Supplemental Table 1. Exact mass of asukamycin and its derivatives.

	Formula	Calculated Mass	Observed Mass	Mass Error (ppm)	Upper Chain Fragment
A1	$C_{31}H_{34}N_2O_7$	546.2366	546.2360	1.19	189.13
A2	$C_{28}H_{30}N_2O_7$	506.2053	506.2062	1.78	149.09
A3, **A4**	$C_{29}H_{32}N_2O_7$	520.2210	520.2228	3.62	163.11
A5	$C_{30}H_{34}N_2O_7$	534.2366	534.2377	2.05	177.13
A6, **A7**	$C_{31}H_{36}N_2O_7$	548.2523	548.2530	1.36	191.14
B1	$C_{31}H_{36}N_2O_7$	548.2523	548.2528	0.91	189.13
B2	$C_{28}H_{32}N_2O_7$	508.2210	508.2229	3.74	149.10
B3, **B4**	$C_{29}H_{34}N_2O_7$	522.2366	522.2382	3.06	163.11
B5	$C_{30}H_{36}N_2O_7$	536.2523	536.2517	1.02	177.13
C1	$C_{31}H_{34}N_2O_5$	514.2468	514.2477	1.73	189.13
C2	$C_{28}H_{30}N_2O_5$	474.2155	474.2163	1.68	149.10
C3, **C4**	$C_{29}H_{32}N_2O_5$	488.2311	488.2317	1.15	163.11
C5	$C_{30}H_{34}N_2O_5$	502.2468	502.2477	1.77	177.13
D1	$C_{31}H_{34}N_2O_6$	530.2417	530.2422	0.90	189.13
D2	$C_{29}H_{32}N_2O_6$	504.2260	504.2254	1.25	149.10
D3, **D4**	$C_{28}H_{30}N_2O_6$	490.2104	490.2110	1.34	163.11
D5	$C_{30}H_{34}N_2O_6$	518.2417	518.2411	1.06	177.13
A8	$C_{20}H_{20}N_2O_7$	400.1271	400.1289	4.73	---
B8	$C_{20}H_{22}N_2O_7$	402.1427	402.1444	4.19	---
C8	$C_{20}H_{20}N_2O_5$	368.1372	368.1394	6.02	---
A1a	$C_{26}H_{29}NO_6$	451.1995	451.2010	3.44	189.13
A2a	$C_{23}H_{25}NO_6$	411.1682	411.1657	6.13	149.09
A3a,**A4a**	$C_{24}H_{27}NO_6$	425.1838	425.1804	8.13	163.11
A5a	$C_{25}H_{29}NO_6$	439.1995	439.1990	1.09	177.12
B1a	$C_{26}H_{31}NO_6$	453.2151	453.2171	4.43	189.13
B2a	$C_{23}H_{27}NO_6$	413.1838	413.1801	8.93	149.09
B3a, **B4a**	$C_{24}H_{29}NO_6$	427.1995	427.1956	9.19	163.10
B5a	$C_{25}H_{31}NO_6$	441.2151	441.2120	7.05	177.12

Supplemental Table 2. NMR connectivities in 4-hydroxyprotoasukamycin (**D1**) observed by ^{1}J- and ^{n}J-heteronuclear carbon-proton shift correlation methods (HSQC and HMBC).

Position	δ_C (ppm)	δ_H (ppm)	C,H-Coupling[a]
1	180.07	--	6-H, 0-NH, 5-H, 3-H
2	140.95	--	--
3	124.57	6.21 (d, *J*=10.1)	0-NH (w)
4	69.63	--	7-H, 3-H, 8-H, 5-H (w), 0-NH (w)
5	152.53	6.93 (dd, *J*=10.0, 3.1)	6-H, 7-H, 3-H (w)
6	130.64	7.62 (d, *J*=2.95)	5-H, 3-H, 7-H, 0-NH
7	137.64	5.80 (d, *J*=15.5)	6-H, 5-H, 3-H, 8-H, 9-H
8	131.12	6.48 (dd, *J*=14.8, 11.8)	9-H, 10-H
9	138.97	6.66 (dd, *J*=14.8, 11.2)	7-H, 8-H, 11-H
10	129.35	6.53 (dd, *J*=15.37, 11.31)	8-H, 11-H
11	141.23	7.07 (dd, *J*=15.0, 11.6)	10-H, 12-H
12	123.47	6.46 (d, *J*=15.2)	11-H, 10-H
13	164.94	--	14-NH
1’	164.94	--	0-NH, 2’-H
2’	123.85	6.51 (d, *J*=15.2)	3’-H, 4’-H
3’	141.23	7.15 (dd, *J*=15.0, 11.6)	2’-H, 4’-H, 5’-H
4’	128.53	6.30 (dd, *J*=14.9, 11.7)	3’-H, 2’-H, 6’-H
5’	140.59	6.63 (dd, *J*=15.0, 10.9)	3’-H, 6’-H, 7’-H
6’	127.67	6.18 (dd, *J*=15.2, 10.9)	4’-H, 5’-H
7’	144.84	5.90 (dd, *J*=15.4, 7.0)	5’-H, 9’/13’-H (w)
8’	40.46	2.08 (m)	6’-H, 7’-H, 9’/13’-H, 10’/12’-H
9’,13’	32.10	1.70 (m), 1.09 (m)	10’/12’-H, 13’/9’-H, 7’-H, 11’-H
10’,12’	25.39	1.26 (m), 1.68 (m)	11’-H, 9’/13’-H, 12’/10’-H
11’	25.60	1.13 (m), 1.63 (m)	10’/12’-H, 9’/13’-H
1’’			
2’’	113.57	--	
3’’			
4’’			
5’’			
NH-14		9.20 (s)	
NH-0’		9.17 (s)	

[a.] Indicated protons show connectivities to the given carbon attributed to ^{2}J-, ^{3}J- or weak ^{4}J-coupling. (w) weak *J*-coupling.

[b.] Coupling with 8’-H (2.08 ppm) was suppressed.

[c.] ^{13}C-NMR and ^{1}H-NMR signals for C1’’, C3’’, C4’’ and C5’’ are undetectable in DMSO-d^6.

