Supporting Information

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Supplementary Methods

Pacidamycin production and isolation. For each gene deletion experiment, three mutants were selected for pacidamycin production analysis after PCR verification (primers listed in table S2). Wild-type *S. coeruleorubidus* was grown side by side as controls with all of the mutants. Starter cultures in TSB medium were inoculated with spores and incubated at 30 °C, 200 rpm for 24 h. Production medium (20 mL; 20.99 g/L MOPS, 10 g/L lactose, 4.41 g/L K_2HPO_4, 2.14 g/L NH_4Cl, 0.6 g/L $MgSO_4$, 10 mg/L $FeSO_4 \cdot 2H_2O$, 10 mg/L $MnCl_2 \cdot 4H_2O$, 10 mg/L $ZnSO_4 \cdot 7H_2O$, 10 mg/L $CaCl_2$, pH 7.0) was inoculated with starter culture (0.5 mL) and incubated at 30 °C, 200 rpm for 5 d. Pacidamycins were extracted from the cell-free broth (10 mL) using XAD-16 resin (5% v/v). The resin was washed with water (20 mL) and the extract was eluted with methanol (5 mL). The solvent was removed by a rotary evaporator and the residue was redissolved in water/methanol 1:1 (500 µL) and subjected to LC-HRMS analysis (25 µL injection).

Cloning, overexpression and purification of NRPSs. *pacH, J, L, N, O, P, U* were PCR amplified from genomic DNA extracted from *S. coeruleorubidus* (primers listed in table S2). Purified PCR products were ligated to pET-30 Xa/LIC (Novagen) following the standard protocol and confirmed by DNA sequencing. The resulting expression constructs were transformed into *E. coli* BL21 or BAP1 cells for protein expression. Expression and purification for all proteins followed the same general procedure and is detailed as follows. In 1 L of liquid culture, the cells were grown at 37 °C in LB medium with 50 µg/mL kanamycin to an OD600 of 0.4. The cells were cooled on ice for 10 min and then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 16 °C. The cells were harvested by centrifugation (6000 rpm, 6 min, 4 °C), resuspended in 30 mL lysis buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM imidazole) and lysed by sonication on ice. Cellular debris was removed by ultracentrifugation (35000 rpm, 30 min, 4 °C). Ni-NTA agarose resin was added to the supernatant (1 mL/L of culture) and the solution was nutated at 4 °C for 1 h. The protein resin mixture was loaded into a gravity flow column, and proteins were eluted with increasing concentrations of imidazole in Buffer A (50 mM Tris-HCl, pH 8.0, 2 mM EDTA). Purified proteins were concentrated and buffer exchanged into Buffer A + 10% glycerol using Amicon Ultra filters. The final proteins were flash-frozen in liquid nitrogen and stored at -80 °C. The approximate protein yields were 16 mg/L for PacH (10 kDa), 6 mg/L for PacJ (8 kDa), 5 mg/L for PacL (90 kDa), 16 mg/L for PacN (63 kDa), 42 mg/L for PacO (57 kDa), 17 mg/L for PacP (90 kDa), and 3 mg/L for PacU (55 kDa).

Supplementary Tables

Table S1. Prediction of the amino acid specificity of adenylation domains in pacidamycin cluster.

Protein	10 AA code	Predicted specificity
PacL	DPGIYGQLAK	Val/Leu
PacO	DLSSVGVCYK	Ala
PacP	DIWQGTADDK	Ser/Arg
PacU	DCHYFALITK	Pro/p-hydroxyphenylglycine

Table S2. Primers used in this study.

