SUPPLEMENTARY INFORMATION (SI) APPENDIX

Hierarchical Routing in Carbon Metabolism Favors Iron-Scavenging Strategy in Iron-Deficient Soil *Pseudomonas* species

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Detailed Methods.

Culturing and nutrient conditions. Freeze-dried cells of *P. putida* KT2440, *P. putida* S12, and *P. protegens* Pf-5 from American Type Culture Collection (ATCC, Manassas, VA) were first plated on agar-solidified Luria-Bertani (LB) medium and stored at 4°C until use. Liquid cell cultures, which were prepared using individual colonies selected from the plates and resuspended in LB medium in 20-mL glass tubes and 125-mL or 250-mL baffled flasks inside of a G24 incubator shaker (New Brunswick Scientific, Edison, NJ) at 220 rpm and 30°C. The volume of the suspended cells did not exceed one-fifth of the culturing vessels, which were covered with sponge caps to enable gas exchange and prevent contamination. The growth medium, which was pH-adjusted (pH 7.0) and filter-sterilized (0.22 µm nylon; Waters Corporation, MA, United States), contained the following major salts: 37 mM NH₄Cl, 0.80 mM MgSO₄, 0.034 mM CaCl₂, 2H₂O, 20 mM K₂HPO₄ and 5.0 mM NaH₂PO₄. The trace metal concentrations were as follows: 0.97 µM H₃BO₃, 13 µM CuSO₄·5H₂O, 70 µM ZnSO₄·5H₂O, 5.7 µM MnSO₄·H₂O, 0.21 µM NiCl₂·6H₂O, and 1.2 µM Na₂MoO₄·2H₂O. We investigated different nutrient conditions by varying the concentration of both solution Fe and the types of carbon substrates. Disodium ethylene-diamine-tetra-acetic-acid (EDTA) was added as a Fe chelator and the EDTA concentration to be used was determined using the software Visual MINTEQ (version 3.1) to simulate metal speciation in the given growth medium composition, including carbon substrate concentrations as detailed below. Prior to EDTA addition, the total dissolved Fe concentrations were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Spectro Analytical). The cells were grown under Fe-replete condition with 30 μM total dissolved unchelated Fe, intermediate Fe-limited condition with 650 nM total dissolved unchelated Fe, or Fe-limited with 38 nM total dissolved unchelated Fe—unchelated here means unchelated by EDTA (See detailed composition of the Fe speciation in Table S1). Total carbon-equivalent substrate concentrations (100 mM C or 300 mM C total) were provided as single-substrate or mixed-substrate scenarios. For the intermediate Fe-limited growth condition, we conducted experiments with glucose alone, succinate alone, citrate alone, glucose with succinate, and glucose with citrate, glucose with acetate, and glucose with benzoate; for the severe Fe-limitation, we performed experiments with glucose alone, fructose alone, succinate alone, malate alone, and glucose with benzoate. For all the substrate scenarios, we conducted Fe-replete growth experiments as reference to the intermediate and severe Fe-limited growth experiments. For the single-substrate scenarios, we used glucose (16.67 mM or 50 mM), fructose (16.67 mM), succinate (25 mM or 75 mM), malate (25 mM), or citrate (50 mM); for the mixed-substrate scenarios, we used 1:1 carbon-equivalent mixture of glucose and succinate (respectively, 25 mM and 37.5 mM), glucose and citrate (respectively, 25 mM and 25 mM), glucose and acetate (respectively, 25 mM and 150 mM), or glucose and benzoate (respectively, 8.33 mM and 7.14 mM).

For each growth condition, the cells (three biological replicates) were transferred twice in the growth medium to ensure the cells were acclimated in the specific medium composition. Biomass growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) using an Agilent Cary UV-visible spectrophotometer (Santa Clara, California). An exponential growth rate (h⁻¹) was determined via regression analysis. Measurement of cell dry weight in grams (gCDW) as a function of exponential OD₆₀₀ was obtained following lyophilization of sample aliquots (1.5-mL) using a Labconco (Kansas City, MO, USA) Freeze-Dryer System.
Table S1. Chemical speciation of Fe in terms of its equilibrium concentration, complexation with chelator EDTA or complexation with respective organic acid in the growth medium. Calculation for speciation of Fe in each experiment were computed using Visual Minteq (Version 3.1). The starting exogenous Fe concentration (~650 nM), provided as Fe$^{2+}$ salts, was kept the same in all conditions.

|                          | Equilibrium Fe concentration (nM) | EDTA bound Fe (nM) | Substrate bound Fe (nM) |
|--------------------------|----------------------------------|--------------------|--------------------------|
| **Intermediate Fe-limited conditions (no EDTA added)** |                                  |                    |                          |
| Succinate-alone$^1$      | 650                              | —                  | 0                        |
| Citrate-alone            | 5.49                             | —                  | 645                      |
| Glucose:Succinate        | 650                              | —                  | 0                        |
| Glucose:Citrate          | 10.2                             | —                  | 640                      |
| Glucose:Acetate          | 563                              | —                  | 86.6                     |
| Glucose:Benzoate         | 650                              | —                  | 0                        |
| **Severe Fe-limited conditions (90 µM EDTA added)** |                                  |                    |                          |
| Succinate$^1$            | 31.1                             | 619                | 0                        |
| Malate                   | 21                               | 611                | 17.7                     |
| Glucose:Benzoate         | 35.8                             | 614                | 0                        |

