Article

Benzylsuccinate synthase is post-transcriptionally regulated in the toluene-degrading denitrifier *Magnetospirillum* sp. strain 15-1

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1. Supplemental Figures and Tables

(a.1)

(a)





3.0











(b.2)

2

3

1

(b)









9 **Figure S1:** Growth of *Magnetospirillum* sp. 15-1 at different conditions. Growth of *Magnetospirillum* sp. 15-1 with:

10 (a) 2.5 mM benzoate as the sole carbon source, 5 mM (a.1) and 10 mM KNO3 (a.2) under anoxic conditions; (b)

11 0.5 mM toluene as the sole carbon source, 5 mM (b.1) and 10 mM KNO3 (b.2) under anoxic conditions; (c) 2.5

12 mM benzoate as the sole carbon source under oxic conditions. Nitrite was below detection limits in each culture.

13 Δ : Nitrate consumption; •: optical density; \Box : carbon source degradation.





Table S1: Identity (%) of proteins involved in anaerobic toluene degradation among different degraders to Magnetospirillum sp. 15-1^a.

Protein	Magnetospirillum sp. TS-6 AB167725.1	Azoarcus tolulyticus ATCC 51758 NZFTMD01000027.1	Azoarcus toluclasticus ATCC 700605 ARJX01.1	Azoarcus sp. CIB DQ988527.1	<i>Azoarcus</i> sp. EbN1 NC006513	Thauera aromatica K172 CP028339.1	Herminiimonas sp. CN AVCC00000000.1	Desulfobacula toluolica Tol2 NC018645	Geobacter metallireducens GS-15 CP000148
BssD	98.8	65.6	64.7	65.6	62.8	62.2	63.1	57.5	48.2
BssC	98.3	62.7	61	61	63.8	62.1	58.6	55.2	56.9
BssA	99.8	83.9	83.6	82.5	81.8	80.6	85.5	76.2	74.1
BssB	100	69	72.3	68.7	73.3	74.7	65.5	53.6	56.3
BssE	98.6	79.2	79.2	79.2	81.7	79.6	82.1	67.7	64.5
BssF		60.7	59.6	61.3	61.4	63.6	63	51.9	48.1
BssG		51.6	51.6	51	52.9	51.8	54.7		
BssI		50.8		50.3			47.7		
BssJ		64.9	61.7	64.9	63.6	63	58.9		
BssK		54.8	69.1	54.8	56.7	58.9	55		
BssL		48.5	48.7	49	48.7	50.1	51.7		
XylR									41.8

Microorganisms	2020,	8, x	FOR	PEER	REVIEW
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2 of 16

TdiR		63.5	63.5	63.2	59.8	65.2		
TdiS	52.3		58.3	58.9	58.5	58.9		
Ycf48		34						
YdfJ		49.5						
BbsH	82.1	81.3	79.4	79.8	80.2	79.8	72	72
BbsG	77.6	77.1	77.4	78.4	77.4	75.2	69.9	71.6
BbsF	73.2	74.6	73.7	74.4	74.6	75.6	67.5	71
BbsE	69.7	71.8	69.4	70.6	70.4	72.8	63.3	64.9
BbsD	74.3	72.3	73.9	73.9	73.9	75.1	67.1	68.3
BbsC	44	43.7	44.8	44	44	45.2	42.9	39.6
BbsB	84.4	84.6	84.6	85.4	85.6	83.1	73.1	74.4
BbsA	74.5	74.5	73.8	71.1	74.5	72.8	61.8	66.2