Primer	Sequence	Note
M119_F	5'- AGACCCTCGTGCTCGTCACG-3'	Used for Δ*pacO* mutant construction; M119_R is also used for Δ*pacO* mutant verification
M119_R	5'- GGACCAACTGGAGTGGCAGC-3'	
M119-apra_F	5'-GCGTCGAGCCATCCTGAAACTCTGAGAGGCAAACCTATGATTCCGGGGATCCGTCGACC-3'	
M119-apra_R	5'-GTACCTGTATACGCGCCATCCTGAACGACAGATGAATTATGTAGGCTGGAGCTGCTTC-3'	
M120_F	5'-ACAAACGCTGAGCTACCGCG-3'	Used for Δ*pacP* mutant construction
M120_R	5'- ACATTGCGGTGCTCGAACGC-3'	
M120-apra_F	5'-AGGCCGCCACTACGTTCGGCCGGAAAGGCTGGCACTGTGATTCCGGGGATCCGTCGACC-3'	
M120-apra_R	5'-AGTCGTCGGGCGCTGCCGCAAGTCGACCGGTCAGTCTCATGTAGGCTGGAGCTGCTTC-3'	
M121_F	5'-AAAAAATCTAGAGCTGCTGGCCGCGGAGAACG-3'	Used for Δ*pacQ* mutant construction
M121_R	5'-AAAAAAGAATTCGTCCAGTGAGGCCGGGAAGG-3'	
M121-apra_F	5'-AACGGCCATCGCGGCCACCCAGGAAGGCGGCACCCGGTGATTCCGGGGATCCGTCGACC-3'	
M121-apra_R	5'-GGTCCCTGGCCGGCTGCTGGACGAGCTGCCGGCTCGTCATGTAGGCTGGAGCTGCTTC-3'	
M125_F	5'-GCGAGATCGAGCAGGTGGTA-3'	Used for Δ*pacU* mutant construction
M125_R	5'-GCGTCGTACAGCAGCATGGT-3'	
M125-apra_F	5'-CTGTTGCCGCACTTCCCCGCCGCCCTCACCCGCTGGATGATTCCGGGGATCCGTCGACC-3'	
M125-apra_R	5'-CTGGCTTCCGTCGCCTTACTGATGCCGACGCCTTGCTCATGTAGGCTGGAGCTGCTTC-3'	
Apra_i	5'-AGTTGTCTCTGACACATTCT-3'	Internal primer for mutants verification
121_PET30R	5'-AGAGGAGAGTTAGAGCCTTATACGACTCCCCCGGCCGTG-3'	External primer for Δ*pacP* mutant verification
122_PET30R	5'-AGAGGAGAGTTAGAGCCTTATTGAAGATCATTACTTGGT-3'	External primer for Δ*pacQ* mutant verification
126_PET30R	5'-AGAGGAGAGTTAGAGCCTTAGGCCTGGGGAGCGCCGTT-3'	External primer for Δ*pacU* mutant verification
119c_NdeI	5'- AAAAAACATATGACCGGGTCTCCTTTTTGATA-3'	Used for *pacO* complementation
119c_EcoRI	5'- AAAAAAGAATTCTTAGGGGCGCCGAGTTCGGC-3'	
111a_PET30F	5'-GGTATTGAGGGTCGCATGAATCTACAGGATCAGAA-3'	Used for PacH expression
111a_PET30R	5'-AGAGGAGAGTTAGAGCCTTATTTCCTGGTGCCGTTCGCA-3'	
113_NdeI	5'-AAAAAACATATGTTCGACGACGAGGGGCG-3'	Used for PacJ expression
113_EcoRI	5'-AAAAAAGAATTCGGGGCGGCTGATCCCTCATCGG-3'	
115_PET30F	5'-GGTATTGAGGGTCGCATGTCGACCACAGATGACGC-3'	Used for PacL expression
115_PET30R	5'-AGAGGAGAGTTAGAGCCTTAGAGCATCGCCGCGAGCTCA-3'	
117_PET30F	5'-GGTATTGAGGGTCGCATGTTCCCGCTGAGCGCCTCA-3'	Used for PacN expression
117_PET30R	5'-AGAGGAGAGTTAGAGCCTTAACGCTGGAGGTCGGGGGTC-3'	
119_PET30F	5'-GGTATTGAGGGTCGCATGCGCGATGCAGTAGCCGC-3'	Used for PacO expression, *pacO* complementation verification
119_PET30R	5'-AGAGGAGAGTTAGAGCCTTAGGGGCGCCGAGTTCGGC-3'	

120_PET30F	5’-GGTATTGAGGGTCGCATGACGGAGCAGGTCGAGGCA-3’	Used for PacP expression
120_PET30R	5’-AGAGGAGAGTTAGAGCCTTACCGGGTGCCGCCTTCCTGG-3’	
125D_PET30F	5’-GGTATTGAGGGTCGCATGTCTCTCACATTGGTCGA-3’	Used for PacU expression
125_PET30R	5’-AGAGGAGAGTTAGAGCCTTAACCGGCCAACCCCGCCGCC-3’	