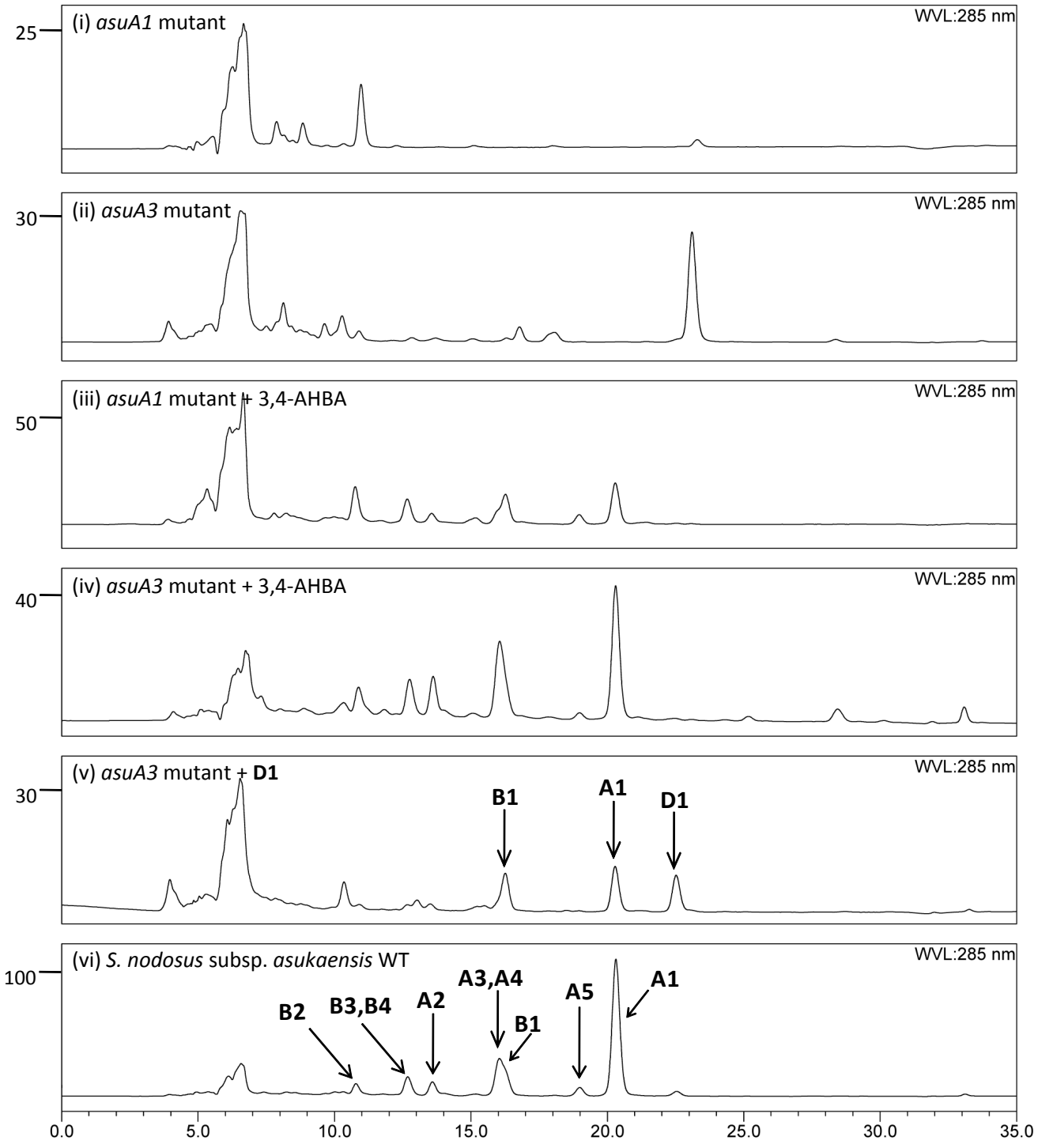
Supplemental Table 3. Oligonucleotide primers used for the mutant constructions and λ-Red recombinations.

Primers	Sequence from 5' to 3'
AsuE1-1	CCCAAGCTTCGAAGACCCGTGGCGTGAGCC
AsuE1-2	CCCAGATCTCCGCGCGCACAGCAGCGC
AsuE1-3	CCCAGATCTCCGGCGGTGAAGGGCGCGCAC
AsuE1-4	CCCCTCGAGACCTGATGGAGGGGTCCAGTA
AsuRED-4	GGTCCGGAAGCGGCCTGCGGAGCGCCCTGCGGGGCGGCTCTAGAGATATCATTCCG GGGATCCGTCGACC
AsuRED-6	AGAACCACGGCTGGACCGAGGGCTGACACCCCCGGGGCGTATCCGGCCGTTGTAGG CTGGAGCTGCTTC
AsuRED-E3F	AGTCGTCAGCCTGCCCTACCACCACCCGTTCATGGTGGCCTCGCGGGCCATTCCGG GGATCCGTCGACC
AsuRED-E3R	AGCCGACGATCGTGCTGCGCCGGTCGATCAGCTCGGCCAGGGCCTTGCGTGTAGGC TGGAGCTGCTTC
S83TF	AAGGATCCGGCGGCACCCGCAACATCGGTGGCACCAA
S83TR	GGGATGGAGGTGGTGAAGATGAAG

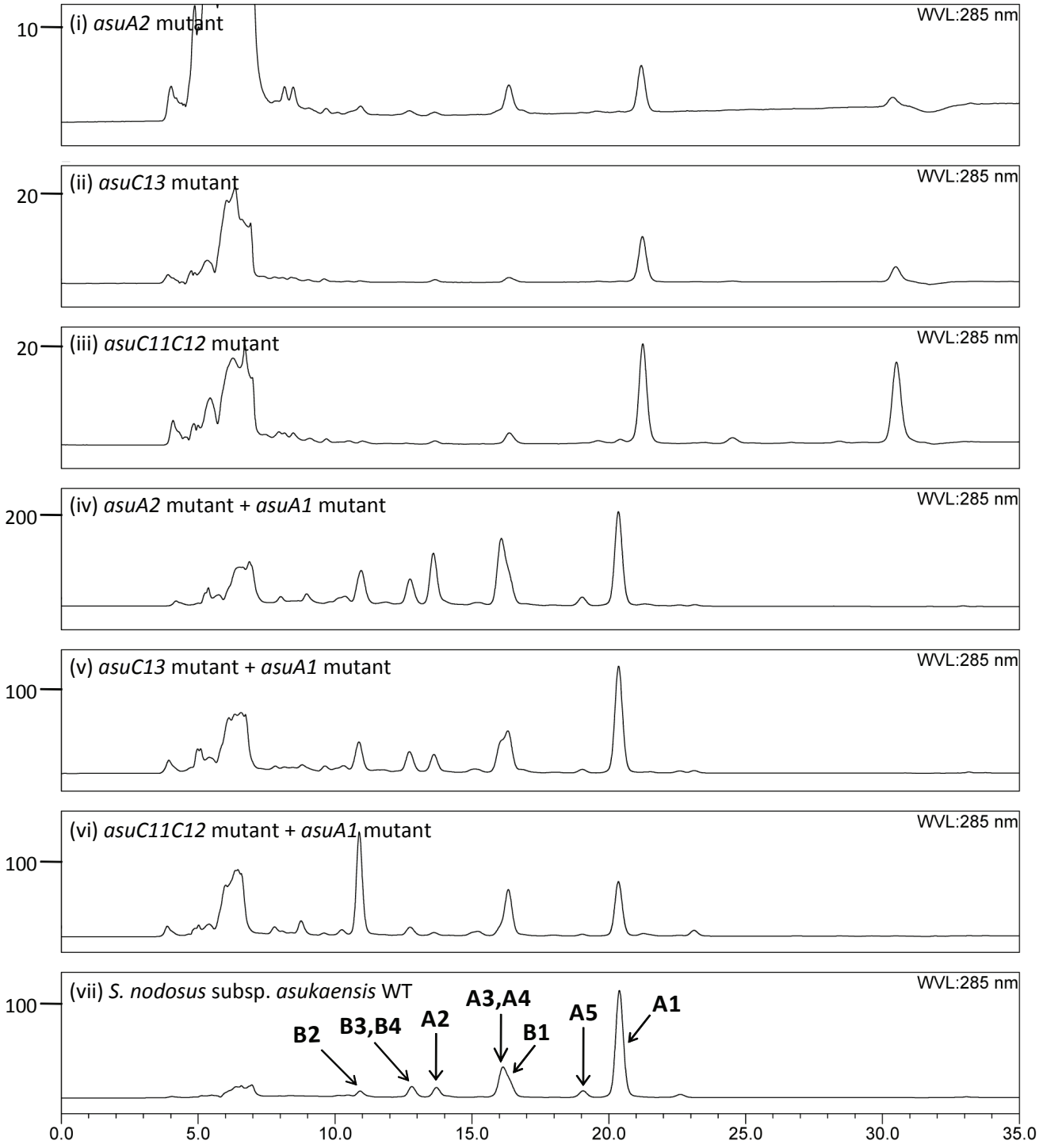
* The underlined regions indicate the DNA sequence matching pART1361.

