

SUPPLEMENTARY MATERIAL

Functional Analysis of a c-di-AMP-specific Phosphodiesterase

MsPDE from *Mycobacterium smegmatis*

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Supplementary figures

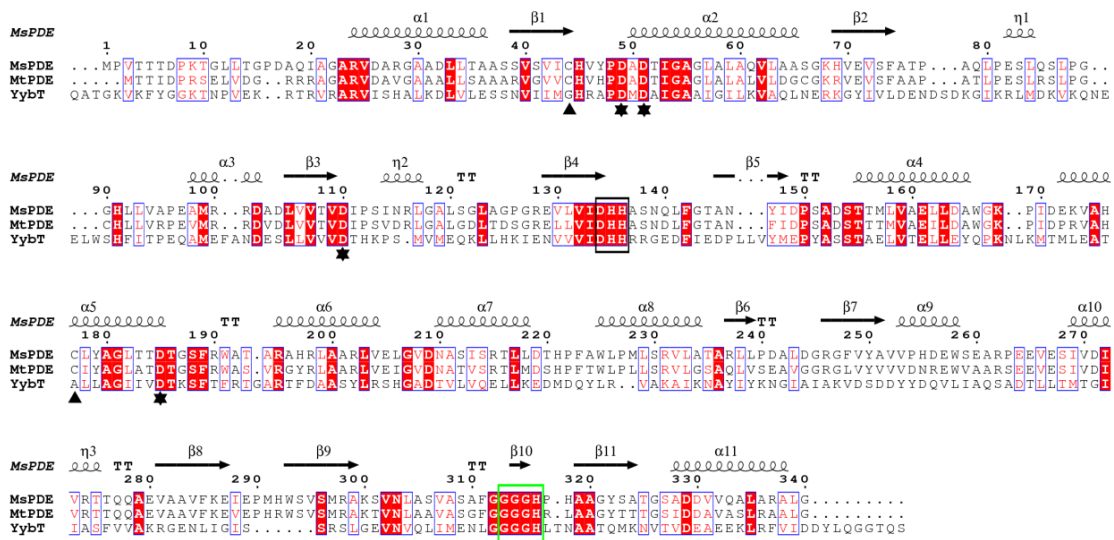


FIGURE S1. Multiple sequence alignment of MsPDE homologues. White letters shaded in red denote identical residues, red letters with white background are similar residues, black letters indicate variable residues and dots represent gaps. The amino acids critical for the phosphodiesterase activity are highlighted by black or green boxes. The secondary structures of MsPDE are shown at the top of the panel (PDB: 4LS9). α : α -helix; β : β -sheet; η : η -turns/coils. The amino acid sequences are from *M. smegmatis* MC² 155 (Accession No.: YP_886967), *M. tuberculosis* H37Rv (Accession No.: CCP45638) and *B. subtilis* strain 168 (Accession No.: P37484).

