Supplemental Data

Functional and Structural Analysis of the Siderophore Synthetase AsbB through Reconstitution of the Petrobactin Biosynthetic Pathway from *Bacillus anthracis*

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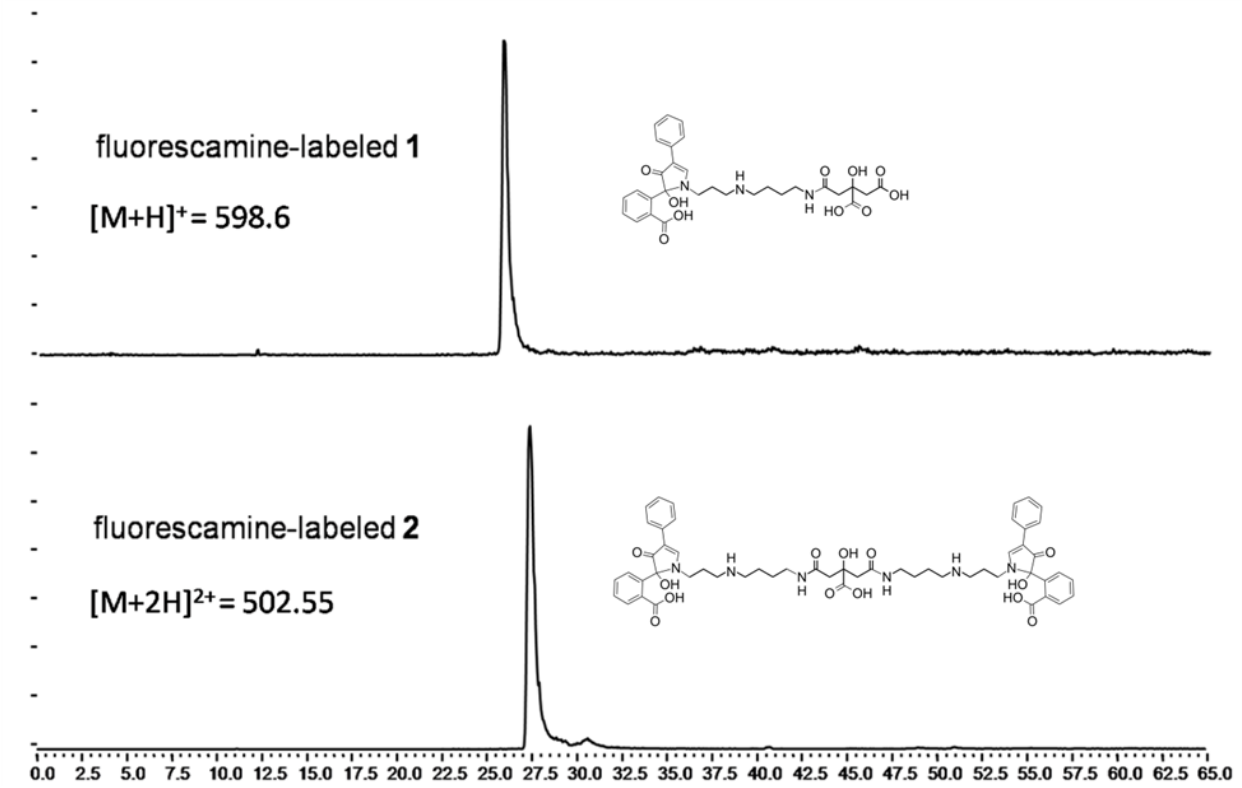
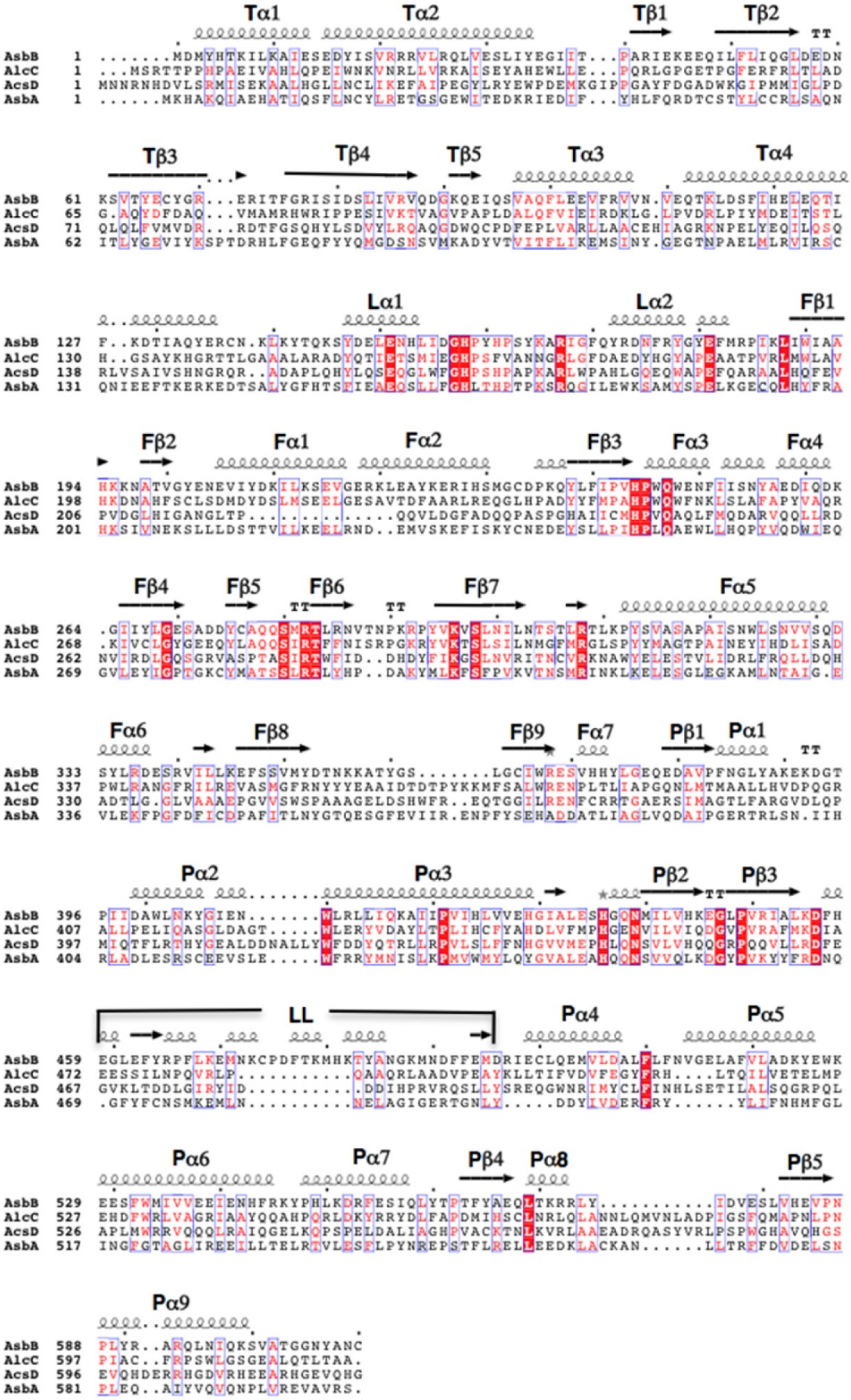
Gene Name	Number	Expression Vector(s)	Cloning Strategy	Source	Antibiotic	Expression Strain
asbA	GBAA_1981	pMCSG26	LIC	this work	Amp, Spect, Tet	BLR (Invitrogen) cont. the pRARE plasmid (Novagen)
asbB	GBAA_1982	pET28b/pMCSG28	NdeI-XhoI/LIC	this work	Kan, Spect, Tet/Amp, Spect	BLR (Invitrogen)/ BL21 (DE3)-Gold (Invitrogen) cont. the pRARE plasmid (Novagen)
asbC	GBAA_1983	pET28b	NdeI-XhoI	ref. 4	Kan, Spect, Tet	BLR (Invitrogen)
asbD	GBAA_1984	pET28b	NdeI-XhoI	ref. 4	Kan, Tet/Kan	BLR (Invitrogen)/ BAP1 (Walsh Group, Harvard Univ)
asbE	GBAA_1985	pET28b	NdeI-XhoI	ref. 4	Kan, Tet	BLR (Invitrogen)