Supplemental Figure 1. HPLC analysis of asukamycin metabolites. The arrows identify the peaks of asukamycin **A1** and related metabolites, **A2-A5**, **B1-B4**, **C1-C4**, **D1-D5**, **A1a-A5a**, **B1a-B4a**.
a. Twenty µL of crude culture extract of the *asuA1* mutant (i), the *asuA3* mutant (ii), the *asuA1* mutant supplemented with 3,4-AHBA (iii), the *asuA3* mutant supplemented with 3,4-AHBA (iv), the *asuA3* mutant supplemented with **D1** (v) and the *S. nodosus* subsp. *asukaensis* wild type strain (vi).
b. Twenty µL of crude culture extract of the *asuA2* mutant (i), the *asuC13* mutant (ii), the *asuC11C12* mutant (iii), the *S. nodosus* subsp. *asukaensis* wild type strain (iv), and 100 µL crude co-culture extract of the *asuA2* and the *asuC1* mutant (v), the *asuC13* and the *asuC1* mutant (vi), the *asuC11C12* and the *asuC1* mutant (vii).
c. Twenty µL of crude culture extract of the *asuD1* mutant (i), the *asuD2* mutant (ii) and the *asuD3* mutant (iii).
d. Twenty µL of crude culture extract of the *asuE1* mutant (i), the *asuE2* mutant (ii), the *asuE3* mutant (iii) and the *S. nodosus* subsp. *asukaensis* wild type strain (iv).