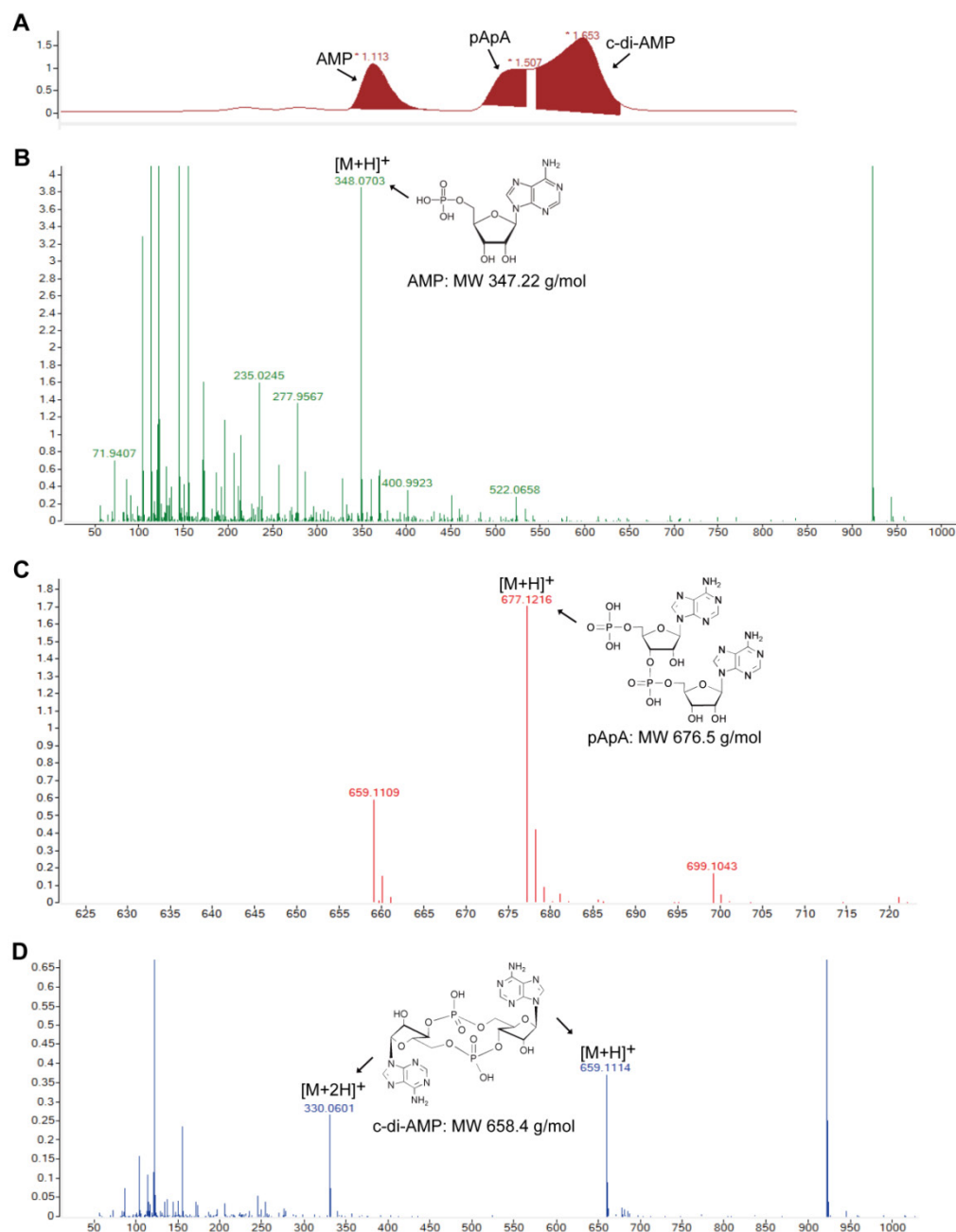


FIGURE S2. Identification the hydrolysis products of c-di-AMP catalyzed by MsPDE through LC/Q-TOF analysis. All of the analyses were performed in positive-ion detection mode. (A) HPLC-DAD analysis of the c-di-AMP hydrolysis products catalyzed by MsPDE. (B) AMP was detected at an m/z of 348.07 $[M+H]^+$. (C) pApA was detected at an m/z of 677.12 $[M+H]^+$. (D) c-di-AMP was detected at m/z of both 659 $[M+H]^+$ and 330 $[M+2H]^+$.

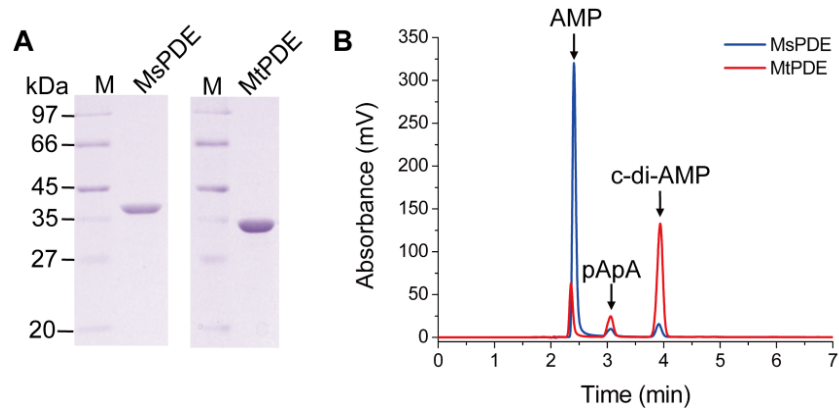


FIGURE S3. MsPDE exhibits higher PDE activity than MtPDE. (A) SDS-PAGE analyses of purified MsPDE and MtPDE. (B) Enzymatic activities of MsPDE and MtPDE in hydrolysis of c-di-AMP. The same amount of MsPDE and MtPDE were incubated at 37 °C for 20 min with 10 nM c-di-AMP in standard reaction mixture. The reaction was terminated by boiling for 15 min and analyzed by HPLC. The standard reaction mixture contains 100 mM Tris (pH 7.5), 100 mM NaCl and 10 mM MnCl₂.

