kbp amplicon including *gcoAB* (see Fig. S5); SBF*51676*[c], pBAC1262/EcoRI X ACN1667[b] selected by high-level Km^R, Gua$^-$	This study
ACN1686	ACIAD1443::*gcoAB51661;* Km^R*51667;* SBF*51676*[c] multiple copies of 9.7 kbp amplicon Derived from ACN1676 by direct selection on a guaiacol plate, Gua$^+$	This study
ACN1687	ACIAD1443::*gcoAB51661;* Km^R*51667;* SBF*51676*[c] multiple copies of 9.7 kbp amplicon Derived from ACN1676 by direct selection on a guaiacol plate, Gua$^+$	This study
ACN1690	ACIAD1443::*gcoAB51661;* Km^R*51667;* SBF*51676*[c] multiple copies of 9.7 kbp amplicon Derived from ACN1676 by direct selection on a guaiacol plate, Gua$^+$	This study
ACN1738	*catA-gcoA51738*; *gcoB51661*; Km^R*51667;* EASy-derived Gua$^+$ isolate from evolving population of ACN1687 (see Fig. S5)	This study
ACN1739	*catA-gcoA51738*; *gcoB51661*; Km^R*51667;* Reconstructed strain, Gua$^+$ pBAC1314/NdeI X ADP1[b] selected by Km^R	This study
ACN1762	*catA-p-gcoA51764*; *gcoB51661*; Km^R*51667;* Reconstructed strain, Gua$^+$ pBAC1337/NdeI X ADP1[b] selected by Km^R	This study
ACN1764	*catA-p-gcoA51764*; *gcoB51661*; Km^R*51667;* EASy-derived Gua$^+$ isolate from evolving population of ACN1690 (see Fig. S5)	This study
ACN1850	ACIAD1443::*gcoA51850* [encodes GcoA(G72D)], *gcoB51850* [encodes GcoB(A4T)]; Km^R*51667;* EASy-derived Gua$^+$ isolate from evolving population of ACN1686 Mutations identified in genome outside the *gcoAB* region (see Table S4)	This study
ACN1863	ACIAD1443::*gcoA51661,gcoB51850* [encodes GcoB(A4T)]; Km^R*51667;* Reconstructed strain, Gua$^-$ pBAC1446/NdeI X ADP1[b] selected by Km^R	This study
ACN1881	ACIAD1443::*gcoA51850* [encodes GcoA(G72D)], *gcoB51661*; Km^R*51667;* Reconstructed strain, Gua$^-$ pBAC1456/NdeI X ADP1[b] selected by Km^R	This study
ACN1886	ACIAD1443::*gcoA51850* [encodes GcoA(G72D)], *gcoB51850* [encodes GcoB(A4T)]; Km^R*5166;* Reconstructed strain, Gua$^-$ pBAC1459/NdeI X ADP1[b] selected by Km^R	This study
ACN1887	*catA-p-gcoA51887; gcoB51661*; Km^R*51667;* Reconstructed strain, Gua$^-$ introduces the 342-bp deletion encoding a fused protein observed in the evolving population of ACN1687 (corresponding to fragment 2 in Fig. 3) pBAC1479/AatII X ADP1[b] selected by Km^R	This study
***P. putida* strains**		
KT2440	Wild type	ATCC 47054
CJ499	*catA*:*gcoAB* (*catA-gcoA* fused), Gua$^+$	This study

Table S2. *Acinetobacter baylyi* [a] and *Pseudomonas putida* strains (continued)		
	Relevant characteristics and descriptions	**Source**
CJ501	Δ*catA2 catA*:*gcoAB* (*catA-gcoA* fused gene), Gua^+	This study
CJ514	Δ*catRBC*::Ptac:*catA*:*gcoAB*, converts guaiacol to muconate	This study
CJ516	Δ*catRBC*::Ptac:*catA*:*gcoAB* (*catA-gcoA* fused gene), converts guaiacol to muconate	This study

[a] *A. baylyi* strains were derived from ADP1, previously known as *Acinetobacter calcoaceticus* or *Acinetobacter* sp.[12]

[b] In strains constructed by allelic replacement, blue text indicates the donor DNA and the restriction enzyme used to linearize the plasmid (pBAC number/Enzyme). The donor DNA transformed (X) the indicated recipient strain.

