Supporting Information

Mechanism of Benzylsuccinate Synthase Probed by Substrate and Isotope Exchange

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Materials and Methods

All experiments were performed at 4 °C in an anaerobic glove box (Coy Laboratory Products) under a 3% H_2/ 97% N_2 atmosphere, with an oxygen concentration no higher than 5 ppm. Unless otherwise stated all solvents and chemicals used were of commercially available analytical grade. Deoxygenation of solvents was effected by bubbling with argon for 30 - 45 minutes. The protein concentration was determined by the Bradford method.[1]

Growth of *Thauera Aromatica* strain T

T. Aromatica strain T was grown at 30 °C under a N_2 atmosphere in the minimal medium described by Coschigano,[2] containing 0.5 mM toluene as the carbon source and 5 mM nitrate as an electron acceptor. Toluene and nitrate were maintained at their approximate starting concentrations by additional feedings during growth. Cells were grown to an OD_{600} of ~ 0.9 and harvested under anaerobic conditions by centrifugation at 6,000 *g* for 30 min in sealed nitrogen-flushed centrifuge tubes. The cell pellet was suspended in degassed buffer containing 10% glycerol, 0.5 mM of sodium dithionite and the cells were pelleted again at 6,000 g for 30 min. These cell pellets were frozen with liquid nitrogen and stored at −80 °C.

Preparation of cell-free extracts

Typically, to study the forward reaction (fumaric acid + toluene/*p*-cresol → benzylsuccinic acid/(4-hydroxybenzyl)-succinic acid, one volume of cell pellet was resuspended in 2.5 volumes of a buffer comprising 40 mM tris/Cl, 10 mM fumarate and 10% glycerol at pH 8.0. The resuspended cells were sonicated at 0 °C with four bursts (13 W × 30 s), with 1 min rest between each burst to allow the solution to cool down. The lysate was centrifuged at 14, 000 *g* at 4 °C for 10 min to remove the cell debris and the supernatant containing the soluble proteins was used immediately. To study the backward reaction (benzylsuccinic acid/(4-hydroxybenzyl)-succinic acid → fumaric acid + toluene/*p*-cresol), one volume of cell pellet was resuspended in 2.5 volumes of a buffer comprising 40 mM tris/Cl, 10 mM benzylsuccinic acid (racemic mixture)/ ~8 mM (4-hydroxybenzyl)-succinic acid and 10% glycerol at pH 8.0 and the lysate prepared as described above.

HPLC separation of benzylsuccinic acid and (4-hydroxybenzyl)-succinic acid.

Chromatography was performed at room temperature with detection at 254 nm using a Vydac reverse-phase C_{18} column (5 μM, 4.6×250). The column was equilibrated in 0.3% formic acid and compounds were eluted with an ascending gradient of CH_3CN: 0-35% over 6 mL; 35-47% over 12 mL; and 47-100% over 5 mL at a flow rate of 1 mL/min. Using this gradient, benzylsuccinic acid eluted at 11.5 min and (4-hydroxybenzyl)-succinic acid eluted at 9.7 min. The peaks containing these products were collected and analyzed by ESI-MS spectroscopy in negative ion mode to verify their identity.

Enzymatic synthesis of the d_8-benzylsuccinic acid and (4-hydroxybenzyl)-succinic acid

To 2.0 mL of the cell extract was added 1.0 mL of d_8-toluene aqueous solution, (5 μL per mL, 5.75 mM) and the reaction mixture was incubated at 4 °C. After reacting for 24 hr, 0.5 mL of 4 M HCl was introduced to quench the reaction. The protein residue was then centrifuged at 14, 000 *g* at ambient temperature for 15 min, and the resulting aqueous solution was analyzed by reverse phase HPLC. The d_8-benzylsuccinic acid, which elutes at 11.5 min using the solvent system described above, was collected and lyophilized for future use. (4-hydroxybenzyl)-succinic acid was synthesized by the same method except that *p*-cresol (16 mM final concentration) was substituted for toluene in the reaction. (4-hydroxybenzyl)-succinic acid eluted at 9.7 min from the HPLC column, and was collected and lyophilized and stored under inert atmosphere (Figure S1). ESI-MS confirmed the identity of this compound: calculated mass for $C_{11}H_{12}O_5$(M-) = 223.07; found: 223.0

Synthesis of (4-hydroxybenzyl)-succinic acid from benzylsuccinic acid and *p*-cresol.