17 ClustalW 2.1 alignment; Cost Matrix: BLOSUM62; Gap open cost:10; Gap extend cost: 0.1

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		PAS domain
Magnetospirillum sp. 15-1	1	MFEYKSILDNAPVGICFLRSRNTIRCNRRFEDLFGYDSD
Herminiimonas CN	1	YEAIFRNTPVAICHLRNRT IRCNTRFEELLGYRRG
Azoarcus sp. CIB	1	MKNDVVAVBFDDEQGEDVSVGYEAIFRNTPVAICHLRNRTIKRCNRRYEELFGYPPG
Thauera aromatica K172	1	MSGNA ASTETEMBA HGNDPAT <mark>GYE</mark> VIFRNTP AICHLRNRAFURCNTRFEELFGYARG
		PAC domain
	40	EINNKSVRILYPSTEMENHIGEVYGHFFEKNLAVRDERPMARKDGTMIWCIVTGCTIDSA
	37	ELDNKSVRLLYPSEDSFUTIGDKEGHFFERHNTFKDERPIIRKDGTLVWCIVTGTLLNPS
	58	ELDNCSVR HYPSDESFSTIGRKYGHFFEREHTFKDERPFYRKDGTLIWCIVTGTLLDPR
	61	ELDNKSVRLLYPTDESFRTIGENYGHFFERHDTFKDERPIIRKDGSVIWCIVTGSLLD <mark>S</mark> S
		`
	100	NPHLCQIWVVQDISEHRRTEDLLKESIEKLELVVQQRTMDLRNHIEALNVEVATRINAEG
	97	NPRLGGIWVVQDISDHKRLEDELKANVEKLEIUVHQRTAELRRHVNSLEQEVATRKKAED
	118	NLQLGSIWVVQDISEHKRIEDELKADVEKLEIVVQQRTVELSRHVASLEEEVATRQKAEE
	121	NPRLGSIWVVQDISEHKRTEDDLKASVEKLEILVHQRTELHKHVNKLEQEVATRKLAEE
		♦ PAS domain
	160	IANETORK OTVEHTVEVG CVTDAAGRILEANHVEDET CNAD KVRDWGDLETRFFLP
	157	IANENREKYQ <mark>TLFHMLPIGISITD</mark> SEG <mark>GILEANCQFVEMVGTKRA</mark> LPMSWQQLPRRFFLH
	178	AANENREKYQKLFHMLPIGISITDPQGDILEANCQFAEMAGLERASPESWRKLPLRFFLH
	181	ANEHREKIEKLEHMLPIGISITUNEGKILEANROFTELVGTPEKPEL WOOLPORFTIS
		PAC domain
	220	DDTALQSONLPWLI NHINSE GSVEIGMRKKRGGKVHWINVSSIALDIAGAPALLTVFT
	217	DGTDIPRQRLPWQIRNYQSSIRH_EIGMRDEKSRKVRWLSVSSSLIEHNDQKMVVAAFT
	238	DGT-IPRHRLPWQIRDFQKDSINNIEIGMRDEKARKIRWLSVSSSLIERKDRQFVVAAFT
	241	DGTR ARREPWRIHDVORDSINNIEIGMR BESKRORWLSVSSSLIELRGORMVVAAFT
		HisKa domain
	280	DITYRRRIEELERLRYAELTRLGRINSMAEMSAALAHQMGQPLVSAINYLNGCRLRL
	277	DITYRKRIEELERLRHAELTRLGRINSM <mark>S</mark> EMCAALAHQMGQPLVSAVNYLNGCRLRLERV
	298	DITYRKRIEELERLRHAELTRLGRINSMAEMAAALAHQMGQPLVSAUNYLNGCMLRLERV
	301	DITTRREEDERERHALIREGRINAMAGMAAALANQMGQFEVSALNILUGCKERED
		<u>→</u>
	340	DGAEEISN SNGLSITYLEQAGEILRRVRDFVCRHDPEKIPEDINAVITGAVSFLNFEIHK
	337	DGVEEISTSLGLAIEYLGQAGJILR VKDFVCKHKPEKIPEDINEVIQDALSFLNFEIHR
	358	RCA ABTS SLOLA THE OAGETLE WKDEVCKHTPD TPNINEVIADALSELSE SPUER
	301	
	400	HRVSVNLDLASNVPLVPLON EIQQVLFNLIKNGMEAMT LEEFERILTIGSS RPDRSE
	397	HOVIVOLOLPPNPPLVPLCKVETQQVLFNLMKNGMEAMISLEABARVUTIGIEVGEGRA
	418	HDVSVDIALIEBPELVPLCAUEIQUVLENLMANGUEAMIGLESBARREVIGSEIVGEGGA
	421	
	460	WEVFVIDNGRGVARSRSHRIFEPMETTKPDGIGIGLTICRCIVESHGGKLSFTPMGRQGS
	457	TKVFVQDHGTGVEKQTAKRVFEPYFSTKPDGMGVGLTICRSIVESHGGELSFSKIGKRGS
	478	MKVFVQDHGTG EKKSAKKVFEPYFSTKPDGMGIGLTICKSIVESHGGQLSFS-IGRGGS MKUEVODHCVGVEKBAEKBAEFPYFSTKPDGMGIGLTICKSIVESHGGQLSFS-IGRGGS
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	520	REQITLE ASPAIAPSTTVPL
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Figure S2: Pairwise alignment of TdiS and TdiR proteins among anaerobic toluene degraders. The alignment of
 TdiS (a) and TdiR (b) are shown for four different toluene degraders. Shaded background represents the