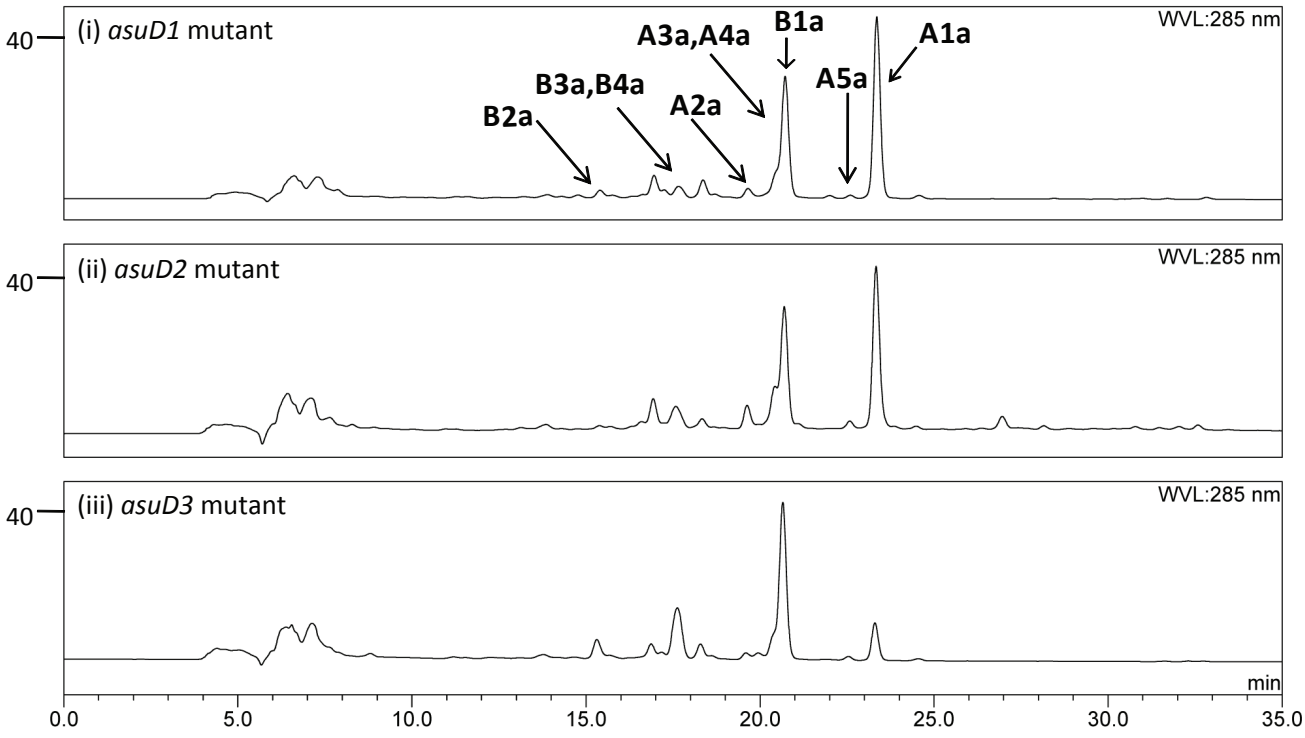
Supplemental Fig. 1a



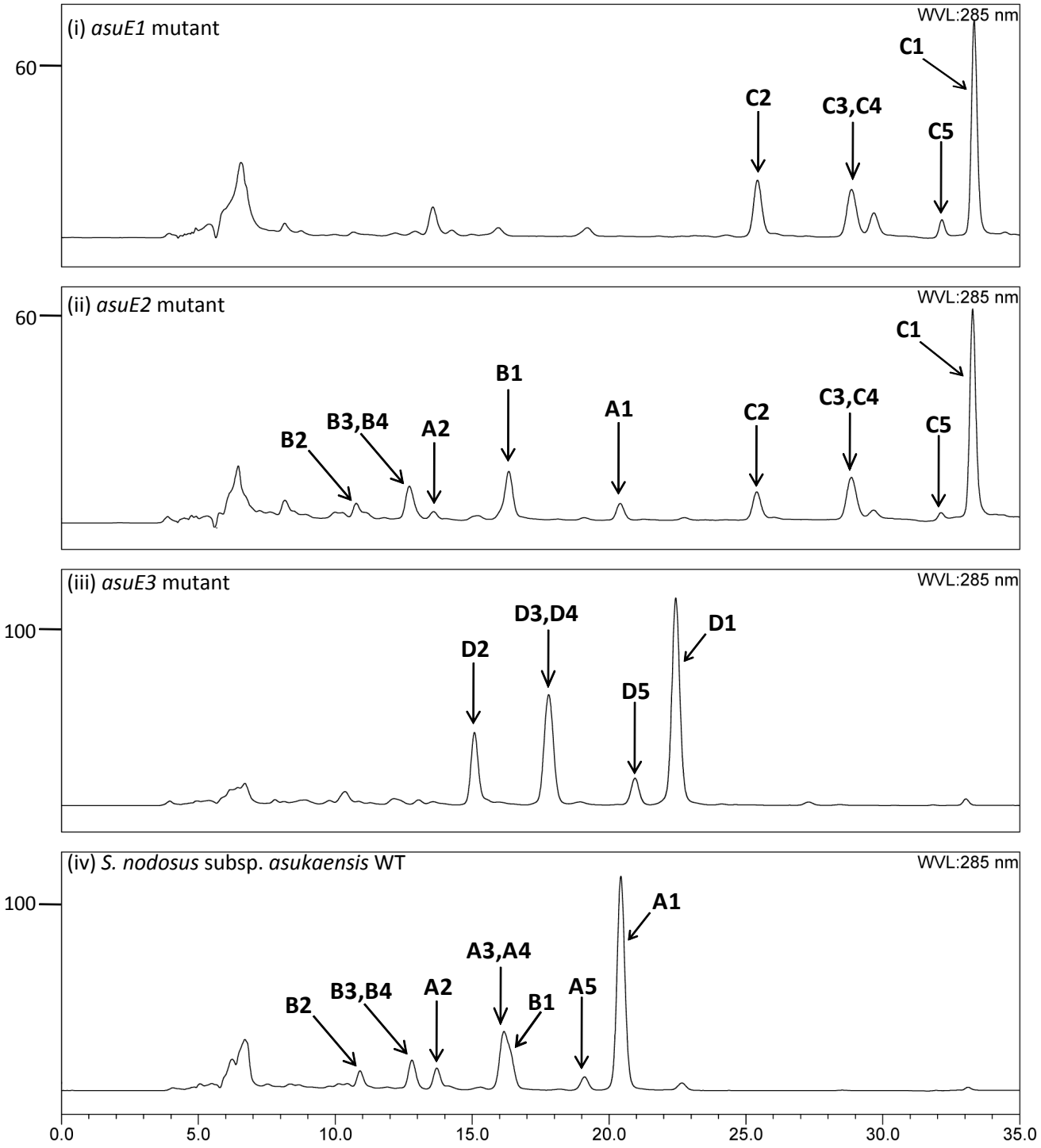
Supplemental Fig. 1b



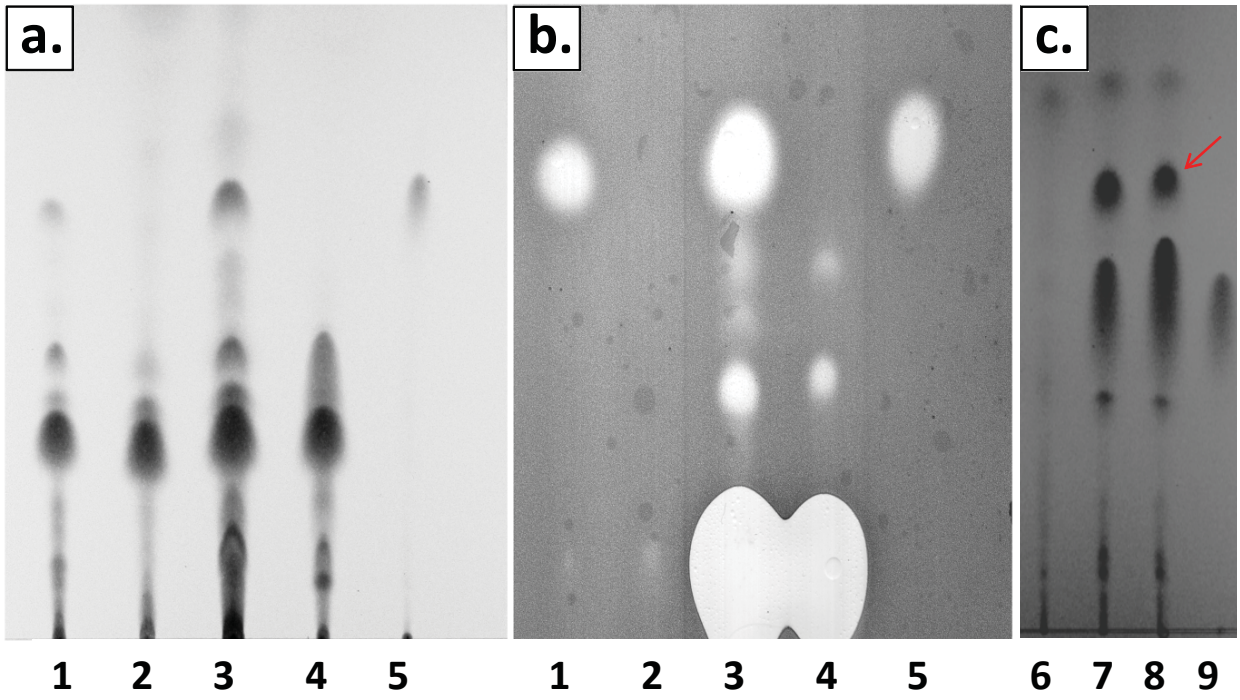
Supplemental Fig. 1c



Supplemental Fig. 1d

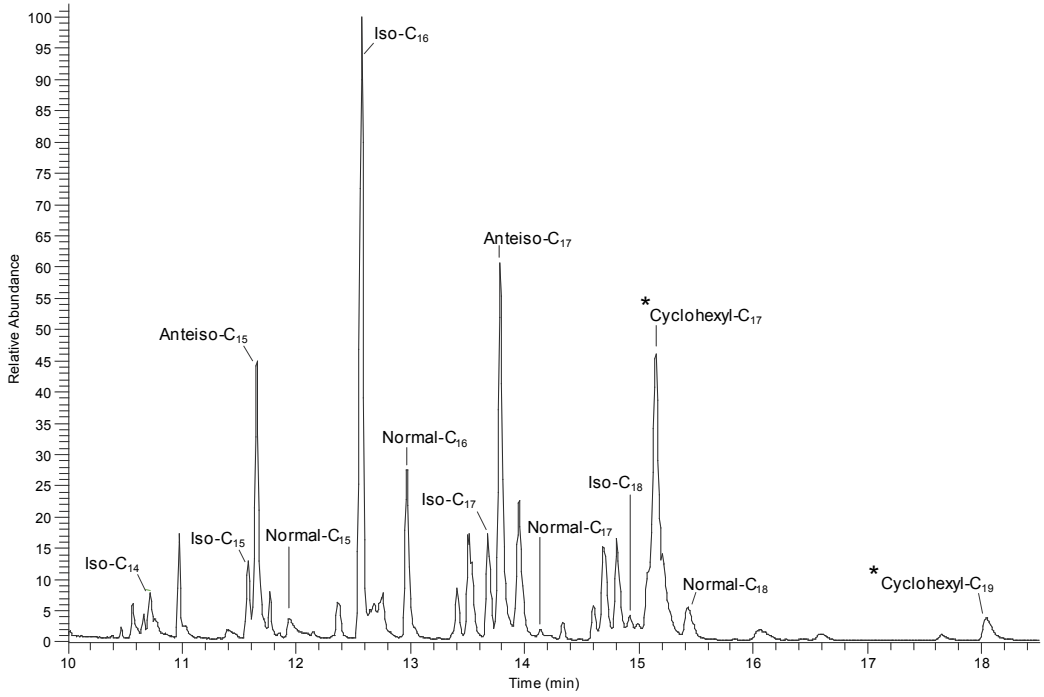


Supplemental Figure 2. TLC analysis and bioassay. **a.** and **c.** Chromatograms visualized under UV 254 nm. **b.** The same TLC plate was further overlayed with *Bacillus subtilis* mixed with nutrient agar and incubated overnight at 37°C. The red arrow shows the newly formed product 2880-II. Lane 