$^1$The software has no constant for Fe$^{2+}$ complexation by succinate.
Extracellular substrate depletion and extracellular metabolite excretion. To quantify substrate depletion by the cells, 0.5-mL of samples of cell suspensions from the three biological replicates were harvested during the different phases of cell growth and filter-centrifuged (Sigma Aldrich Spin-X 0.22 µm filters) at 9391g for 5 mins at 4°C (Centrifuge 5423 R, Eppendorf, Hauppauge, NY). Specifically, samples at the beginning of the lag phase, the substrate concentrations for were obtained at the beginning of the lag phase, at mid-exponential phase, and at the onset of stationary phase during growth on glucose:succinate, glucose:citrate, and glucose:acetate mixture scenarios; for the glucose:benzoate mixture conditions, samples were obtained at multiple timepoints over the entire course of cell growth phases. The supernatants were stored at 4°C until analysis for simultaneous quantification by 1H nuclear magnetic resonance (NMR) using a Varian Unity INOVA 600-MHz NMR spectrometer at 25°C, as described previously3. Substrate assimilation was confirmed by metabolite labeling of substrate-derived carbons as detailed below.

Extracellular metabolite levels were measured by centrifuging cell suspensions and filtering the supernatants (50-µL) followed by 1:10 or 1:100 dilution with LC-MS grade water (Fisher Scientific, Pittsburgh, PA) before analysis via LC-MS. Dilution ratios are adjusted depending on the concentrations of the extracellular metabolites. For measuring the excretion rate of a metabolite, samples (three biological replicates) were harvested at several time points throughout the growth phase. The excretion rate (µmol gCDW⁻¹ h⁻¹) was then computed via regression analysis. All samples were stored at 4°C prior to processing via LC-MS.

Kinetic and long-term intracellular ¹³C labeling. Labeled substrates were purchased from Cambridge Isotopes (Tewskbury, MA, USA). We tracked kinetic isotopic incorporation of [U-¹³C₆]-glucose or [U-¹³C₄]-succinate into intracellular metabolites to capture in vivo cellular fluxes into siderophore precursors1,4. In liquid cultures at mid-exponential-phase when half of the substrate was consumed, a matching amount of labeled substrate was added to the nonlabeled substrate remaining in the media to reach a 1:1 ratio. Samples were then taken at short time intervals (2, 5, or 15 mins) and the incorporation of ¹³C-labeled glucose or ¹³C-labeled succinate into metabolites was monitored using LC-MS. Data from samples taken immediately before the addition of the labeled substrate are plotted for time = 0.

We also monitored long-term intracellular incorporation of substrates by growing liquid cultures for at least two doubling times in media solutions containing [1,4-¹³C₂]-succinate alone, [¹³C₄]-succinate with unlabeled glucose, [U-¹³C₆]-glucose with unlabeled succinate, [U-¹³C₆]-glucose with unlabeled citrate, or [U-¹³C₆]-glucose with unlabeled benzoate under the different aforementioned Fe-replete and Fe-limited conditions. Samples were taken during exponential phase. Method details on sample extraction prior to LC-MS analysis are provided elsewhere3.
Metabolomic analysis via LC-MS. Samples were analyzed by reversed-phase ion-pairing LC via ultra-high performance LC (UHPLC; Thermo Scientific DionexUltiMate 3000) coupled to a high-resolution accurate-mass mass spectrometer (MS; Thermo Scientific Q Exactive) with electrospray ionization operated in full-scan negative mode (m/z range 70-900). Details on the LC parameters, including the solvent gradient and injection volume, were reported elsewhere. The following metabolites and amino acids were monitored: aspartate, ornithine, 3-phosphoglycerate, α-KG, pyruvate, fumarate, succinate, gluconate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, dihydroxyacetone, phosphoenolpyruvate, 6-phosphogluconate, citrate, malate, sedoheptulose-7-phosphate, ribose-5-phosphate and xylulose-5-phosphate. Metabolite levels and 13C labeling patterns were retrieved using the Metabolomics Analysis and Visualization Engine (MAVEN) software packag. For the labeled data, 13C-labeled fractions were corrected for natural 13C abundance. Aspartate labeling was taken as a surrogate for oxaloacetate (OAA) labeling due to the direct synthesis of aspartate from OAA.