Supplementary Figures

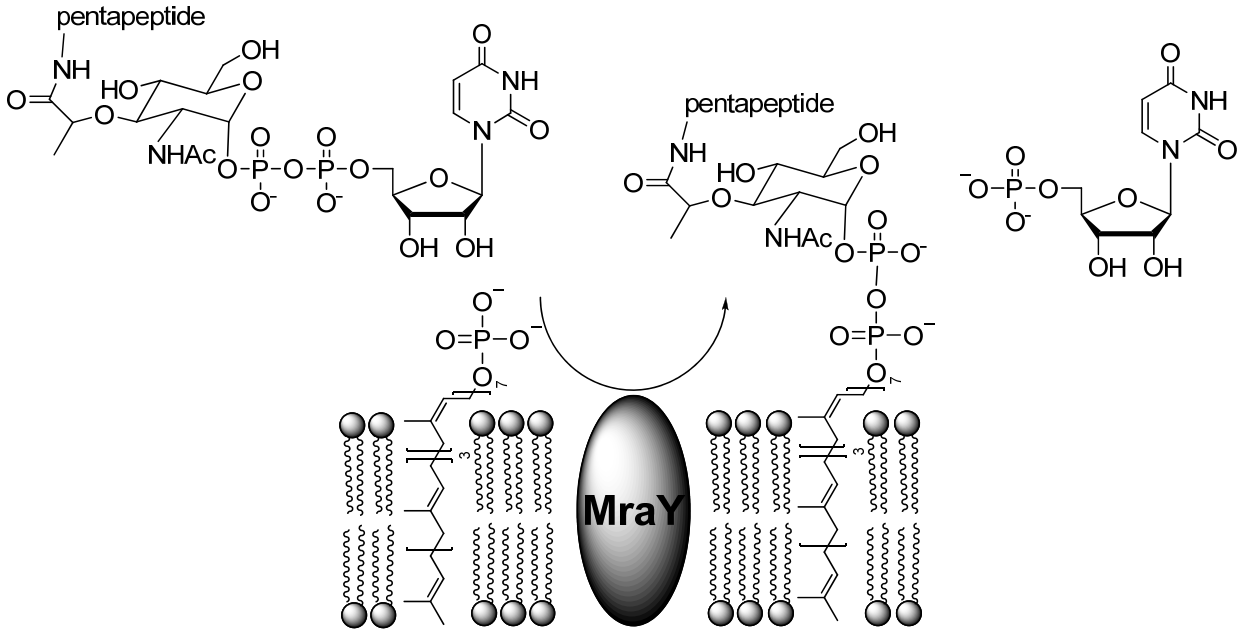


Fig. S1. Translocase MraY-catalyzed reaction: UDPMurNAc-pentapeptide is cleaved to release UMP, and phospho-*N*-acetylmuramoyl-pentapeptide motif is transferred onto undecaprenyl phosphate carrier lipid to form lipid intermediate I during bacterial cell wall assembly.

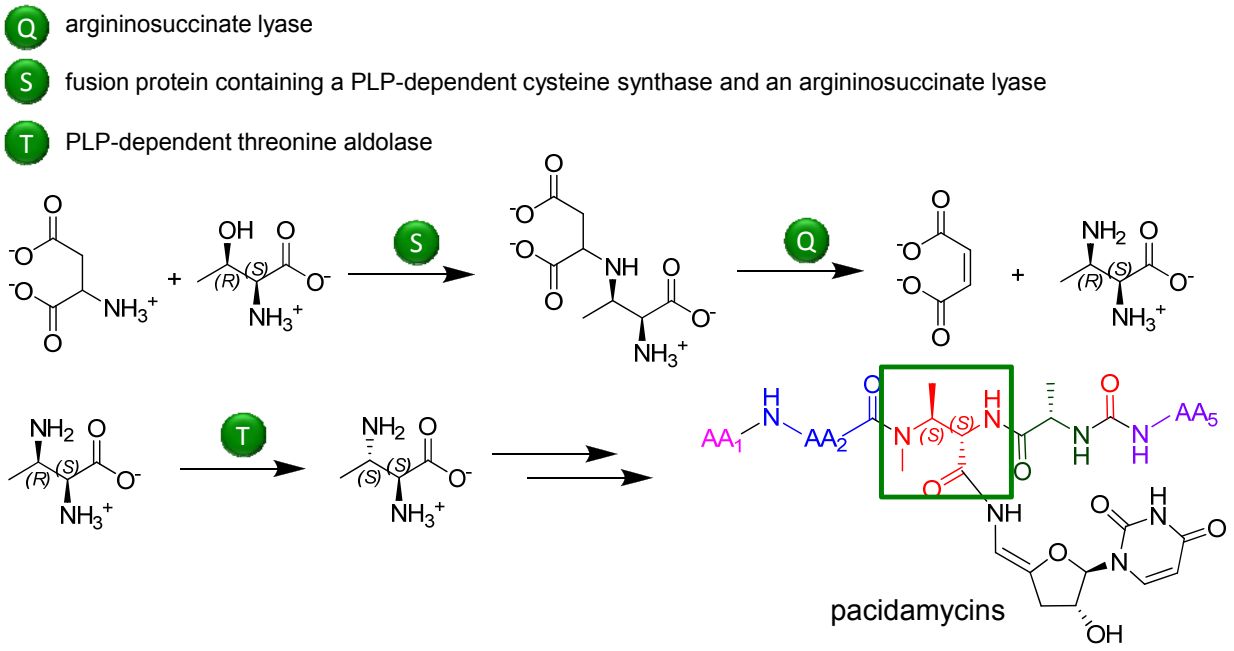


Fig. S2. Proposed biosynthetic pathway for *2S,3S*-DABA.