1, the wild type strain; Lane 2, the *asuD2* mutant; Lane 3, the *asuD2* mutant/pALS4; Lane 4, the *asuD2* mutant/pALS4-S83T; Lane 5, standard asukamycin (10 μg); Lane 6, *S. lividans*/pAS9A1; Lane 7,8, *S. lividans*/pAS9A1 supplemented with ferulic acid (2.5 mg/ml); Lane 9, standard ferulic acid (10 μg).

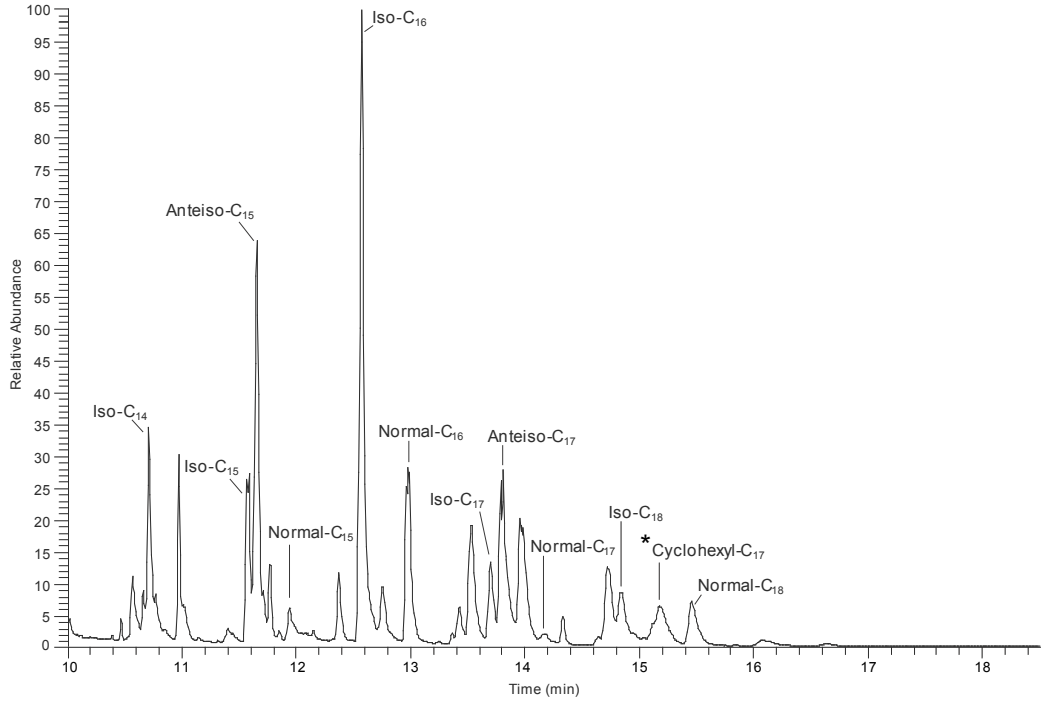


Supplemental Figure 3. Fatty acid profiles generated by GC-MS analysis. **a.** The *asuC15* mutant. **b.** The *S. nodosus* subsp. *asukaensis* wild type strain. The identified fatty acid derivatives are indicated.