[C]The synthetic bridging fragment (SBF*51676*) corresponds to linearized pBAC1262

[d]ΩK indicates the omega drug-resistance cassette for Km^R from pUI1637

Table S3. Plasmids		
Plasmid	**Relevant characteristics**	**Source**
pUC18, pUC19	Ap^R; cloning vectors	13
pUI1637	Source of omega Km^R cassette	14
pRMJ1	Source of *sacB*-Km^R cassette	15
pBAC1152	Ap^R; SacI-SphI DNA from ADP1 (1,440,498-1,442,424)[a], PCR product (primers ST69 and ST70) in pUC19 (partial *catA,* ACIAD1443*,* partial ACIAD1444*)*	This study
pBAC1153	Ap^R; Changed GGATCA (1,441,335-1,441,340)[a] in ACIAD1443 on pBAC1152 to GGATCC (BamHI site) by site-directed mutagenesis. This change encodes a P instead of Q in the encoded protein.	This study
pBAC1215	Ap^R, Km^R; ACIAD1443::*sacB*-Kan^R*51643*; *sacB*-Km^R excised from pRMJ1 as a BamHI fragment, inserted into the BglII site of pBAC1152	This study
pBAC1250	Ap^R; Derived from pBAC1153 by site-directed mutagenesis to introduce two restriction sites. An EcoRV site (GATATC) was created (T→C, 1,441,050[a]) with primers MTV343 and MTV344. An SpeI site (ACTAGT) was created (C→G, 1,441,024[a]) with primers MTV341 and MTV342.	This study
pBAC1255	Ap^R; ACIAD1443::*gcoAB51661*; *gcoAB* DNA was excised from pCJ021 by digestion with XbaI and EcoRV and ligated to pBAC1250 digested with SpeI and EcoRV. *gcoAB* are inserted in *A. baylyi* DNA downstream of *catA*.	This study
pBAC1261	Ap^R, Km^R; ACIAD1443::*gcoAB51661;* Km^R*51667*; ΩKm^R cassette was excised from pUI1637 as an EcoRV fragment and ligated to EcoRV-digested pBAC1255	This study
pBAC1262	Ap^R; SBF*51676*. ACIAD1444 region (1,442,139-1,443,156)[a] upstream of *benE* region (1,438,049-1,439,238)[a], constructed by SOEing[20] PCR using primers MTV362-MTV365 cloned into SacI and PstI sites of pUC18	This study
pBAC1278	Ap^R, Made by excising the internal EcoRI fragment of pBAC1261; carries part of ACIAD1443 and ACIAD1444 from pBAC1261. Also carries EcoRV and BamHI sites introduced by site directed mutagenesis (see pBAC1153 and pBAC1250). Used to construct the gap-repair plasmid pBAC1282.	This study
pBAC1280	Ap^R; *catA*-ACIAD1443 region (1,439,827-1,441,020)[a] with an engineered stop codon [TGA] introduced by changing C→T at position 1,441,018.[a] cloned into EcoRI and EcoRV sites of pBAC1278 by incorporating these same sites at the ends of this region with PCR primers MTV388 and MTV390.	This study
pBAC1282	Ap^R, Km^R; ΩKan^R cassette from pUI1637 cut by Ecl136II and XmnI was cloned into EcoRV-digested pBAC1280. This plasmid, linearized with XbaI and EcoRV, allows capture of the *A. baylyi* chromosomal region containing *gcoAB* using the gap-repair method.	This study
pBAC1314	Ap^R, Km^R; *catA-gcoA51738*; *gcoB51661*; Km^R*51667;* DNA recovered from ACN1738 by the gap-repair method using linearized pBAC1282	This study