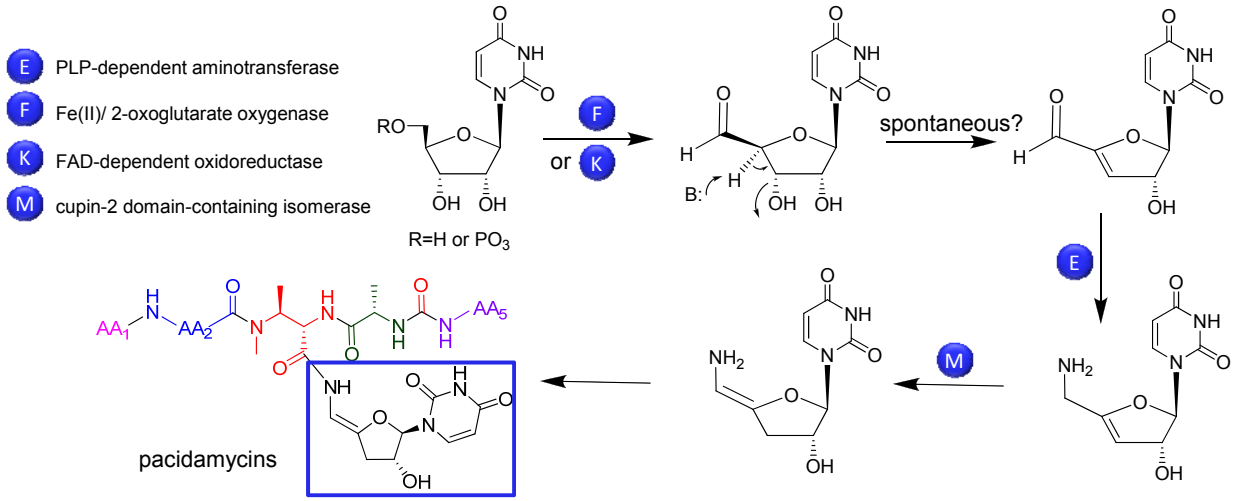


Fig. S3. Proposed biosynthetic pathway for 3'-deoxy-4',5'-enamino-uridine.

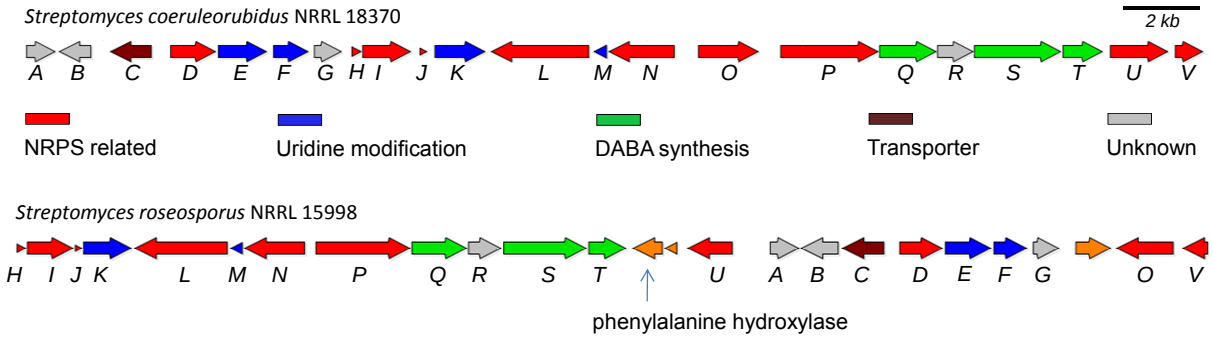
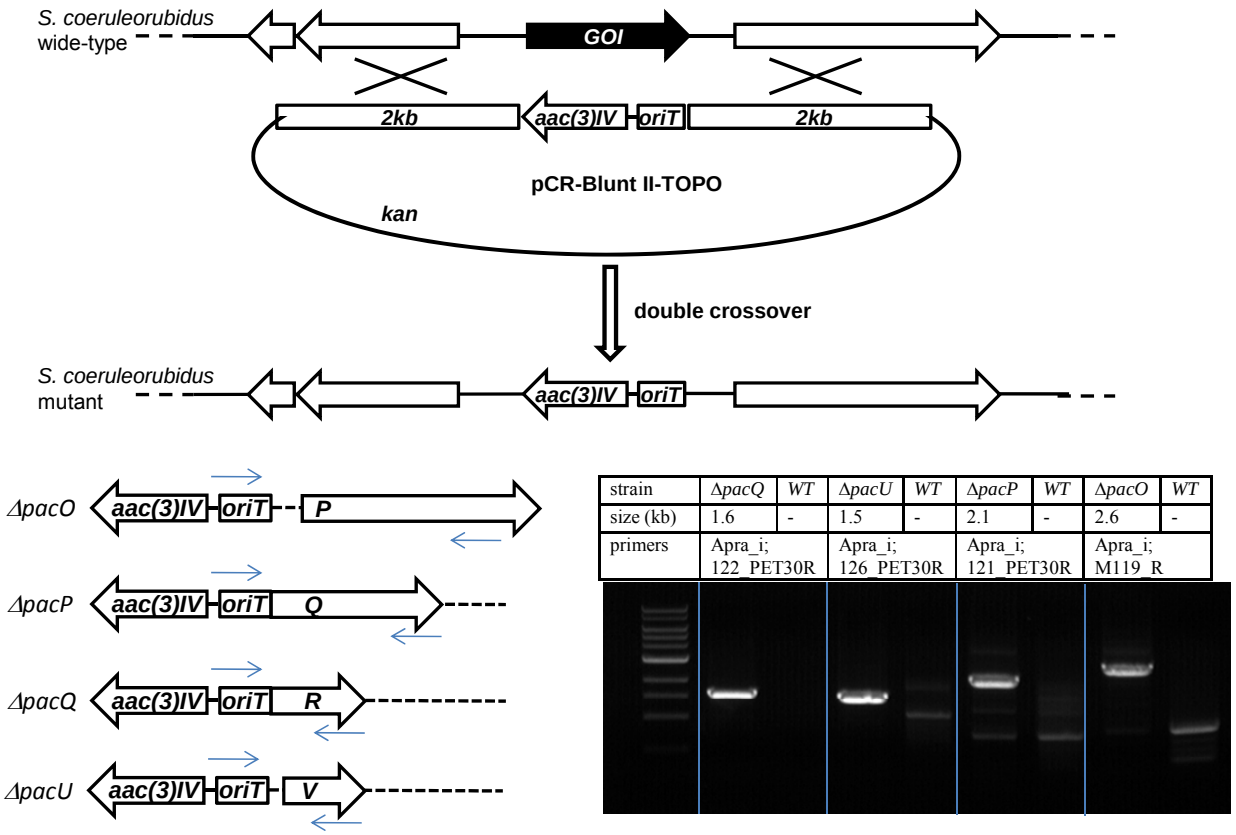