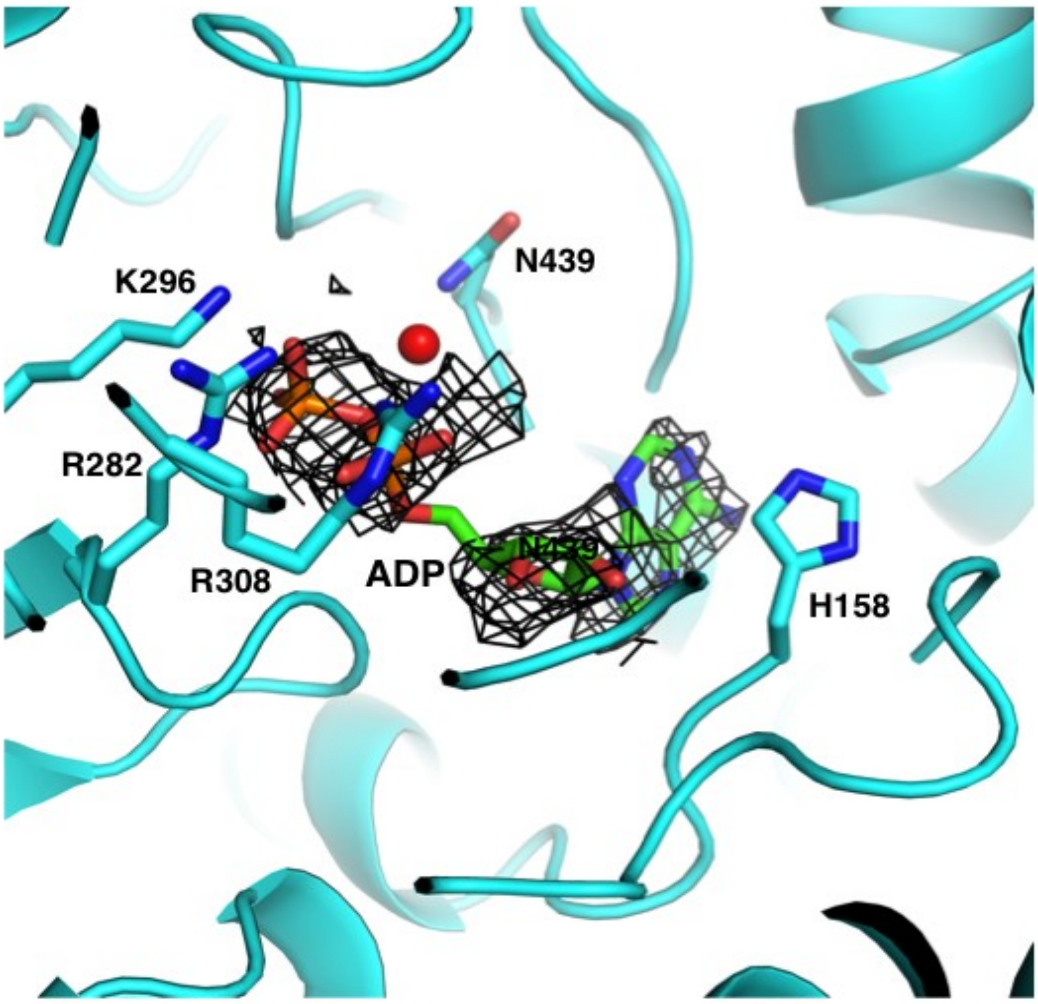
Supplemental Table 1. Gene, vector, strain, and expression conditions. LIC = Ligation independent cloning. Primer sequences and PCR conditions required for amplification of fragments is available upon request.