Table S3. Plasmids (continued)		
Plasmid	**Relevant characteristics**	**Source**
pBAC1337	Ap^R, Km^R; *catA-p-gcoA51764*; *gcoB51661*; Km^R*51667;* DNA recovered from ACN1764 by the gap-repair method using linearized pBAC1282	This study
pBAC1446	Ap^R, Km^R; *gcoB51850*; [encodes GcoB(A4T)] made by replacing the SbfI-PspOMI fragment of pBAC1261 with comparable DNA carrying the *gcoB* mutation discovered in ACN1850	This study
pBAC1456	Ap^R, Km^R; ACIAD1443::*gcoA51850* ; [encodes GcoB(A4T)] made by replacing the SbfI-BsiWI fragment of pBAC1261 with comparable DNA carrying the *gcoA* mutation discovered in ACN1850	This study
pBAC1459	Ap^R, Km^R; ACIAD1443::*gcoA51850; gcoB51850* [encodes GcoA(G72D) and GcoB(A4T)] constructed by replacing the BbvCI-BsiWI fragment of pBAC1261 with comparable DNA carrying both mutations discovered in ACN1850	This study
pBAC1479	Ap^R, Km^R; *catA-p-gcoAB51887; gcoB51661*; Km^R*51667;* the deletion revealed by Fragment 2 at day 23 (Sample B) in the evolving ACN1687 culture (Figure 3C) was reconstructed on a plasmid. A PCR product (generated with primers MTV388 and MTV704) was isolated and digested with BsiWI and EcoRI. This DNA was cloned by ligating to DNA with the same sequence as the largest fragment resulting from digestion of pBAC1337 with BsiWI and EcoRI. Essentially this method swaps the DNA sequence of the tethered fusion.	This study
pBTL-2	Km^R; pBBR1-based broad host range plasmid used for expression in *P. putida* KT2440. (Addgene plasmid # 22806). A gift from Ryan Gill.	16
pCJ004	Cm^R,Tc^R; For deletion of *catA2* in *P. putida* KT2440	17
pCJ021	Km^R; The genes encoding GcoA (WP_020419855.1) and GcoB (WP_020419854.1) were amplified from *Amycolatopsis* sp. ATCC 39116 gDNA with primers oCJ169 and oCJ170 (2,291 bp) and assembled into pBTL-2 amplified linearly with primers oCJ160 and oCJ161 (2,595 bp).	This study
pCJ114	Km^R; The genes encoding GcoA (WP_011595125.1) and GcoB (WP_011595126) were amplified from *Rhodococcus jostii* RHA1 gDNA with primers oCJ173 and oCJ174 (2262 bp) and assembled into pBTL-2 amplified linearly with primers oCJ160 and oCJ161 (2,595 bp).	This study
pCJ120	Km^R; The genes encoding GcoA (EHK82401) and GcoB (EHK82400) from *Rhodococcus pyridinivorans* AK37 were codon optimized for expression in *P. putida* KT2440 and synthesized as two dsDNA fragments (1,159 and 1,160 bp) by SGI-DNA and assembled into pBTL-2 amplified linearly with primers oCJ160 and oCJ161 (2,595 bp).	This study
pCJ121	Km^R; Plasmid for integration of genes encoding the CYP255A guaiacol O-demethylase and reductase from *Amycolatopsis* sp. ATCC39116 downstream of *catA* in *P. putida* KT2440. The genes encoding GcoA (WP_020419855.1) and GcoB (WP_020419854.1) were amplified from *Amycolatopsis* sp. ATCC 39116 gDNA with primers oCJ617/oCJ618 (2,308 bp) and assembled into pMFL56,[18] which contains the targeting regions for integration downstream of *catA*, amplified linearly with oCJ615 and oCJ616 (7,655 bp).	This study
pCJ126	Km^R; Plasmid for integration of genes encoding the CYP255A guaiacol O-demethylase and reductase from Amycolatopsis sp. ATCC39116 downstream of *catA* in *P. putida* KT2440, with *gcoA* fused to *catA*. The fusion was created by amplifying pCJ121 with primers oCJ619 and oCJ620 (9,878 bp) and using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) to circularize the product according to the manufacturer's instructions.	This study
pMFL22	Cm^R,Tc^R; For deletion of *catBC* and integration of the tac promoter upstream of *catA* in *P. putida* KT2440.	18
pCJ021	Km^R; The genes encoding GcoA (WP_020419855.1) and GcoB (WP_020419854.1) were amplified from *Amycolatopsis* sp. ATCC 39116 gDNA with primers oCJ169 and oCJ170 (2,291 bp) and assembled into pBTL-2 amplified linearly with primers oCJ160 and oCJ161 (2,595 bp).	This study