Fig. S4. Comparison of two putative *pac* gene clusters from *S. coeruleorubidus* and *S. roseosporus*. All *pac* genes identified from *S. coeruleorubidus* are present in *S. roseosporus*. Three additional genes are found in the cluster of *S. roseosporus*, one encoding a phenylalanine hydroxylase homolog and the other two encoding hypothetical proteins.



strain	Δ*pacQ*	*WT*	Δ*pacU*	*WT*	Δ*pacP*	*WT*	Δ*pacO*	*WT*
size (kb)	1.6	-	1.5	-	2.1	-	2.6	-
primers	Apra_i; 122_PET30R		Apra_i; 126_PET30R		Apra_i; 121_PET30R		Apra_i; M119_R	

Fig. S5. Inactivation of *pacO*, *pacP*, *pacQ*, and *pacU* in *S. coeruleorubidus.* All genes were deleted in-frame by homologous recombination as illustrated above. The genomic DNA of wide-type and mutant strains was extracted and used as templates for subsequent PCR verification of mutants. PCR was carried out using one internal primer from *acc(3)IV-oriT* cassette and one external primer from an adjacent gene (Table S2). The size of PCR product was consistent with the expected size of each mutant.

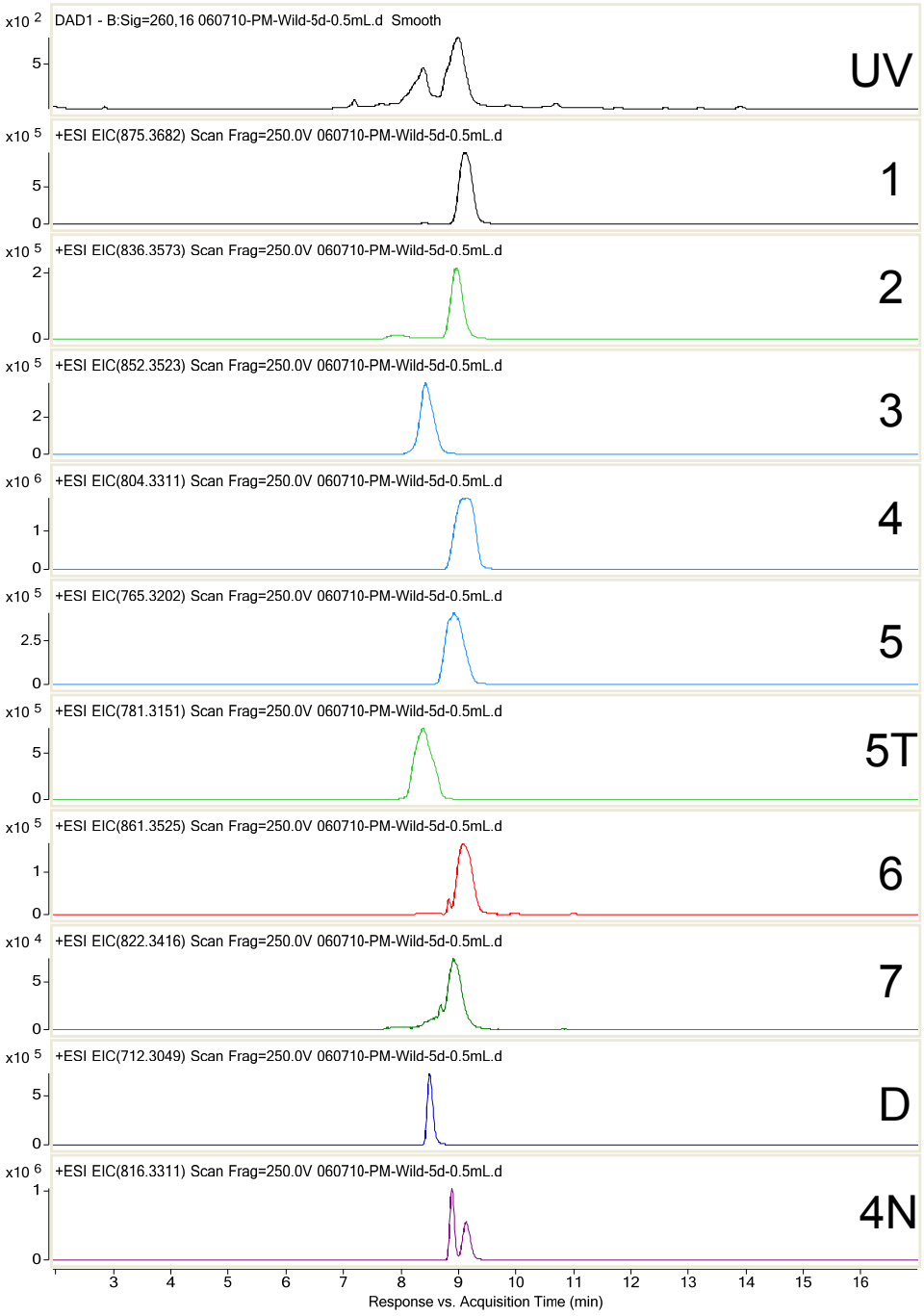


Fig. S6. Extracted ion chromatograms of pacidamycins extracted from wide-type *S. coeruleorubidus* culture.