To 500 μL of the cell extract was added 250 μL (47.8 mM) *p*-cresol aqueous solution and the reaction mixture was incubated at 4 °C. This mixture was allowed to react for 24 hr and quenched by addition of 200 μL 4 M HCl. The protein residue was then removed by centrifugation at 14, 000 *g* at ambient temperature for 15 min, and the subsequent aqueous solution was analyzed by reverse phase HPLC. Formation of the (4-hydroxybenzyl)-succinic acid was confirmed by collecting the corresponding HPLC peak and analyzing it by ESI/MS.

Synthesis of benzylsuccinic acid from (4-hydroxybenzyl)-succinic acid and toluene.

To 500 μL of cell-free extract was added 500 μL of an aqueous solution containing 5.75 mM toluene (50 μL/L) and ~ 8 mM (4-hydroxybenzyl)-succinate, and the reaction mixture was incubated at 4 °C. This mixture was allowed to react for 24 hr and quenched by addition of 200 μL 4 M HCl. The protein residue was removed by centrifugation at 14, 000 *g* at ambient temperature for 15 min, and the resulting aqueous solution was analyzed by reverse phase HPLC. Formation of the benzylsuccinic acid was confirmed by collecting the corresponding HPLC peak and analyzing by ESI/MS in negative ion mode (Figure S1). Mass calculated for $C_{11}H_{12}O_4$(M-) = 207.07, found: 207.0; Mass calculated for $C_{11}H_4D_8O_4$(M-) 215.12, found: 215.1.

Control reactions: synthesis of (4-hydroxybenzyl)-succinic acid in the absence of co-substrate.

To rule out the possibility that fumaric acid present in the cell-free extract was contributing to the exchange reactions, a control reaction was performed with only *p*-cresol in the absence of externally added benzylsuccinic acid. Cell-free extracts were prepared as described above except that the buffer lacked either substrate. To 300 μL of cell free extract was added an equal volume of lysis buffer containing *p*-cresol (47.8 mM). The reaction was incubated at 4 °C for 24 hrs before quenching with 200 μL 4 M HCl. The protein residue was then spun down at 14, 000 *g* at ambient temperature for 15 min, and the subsequent aqueous solution was analyzed by reverse phase HPLC. The HPLC peak corresponding to (4-hydroxybenzyl)-succinic acid was collected and analyzed by ESI/MS to confirm its identity. The amount of (4-hydroxybenzyl)-succinic acid produced in this reaction was only ~ 0.5 % of that produced in a similar reaction containing 10 mM benzylsuccinic acid and overlaid with 400 μL oxygen-free hexane (Figure S2).

Determination of the rate of (4-hydroxybenzyl)-succinate formation from fumarate and *p*-cresol.

To 1000 μL of cell-free extract prepared as described above was added 500 μL (47.8 mM, 5 μL/ ml) *p*-cresol dissolved 40 mM Tris/Cl buffer containing, 10 mM fumaric acid and 10% glycerol at pH 8.0. The reaction mixture was incubated at 4 °C in an anaerobic glove box. After reacting for 5, 10, 15, 20, 25 and 30 min, 200 μL solution was removed and quenched by addition of 50 μL 4 M HCl. The protein residue was then removed by centrifugation at 14, 000 *g* for 15 min, and the resulting aqueous solutions were analyzed by reverse phase HPLC. The reaction rate was calculated by integration of peak areas after correcting the base line for background absorbance. Authentic (4-hydroxybenzyl)-succinic acid is not commercially available, therefore authentic 3-(4-hydroxyphenyl)-propionic acid, which has an indistinguishable u.v. spectrum, was used to construct a standard curve (Figure S3 and S4).