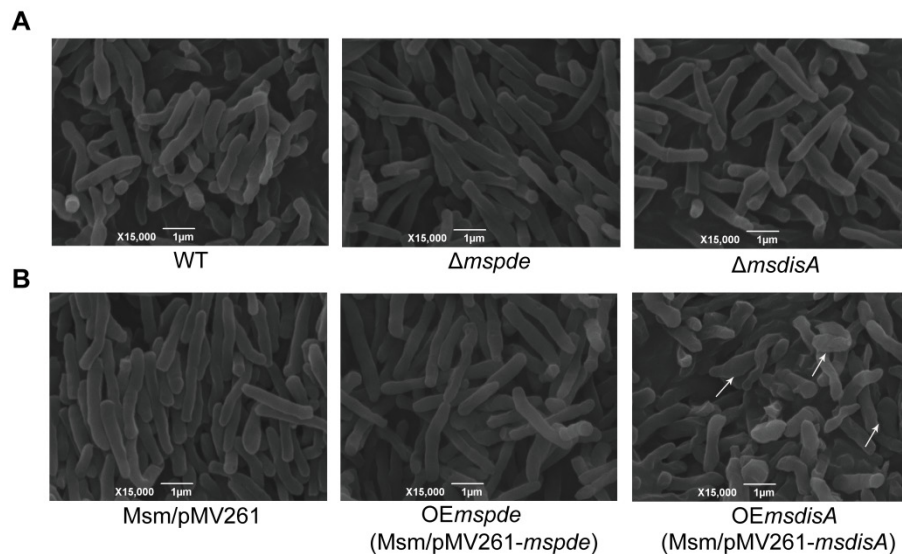


FIGURE S4. The effect of *mspde* and *msdisA* mutants on cell morphology of *M. smegmatis*. (A) Scanning electron microscopy assays of $\Delta mspde$ and $\Delta msdisA$ and WT strain. (B) Scanning electron microscopy assays of OEmspde, OEmsdisA and the control strain. The cells with swelling morphology were highlighted by arrows.

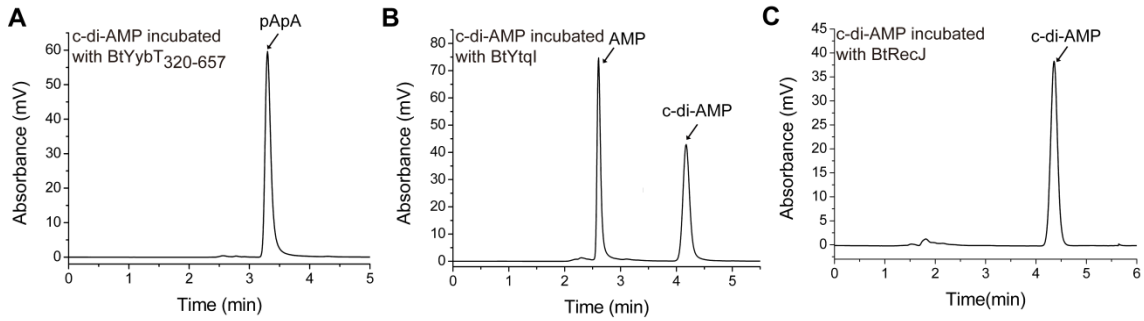


FIGURE S5. DHH family proteins exhibited significant difference when hydrolyze c-di-AMP. (A) BtYybT₃₂₀₋₆₅₇ hydrolyzed c-di-AMP in to pApA with high efficiency. (B) BtYtqI could hydrolyze c-di-AMP into AMP. (C) BtRecJ was incapable of hrdolyzing c-di-AMP.