35 similarity of the aminoacidic residue between the different aminoacidic sequences. Arrows placed above the first

36 aminoacidic sequence highlights conserved protein domain.

Microorganisms 2020, 8, x FOR PEER REVIEW



Data Set S1: qPCR raw data of *bssA, bcrC* and 16S rRNA from AN and AB cultures.

Data Set S2: Copy numbers of *bssA, bcrC* and 16S rRNA from AN and AB cultures.





Table S2: Detection of CRP/FNR superfamily members. Regulation of each protein is indicated (log2 fold) and their significance (p-value) in anaerobic and aerobic cultures. AN_T
 represents cultures growing anaerobically with toluene and either 2.5 mM (AN_T2.5), 5 mM (AN_T5), or 10 mM (AN_T10) of KNO₃. AB_T represents cultures growing aerobically

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with benzoate and supplied additionally with either toluene alone (AB_T) or toluene and 5 mM of KNO3 (AB_TN).

				Anaerobio	cultures				Aerobi	c cultures	
Gene	Locus	log2	p-value	log2	p-value	log2	p-value	log2	p-value	log 2	p-value
		AN_T2.5	AN_T2.5	AN_T5	AN_T5	AN_T10	AN_T10	AB_T	AB_T	AB_TN	AB_TN
contig01_gene37	39763-40416	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
contig01_gene283	296369-295611	-0.123	0.797	-1.353	0.172	-1.213	0.099	ND	ND	ND	ND
contig01_gene551	568946-569650	-0.861	0.022	-0.331	NC	-0.178	0.724	ND	ND	ND	ND
contig04_gene2311	291845-292429	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
contig04_gene2534	518994-519695	-0.307	0.358	-0.344	0.588	-0.323	0.211	-0.230	0.604	0.611	0.643
contig06_gene3113	217618-218352	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
contig07_gene3349	117592-116894	-0.048	0.847	0.065	0.724	-0.226	0.439	0.355	0.642	0.882	0.135
contig07_gene3350	118295-117597	-0.056	0.900	-0.231	0.267	-1.105	0.028	0.054	NC	0.088	NC
contig09_gene3888	164717-165376	ND	ND	ND	ND	NC	NC	ND	ND	ND	ND
contig09_gene3893	170577-169882	-0.410	0.612	-0.953	NC	-1.182	0.050	ND	ND	ND	ND
contig10_gene4043	99521-96201	-1.371	NC	-0.788	NC	0.605	NC	0.206	NC	0.558	NC
contig10_gene4044	96209-97012	2.421	0.073	ND	ND	-1.268	NC	-0.381	NC	-0.471	NC









- Figure S4: Fold change averages of *bcrC* expression in anaerobic and aerobic cultures of *Magnetospirillum* sp 15AN cultures growing with 2.5 mM, 5 mM, or 10 mM of KNO₃ are shown as "Anaerobic" and AB cultures
- 54 growing in the presence of toluene and toluene/nitrate are shown as "Aerobic".

55

56 Data Set S3: Proteins identification and abundances from AN and AB cultures.







Figure S5: Metabolic pathways detected in anaerobic and aerobic cultures of *Magnetospirillum* sp. 15-1. KEGG pathways mapping shows pathways unique to AN cells (purple),
 unique to AB cells (green), and shared between both conditions (orange).