[a]Genomic positions in ADP1 (NCBI reference NC_005966)

Table S4. Primers		
Name	**Sequence (5' → 3')**	**Uses and Notes**
MTV274	GCTCGACGCCTTCTATTTCAA	For *rpoA* copy number analysis; used with MTV275 for qPCR
MTV275	TTTACGTCGCATTCTATTGTCTTCTT	For *rpoA* copy number analysis; used with MTV274 for qPCR
MTV302	GCGTTGGCTACCCGTGATA	For Km copy number analysis; used with MTV303 for qPCR
MTV303	GGAAGCGGTCAGCCCATT	For Km copy number analysis; used with MTV302 for qPCR
MTV341	TCATGGTAAAAGCCGACTAGTCAATGAGCTGATCG	With MTV342 for C → G mutagenesis; creates SpeI site to change pBAC1153 to pBAC1250
MTV342	CGATCAGCTCATTGACTAGTCGGCTTTTACCTGA	With MTV341 for C → G mutagenesis; creates SpeI site to change pBAC1153 to pBAC1250
MTV343	ATGAGCTGATCGGGACAGATATCTTTTCTGTTGCCG	With MTV344 for T → C mutagenesis; creates EcoRV site to change pBAC1153 to pBAC1250
MTV344	CGGCAACAGAAAAGATATCTGTCCCGATCAGCTCAT	With MTV343 for T → C mutagenesis; creates EcoRV site to change pBAC1153 to pBAC1250
MTV357	CTGGGGCTGGGGCTTCC	With MTV358 amplifies 468 bp of *gcoAB* region
MTV358	CGACCAGCTCGACGTACTGC	With MTV357 amplifies 468 bp of *gcoAB* region
MTV362	GAGTCAGAGCTCACATTCAATTGTTTATCGAAC	Used to make SBF (pBAC1262); with MTV363 amplifies 1018 bp of ACIAD1444; SacI site
MTV363	GTTTTTAACAGGGTTGTCATGGTAAAACCTTAAACCTTCAA	Used to make SBF (pBAC1262); with MTV362 amplifies 1018 bp of ACIAD1444; overlapping sequence (*benE*) for SOEing[20] PCR
MTV364	TTGAAGGTTTAAGGTTTTACCATGACAACCCTGTTAAAAAC	Used to make SBF (pBAC1262); with MTV365 amplifies 1190 bp of *benE*; overlapping sequence (ACIAD1444) for SOEing[20] PCR
MTV365	GATGATCTGCAGTTAGTTTAGCTGGCTTTGG	Used to make SBF (pBAC1262); with MTV364 amplifies 1190 bp of *benE*; PstI site
MTV379	TTTAAGCGTATTGCCGAATCAG	For fragment 62 (F_{62}) copy number analysis. Used with MTV380 for qPCR
MTV380	CAAACTGCATCAGACGAACCA	For fragment 62 (F_{62}) copy number analysis. Used with MTV379 for qPCR
MTV388	GAGTCAGAATTCGGCGATTTCAAG	Used with MTV390 to amplify *catA*-ACIAD1443 DNA and to introduce an EcoRI site for cloning. Also used with MTV704 to amplify fragment 2 (Figure 3C) for construction of pBAC1479.
MTV390	GATGATGATATCTCAGCTTTTACCATGATTATATTTACC	Used with MTV388 to amplify *catA*-ACIAD1443 DNA and introduce a stop codon in ACIAD1443. Introduces an EcoRV site for cloning; C→T at position 1,441,018
MTV459	GATGATTCTAGATTACACCGCTAGACGTG	Used with MTV388 to amplify *catA* region and to introduce an XbaI site for cloning
MTV704	AGCCGCTCGTACACCTCGTA	Used with MTV388 to amplify fragment 2 (Figure 3C) from DNA of evolving ACN1687 (isolated at day 23). Used to make pBAC1479
MTV706	ATCAGACGAACCAAGCGTG	Binds to ACIAD1443. Used with MTV707 to detect deletions that occur during evolution, including some that create tethered fusions (*catA-p-gcoA*).
MTV707	CGATCTAAAATTGACGCGTTTG	Binds to *catA*. Used with MTV706 to detect deletions that occur during evolution, including some that create tethered fusions (*catA-p-gcoA*).
MTV714	CGACCCAACAGGCGAGCAGCA	Binds to *catA*. With MTV715 used to detect deletions that occur during EASy evolution of strains growing on guaiacol.
MTV715	CGAGATCGGGCCGTTCGGTTTGG	With MTV714 used to detect deletions that occur during EASy evolution of strains growing on guaiacol.
MTV751	GAGATCGGGCCGTTCGGT	Binds to *gcoA*. With MTV770 used to detect deletions that occur during evolution, including some that create fusions (*catA-gcoA* or *catA-p-gcoA*).
MTV770	TGCTGAATCAGTTGGGCCG	Binds to *catA*. With MTV751 used to detect deletions that occur during evolution, including some that create fusions (*catA-gcoA* or *catA-p-gcoA*).
ST69	GTTATCGAGCTCATGGTAACCGCCCTG	With ST70 amplifies region between *catA-ACIAD1444* (1,440,498-1,442,425). ST69 introduces a SacI site to *catA*. Used to make pBAC1152
ST70	GTCTAAGCATGCAAGCAACATCACCCGATC	With ST69 amplifies region between *catA*-ACIAD1444 (1,440,498-1,442,425). ST70 introduces a SphI site to ACIAD1444 Used to make pBAC1152