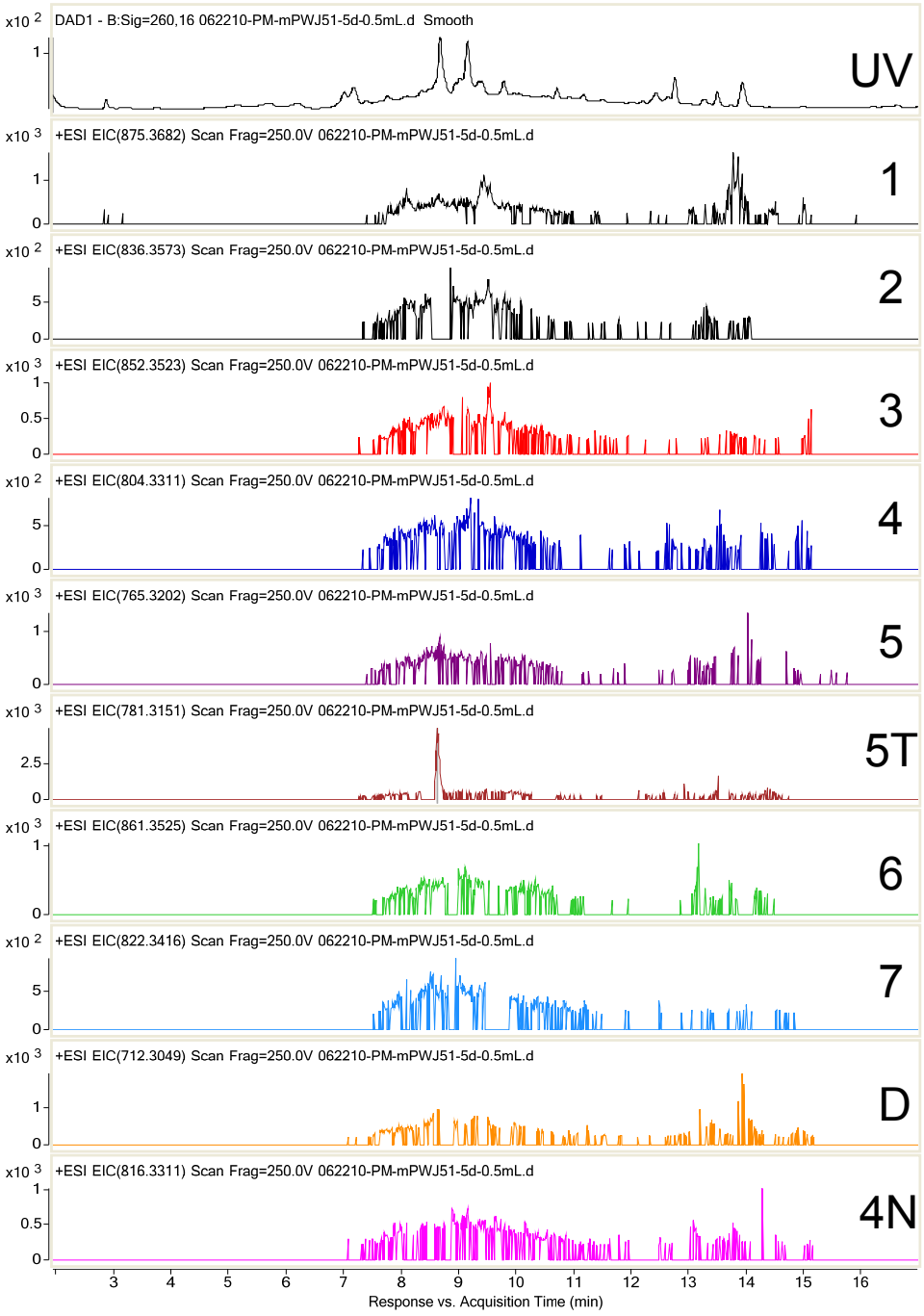


Fig. S7. Extracted ion chromatograms of pacidamycins extracted from culture of Δ*pacO* mutant. The change in the y-axis of the mass signal scale (compared to Fig. S6) led to the appearance of mass noise.