Metabolic flux ratio analysis. To determine metabolic reprogramming of cells grown on a glycolytic substrate (glucose) and a gluconeogenic substrate (benzoate) in response to Fe deficiency, we conducted a metabolic flux ratio analysis across metabolite nodes during exponential growth of cells simultaneously on [U-13C6]glucose and unlabeled benzoate under Fe-replete and Fe-limited conditions. Due to their location at important junctures in the network of central carbon metabolism, we focused on the following four metabolic nodes in the metabolic network: succinate, glyceraldehyde-3-phosphate, phosphoenolpyruvate, and pyruvate. We used the experimental profiling of the intracellular metabolite labeling patterns to quantify the fractional contribution of different metabolites and pathways towards each node. We obtained the optimized flux ratios by iterative optimization with the objective of obtaining the minimum error difference between experimental and simulated labeling data. An example for the equations used to simulate the labeling data for metabolite GAP is provided below:

\[
\text{GAP}_0 = f_1(6\text{-PG}_0) + f_2(3\text{-PG}_0) \\
\text{GAP}_3 = f_1(6\text{-PG}_3) + f_2(3\text{-PG}_3)
\]

where, \(f_1\) and \(f_2\) refers to the fraction of GAP origination from 6-PG and 3-PG respectively; metabolite\(_0\) and metabolite\(_{3,6}\) refers to the non-labeled and fully-labeled fractions of the respective metabolite. We also estimated changes in the ATP, NAD(P)H, and UBQH\(_2\) yield using the resulting Fe-dependent metabolic flux ratios. When a metabolic flux contribution can involve enzymes with different co-factors, equal contribution of each enzyme was assumed.
**Siderophore characterization and Quantitation.** Using LC-MS-MS analysis, we followed a published procedure for isolation and characterization of PVD siderophore secreted by exponentially-growing *P. putida* KT2440 grown on unlabeled glucose, unlabeled succinate, unlabeled citrate, 1:1 U-13C6 glucose:unlabeled succinate (carbon-equivalent) mixture, and 1:1 U-13C6 glucose:unlabeled citrate (carbon-equivalent) mixture under intermediary Fe-limited condition. The peaks for PVD candidates in the bacterial supernatants were identified from the full scan total ion chromatogram by extracting the fragments of the 2,3-diamino-6, 7-dihydroxyquinoline present in all PVD siderophores. The PVD structures were elucidated following collision-induced MS/MS fragmentation. Both the doubly charged ion [M+2H]^{2+} and the single charge ion [M+H]^+ were used as precursors in the collision cell. Following the structure elucidation, we compared the distribution of isotopologue m/z values of [M+H]^+ PVD ions in the secretion under the different nutrient growth conditions.

The extracellular amount of total secreted PVD siderophores was determined in pH-adjusted (pH 7) bacterial supernatants as done previously using absorbance measurements at 400 nm. A PVD standard (Sigma Aldrich) isolated from a *P. fluorescens* strain was used for quantitation, based on previous LC-MS verification that structures of PVD from the different *Pseudomonas* species have the same dihydroxyquinoline-type chromophore. We determined maximum yield of secreted PVD or PVD excretion rate (µmol g_{CDW}^{-1} h^{-1}) from regression analysis of time-course data taken from exponentially-growing liquid cultures with glucose alone, fructose alone, succinate alone, malate alone, or glucose with benzoate.

**Mineral Dissolution experiments.** Mineral dissolution experiments of three Fe-bearing minerals were conducted with both Fe-replete and Fe-limited bacterial secretions following growth on different substrates (glucose alone, succinate alone, or a mixture with glucose and benzoate). These experiments (10 mL) were performed in 50-mL propylene tubes with the following Fe (oxyhydr)oxide minerals at 1 g L^{-1}: hematite (Fe_{2}O_{3}), goethite [FeO(OH)], or magnetite (Fe_{3}O_{4}). Cell-free supernatants (from three biological replicates) were obtained at the end of exponential phase by centrifugation (~20,000 g, 5 min) followed by filtering. Prior to the mineral addition, all solutions were adjusted to pH 7.5. Experiments were conducted in an incubator shaker as described above for the cell culturing, and the tubes were wrapped in aluminum foil to exclude light-mediated reactions. After 100-h reaction time, samples were centrifuged, filtered and was adjusted to pH 4.5 with acetate buffer prior to analysis. The dissolved Fe concentration was measured via ICP-AES analysis, with a detection limit of 43.7 nM. Metal concentrations were blank-corrected with control experiments conducted with cell extracts without minerals.
Protein extraction. At mid-exponential phase (OD\textsubscript{600} = 0.5-0.7), sample aliquots (25-mL) were harvested from biological replicates (n = 3) and centrifuged (at 4 °C). Cell pellets were washed twice with high-purity deionized (Milli-Q grade) water and store at 20 °C for further use. Prior to analysis, pellets were thawed, resuspended in 4-mL of 2.5 mM phosphate buffer (pH 7) and vortexed. Cell lysis was then conducted in a French Pressure cell press (Thermo Spectronics, Waltham MA) with double 3-min cycles at 900 psi. Following cell lysis, aliquots were centrifuged (16000g, 10-min, 4 °C), and supernatant (2 mL) was concentrated to 200-µL using a Spin-X® UF 500 Concentrator (Corning, Tewksbury, MA). Proteins were then precipitated overnight at -20 °C in 9 volumes of cold 10% trichloroacetic acid in acetone and centrifuged (16000g, 10-min, 4 °C) to form a protein pellet. The pellets were washed twice in cold acetone following centrifugation, and urea (7 M) was added to initiate protein dissolution. Dissolved pellets were then diluted in 50 mM triethylammonium bicarbonate (TEAB) buffer (pH 8). The urea contamination in the final sample was less than 1 M. 