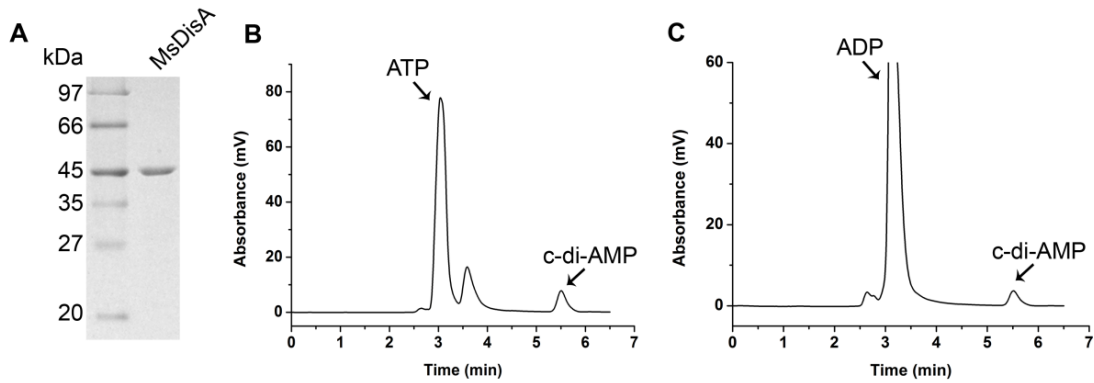


FIGURE S6. MsDisA synthesized c-di-AMP using both ATP and ADP as substrate. (A) SDS-PAGE analysis of purified MsDisA. (B) MsDisA synthesizes c-di-AMP using ATP as substrate. Purified MsdisA protein (20) was incubated at 37 °C overnight with 20 nM ATP in standard reaction mixture. (C) MsDisA converts ADP to c-di-AMP. MsdisA was incubated at 37 °C overnight with 20 nM ADP in standard reaction mixture. The reaction products were analyzed by HPLC.

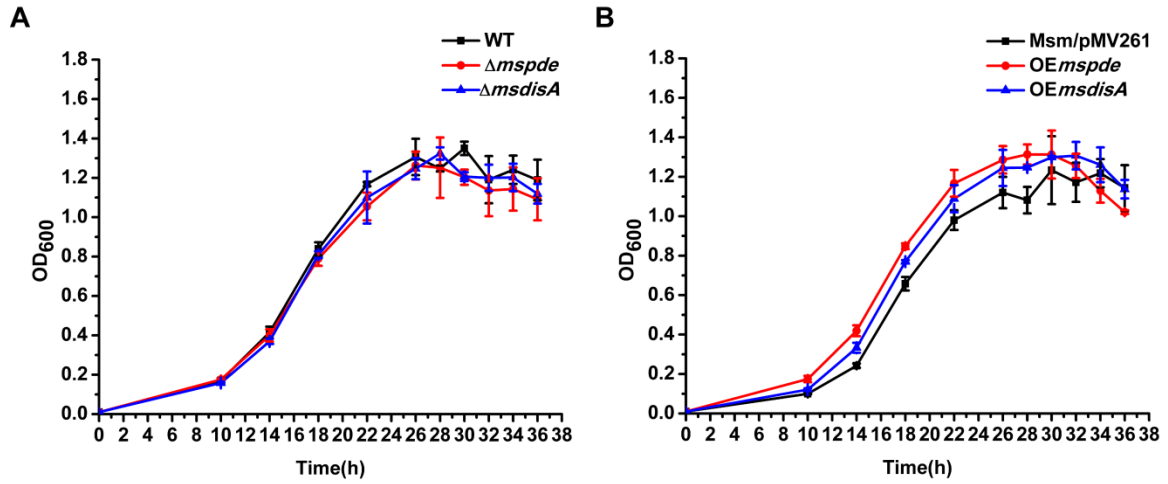


FIGURE S7. Broth growth curves of wild type (WT), $\Delta mspde$, $\Delta msdisA$, OEmspde and OEmsdisA strains. Strains from overnight-grown cultures were diluted into 7H9 broth to an OD₆₀₀ of 0.02 and incubated in flask at 37 °C. 20 μ g/ml of kanamycin was added to Msm/pMV261, OEmspde and OEmsdisA cultures to maintain the overexpression plasmids.