Determination of the rate of (4-hydroxybenzyl)-succinate formation from benzylsuccinic acid and *p*-cresol.

To 1000 μL of cell-free extract prepared as described above was added 500 μL (47.8 mM, 5 μL/ ml) *p*-cresol dissolved 40 mM Tris/Cl buffer containing, 10 mM benzylsuccinic acid and 10% glycerol at pH 8.0. After addition of 500 μL O_2-free hexane, the mixture was gently stirred to allow the reaction to occur. After reacting for 12, 21, 27 and 31 hr, 200 μL of the reaction mixture was removed and quenched by addition of 50 μL 4 M HCl. The amount of (4-hydroxybenzyl)-succinic acid formed was analyzed by reverse phase HPLC as described above.

Analysis of deuterium-labeled toluene formed from the reverse enzymatic reaction.

Cell-free extracts were prepared as described above in 40 mM tris/Cl buffer containing ~ 8 mM d_8-(R)-benzylsuccinate and 10% glycerol at pH 8.0. To 300 μL of cell-free extract, 300 μL of either the same buffer (lacking benzylsuccinate), or 300 μL of buffer containing *p*-cresol (5 μL/1 mL), were added, and each reaction overlaid with 400 μL of oxygen-free hexane. The reactions were allowed to stir at 4 °C for 24 hrs before 200 μL 4 M HCl solutions were added respectively to quench both reactions. The hexane

layers were separated and collected. The formation of C_7D_8 was confirmed by GC-MS analysis (Figure S5).

References

1) Bradford, M. M. *Analytical Biochemistry* **1976**, 72, 248-254.
2) Coschigano, P. W. *Applied and Environmental Microbiology* **2000**, *66*, 1147-1151.