Protein concentration was determined by Bradford assay and further quantified by running a precast NOVEX 12% Tris/Glycine mini-gel (Invitrogen Carlsbad, CA) along with an E. coli lysate gradient (2, 5, 10, 20 µg/lane). Visualization for the SDS gel was carried out using colloidal Coomassie blue stain (Invitrogen) imaged by Typhoon 9400 scanner followed by ImageQuant TL 8.1 (GE Healthcare). Further processing was performed according to Thermo Scientific’s TMT Mass Tagging Kits and Reagent protocol with minor modification\textsuperscript{12,13}. Briefly, a total of 200 µg protein of each sample in 70-µL of 7 M urea and 50 mM TEAB was reduced [with 11 mM tris (2-carboxyethyl)phosphine], 1 h], alkylated [with 37 mM iodoacetamide, 1 h in the dark] and quenched [with 40 mM dithiothreitol (DTT)]. Following quenching, dilutions with 240-µL of 100 mM TEAB were carried out, and samples were digested (with 18 µg trypsin, 18 h, 35 °C); an additional 2 µg trypsin was then added and incubated for 4 h. The TMT 10-plex labels were reconstituted with 45-µL of anhydrous ACN prior to labeling and added with 1:2 ratio to each of the tryptic digest samples. In order to achieve TMT labeling, the samples were incubated for 1 h at room temperature. The peptides from 10 samples were mixed with each tag of TMT10-plex separately. After mixing 1-µL aliquots from each sample and desalting with SCX ziptip (Millipore, Billerica, MA), the label incorporation was checked using Orbitrap Fusion (Thermo-Fisher Scientific, San Jose, CA). Equal amounts of peptides from the 10 digested samples were then pooled together and evaporated to 200-µL and subsequently purified by solid-phase extraction on Sep-Pak Cartridges (Waters, Milford, MA) followed by drying at reduced pressure in a Speed-Vac concentrator (Savant).
Proteomics analysis using nano-scale reverse phase LC-MS. First dimensional fractionation was carried out on the above described tryptic peptides via high-pH reverse-phase chromatography using a Dionex UltiMate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA) as reported previously\textsuperscript{12,13}. Briefly, the TMT 10-plex tagged tryptic peptides were reconstituted in 20 mM ammonium formate at pH 9.5 (eluent A) and loaded onto an XTerra MS C18 column (3.5 µm, 2.1x 150 mm) from Waters, (Milford, MA). The chromatography was carried out using a gradient from 10-45% eluent B over 30-min at a flow rate 200-µL/min. Forty-eight fractions were collected at 1-min intervals, and based on the absorbance at 214 nm and with multiple fraction concatenation strategy\textsuperscript{12}, were pooled into a total of 10 fractions. Ten percent of each fraction was pooled, dried, and reconstituted in 120 µL of 2% ACN/0.5% FA for low pH nano-scale LC-MS/MS analysis. The remaining 90% of each sample was pooled into 5 fractions, dried and subjected to titanium dioxide (TiO2) enrichment using a TiO2 Mag Sepharose kit (from GE Healthcare). Specifically, the TMT 6-plex tagged tryptic peptides were reconstituted in 400 µL of binding buffer (1 M glycolic acid in 80% ACN, 5% TFA). The TiO2 slurry (75 µL) was used and incubated with the sample for 30-min at 1,800 rpm vortex. After washing the beads with washing buffer (80% acetonitrile, 1%TFA), the phosphopeptides were eluted with 100 µL of elution buffer (5% ammonium hydroxide) twice. The eluted fractions were subsequently dried and reconstituted in 25 µL of 0.5% formic acid (FA) for nano-scale LC-MS/MS analysis using an Orbitrap Fusion (Thermo-Fisher Scientific, San Jose, CA) mass spectrometer (MS) equipped with a nanospray Flex Ion Source similar to previous reports\textsuperscript{14,15}.