Supplementary tables

TABLE S1 Primers used in this study

Primers	Primer sequences (5'-3')	Purpose
<i>mispde</i> -F	CATGCCATGGGGCCGGTGACGACAACCGATC	Protein purification
<i>mispde</i> -R	CCGCTCGAGGCCAAGGGCCCGTGCGAG	Protein purification
<i>mtpde</i> -F	ATCCATGGGGACGACGATCGACCCAAGG	Protein purification
<i>mtpde</i> -R	GACTCGAGACCAAGCGCCGCGCGCAACGAC	Protein purification
<i>msdisA</i> -F	CATGCCATGGGGCCGGTGAAGTCCGGCGC	Protein purification
<i>msdisA</i> -R	TAAGAATGCGGCCCGGCCAGCCGGTCCGGCG	Protein purification
DHH-AAA-F	GCTGGTCATCGCCGCGGCCGGTCCAACCAACT	Site-directed mutation
DHH-AAA -R	GGTTGGACGCGGCCGCGGGATGACCAGCACCTC	Site-directed mutation
GGGH-AAAA-F	CGCCAGCGGTTCCGCGGCCGCGCATCCGCACG	Site-directed mutation
GGGH-AAAA-R	GCGGATGCGCGGCCGCGGCGAACGCGCTGGCGACCGA	Site-directed mutation
<i>mispdeU</i> -F	TTAATTAAGCGCGCAAGCGCAACGCGCTG	Protein purification
<i>yybT</i> -F	CCATGGAGTACGCGCCAGGGTCATTTCCG	Protein purification
<i>yybT</i> -R	CTCGAGTCAATTGTGTGCTCCCTGTA	Protein purification
<i>ytqI</i> -F	CCATGGGGCATGAGCAAATTTAGGAG	Protein purification
<i>ytqI</i> -R	CTCGAGTTTACAAATCTCACGTAAATC	Protein purification
<i>recJ</i> -F	CCATGGGGTTACAACCGAAGACGCG	Protein purification
<i>recJ</i> -R	CTCGAGCCCTAACTGTTCTACTTCT	Protein purification
<i>mispdeU</i> -F	TTAATTAAGCGCGCAAGCGCAACGCGCT	Gene knockout
<i>mispdeU</i> -R	ACTAGTCCTCAGCGTTCGTCCCCGTC	Gene knockout
<i>mispdeD</i> -F	AAGCTTTGGCTGATGCGGCGGGCGAC	Gene knockout
<i>mispdeD</i> -R	GCTAGCGCGACCGGAAGTTGAGCCAC	Gene knockout
O <i>E</i> <i>mispde</i> -F	GGATCCACCGGTGACGACAACCGATC	Overexpression
O <i>E</i> <i>mispde</i> -R	AAGCTTTCAGTGGTGGTGGTGGTGGTGGCCGCAAGGGCCCGTGCGAGCGCT	Overexpression
O <i>E</i> <i>msdisA</i> -F	GGAATTCATGGCCGTGAAGTCCGGCGCGAG	Overexpression
O <i>E</i> <i>msdisA</i> -R	CCCAAGCTTTCAGTGGTGGTGGTGGTGGTGGGCCAGCCGGTCCGGCGATCGTC	Overexpression

TABLE S2 Intracellular fatty acids were up-regulated in *Amspde* and *OEmdsiA* strains