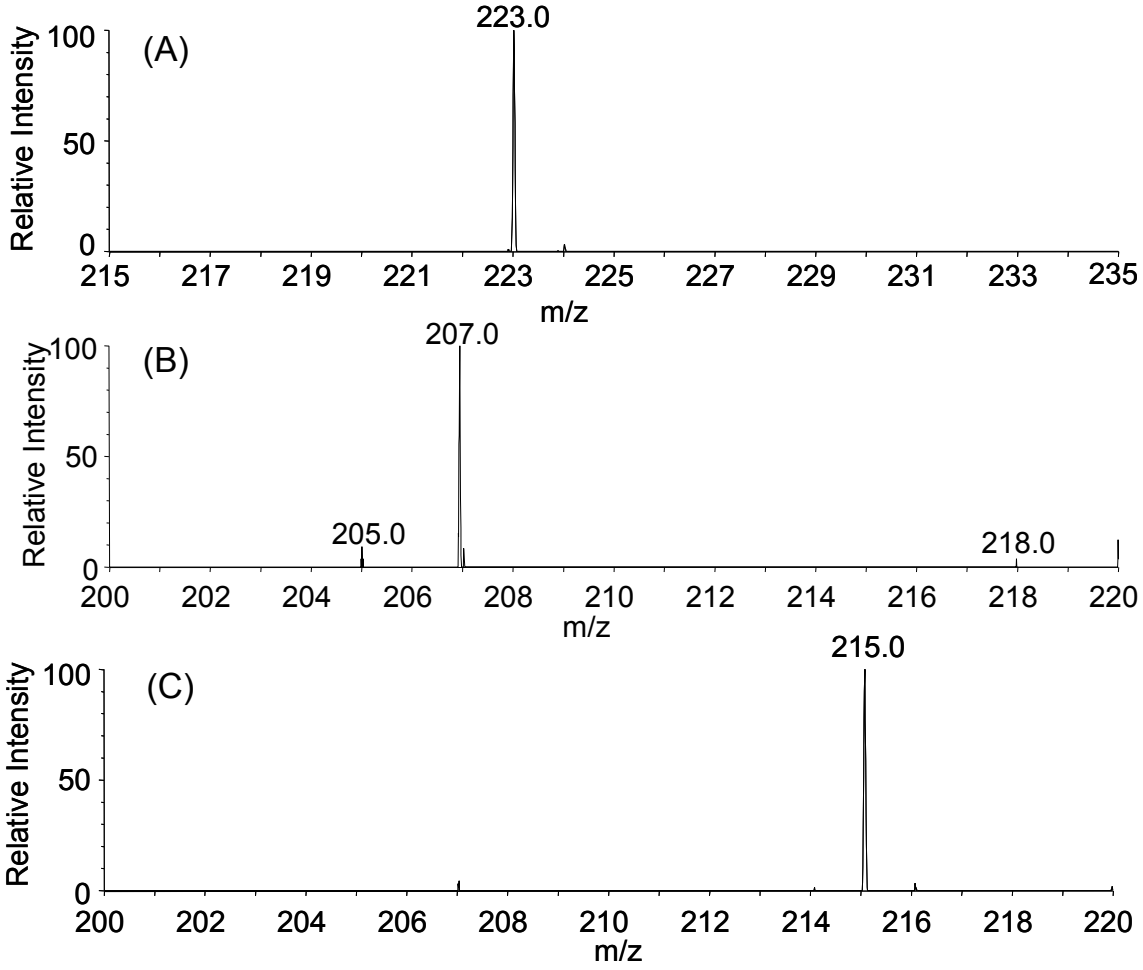

Figure S1. Confirmation of the identities of products synthesized by BSS. (A) Mass spectrum of (4-hydroxybenzyl)-succinic acid (HBS). (B) Mass spectrum of benzylsuccinic acid. (C) Mass spectrum of d_8-benzylsuccinic acid.

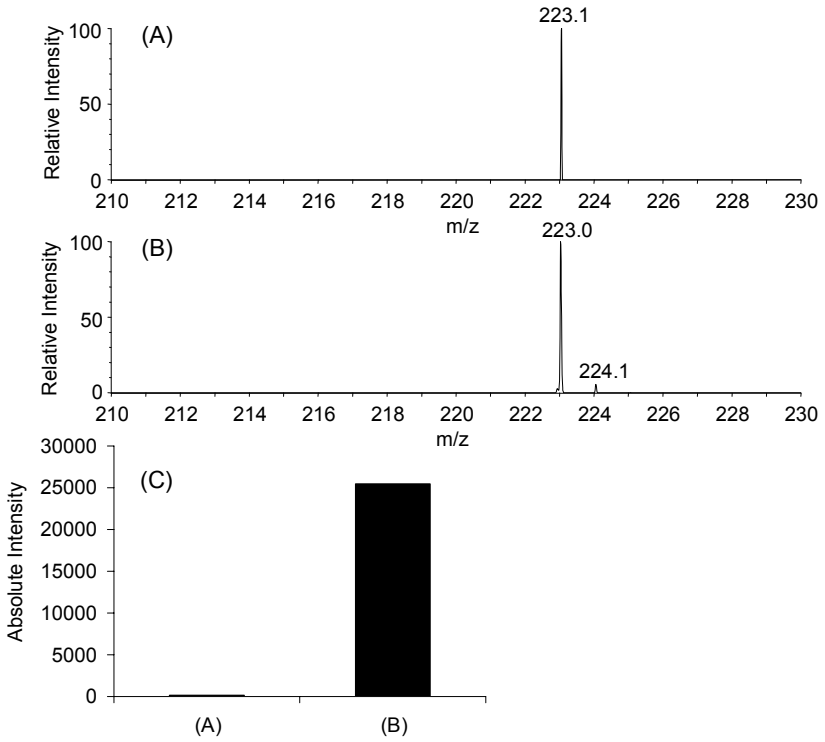

Figure S2. (A) Mass spectrum of HBS synthesized by cell-free extract and *p*-cresol in the absence of benzylsuccinic acid. (B) Mass spectrum of HBS synthesized from cell-free extract with both benzylsuccinic acid and *p*-cresol in the presence of hexane. (C) Comparison of the absolute intensities of the peaks corresponding to 4-HBS (m/z = 223.0) in spectra (A) and (B); the intensity of (B) is ~ 200-fold greater than (A).