The MS was coupled to an UltiMate3000 RS-LC nano HPLC (Thermo Scientific, Sunnyvale, CA). Specific amounts of the reconstituted fraction (2-3 µL for global proteomics fractions and 5-8 µL for enriched phosphor-fractions) was injected onto a PepMap C-18 RP nano-trap column (100 µm x 20 mm dimensions, 3-µm particle size, Dionex) at 20 µL/min flow rate for concentration and on-line desalting. Bound peptides were separated on a PepMap C-18 RP column (75 µm x 25 cm, 3.5 µm particle size) by elution at a flow rate of 300 nL/min using a gradient of 5% to 35% acetonitrile (ACN) in 0.1% formic acid. Prior to subsequent runs, the column was re-equilibrated with 5% ACN-0.1% FA for 25 min. The Orbitrap Fusion was operated in positive ion mode with the nano spray voltage set at 1.6 kV and the source temperature at 275 °C. The Fourier transform (FT), ion-trap (IT), and quadrupole mass analyzers were externally calibrated; the MS was operated in the data-dependent acquisition (DDA) mode, and the FT mass analyzer was used for survey scans to select precursor ions. Subsequently, 3 second top speed data-dependent HCD-MS/MS scans were run using a normalized collision energy of 37.5% for all precursor ions with 2-7 charges per ion. The threshold ion count was > 10,000. MS survey scans were conducted at a resolving power of 120,000 FWHM (measured at m/z 200) for all the masses in the range of m/z 400-1600 using the settings AGC = 3e5 and Max IT = 50 ms. The MS/MS scans were conducted using higher-energy collision dissociation (HCD) at 60,000 FWHM resolution for the mass range m/z 105-2000. The AGC setting, Max IT, and Q isolation window were set to 1e5, 120 ms and ±1.6 Da, respectively. The dynamic exclusion parameters were set at 1 with a 45 second exclusion duration with a ±10 ppm exclusion mass width. All data were acquired using the Xcalibur 3.0 operation software and Orbitrap Fusion Tune 2.0 (Thermo-Fisher Scientific).
Data interpretation and protein identification. Raw spectral data (MS and MS/MS) were processed and searched using Proteome Discoverer 2.2 (Thermo) with Sequest HT. Data was queried using the Pseudomonas_putidaKT2440.fasta database containing 5528 entries (downloaded June 12, 2017). The default search settings used for 10-plex TMT quantitative processing and protein identification were as follows: tryptic proteolysis, allowing two missed cleavages, with fixed carbamidomethyl modification of cysteine, fixed 10-plex TMT modifications of lysine and N-terminal amines and variable modifications of methionine oxidation and deamidation of asparagine/glutamine residues. The peptide and fragment mass tolerance values were 10 ppm and 50 mDa, respectively. The identified peptides were filtered for a maximum 1% False Discovery Rate using the Benjamini Hochberg procedure\textsuperscript{16} incorporated in the Percolator algorithm in PD 2.2 with the peptide confidence was set to high. The TMT10-plex quantification method within the PD 2.2 software was used to calculate the reporter ratios with mass tolerance ±10 ppm without applying the isotopic correction factors. The peptide spectra containing all reporter ions were designated as quantifiable and used for peptide/protein quantitation. Summed intensity normalization was used to estimate expression ratios. Expected ratio distortion due to the co-isolation of near isobaric precursor ions was minimized by applying a co-isolation filter of 50%.

Statistical Analysis. Statistical analysis of growth, physiological, and metabolomics experiments were carried out using One-way Analysis of Variance (ANOVA) test. A Tukey analysis was subsequently used as a post hoc analysis. Statistically-significant difference was determined at \( p \leq 0.05 \). For the proteomics data, the thresholds for significant change was estimated by carrying out log2 transformations of the expression ratios from each of the data sets and consequently fitting them to 60 different statistical distributions using the program EasyFit (MathWave Technologies). The goodness of fit was determined by the Kalmogrov/Smirnov, Anderson Darling, and Chi-Squared tests\textsuperscript{17–19}. In each case the best fit was determined to be to the four- parameter Dagum distribution\textsuperscript{20}. The 95% confidence intervals were estimated by generating distributions randomly using the exact parameters of the experimentally derived distributions. to evaluate the difference between the means of the samples being compared, a two tailed heteroscedastic t-test was conducted. An expression ratio was considered to have changed significantly if the magnitude of the change was greater than what would be expected to be observed by chance with a probability of 0.05, and if its associated p-value was < 0.01.
Data availability. Source data sets are uploaded in open-source repositories for free accessibility. All MS proteomics data are deposited in the ProteomeXchange (data set identifier PXD013605).

Uploaded on the MetaboLights database (study identifier MTBLS1715) are the LC-MS data along with the methodological details for the intracellular metabolite labeling experiments with *P. putida* KT2440 grown on [U-13C6] glucose and unlabeled benzoate under Fe-replete, intermediate-Fe limited, Fe-limited Phase 1 and Fe-limited Phase 2 conditions. Also uploaded on Metabolites database are the following datasets obtained with *P. putida* KT2440: kinetic labeling in glucose-grown and succinate-grown Fe-replete and intermediate Fe-limited cells, long-term labeling with [1,4-13C2-succinate] in Fe-replete and intermediate Fe-limited cells, long-term labeling with [U-13C4-succinate] and unlabeled glucose in Fe-replete and intermediate Fe-limited cells, long-term labeling with [U-13C6-glucose] and unlabeled citrate in intermediate Fe-limited cells, and mass spectra of major pyoverdine ion in Fe-limited cells grown on glucose alone, succinate alone, citrate alone, 13C-glucose:succinate, or 13C-glucose:citrate. To access these files on MetaboLights: 1. Please visit [http://www.ebi.ac.uk/metabolights/](http://www.ebi.ac.uk/metabolights/), 2. Navigate to the search bar on the top-right of the page, and 3. Enter study identifier MTBLS1715.
Table S2. Fold changes of protein abundance in glucose:benzoate grown *P. putida* KT2440 cells grown under Fe-limited conditions with respect to cells grown under Fe-replete conditions. Grey boxes indicate the lack of quantifiable proteins. Refer to the legends of Fig. 1 in the main text for the metabolite abbreviations. All the data obtained from biological replicates (n=3) are shown.