Table S4. Primers (continued)		
Name	**Sequence (5' → 3')**	**Uses and Notes**
ST71	GATCAGATACCGGATCCGACAATTTTAACCCAG	Used with ST72 to introduce a BamHI site in pBAC1153
ST72	CTGGGTTAAAATTGTCGGATCCGGTATCTGATC	Used with ST71 to introduce a BamHI site in pBAC1153
oCJ160	GATATCATTCAGGACGAGCCTCAGACTCC	Forward primer for linear amplification of pBTL-2
oCJ161	CTCTAGAGTGTGAAATTGTTATCCGCTCACAATTCC	Reverse primer for linear amplification of pBTL-2
oCJ169	aacaatttcacacTCTAGAgAGGAGGACAGCTATGACGACGACCGAACGGCC	Forward primer for amplification of the genes encoding GcoA and GcoB from *Amycolatopsis* sp. ATCC 39116 gDNA, incorporating a Shine-Dalgarno consensus RBS and overlapping sequence for assembly into pBTL-2
oCJ170	ggctcgtcctgaatGATATCTCACGAGGCCGGCGTG	Reverse primer for amplification of the genes encoding GcoA and GcoB from *Amycolatopsis* sp. ATCC 39116 gDNA with overlapping sequence for assembly into pBTL-2
oCJ173	aacaatttcacacTCTAGAgAGGAGGACAGCTATGACCGCCACCCTGTCTTGG	Forward primer for amplification of the genes encoding GcoA and GcoB from *R. jostii* RHA1 gDNA, incorporating a Shine-Dalgarno consensus RBS and overlapping sequence for assembly into pBTL-2
oCJ174	ggctcgtcctgaatGATATCTCACGCGGCGGGCG	Reverse primer for amplification of the genes encoding GcoA and GcoB from *R. jostii* RHA1 gDNA with overlapping sequence for assembly into pBTL-2
oCJ615	gttcgaggttatgtcactgtgattttgcctg	Forward primer for amplification of the downstream targeting region for integration downstream of *catA* from pMFL56
oCJ616	CTCGAGtcagccctcctgcaac	Reverse primer for amplification of the upstream targeting region for integration downstream of *catA* from pMFL56
oCJ617	cgcgggcgttgcaggagggctgaCTCGAGAGGAGGTAATACatgacgacgaccgaacggc	Forward primer for amplification of the genes encoding GcoA and GcoB from *Amycolatopsis* sp. ATCC 39116, incorporating a RBS calculated using the Salis RBS calculator[19] and overlapping sequence for assembly with the upstream targeting region for integration downstream of *catA* in *P. putida* KT2440
oCJ618	aggcaaaatcacagtgacataacctcgaacGTTTAAACtcacgaggccggcgtgaac	Reverse primer for amplification of the genes encoding GcoA and GcoB from *Amycolatopsis* sp. ATCC 39116 with a PmeI site and overlapping sequence for assembly with the downstream targeting region for integration downstream of *catA* in *P. putida* KT2440
oCJ619	accgaacggcccgatctcg	Forward primer for site directed mutagenesis of pCJ121 to generate the CatA-GcoA fusion in *P. putida* KT2440
oCJ620	cgcctcggcgtcaggc	Forward primer for site directed mutagenesis of pCJ121 to generate the CatA-GcoA fusion in *P. putida* KT2440