Retention time (Min)	WT			<i>Amspde</i>			<i>OEmdsiA</i>			Response/ Response ratio	
	Response	ECL	Peak Name	Response	ECL	Peak Name	Response	ECL	Peak Name	<i>Amspde</i> / WT	<i>OEmdsiA</i> / WT
1.79	2119	7.25	---	---	---	---	---	---	---	0.00	0.00
1.89	316	7.45	---	343	7.45	---	494	7.45	---	1.09	1.56
1.92	482	7.52	---	561	7.51	---	451	7.50	---	1.16	0.94
1.995	364	7.67	---	447	7.66	---	404	7.65	---	1.23	1.11
2.037	910	7.75	---	1090	7.75	---	1113	7.74	---	1.20	1.22
2.08	1538	7.84	---	1722	7.83	---	1549	7.83	---	1.12	1.01
2.19	494	8.05	---	566	8.05	---	475	8.04	---	1.15	0.96
2.253	1592	8.18	---	1503	8.18	---	927	8.17	---	0.94	0.58
2.32	3289	8.31	---	4018	8.31	---	3374	8.30	---	1.22	1.03
2.482	283	8.63	---	280	8.63	---	285	8.63	---	0.99	1.01
2.574	1465	8.82	---	1672	8.82	---	1079	8.81	---	1.14	0.74
2.671	888	9.01	9:00	1410	9.01	9:00	1351	9.00	9:00	1.59	1.52
2.905	273	9.47	---	225	9.47	---	---	---	---	0.82	0.00
3.044	---	---	---	172	9.75	---	---	---	---	∞	---
3.111	436	9.88	---	798	9.88	---	812	9.88	---	1.83	1.86
3.174	1714	10.00	10:00	2387	10.00	10:00	2351	10.00	10:00	1.39	1.37
3.862	738	11.00	11:00	786	11.00	11:00	633	11.00	11:00	1.07	0.86
4.054	---	---	---	262	11.20	---	---	---	---	∞	---
4.319	631	11.49	---	671	11.49	---	643	11.49	---	1.06	1.02
4.784	3395	12.00	12:00	5370	12.00	12:00	4060	12.00	12:00	1.58	1.20
5.035	1215	12.22	---	1443	12.21	---	861	12.21	---	1.19	0.71
5.258	---	---	---	---	---	---	451	12.41	---	---	∞
5.612	---	---	---	---	---	---	232	12.72	---	---	∞
5.946	936	13.01	13:00	1407	13.01	13:00	725	13.01	13:00	1.50	0.77
6.252	1354	13.23		1587	13.23		1228	13.23		1.17	0.91
6.701	1215	13.56	unknown 13.565	1379	13.56	unknown 13.565	---	---	---	1.13	0.00
6.982	436	13.77	---	461	13.77	---	638	13.77	---	1.06	1.46
7.096	3066	13.85	---	2822	13.85	---	3162	13.85	---	0.92	1.03
7.298	31002	14.00	14:00	52666	14.00	14:00	48868	14.00	14:00	1.70	1.58
8.837	1782	15.00	15:00	548	14.84		1208	14.84	---	0.31	0.68
8.591	---	---	---	2714	15.00	15:00	2658	15.00	15:00	∞	∞

10.04	901	15.72	16:0 ANTEISO	1539	15.72	16:0 ANTEISO	2008	15.72	16:0 ANTEISO	1.71	2.23
10.127	7515	15.77	16:1 w9c	12089	15.77	16:1 w9c	15574	15.78	16:1 w9c	1.61	2.07
10.202	11199	15.82	Sum In Feature 3	15937	15.82	Sum In Feature 3	17608	15.82	Sum In Feature 3	1.42	1.57
10.263	45257	15.86	Sum In Feature 3	73419	15.86	Sum In Feature 3	71218	15.86	Sum In Feature 3	1.62	1.57
10.506	220284	16.00	16:00	368950	16.00	16:00	388366	16.01	16:00	1.67	1.76
10.897	---	---	---	1262	16.23	15:0 2OH	786	16.24	---	∞	∞
10.97	---	---	---	---	---	---	1075	16.27	---	---	∞
11.249	3628	16.43	16:0 10 methyl	5560	16.43	16:0 10 methyl	4786	16.43	16:0 10 methyl	1.53	1.32
11.753	---	---	---	391	16.72	17:0 ANTEISO	338	16.72	17:0 ANTEISO	∞	∞
11.874	994	16.80	17:1 w8c	2353	16.79	17:1 w8c	2231	16.79	17:1 w8c	2.37	2.24
12.231	1932	17.00	17:00	3124	17.00	17:00	2701	17.00	17:00	1.62	1.40
12.949	---	---	---	585	17.41	17:0 10 methyl	455	17.41	17:0 10 methyl	∞	∞
13.491	---	---	---	---	---	---	1537	17.72	Sum In Feature 5	---	∞
13.588	94902	17.77	18:1 w9c	150069	17.77	18:1 w9c	193270	17.78	18:1 w9c	1.58	2.04
13.684	5355	17.83	18:1 w7c	9414	17.83	18:1 w7c	12402	17.83	18:1 w7c	1.76	2.32
13.988	19506	18.00	18:00	42605	18.00	18:00	41209	18.00	18:00	2.18	2.11
14.333	4583	18.20	---	5048	18.20	---	6178	18.20	---	1.10	1.35
14.483	---	---	---	---	---	---	1449	18.29	---	∞	∞
14.68	59712	18.40	TBSA 10Me18:0	107581	18.40	TBSA 10Me18:0	102229	18.40	TBSA 10Me18:0	1.80	1.71
14.98	4326	18.57	---	5135	18.57	---	5855	18.57	---	1.19	1.35
15.096	2212	18.64	19:0 ISO	3051	18.63	19:0 ISO	2800	18.63	19:0 ISO	1.38	1.27
15.882	1867	19.09	18:1 2OH	2446	19.08	18:1 2OH	1457	19.09	18:1 2OH	1.31	0.78
16.6	---	---	---	---	---	---	687	19.50	---	---	∞
16.722	994	19.57	---	2394	19.58	---	2256	19.58	---	2.41	2.27
16.808	---	---	---	2319	19.62	---	2540	19.62	---	∞	∞
16.941	---	---	---	4649	19.70	---	3703	19.69	---	∞	∞
17.137	1332	19.81	---	3309	19.81	---	2416	19.81	---	2.48	1.81
17.461	3777	20.00	20:00	7788	20.00	20:00	6270	20.00	20:00	2.06	1.66
17.658	---	---	---	671	20.11	---	821	20.11	---	∞	∞
17.77	945	20.18	---	1513	20.17	---	1567	20.17	---	1.60	1.66
17.886	---	---	---	3345	20.24	---	3240	20.23	---	∞	∞
17.969	---	---	---	---	---	---	2895	20.29	---	---	∞