| Reactions                        | ORF number | Proteins | Protein abundance (Average) (p-values) (-Fe/+Fe) |
|----------------------------------|------------|----------|-----------------------------------------------|
| Gluc_{peri} \rightarrow Gluc_{peri} | PP_1019    | oprB-1   | 0.826 0.303                                   |
| Gluc_{peri} \rightarrow Gluc    | PP_1015    | gtsA     |                                               |
| Gluc_{peri} \rightarrow Gluc    | PP_1016    | gtsB     | 0.62 0.011                                    |
| Gluc_{peri} \rightarrow Gluc    | PP_1017    | gtsC     |                                               |
| Gluc_{peri} \rightarrow Gluc    | PP_1018    | gtsD     |                                               |
| Gluc \rightarrow G6P            | PP_1444    | gcd      | 1.171 0.406                                   |
| Gluc_{peri} \rightarrow Glucn_{peri} | PP_3417   | gntP     | 0.861 0.438                                   |
| Glucn_{peri} \rightarrow Glucn  | PP_3382    | PP3382   | 0.795 0.175                                   |
| Glucn_{peri} \rightarrow 6-PG   | PP_3384    | PP3384   | 0.903 0.580                                   |
| Glucn_{peri} \rightarrow 2KGlcun_{peri} | PP_3383   | PP3383  |                                               |
| Glucn_{peri} \rightarrow 2KGlcun_{peri} | PP_3377   | kguT     |                                               |
| Glucn_{peri} \rightarrow 2KGlcun_{peri} | PP_3378   | kguK     | 0.896 0.508                                   |
| 2KGlcun_{peri} \rightarrow 2KGlcun_{peri} | PP_3376   | kguD     |                                               |
| 2-KGlucP \rightarrow 2KGlcnP | PP_1011    | glk      | 0.869 0.362                                   |
| 2KGlcnP \rightarrow 6-PG        | PP_3416    | gnuK     | 0.797 0.190                                   |
| G6P \rightarrow 6-PG            | PP_1024    | pgl      | 0.709 0.077                                   |
| G6P \rightarrow 6-PG            | PP_4042    | zwfB     |                                               |
| G6P \rightarrow 6-PG            | PP_5351    | zwf      |                                               |
| G6P \rightarrow 6-PG            | PP_1022    | zwfA     |                                               |
| 6-PG \rightarrow KDPG           | PP_1010    | edd      |                                               |
| KDPG \rightarrow Pyruvate + GAP | PP_1024    | eda      | 0.715 0.085                                   |
| GAP \rightarrow 1,3-bisPG       | PP_2149    | gap-2    | 0.817 0.319                                   |
| GAP \rightarrow 1,3-bisPG       | PP_1009    | gap-1    | 0.781 0.214                                   |
| 1,3-bisPG \rightarrow 3-PG     | PP_4963    | pgk      | 0.93 0.775                                    |
| 2-PG \leftrightarrow 3-PG       | PP_5056    | pgm      |                                               |
| 2-PG \rightarrow PEP            | PP_1612    | eno      |                                               |
| Pyruvate \rightarrow PEP        | PP_1362    | pykA     | 1.07 0.632                                    |
| PEP \rightarrow Pyruvate        | PP_4301    | pykF     | 0.964 0.767                                   |
| PEP \rightarrow Pyruvate        | PP_2082    | ppsA     | 1.022 0.820                                   |
| DHAP \rightarrow GAP            | PP_4715    | tpiA     |                                               |
| FBP \rightarrow DHAP + GAP     | PP_4960    | fda      | 1.049 0.713                                   |
| FBP \rightarrow F6P             | PP_5040    | fbp      |                                               |
| G6P \rightarrow F6P             | PP_1808    | pgi1     |                                               |
| G6P \rightarrow F6P             | PP_4701    | pgi2     | 0.926 0.754                                   |
| 6-PG \rightarrow Ru5P + CO2    | PP_4043    | gnd      |                                               |
| Ru5P \rightarrow R5P            | PP_5150    | rpiA     |                                               |
| Ru5P \leftrightarrow Xu5P       | PP_0415    | rpe      | 0.865 0.410                                   |
| Xu5P + R5P \rightarrow GAP + S7P | PP_4965   | tktA     | 0.902 0.650                                   |
| S7P + GAP \rightarrow E4P + F6P | PP_2168    | tal      | 1.3 0.124                                     |
| F6P + GAP \rightarrow E4P + Xu5P | PP_4965   | tktA     | 0.902 0.650                                   |
| Pyruvate \rightarrow Acetyl-CoA + CO2 | PP_0339 | aceE | 0.973 0.966                                   |
| Pyruvate \rightarrow Acetyl-CoA + CO2 | PP_0338 | aceF | 0.981 0.999                                   |
| OAA \rightarrow Pyruvate + CO2  | PP_5347    | accC2    | 0.786 0.227                                   |
| Pyruvate + CO2 \rightarrow OAA  | PP_5346    | oadA     | 0.684 0.049                                   |
| OAA \rightarrow PEP + CO2      | PP_1389    | PP1389   | 1.188 0.368                                   |
| OAA \rightarrow PEP + CO2      | PP_1505    | ppc      | 1.248 0.219                                   |
| OAA + Acetyl-CoA \rightarrow Citrate | PP_4194 | gtaA | 0.899 0.634                                   |
| Reaction                                      | PR | Description | Value | p-Value |
|-----------------------------------------------|----|-------------|-------|---------|
| Cis-aconitate → Isocitrate                    |    |             |       |         |
| Isocitrate → α-KG                            |    |             |       |         |
| Isocitrate → α-KG                            |    |             |       |         |
| α-KG → Succinyl-CoA + CO₂                    |    |             |       |         |
| α-KG → Succinyl-CoA + CO₂                    |    |             |       |         |
| α-KG → Succinyl-CoA + CO₂                    |    |             |       |         |
| α-KG → Succinyl-CoA + CO₂                    |    |             |       |         |
| Succinyl-CoA → Succinate                     |    |             |       |         |
| Succinyl-CoA → Succinate                     |    |             |       |         |
| Succinate → Fumarate                         |    |             |       |         |
| Succinate → Fumarate                         |    |             |       |         |
| Succinate → Fumarate                         |    |             |       |         |
| Fumarate → Malate                            |    |             |       |         |
| Malate + NAD(P)+ → OAA + NAD(P)H + H+        |    |             |       |         |
| Malate + a quinone → oxaloacetate + a quinol |    |             |       |         |
| Malate + a quinone → oxaloacetate + a quinol |    |             |       |         |
| Malate + a quinone → oxaloacetate + a quinol |    |             |       |         |
| Malate → Pyruvate + CO₂                      |    |             |       |         |
| Benzoate_ext → Benzoate_peri                 |    |             |       |         |
| Benzoate_ext → Benzoate_peri                 |    |             |       |         |
| Benzoate_peri → Benzoate                     |    |             |       |         |
| Benzoate → Benz-1,2-diol                      |    |             |       |         |
| Benzoate → Benz-1,2-diol                      |    |             |       |         |
| Benz-1,2-diol → Catechol + CO₂               |    |             |       |         |
| Catechol → cis,cis-Muconate                  |    |             |       |         |
| Catechol → cis,cis-Muconate                  |    |             |       |         |
| cis,cis-Muconate → Muconolactone             |    |             |       |         |
| Muconolactone → 4-ketoacidipate enol-lactone |    |             |       |         |
| 4-ketoacidipate enol-lactone → 3-ketoacidipate|    |             |       |         |
| 3-ketoacidipate → 3-ketoacidipyl-CoA          |    |             |       |         |
| 3-ketoacidipyl-CoA → Succinyl-CoA + Acetyl-CoA|    |             |       |         |
| Citrate → Glyoxylate + Succinate             |    |             |       |         |
| Glyoxylate + Acetyl-CoA → Malate              |    |             |       |         |
Estimation of CO₂ production. To close the carbon balance within our systems, we estimated the amount of CO₂ generated in the nine CO₂ producing metabolic reactions within substrate catabolism and central carbon metabolism. We computed these estimations for *P. putida* KT2440 cells grown on 1:1 glucose:benzoate mixture under Fe-replete, intermediate Fe-limited and severe Fe-limited conditions. For the Fe-replete condition, we calculated the CO₂ produced based on a previous metabolic flux analysis performed on the same strain, growth media and Fe condition³. The estimations of CO₂ produced in the intermediate Fe-limited condition and first phase of the severe Fe-limited condition was based on the intracellular labeling patterns of *P. putida* cells grown on [U-¹³C₆]-glucose with unlabeled benzoate, as well as flux ratio analysis (described above) for their respective Fe conditions. For the second phase of the severe Fe-limited condition, CO₂ generated was computed based on a reported metabolic flux analysis performed on *P. putida* KT2440 cells grown on Fe-limited glucose-only media⁴. When relevant, carbon effluxes (metabolite secretion, PVD production, and biomass production) were subtracted from the model at the respective metabolite nodes. For instance, the gluconate secretion rate was reflected by the loss of gluconate from the periplasm, and subsequent reduced flux of gluconate towards 6-phosphogluconate. Additionally, the carbon consumption rate was used to obtain CO₂ produced during substrate catabolism (Benz-1,2-diol → Catechol + CO₂) (Table S2).