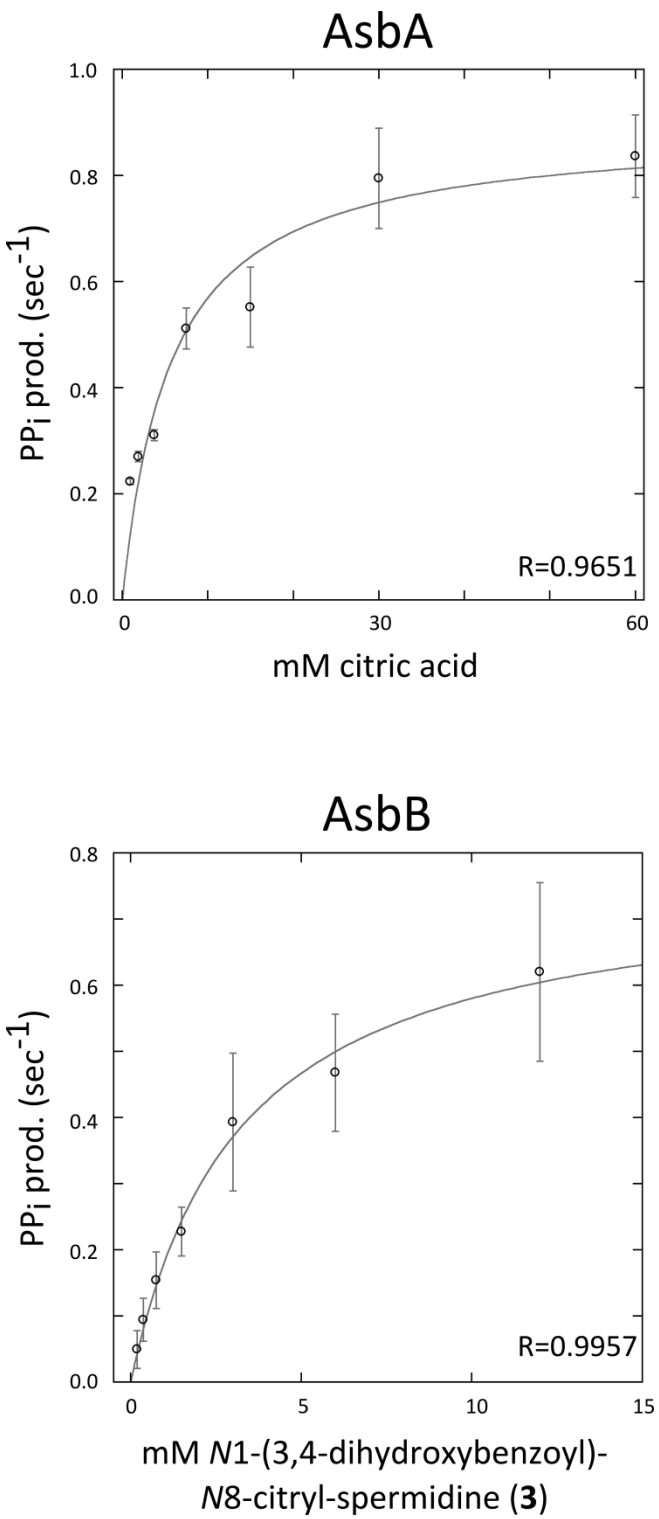
Supplemental Figure 1. Fluorescamine derivatization of prepared standards. Zwitterionic intermediates of petrobactin biosynthesis were derivatized with fluorescamine and analyzed by LC-MS using selected ion monitoring of predicted *m/z* of products.