a. *asuC15* mutant

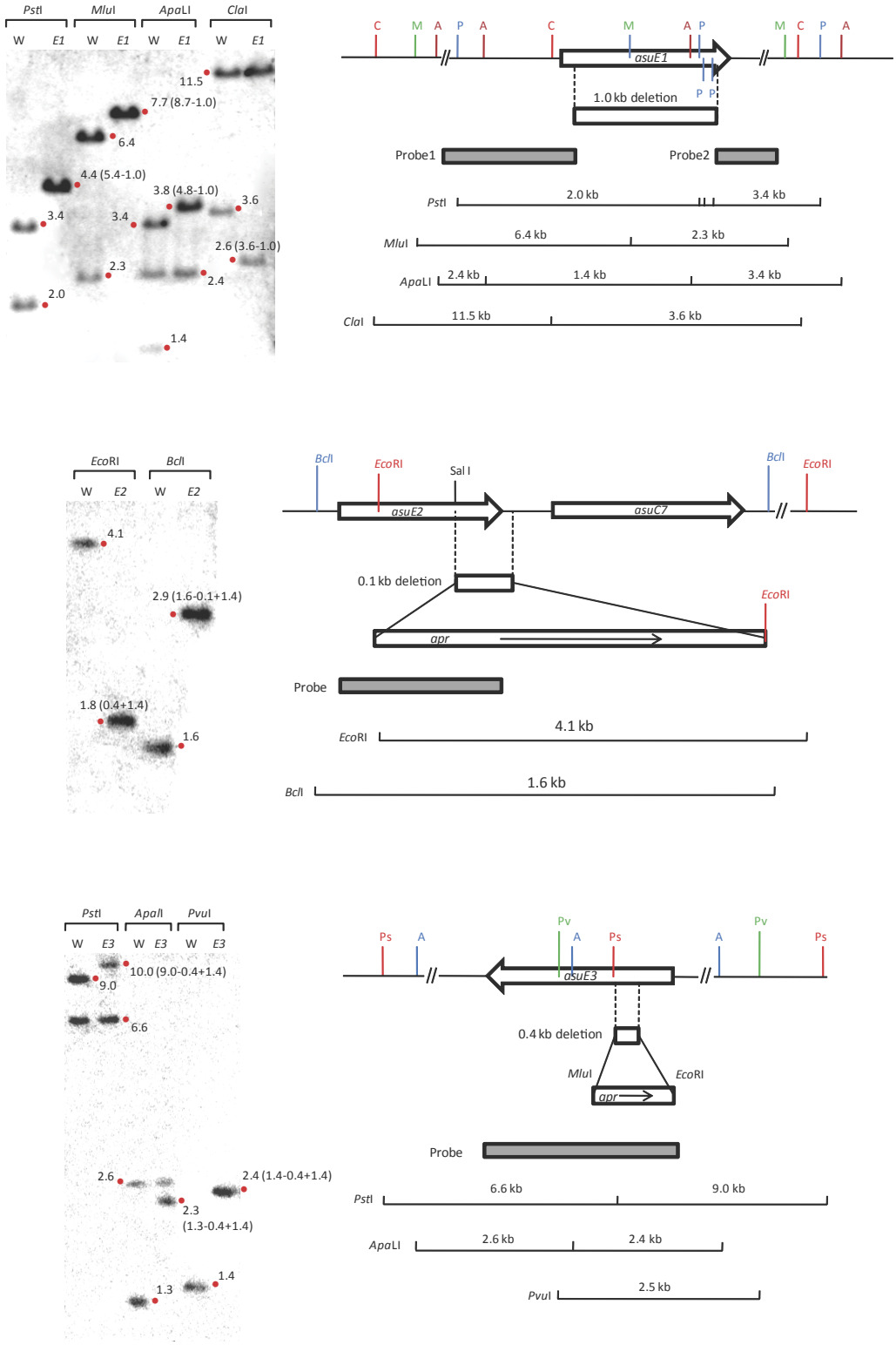


b. *S. nodosus* subsp. *asukaensis* wild type

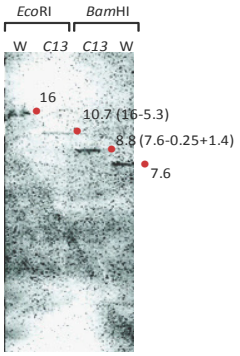


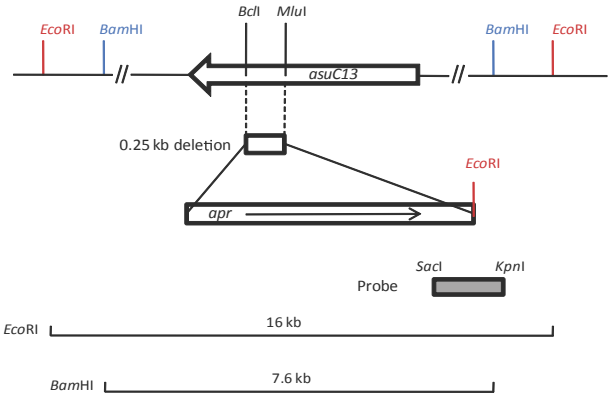
* ω-cyclohexyl fatty acids

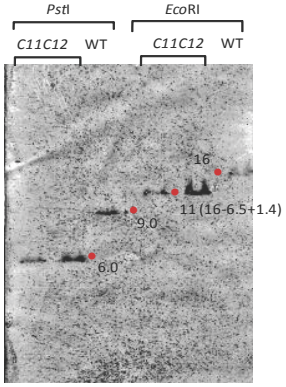
Supplemental Figure 4. Southern blots of thirteen constructed mutants. Hybridization probes and anticipated restriction digestion patterns are indicated.