18.1	52714	20.37	---	75612	20.37	---	1065	20.44	---	1.43	0.02
18.334	3302	20.5	---	4748	20.50	---	2543	20.50	---	1.44	0.77
18.398	4689	20.54	---	6487	20.53	---	2741	20.53	---	1.38	0.58
18.478	7005	20.59	---	7614	20.59	---	2512	20.58	---	1.09	0.36

Abbreviations: ECL, equivalent chain length.

* Response/Response ratio > 1.25, up-regulated (highlighted in red); Response/Response ratio < 0.8, down-regulated (highlighted in green).

TABLE S3 Intracellular fatty acids in *ΔmsdisA* and *OEmspde* strains were down-regulated

Retention time (Min)	WT			<i>ΔmsdisA</i>			<i>OEmspde</i>			Response/ Response ratio	
	Response	ECL	Peak Name	Response	ECL	Peak Name	Response	ECL	Peak Name	<i>ΔmsdisA</i> /WT	<i>OEmspde</i> /WT
1.643	---	---	---	222466	6.95	---	215707	6.95	---	∞	∞
1.79	2119	7.25	---	2083	7.25	---	1845	7.26	---	0.98	0.87
1.89	316	7.45	---			---	268	7.45	---	0.00	0.85
1.92	482	7.52	---	161	7.51	---	533	7.51	---	0.33	1.11
1.995	364	7.67	---	214	7.65	---	400	7.66	---	0.59	1.10
2.037	910	7.75	---	761	7.75	---	988	7.75	---	0.84	1.09
2.08	1538	7.84	---	1185	7.83	---	1772	7.83	---	0.77	1.15
2.19	494	8.05	---			---	793	8.05	---	0.00	1.61
2.253	1592	8.18	---	1588	8.17	---	1362	8.17	---	1.00	0.86
2.32	3289	8.31	---	2734	8.31	---	3246	8.31	---	0.83	0.99
2.482	283	8.63	---	192	8.64	---	294	8.63	---	0.68	1.04
2.574	1465	8.82	---	1352	8.81	---	1469	8.82	---	0.92	1.00
2.671	888	9.01	9:00	932	9.01	9:00	1076	9.01	9:00	1.05	1.21
2.905	273	9.47	---	247	9.48	---	324	9.48	---	0.90	1.19
3.111	436	9.88	---	395	9.89	---	721	9.88	---	0.91	1.65
3.174	1714	10.00	10:00	1231	10.00	10:00	1959	10.00	10:00	0.72	1.14
3.862	738	11.00	11:00	---	---	---	505	11.00	11:00	0.00	0.68
4.319	631	11.49	---	536	11.49	---	537	11.49	---	0.85	0.85
4.784	3395	12.00	12:00	2096	12.00	12:00	3358	12.00	12:00	0.62	0.99
5.035	1215	12.22	---	501	12.21	---	1001	12.22	---	0.41	0.82
5.946	936	13.01	13:00	315	13.01	13:00	527	13.01	13:00	0.34	0.56
6.252	1354	13.23	---	667	13.23	---	1000	13.23	---	0.49	0.74
6.643	---	---	---	458	13.52	---	575	13.52	---	∞	∞
6.701	1215	13.56	unknown 13.565	---	---	---	---	---	---	0.00	0.00
6.982	436	13.77	---	---	---	---	391	13.77	---	0.00	0.90
7.096	3066	13.85	---	---	---	---	---	---	---	0.00	0.00
7.103	---	---	---	---	---	---	2995	13.85	---	---	∞
7.159	---	---	---	2282	13.90	14:1 w5c	---	---	---	∞	---
7.298	31002	14.00	14:00	15159	14.00	14:00	27275	14.00	14:00	0.49	0.88
8.837	1782	15.00	15:00	567	15.00	15:00	1311	15.00	15:00	0.32	0.74
10.04	901	15.72	16:0 ANTEISO	---	---	---	667	15.72	16:0 ANTEISO	0.00	0.74
10.127	7515	15.77	16:1 w9c	3123	15.78	16:1 w9c	5724	15.77	16:1 w9c	0.42	0.76
10.202	11199	15.82	Sum In Feature 3	3519	15.82	Sum In Feature 3	8498	15.82	Sum In Feature 3	0.31	0.76