Tα1 Tα2 Tβ1 Tβ2
AsbB 1 .....MDMYHTKILKAIESEDYISVRRRVLRQLVESLIYEGIIT...PARIEKEEQILFLIQGLDEDN
AlcC 1 ...MSRTTPPHPAEIVAHLQPEIWNKVNRLLVRKAISEYAHEWLLE...PQRLGPGETPGFERFRLTLAD
AcsD 1 MNNRNHDVLSRMISEKAALHGLLNCLIKEFAIPEGYLRYEWPDEMKGIPPGAYFDGADWKGIPMMIGLPD
AsbA 1 ......MKHAKQIAEHATIQSFLNCYLRETGSGEWITEDKRIEDIF...YHLFQRDTCSTYLCCRLSAQN
Tβ3 Tβ4 Tβ5 Tα3 Tα4
AsbB 61 KSVTYECYGR...ERITFGRISIDSLIVRVQDGKQEIQSVAQFLEEVFRVVN.VEQTKLDSFIHELEQTI
AlcC 65 G.AQYDFDAQ...VMAMRHWRIPPESIVKTVAGVPAPLDALQFVIEIRDKLG.LPVDRLPIYMDEITSTL
AcsD 71 QLQLFVMVDR...RDTFGSQHYLSDVYLRQAQGDWQCPDFEPLVARLLAACEHIAGRKNPELYEQILQSQ
AsbA 62 ITLYGEVIYKSPTDRHLFGEQFYYQMGDSNSVMKADYVTVITFLIKEMSINY.GEGTNPAELMLRVIRSC
Lα1 Lα2 Fβ1
AsbB 127 F..KDTIAQYERCN.KLKYTQKSYDELENHLIDGHPYHFSYKARIGFQYRDNFRYGYEFMRPIKLIWIAA
AlcC 130 H..GSAYKHGRTTLGAAALARADYQTIETSMIEGHPSFVANNGRLGFDAEDYHGYAPEAATPVRLMWLAV
AcsD 138 RLVSAIVSHNGRQR..ADAPLQHYLQSEQGLWFGHPSHPAPKARLWPAHLGQEQWAPEFQARAALHQFEV
AsbA 131 QNIEEFTKERKEDTSALYGFHTSFIEAEQSLLFGHLTHPTPKSRQGILEWKSAMYSPELKGECQLHYFRA
Fβ2 Fα1 Fα2 Fβ3 Fα3 Fα4
AsbB 194 HKKNATVGYENEVIYDKILKSEVGERKLEAYKERIHSMGCDPKQYLFIPVHPWQWENFIISNYAEDIQDK
AlcC 198 HKDNAHFSCLSDMDYDSLMSEELGESAVTDFAARLREQGLHPADYYFMPAHPWQWFNKLSLAFAPYVAQR
AcsD 206 PVDGLHIGANGLTP.....QQVLDGFADQQPASPGHAIICMHPVQAQLFMQDARVQQLLRD
AsbA 201 HKSIVNEKSLLLDSTTVILKEELRND..EMVSKEFISKYCNEDEYSLLPIHPLQAEWLLHQPYVQDWIEQ
Fβ4 Fβ5 Fβ6 Fβ7 Fα5
AsbB 264 .GIIYLGESADDYCAQQSMRTLRNVTNPKRPYVKVSLNILNTSTLRTLKPYSVASAPAISNWLSNVVSQD
AlcC 268 .KIVCLGYGEEQYLAQQSIRTFFNISRPGKRYVKTSLSILNMGFMRGLSPYYMAGTPAINEYIHDLISAD
AcsD 262 NVIRDLGQSGRVASPTASIRTWFID..DHDYFIKGSLNVRITNCVRKNAWYELESTVLIDRLFRQLLDQH
AsbA 269 GVLEYIGPTGKCYMATSSLRTLYHP..DAKYMLKFSFPVKVTNSMRINKLKELESGLEGKAMLNTAIG.E
Fα6 Fβ8 Fβ9 Fα7 Pβ1 Pα1
AsbB 333 SYLRDESRVILLKEFSSVMYDTNKKATYGS.......LGCIWRESVHHYLGEQEDAVPFNGLYAKEKDGT
AlcC 337 PWLRANGFRILREVASMGFRNYYYEAAIDTDTPYKKMFSALWRENPLTLIAPGQNLMTMAALLHVDPQGR
AcsD 330 ADTLG.GLVAAAEPGVVSWSPAAAGELDSHWFR..EQTGGILRENFCRRTGAERSIMAGTLFARGVDLQP
AsbA 336 VLEKFPGFDFICDPAFITLNYGTQESGFEVIIR.ENPFYSEHADDATLIAGLVQDAIPGERTRLSN.IIH
Pα2 Pα3 Pβ2 Pβ3
AsbB 396 PIIDAWLNKYGIEN.......WLRLLIQKAIIPVIHLVVEHGIALESHGQNMILVHKEGLPVRIALKDFH
AlcC 407 ALLPELIQASGLDAGT.....WLERYVDAYLTPLIHCFYAHDLVFMPHGENVILVIQDGVPVRAFMKDIA
AcsD 397 MIQTFLRTHYGEALDDNALLYWFDDYQTRLLRPVLSLFFNHGVVMEPHLQNSVLVHQQGRPQQVLLRDFE
AsbA 404 RLADLESRSCEEVSLE.....WFRRYMNISLKPMVWMYLQYGVALEAHQQNSVVQLKDGYPVKYYFRDNQ
LL Pα4 Pα5
AsbB 459 EGLEFYRPFLKEMNKCPDFTKMHKTYANGKMNDFFEMDRIECLQEMVLDALFLFNVGELAFVLADKYEWK
AlcC 472 EESSILNPQVRLP.....QAAQRLAADVPEAYKLLTIFVDVFEGYFRH....LTQILVETELMP
AcsD 467 GVKLTDDLGIRYID.....DDIHPRVRQSLLYSREQGWNRIMYCLFINHLSETILALSQGRPQL
AsbA 469 .GFYFCNSMKEMLN.....NELAGIGERTGNLY.....DDYIVDERFRY.....YLIFNHMFGL
Pα6 Pα7 Pβ4 Pα8 Pβ5
AsbB 529 EESFWMIVVEEIENHFRKYPHLKDRFESIQLYTPTFYAEQLTKRRLY.....IDVESLVHEVPN
AlcC 527 EHDFWRLVAGRIAAYQQAHPQRLDKYRRYDLFAPDMIHSCLNRLQLANNLQMVNLADPIGSFQMAPNLPN
AcsD 526 APLMWRRVQQQLRAIQGELKQPSPELDALIAGHPVACKTNLKVRLAAEADRQASYVRLPSPWGHAVQHGS
AsbA 517 INGFGTAGLIREEILLTELRTVLESFLPYNREPSTFLRELLEEDKLACKAN.....LLTRFFDVDELSN
Pα9
AsbB 588 PLYR..ARQLNIQKSVATGGNYANC
AlcC 597 PIAC..FRPSWLGSGEALQTLTAA.
AcsD 596 EVQHDERRHGDVRHEEARHGEVQHG
AsbA 581 PLEQ..AIYVQVQNPLVREVAVRS.