Table S5. Synthesized dsDNA fragments containing genes encoding GcoA and GcoB *Rhodococcus pyridinivorans* AK37, optimized for expression in *P. putida* KT2440

Name	**Sequence (5' → 3').** Overlaps for assembly together into pBTL-2 are in gray, RBSs calculated using the Salis[19] RBS calculator are underlined, and start and stop codons are in **bold**.
Fragment 1 (GcoA)	gtgagcggataacaatttcacacTCTAGAgGACGAAAGTATT**atg**accagcaccctctcgtggctcgacgagatcaccatggaggagctggagcg taacccgtatccggtgtacgaacgtctgcgcgcagaagcaccagtggcctttgttccagtgctgggcgcctatgtggccagtaccactgaagcct gccgtgcagtagcagccggcgatgatttcgacggcatcatcaccccagccggtggtcgtaccttcggtcatccagccatcatcggcgtgaacggc gacatccatcgcgatctgcgcagcatggtggaaccagcactgcaaccagccgaggtggatcgttggatcgaggatctggtgcgcccaattgcccg ccgttacgtagaagccttcgagagcgatggccgcgcagatttggtggcccaattctgcgaaccggtgagcgtgcgtagcctgggtgatttgctgg gcctgaaggacgtgagcagcgacaaactgcgcgagtggttccacaagctgagcgacagcttcaccaacgccgccatggacgaagacggcaacttc ctgaaccaggagcgcttcgacgagggcgatcgtgccaaggaagagatccgtagcgtggtggatccgctgatcgaccactggatcgaacacccgga cgatagcgccatcagccattggctgcatgacggtatgccggaaggccaaacccgcgatcgcgactacatctatccgaccctgtacgtgttcctgc tgggcgcaatgcaggaaccgggtcatgcaatgagcagcaccctggcaggcctgtttacccgtccggaacagttcgaagccgtggtggatgaaccg ggcctgattccacgtgccatcgcagaaggcatgcgctggactgccccaatttggagtggtaccgcccgtatcgccaagcgtgataccgtggtgag cggcatcgagatcagcgaaggtagcgtggtgatgctgagctacggtagcgccaaccacgacatcgacgtgttcgatgccccgagccgctacgatt tgaccagaccaccactgccgcacctggcctttggtgcaggcaaacatgcatgcgccggcatctacttcgccaacaacgtgagccgcatcggcctg gaagagctgttggagacca
Fragment 2 (GcoA, GcoB)	gcatcggcctggaagagctgttggagaccatcccgaacctggaacgcgacaccagcgaagacgtggagttctggggttggggcttcagaggcccg aaaaccctgcatgcacgctgggagatc**tga**AGGAGGAGAACT**atg**ccgtacaccctcaccgctggaaccggtgttgtgccatgcgaaccaggtcg taccgtgctggaagccttcctgcgtaacggcaactggatgccgaacagctgcaaccagggcacctgtggtacctgcaagatcaaggtgctggacg gcgagctggaccatcgtaatagcccggaagagaccctgaccgccgacgaattagcagccggtttcgtgctggcctgccaagcaactccacgtggt gatgtggccttcgaaaccccagccaccgaagaaccagccggtacccatgtgctgcgcgatgtggttgtgaccgtgaccgaagtgcgcgatattgc