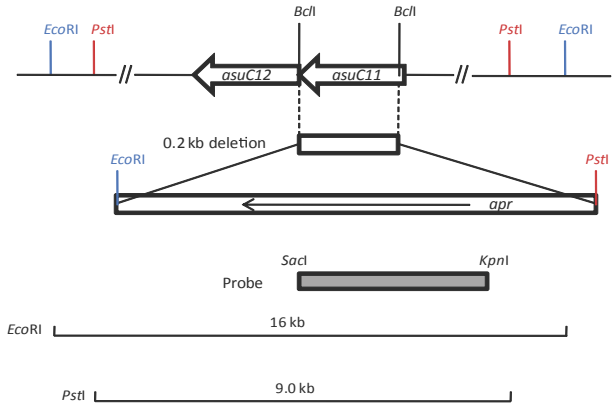


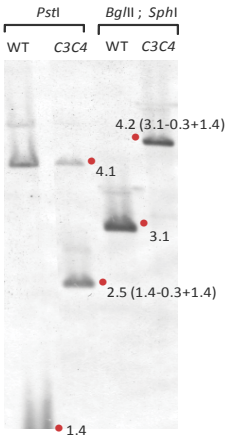
Supplemental Fig. 4 continued

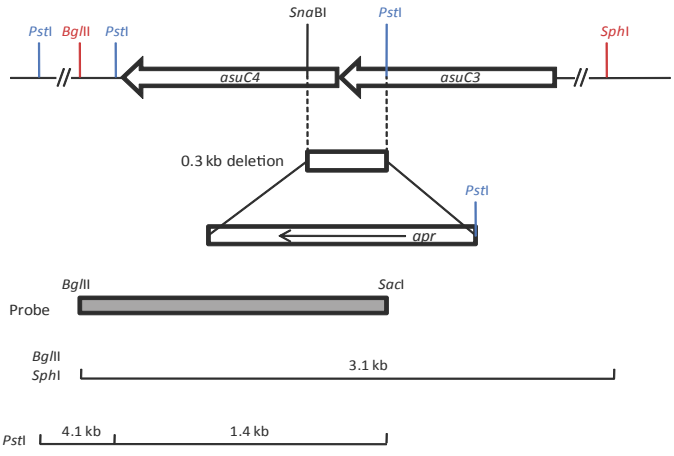




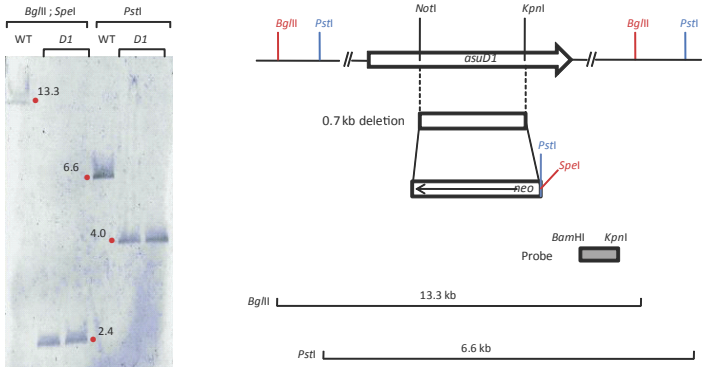


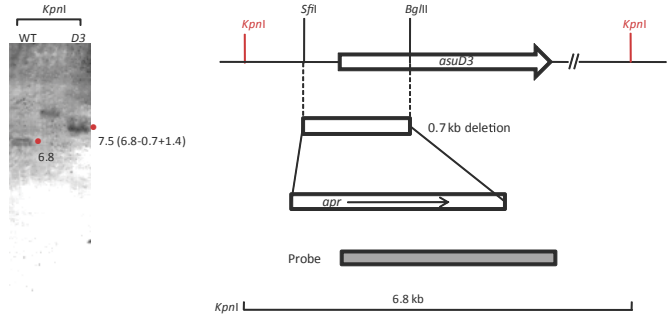


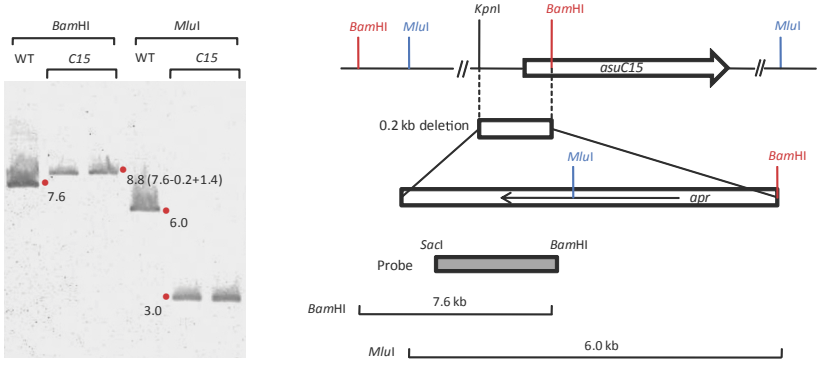


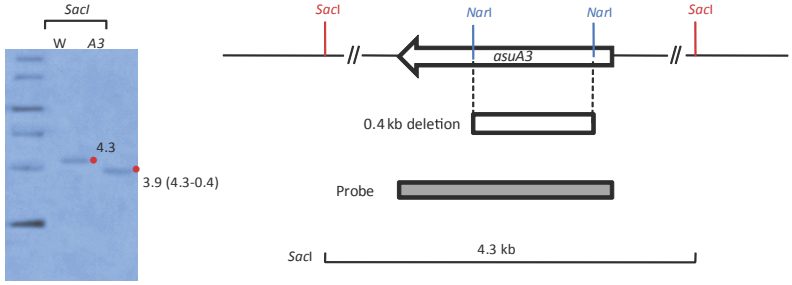


Supplemental Fig. 4 continued

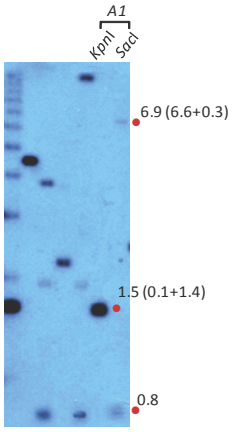


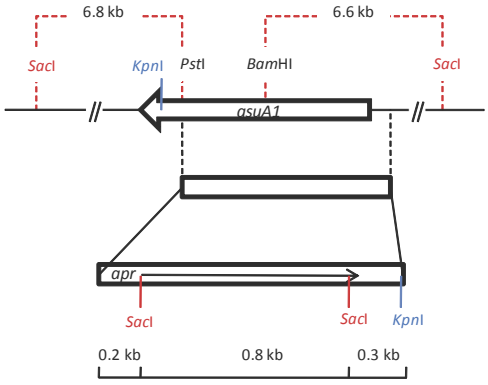




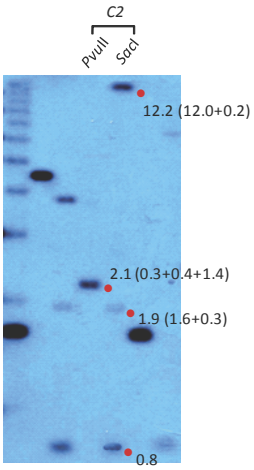


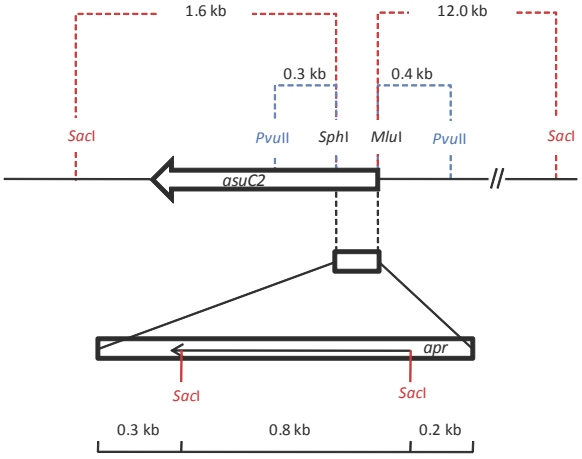
Supplemental Fig. 4 continued



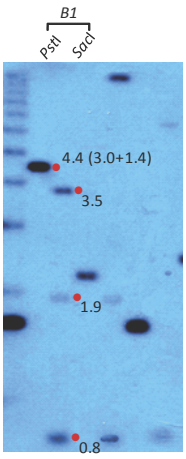


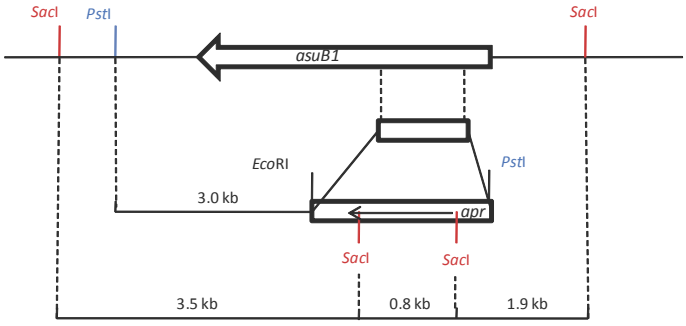
Notes: probed with apramycin resistance gene.