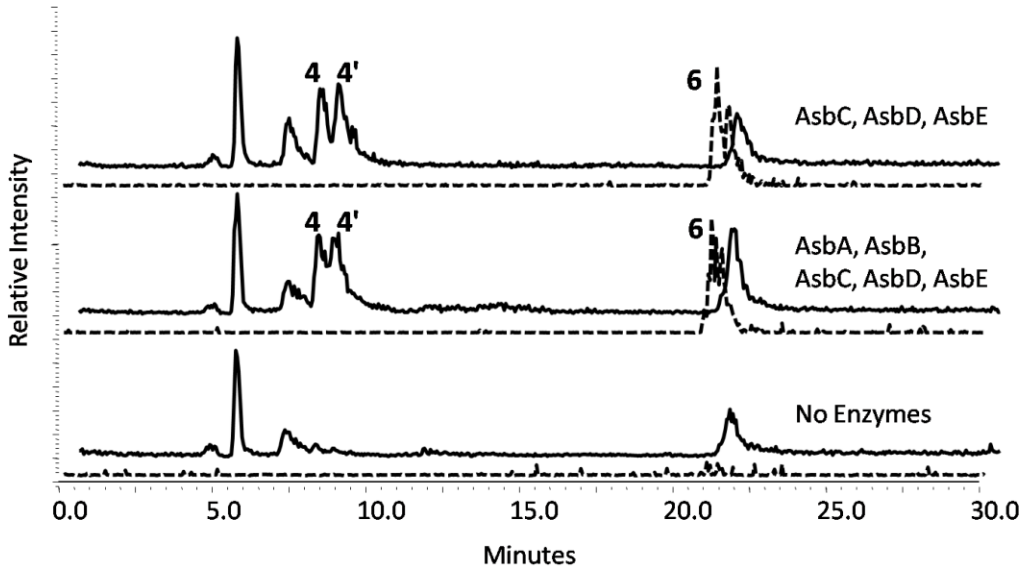
Supplemental Figure 2. Sequence comparison of AsbB, AlcC, AcsD and AsbA. The secondary structure of AsbB is annotated at the top. Highly conserved residues include H158, H161, R282, K308, E434, and N439 of AsbB. Hypothesized structural interactions with substrates are depicted in Fig. 3D.