cgccgatacccgcaaagtgctgctgactgccgacgaaccgctggaatttagcgccggccagtacgtggaggtgaccgtaccaggtaccgagatcc gtcgccagtacagcctggccaatccaccaagcgaggccaaggaactggagctgcatatccgccgtcagccaggtggtgtggcaagtgaatgggtg ttcgagcgcatcgacgtgggcgaacgtgtaaccgtaaccggcccgtatggcgacttcaccttcgacctggaaggcaccgacccgattgtgttgct gggtggtggtaccggtctggcaccattggaagccatcgtgcgtcaagccctgagcttggtgccggatcgtcagattctgctgtaccacggcgtgc gtacctgcgcagatctgtacgacgtggagttcctgcgcgagctggaaacccgccatcatggtttccgctacatcacctgcgtgagccgcgaaagt attcaggacgagcctcagactcca

Table S6. **Mutations in ACN1850 outside the *gcoAB* region**[a,b]

Genes with mutations	**ADP1 Genome position of mutation**[c]	**Nucleotide change Codon change**	**Amino acid change Position of change/ Total residues**	**Potential function of gene product**
ACIAD1064	1,061,077	C→T GGC→GAC (reverse strand)	G→D 141/312	Putative ester hydrolase (EstB)
pbpA ACIAD1101	1,091,644	C→T UGG→UAG (reverse strand)	W→stop 366/675	Penicillin-Binding Protein 2
lon ACIAD1115	1,103,385- 1,103,423	39 bp deletion (reverse strand)	In frame deletion of 13 residues (EYFLNEKMKVIQR) between R237 and E251/808 aa WT protein	DNA-binding ATP-dependent protease
ACIAD1354	1,350,431	G→A	W→stop 385/386	Putative phospholipase A1 precursor (PldA)
ACIAD1807	1,814,453- 1,814,499	47 bp deletion	After Y238, frame shift adds 5 residues (LGCKP) and stop 386 aa WT protein	Dipeptidyl-aminopeptidase/ acylaminoacyl-peptidase
ACIAD3076	3,003,892- 3,004,098	207 bp deletion	Prevents translation 117 aa WT protein	Small hypothetical protein of unknown function (12 kDa)
ACIAD3450	3,372,250	1 bp deletion	After G345, frame shift adds 11 residues (AFLAVRWVGNC) and stop 454 aa WT protein frame shift occurs in the 11th of 12 transmembrane helices	Putative transport protein, unknown function, major facilitator superfamily (MFS) member

[a]Mutations were identified as differences between the sequence of the parent strain and the evolved strain by whole genome resequencing
[b]Sequence changes were observed in 80% or more of the reads
[C]Numbers correspond to positions in the ADP1 genome deposited in NCBI (NC_005966)

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