**Table S3.** List of nine CO₂-producing and CO₂-consuming metabolic reactions considered for the CO₂ efflux estimation (see Fig. 7A in the main text).

| Pathway                                  | Reactions                      |
|------------------------------------------|--------------------------------|
| Pentose Phosphate Pathway                | 6-PG → Ru5P + CO₂              |
| Downstream Entner-Doudoroff Pathway      | Pyruvate → Acetyl-CoA + CO₂    |
|                                          | OAA → Pyruvate + CO₂           |
|                                          | Pyruvate + CO₂ → OAA           |
|                                          | OAA → PEP + CO₂                |
|                                          | Malate → Pyruvate + CO₂        |
| Gluconeogenesis/Anaplerosis              | α-KG → Succinyl-CoA + CO₂      |
|                                          | Isocitrate → α-KG              |
| Tricarboxylic Acid Cycle                 | Benz-1,2-diol → Catechol + CO₂ |
| Initial Aromatic Substrate Catabolism    |                                |
Table S4. Secretion rates of organic acid metabolites during growth of *P. putida* KT2440 on the glucose:benzoate mixture under the Fe-replete, intermediate Fe-limited, and severe Fe-limited conditions. Grey boxes indicate the lack of quantifiable metabolites in the extracellular medium. All the data obtained from biological replicates (n=3) are shown.