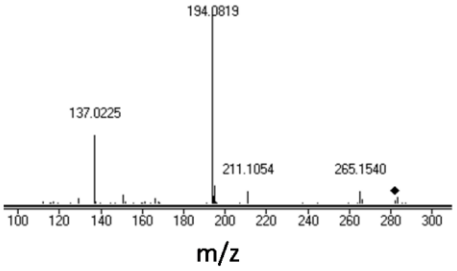


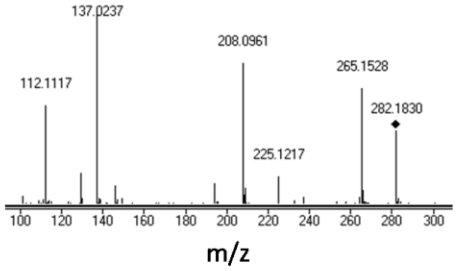
Supplemental Figure 3. Modeling of ADP binding in the AsbB active site. ADP conformation is modeled and refined with partial occupancies based upon loose electron density data as demonstrated by the difference (Fo-Fc) electron density contoured at 3.0 σ (black mesh). Potential protein-substrate interactions are detailed in Figure 3D.

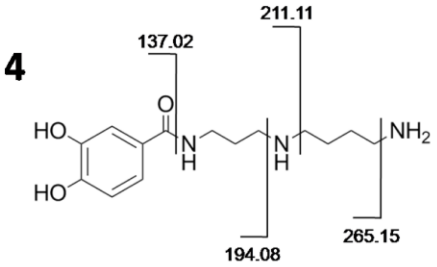


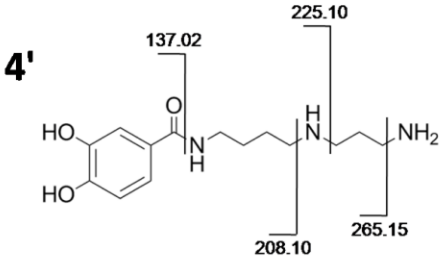
Supplemental Figure 4. Activity plots for quantification of enzymatic efficiency. His_6-AsbA and His_6-AsbB kinetic parameters were determined through plotting initial reaction rates over varying starting concentrations of adenylation substrate. The additional substrates of ATP and spermidine are at near-saturating levels of 6 mM and 40 mM, respectively. Reaction progress was monitored by observing release of pyrophosphate (PP_i) coupled to hydrolysis of the reporter molecule MESG to establish initial rates that were normalized to enzyme concentration. Only data-points not displaying substrate-associated background interference were used to extrapolate the curves. Nonlinear regression of these data using the Michaelis-Menten equation enabled prediction of V_{max} and K_m for approximation of V/K.

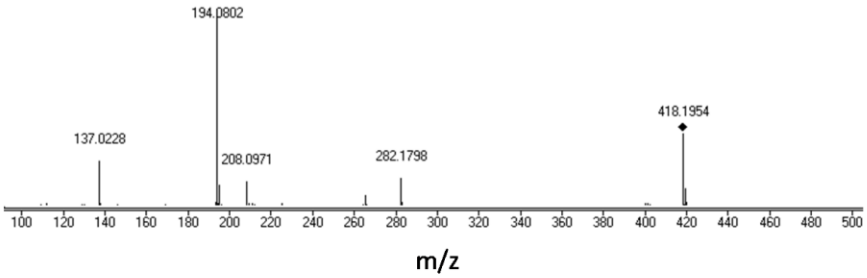

4
4'
6
AsbC, AsbD, AsbE
AsbA, AsbB,
AsbC, AsbD, AsbE
No Enzymes
Relative Intensity
0.0
5.0
10.0
15.0
20.0
25.0
30.0
Minutes