Tree scale: 0.1 Fnr Rhizobium etli CFN42 gi|499365890 HbaR Rhodopseudomonas palustris CGA009 gi|6118088 Nnr Paracoccus denitrificans DSM413 gi|8119332 contig07 gene3350 contig10 gene4043 ntig01 gene551 tig10 gene40 8857 Magnetospirillum magnetotacticum MS-1 gil23016270 contig07_gene3349 929 Desulfovibrio desulfuricans G20 gi|23474021 Dnr Synechocystis sp.gil499176690 966 Ralstonia solanacearum GMI1000 gi|499313483 DnrD Pseudomonas stutzeri ZoBell ATCC14405 gi|4585795 Dnr Pseudomonas aeruginosa PAO1 gil15595724 Dnr Pseudomonas aeruginosa CLJ1 gi| 1373666350 ntig01_gene37 04_gene2311 1148 Corynebacterium glutamicum ATCC13032 gi 19552398 Nnr Rhodobacter sphaeroides ssp. denitrificans IL NnrR Rhodobacter sphaeroides 2.4.3 gi| 1458102 IL106 gi|4680331 NnrR Bradyrhizobium diazoefficiens USDA110 gi|27382195 NnrR Brucella sp. gi|490827980 NnrN Agrobacterium fabrum C58 gi|159186141 NnrN Pseudomonas sp. G179 gi|3925389 Contig09_gene3893 OrfA Pseudomonas putida U gi[22711871 FnrB Pseudomonas putida KT2440 gi[26989951 Fnr Escherichia coli K12-MG1655 gjl16129295 Fnr Shigella flexneri 2a-301 gil56479971 FnrA Pseudomonas stutzeri ZoBell ATCC14405 gil398391 Anr Pseudomonas aeruginosa PAO1 gil15596741 Anr Pseudomonas putida KT2440 gil26990956 Fnr Xanthomonas citri gil499363136 ntig01 gene283 AadR Rhodopseudomonas palustris CGA009 gi|398982 FixK1 Bradyrhizobium diazoefficiens USDA110 gi|27381172 FnrN Paracoccus denitrificans gil75429481 FnrL Rhodobacter sphaeroides 2.4.1 gi| 77464271 FnrN Agrobacterium fabrum C58 gi|15888914 FnrNC Rhizobium etli CE3 gi|3462873 FnrND Rhizobium etli CE3 gi|12083693 FnrN Rhizobium leguminosarum bv. viciae UPM791 gi|120623 contig06_gene3113 contig09_gene3888 no253/ 5554 Bradyrhizobium diazoefficiens USDA110 gi|27380665 395 Bradyrhizobium diazoefficiens USDA110 gj/27375506 1169 Synechocystis sp. PCC6803 gi/499175324 2110 Mesorhizobium japonicum gi/499213134 -5066 Magnetospirillum magnetotacticum MS-1 gil23012191

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Figure S6: Phylogenetic tree of CRP/FNR regulators. Aminoacidic sequences were obtained from the genome of *Magnetospirillum* sp. 15-1 (shown in red) and other amino acid sequences from the NCBI database. Dnr, Fnr, Nnr and C represent branches of the family and their inducers and/or repressors are shown (NO, O₂). The phylogenetic relationship was conducted with the Neighbor-Joining method (1000 bootstrap replicates). The tree scale represents amino acids substitution per site.

81

82 2. Supplemental Materials and Methods

83 2.1. Benzoate detection

84 Degradation of benzoate was quantified through a gradient HPLC with a UV detection of 228 85 nm. Benzoate was separated from other compounds with a LiChrospher 100 RP-18e (5 μ m), 86 LiChroCART 125-4, column. Methanol at 100 % (Rotisolv HPLC Gradient Grade 99 %, Carl Roth, 87 Karlsruhe, BW, Germany) and phosphoric acid at 0.1 % (Chemsolute for analysis 85 %, Th. Geyer, 88 Höxter, NW, Germany) were used as eluents of the mobile phase. Methanol was increased from 20 89 to 100% until minute 20 and remained constant for 5 min. Then methanol was decreased again to 20% 90 and remained constant for 3 min. The flow rate was set at 0.5 mL/min. Quantification was performed 91 by using calibration curves.

92 2.2. RNA quality and purity analysis

To assess the quality of total RNA from each elute, 1 µL of sample was measured by Nanodrop
(Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA) to determine
purity by 260/230 ratios and to check contaminants such as guanidine salts and phenol. To analyse
RNA quality, 5 µL of each RNA elute was run in a 1.2 % agarose gel for 45 min at 90 v.