|                      | Fe-replete          | Intermediate Fe-limited | Fe-limited Phase 1 | Fe-limited Phase 2 |
|----------------------|---------------------|-------------------------|--------------------|--------------------|
| Gluconate (in mmol C gCDW⁻¹ h⁻¹) | 0.29 ± 0.027        | 1.83 ± 0.220            | 0.10 ± 0.03        | 13.9 ± 1.8         |
| Other organic acids (µmol C gCDW⁻¹ h⁻¹) |                     |                         |                    |                    |
| Succinate            | 35.4 ± 4.05         | 32.8±3.99               | 12.5 ± 2.64        |                    |
| Oxaloacetate         |                      | 6.26±1.90               | 35.4 ± 13.9        |                    |
| Fumarate             | 0.95 ± 0.20         | 1.34 ± 0.59             |                    |                    |
| Pyruvate             |                      | 8.79 ± 3.33             |                    |                    |
| α-ketoglutarate      | 95.9 ± 8.16         | 18.5 ± 4.25             |                    |                    |
Fig. S1. A, MS full scan total ion chromatogram (black line) and extracted ion chromatogram (red line) of chromophoric fragment (m/z 204.0773) of all ion fragmentation obtained from extracellular media of P. putida KT2440 grown on, from top to bottom, succinate, citrate, and glucose. B, Chemical structures of the four major PVDs characterized; these structures are similar in terms of the metabolite precursors. In B, the structural differences (highlighted in blue) are the carboxylic acid or amide versions of the same side chain (succinic acid or succinimide) or linear versus cyclic version of the same amino acid (ornithine).
**Fig. S2.** A, Chemical structure of PVDs characterized in the bacterial secretion of *P. protegens* Pf-5, *P. putida* S12, and *P. putida* KT2440, and the biosynthetic investments of the respective metabolite precursors. B, (left) Growth rate and (right) consumption rate for succinate-grown *P. putida* KT2440 under Fe-replete [(+)Fe] and Fe-limiting [(-)Fe] conditions. Color code for A: pink denotes the pentose phosphate (PP) pathway, yellow represents the downstream Entner-Doudoroff pathway (ED) pathway and blue represents the tricarboxylic acid (TCA) cycle. The abbreviations are as follows: succinate, Succ; erythrose 4-phosphate, E4P; 3-phosphoglycerate, 3-PG; phosphoenolpyruvate, PEP; pyruvate, Pyr; oxaloacetate, OAA; α-ketoglutarate, α-KG. Data (average ± standard deviation) for B are from three biological replicates.
Fig. S3. Investigation of Fe-dependent substrate incorporation through the assimilation of [U-13C6]-glucose and unlabeled benzoate into the intracellular metabolites of P. putida KT2440 on, from left to right, Fe-replete [(+) Fe], intermediate-Fe limited [(Int(-)Fe), Fe-limited Phase 1 [(-)Fe P1] and Fe-limited Phase 2 [(-)Fe P2] Glucose:Benzoate mixture. Carbon mapping (A) and metabolite labeling (B) in the periplasm, the Embden–Meyerhof–Parnas (EMP) pathway, the Entner–Doudoroff (ED) pathway and the tricarboxylic acid (TCA) cycle. Labeling patterns: nonlabeled (light blue), singly labeled (orange), doubly labeled (yellow), triply labeled (green), quadruply labeled (pink), quintuply labeled (purple), sextuply labeled (dark blue) and septuply labeled (grey). Refer to the legends of Fig. 1 in the main text for the metabolite abbreviations. Isotopologue data (mean ± s.d.) were from biological replicates (n = 3).
**Fig. S4.** A, Gluconate secretion rates of *P. putida* KT2440 cells grown on, from top to bottom, Fe-replete [(+) Fe], intermediate-Fe limited [(Int(-)Fe], Fe-limited Phase 1 [(-)Fe P1] and Fe-limited Phase 2 [(-)Fe P2] Glucose:Benzoate mixture. B, $^{13}$C-labeled fractions of metabolites participating in the biosynthesis of PVD. Color code for pathway designation: pentose phosphate (PP) pathway in red, downstream Entner-Doudoroff pathway (ED) in light orange, tricarboxylic acid (TCA) cycle in blue. Refer to the legends of Fig. 1 for the metabolite abbreviations. All the data obtained from biological replicates (n=3) are shown.
Fig. S5. PVD production rates (mmol C g<sub>cdw</sub>⁻¹ hr⁻¹) and predictive fluxes from metabolite precursors. PVD production rates are provided for <i>P. putida</i> KT2440 cells grown on glucose-only, glucose:benzoate Fe-limited [(−)Fe] or intermediate-Fe (Int-Fe) nutrient medium. P1 and P2 refer, respectively, to phase 1 (benzoate-only) and phase 2 (glucose-only) of the substrate consumption pattern.
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