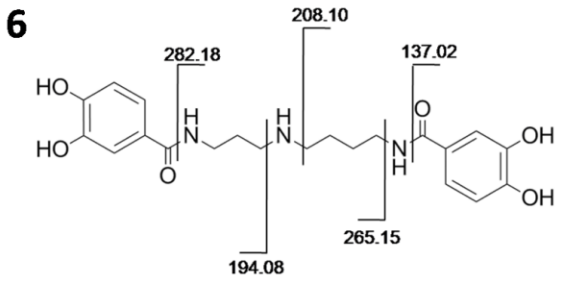

194.0819
137.0225
211.1054
265.1540
m/z


137.0237
208.0961
265.1528
112.1117
282.1830
225.1217
m/z


4
211.11
137.02
HO
HO
O
N
H
N
H
NH2
194.08
265.15


4'
225.10
137.02
HO
HO
O
N
H
H
N
NH2
208.10
265.15


194.0802
137.0228
208.0971
282.1798
418.1954
m/z


6
208.10
282.18
137.02
HO
HO
O
H
N
H
N
H
N
O
OH
OH
265.15
194.08

Supplemental Figure 5. Reaction of AsbE with spermidine and 3,4-DHBA. Initial reactions were analyzed by LC-MS as described in the text. Selective ion monitoring (SIM) was conducted in positive mode for predicted *m/z* of **4** and **4'** ($[M+H]^+$=282.2, solid traces). An extracted ion chromatogram (EIC) was rendered to detect compound **6** with a predicted *m/z* of $[M+H]^+$=418.15 (dashed traces). Corresponding peaks are observed in chromatograms of the petrobactin reconstitution enzymatic reaction and in instances where only the 3,4-DHBA - AMP ligase AsbC, the aryl-carrier protein AsbD, and the aryl transferase AsbE are the only proteins present. These results and previous research (1,2) suggest AsbC, AsbD, and AsbE are necessary and sufficient for formation of **4** and **4'** as well as **6**. The accumulation of these molecules during *in vitro* reconstitution of petrobactin biosynthesis (second trace) demonstrates a lack of incorporation into the final product petrobactin. Unlabeled peaks are consistently observed during LC-MS analysis of all reactions, including the no-enzyme control, and represent artifacts from the reaction. Predicted $[M+H]^+$*m/z* of **4**, **4'**, and **6** were used to target corresponding species for MS/MS analysis. Fragmentation spectra confirm the 3,4-dihydroxybenzoylation pattern for all three compounds.

MS/MS settings are described in the main text. Injected reaction samples were separated on a Phenomenex Synergi Hydro-RP (150x4.6 mm, 4 μm) column in-line with the instrument at a flow rate of 0.3 ml/min with mobile phase supplemented with 0.1% formic acid. 100% ddH_2O was applied for 5 minutes followed by a linear gradient of 0% to 95% MeCN over the course of 20 minutes. 95% MeCN was then applied for an additional 10 minutes.

Supplemental Methods

Protein Purification for Enzymology

All steps were conducted at 4°C. Briefly, harvested cell pellets were resuspended in 5 ml of lysis buffer (20 mM imidazole, 20 mM HEPES, 150 mM NaCl, 1 mM Tris(2-carboxyethyl) phosphine [TCEP], 10% v/v glycerol, pH 8) per 100 ml of original over-expression culture and lysed by sonication. Insoluble material was removed by ultracentrifugation at 30000 x g for 45 min, and the supernatant was batch-bound for 2 hours to 1 ml of Ni^{2+}-NTA slurry (Novagen) that was previously equilibrated in lysis buffer. This batch-binding mixture was poured through a 5 ml fritted glass column where the retained resin was washed with 1 column volume of lysis buffer, 2 column volumes of wash buffer (40 mM imidazole, 20 mM HEPES, 150 mM NaCl, 1 mM TCEP, 10% glycerol, pH 8), and finally 3 ml of elution buffer (250 mM imidazole, 20 mM HEPES, 50 mM NaCl, 1 mM TCEP, 10% v/v glycerol, pH 8). Protein in the eluate was both exchanged into storage buffer (20 mM HEPES, 5 mM NaCl, 1 mM TCEP, 20% v/v glycerol, pH 8) and concentrated using Amicon Ultra centrifugal molecular weight cutoff filters (Millipore). Resulting samples were flash frozen with liquid N_2 and stored at -80°C prior to analysis.

Protein Purification for Crystallization

Harvested overexpression cells were lysed by sonication in the presence of 1 mg/ml lysozyme and a protease inhibitor cocktail tablet (Complete, Roche) in 35 ml of lysis buffer containing 50 mM HEPES pH 8.0, 500 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, and 5% v/v glycerol. The lysate was clarified by centrifugation at 30000 x g for 75 min, followed by filtration through a 0.45 μm filter. Protein was purified by two-step Ni^{2+}-affinity chromatography following the standard protocol described previously (3). Immobilized metal affinity chromatography (IMAC) was conducted using a 5ml HisTrap Chelating HP column charged with Ni^{2+} ions and buffer-exchange chromatography was performed on a HiPrep 26/10 desalting column (GE Healthcare) on an ÄKTAxpress™ (GE Healthcare).

References

1. Lee, J. Y., Janes, B. K., Passalacqua, K. D., Pfleger, B. F., Bergman, N. H., Liu, H., Hakansson, K., Somu, R. V., Aldrich, C. C., Cendrowski, S., Hanna, P. C., and Sherman, D. H. (2007) *J. Bacteriol.* **189**, 1698-1710
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3. Kim, Y., Dementieva, I., Zhou, M., Wu, R., Lezondra, L., Quartey, P., Joachimiak, G., Korolev, O., Li, H., and Joachimiak, A. (2004) *J. Struct. Funct. Genomics* **5**, 111-118