97 To test for DNA residuals within the samples, 1 μL of each elute was subjected to PCR 98 amplification with a HotStar Taq ® according to manufacturer's instructions and by using the 99 universal primers 27F and 1492R. Then, 5 μL of each amplicon was run in a 1.2 % agarose gel for 30 100 min at 100 V.

101

102 2.3. cDNA synthesis

103 Total RNA (150 ng) was used to synthesize cDNA through reverse transcriptase technique using 104 the Ominiscript® Reverse Transcription Kit (Qiagen, Hilden, NW, Germany). First, the RNA samples 105 were dissolved each in RNAse free-water to make 12.75 µL and incubated for 10 min at 65 °C to 106 denature RNA secondary structure. Then a master mix was prepared and each reaction consisted of 107 2 μL 10x buffer RT, 2 μL dNTP mix (5 mM each), 2 μL random hexamer primers (10 μM), 1 μL RNase 108 inhibitor (10 units/ μ L) and 1 μ L Ominiscript Reverse Transcriptase. Negative controls without the 109 reverse transcriptase (NRTs) enzyme were prepared per each sample. Each reaction had a final 110 volume of 20 µL. cDNA synthesis was performed by incubating the samples at 37 °C for 60 min. 111 Samples were then stored at -20 °C until usage.

112 2.4. Preparation of the bssA, bcrC and 16S rRNA standards for qPCR

113 The genes bssA, bcrC and 16S rRNA were amplified by PCR from extracted DNA of 15-1 cells 114 growing exponentially with toluene. The primer pairs used for amplification are shown in Table 1. 115 The PCR reaction consisted of 12.5 µL HotStarTaq Master Mix 2x (Qiagen, Hilden, NW, Germany), 1 116 μ L of each primer (0.4 μ M final concentration), 9.5 μ L of RNase free-water and 1 μ L template. The 117 amplicons were further run in an 1.2% agarose gel and subsequently purified using the Qiagen 118 QIAquick PCR purification kit (Qiagen, Hilden, NW, Germany) according to the manufacturer's 119 instructions. The concentration of the purified DNA was measured with a Nanodrop (Nanodrop ND-120 1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Based on the concentration, 121 the number of gene copies per μ L was calculated with the following equation (eq.1):

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123

$$\frac{Copies}{\mu L} = \frac{\text{PCR product } \left(\frac{\text{ng}}{\mu L}\right) \cdot 10e^{-9}}{\left(\text{Length PCR product } [\text{bp}] \cdot 660 \left[\frac{\text{Da}}{\text{bp}}\right]\right) \cdot 6.022 \cdot 10e^{23}} \quad (\text{eq. 1})$$

124 125

660 Da/bp: Mass of double- stranded DNA; 6.022 • 10e23: Avogadro constant

126 2.5. qPCR reaction for the amplification of bssA, bcrC and 16S rRNA

127 The calibration curve for each gene was prepare by diluting serially the standards with RNase 128 free-water (Qiagen, Hilden, NW, Germany) from 10^8 to 10^1 copies/ μ L. cDNA templates were 129 undiluted or diluted 1:10 for aerobic and anaerobic experiments respectively. cDNA templates for 130 16S rRNA amplification were always diluted 1:10. Negative controls and qPCR blanks were not 131 diluted. To avoid degradation, the samples were kept on ice all time.

A PCR reaction consisted of: 6.25 μL KAPA SYBR FAST, 4.75 μL Rnase free-water, 0.25 μL of
each primer (Table 1) with a final concentration of 200 nM for the 16S_F/16S_R primer pairs and 500
nM for bssA_F3/bssA_R3 and 2bcrC_F/2bcrC_R primer pairs. For the amplification of *bssA* and *bcrC*the amount of cDNA template used was 1.5 μL and for 16S rRNA, 1.0 μL. Amplification was

136 performed in a Step One Plus Real Time PCR System (Applied Biosystems, Thermo Fischer Scientific,

137 Waltham, MA, USA).

138 2.6. Gene expression analysis

The initial abundances (in 30 mL of culture) of *bssA*, *bcrC* and 16S rRNA transcripts were
obtained by calculating the amount of copies per μL (eq. 2) contained in 150 ng of RNA.

$$\frac{Copies}{\mu L} = \left[Quantity \cdot \left(\frac{1 \ mL}{RNA}\right) \cdot \left(\frac{l}{rt_i}\right) \cdot \left(\frac{rt_f}{cDNA}\right) \cdot DF \right] \quad (eq. 2)$$

142 143

144Quantity: qPCR copies/ μ L; RNA : volume (mL) harvested for RNA extraction; l: elute (μ L) after RNA145extraction; rt: total RNA (μ L) used for cDNA synthesis; rtr: final volume (μ L) of the reverse146transcription reaction; cDNA: cDNA (μ L) used for qPCR reaction; DF: dilution factor used in qPCR147reaction.