10.263	45257	15.86	Sum In Feature 3	17505	15.86	Sum In Feature 3	33856	15.86	Sum In Feature 3	0.39	0.75
10.506	220284	16.00	16:00	81123	16.00	16:00	171547	16.00	16:00	0.37	0.78
11.249	3628	16.43	16:0 10 methyl	738	16.43	16:0 10 methyl	2664	16.43	16:0 10 methyl	0.20	0.73
11.874	994	16.80	17:1 w8c	---	---	---	---	---	---	0.00	0.00
12.231	1932	17.00	17:00	27942	17.11	---	1435	17.00	17:00	14.46	0.74
13.588	94902	17.77	18:1 w9c	27623	17.78	18:1 w9c	69673	17.77	18:1 w9c	0.29	0.73
13.684	5355	17.83	18:1 w7c	1613	17.83	18:1 w7c	3863	17.83	18:1 w7c	0.30	0.72
13.988	19506	18.00	18:00	8148	18.00	18:00	15269	18.00	18:00	0.42	0.78
14.333	4583	18.20	---	3714	18.20	---	---	---	---	0.81	0.00
14.68	59712	18.40	TBSA 10Me18:0	19765	18.40	TBSA 10Me18:0	44972	18.40	TBSA 10Me18:0	0.33	0.75
14.98	4326	18.57	---	2370	18.57	---	3701	18.57	---	0.55	0.86
15.096	2212	18.64	19:0 ISO	---	---	---	1470	18.64	19:0 ISO	0.00	0.66
15.882	1867	19.09	18:1 2OH	---	---	---	1010	19.09	18:1 2OH	0.00	0.54
16.722	994	19.57	---	---	---	---	3306	19.62	---	0.00	3.33
17.137	1332	19.81	---	---	---	---	857	19.81	---	0.00	0.64
17.461	3777	20.00	20:00	843	20.00	20:00	2741	20.00	20:00	0.22	0.73
17.77	945	20.18	---	---	---	---	643	20.17	---	0.00	0.68
17.89	---	---	---	---	---	---	1390	20.24	---	---	∞
18.1	52714	20.37	---	135370	20.38	---	39551	20.38	---	2.57	0.75
18.334	3302	20.50	---	---	---	---	---	---	---	0.00	0.00
18.398	4689	20.54	---	---	---	---	---	---	---	0.00	0.00
18.478	7005	20.59	---	---	---	---	---	---	---	0.00	0.00

Abbreviations: ECL, equivalent chain length.

* Response/Response ratio > 1.25, up-regulated (highlighted in red); Response/Response ratio < 0.8, down-regulated (highlighted in green).