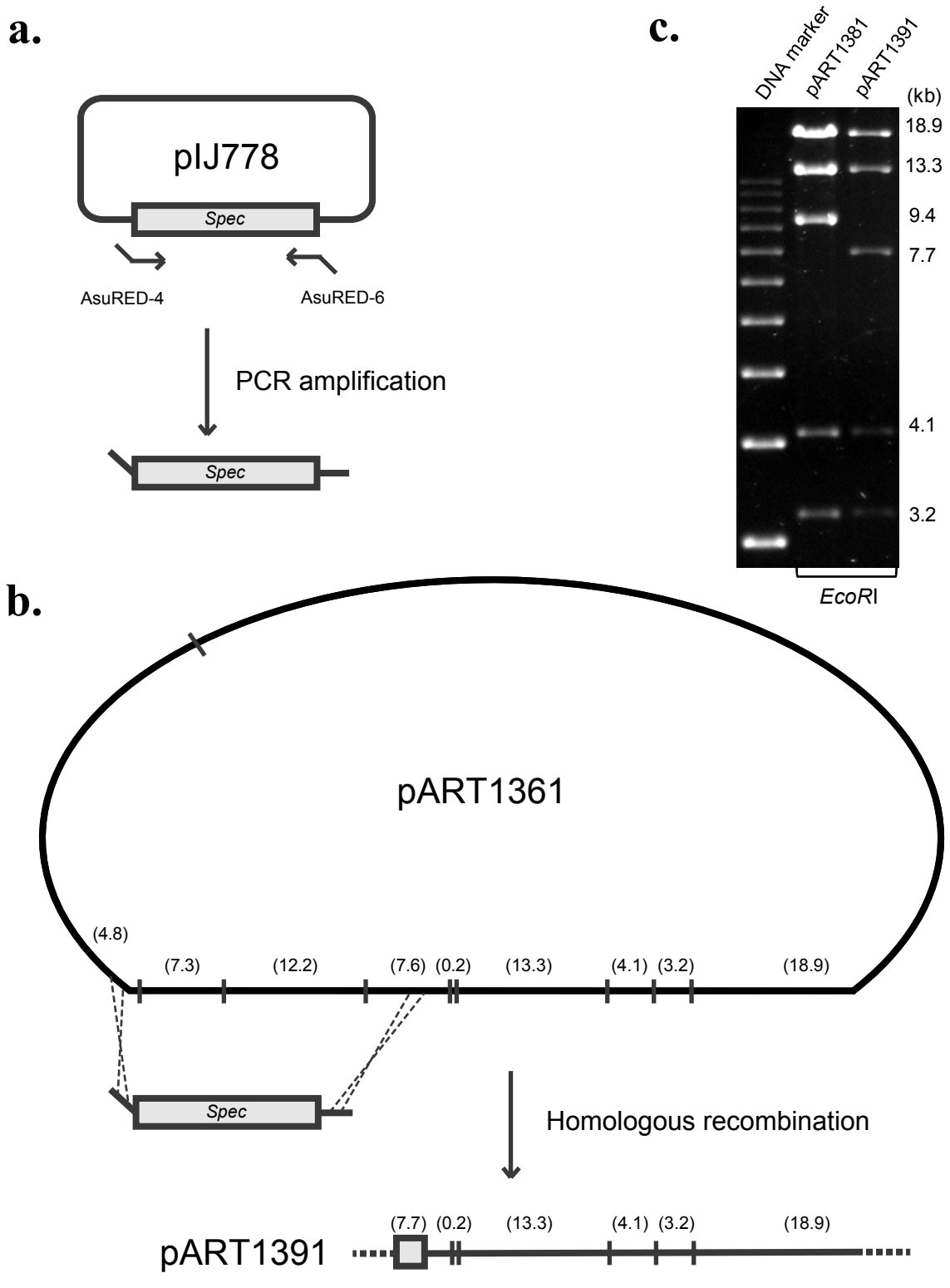
Notes: probed with apramycin resistance gene.



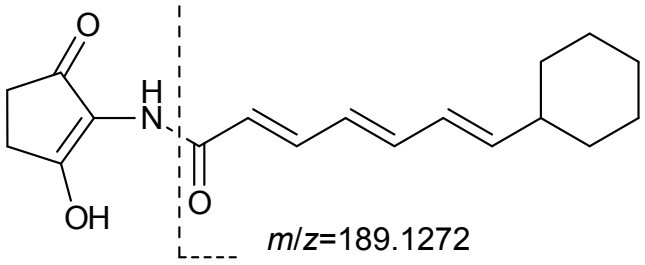


Notes: probed with apramycin resistance gene.

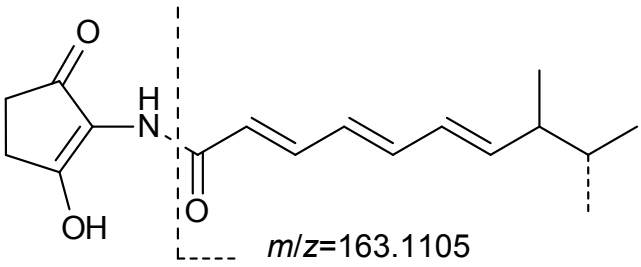
Supplemental Figure 5. Construction of pART1391. **a.** Spectinomycin resistance gene cassette (*spec*) was PCR amplified from pIJ778 using primers AsuRED-4 and -6. **b.** The λ-RED based recombination was conducted to replace the 25.4 kb insert region of pART1361 with a *spec* cassette to create pART1391. The predicted *EcoR*I digestion fragments are indicated (kb).**c.** Comparison of the *EcoR*I digestion pattern by DNA gel electrophoresis. Note: Lane pART1381 indicates another pART1361 derived cosmid clone which is not related to this study.



Supplemental Figure 6. Proposed **E1** and **E3**/**E4** structures. The exact mass of **E1** is 301.1670 (calculated for $C_{18}H_{23}NO_3$, 301.1678; mass error, 3.30 ppm) with a fragment 189.1272 (calculated for $C_{13}H_{17}O$, 189.1279). The exact mass of **E3**/**E4** is 275.1501 (calculated for $C_{16}H_{21}NO_3$, 275.1521; mass error, 7.58 ppm) with a fragment 163.1105 (calculated for $C_{11}H_{15}O$, 163.1123).



Compound **E1**



Compound **E3**/**E4**