148

The calculated *bssA* and *bcrC* copies/µL, were further normalized by using the 16S rRNA gene as the calibrator: the ratio between *bssA* or *bcrC* and the normalizer,16S rRNA, was calculated. Fold changes were calculated by obtaining the ratio between normalized *bssA* or *bcrC* values of toluenegrown cells to normalized *bssA* or *bcrC* values of benzoate-grown cells (reference cultures) which are subjected to the same oxygen conditions and nitrate concentrations (anaerobic cultures) e.g. AN_T2.5/AN_B2.5, AN_T5/AN_B5, etc (Table S3).

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Table S3: Culture pairs used to assess *bssA* and *bcrC* expression from toluene-grown cells relative to *bssA* and *bcrC* expression
 from benzoate-grown cells.

		Anaerobic conditions
Experiment	Toluene-grown cells	Benzoate-grown cells
1	AN_T2.5	AN_B2.5
2	AN_T5	AN_B5
3	AN_T10	AN_B10
		Aerobic conditions
	Toluene-grown cells	Benzoate-grown cells
4	AB_T	AB_B
5	AB_TN	AB_B

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159 2.7. Peptide detection by LC-MS

Purified peptides were dissolved in 20 µL of acetonitrile (100%), incubated for 10 min at room
temperature and consecutively in an ultrasonic bath 3 x 10 s. Further, the samples were centrifuged
and the supernatants were transferred into 12 x 3 mm LC-MS glass vials (Waters Corporation,
Milford, CT, USA).

164 Peptides were then injected into a Nano-HPLC and trapped in a C18-reverse phase column 165 (Acclaim PepMap® 100, 75 μ m x 2 cm, particle size 3 μ M, nanoViper, Thermo Fisher Scientific, 166 Waltham, MA, USA) for 5 min. Peptide separation by a two-step gradient consisted in 90 min from 167 4% to 30% of B (B: 80% acetonitrile, 0.1 % formic acid in MS-grade water) and then 30 min from 30% 168 to 55% of B. The temperature of the separation column was set to 35 °C and the flow rate was 0.3 μ L 169 /min.

170The eluted peptides were later ionized and measured. The MS was set to a full MS/dd-MS2 mode171scan with positive polarity. The full MS scan was adjusted to a MS resolution of 120,000, MS automatic172gain control (AGC) target of $3 \cdot 10^6$ ions, maximum injection time for MS of 80 ms and a scan range of173350 to 1550 m/z. The dd-MS2 scan was set to a resolution of 15,000, AGC target of $2 \cdot 10^5$ ions, a174maximum injection time for 120 ms, TopN 20, isolation window of 1.6 m/z, scan range of 200 to 2000175m/z and dynamic exclusion of 30 s.

- 177 2.8. Proteome data analysis
- 178

179 Proteomic quantification was done using the Top-3 peptide area for approach followed by log2 180 transformation. Transformed values were normalized manually by dividing the transformed values 181 by the median of each sample. The ratio was further multiplied by the mean of the entire proteome 182 data set (aerobic conditions separately from anaerobic conditions). Fold changes were calculated 183 relative to benzoate-grown cells as described in section 2.6 (Table S3). Down-regulated proteins 184 (negative values) indicate that the protein is more abundant in benzoate-grown cells than in toluene-185 grown cells. Contrary, up-regulated proteins indicate that the protein is more abundant in toluene-186 grown cells rather than in benzoate-grown cells. Since fold change of 2 was defined as the threshold, 187 up-regulated proteins were defined by $n \ge +1$ and down-regulated proteins were defined by $n \le -1$. 188 Student's T- test was the statistical method chosen to calculate significance between data. All T-test 189 values below 0.05 were considered as statistically significant.

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