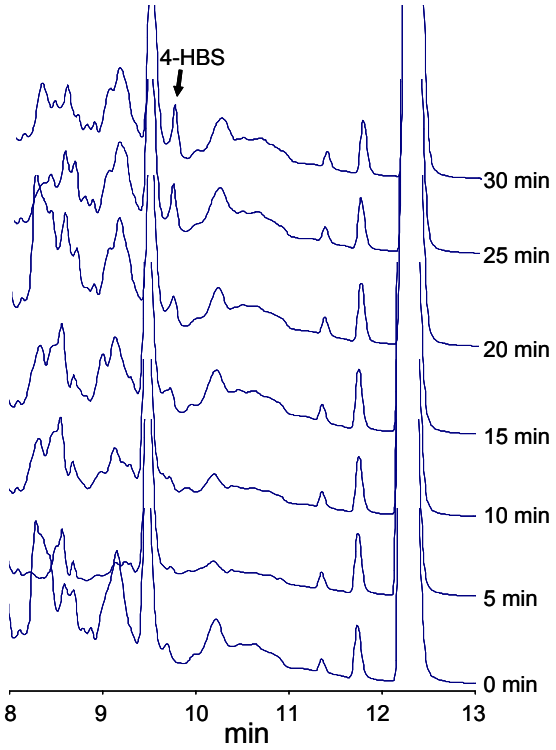

Figure S3: HPLC chromatographs demonstrating the formation of (4-hydroxybenzyl)-succinic acid (HBS) in the presence of saturating concentrations of fumaric acid (10 mM) and *p*-cresol (16 mM). Under the experimental conditions, HBS elutes at 9.7 min.

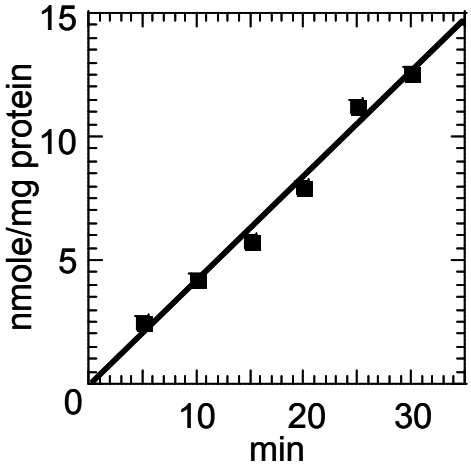

Figure S4: Plot showing the formation of 4-hydroxybenzylsuccinic acid (4-HBS) in the presence of fumaric acid and *p*-cresol as a function of time. The data is derived by integration of the corresponding HPLC chromatographs shown in figure S3. From the data, k_{obs} = 0.422 nmol/min/mg protein.

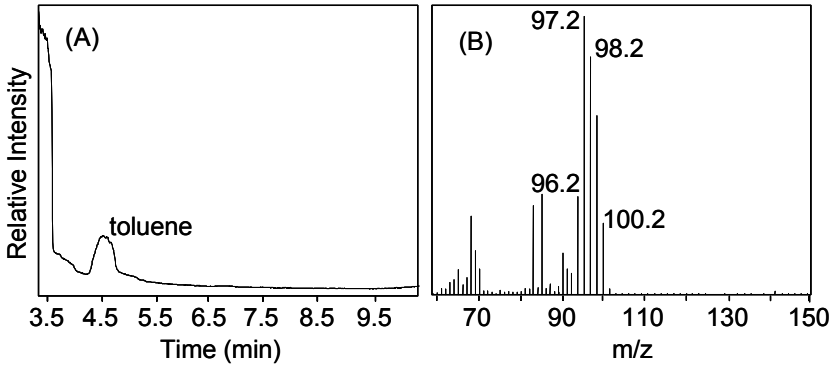

Figure S5: (A) GC chromatograph of the hexane layer extracted from the reaction of d_8-benzylsuccinic acid with cell-free extracts in the absence of *p*-cresol. Under the experimental conditions, toluene is eluted at 4.5 min. (B) Mass spectrum of the toluene peak eluted at 4.5 min.