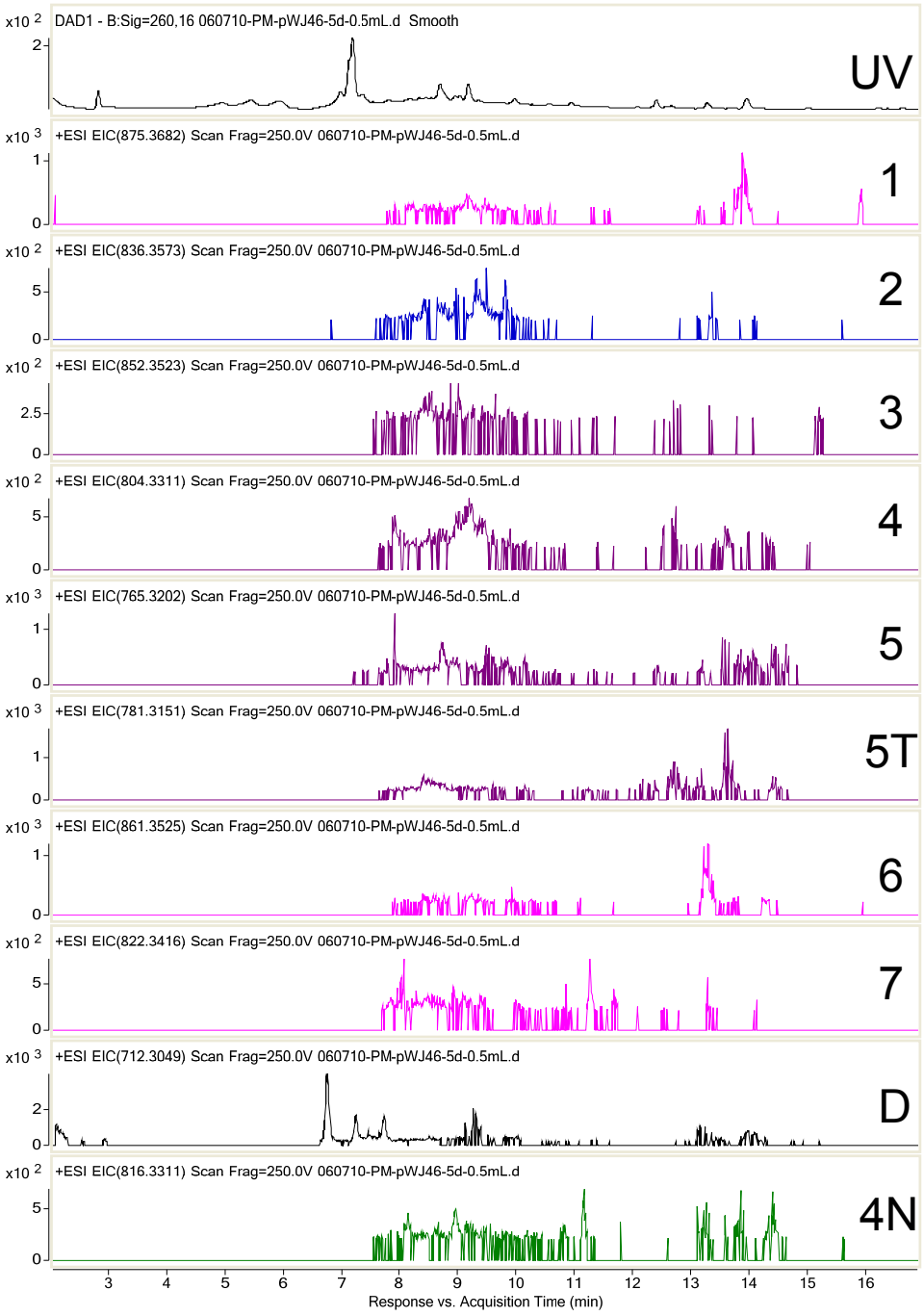


Fig. S8. Extracted ion chromatograms of pacidamycins extracted from culture of Δ*pacP* mutant. The change in the y-axis of the mass signal scale (compared to Fig. S6) led to the appearance of mass noise.

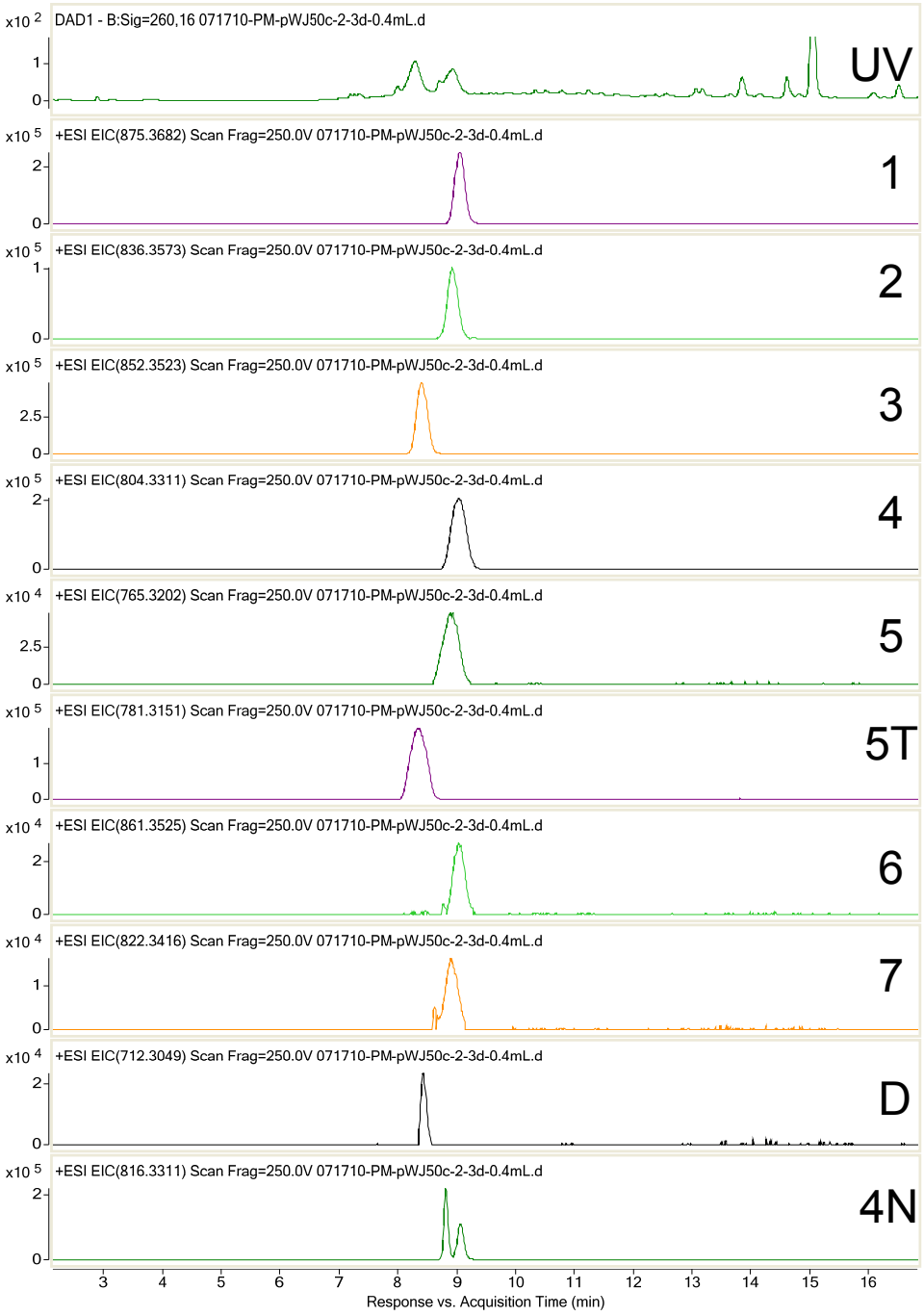


Fig. S9. Extracted ion chromatograms of pacidamycins extracted from culture of Δ*pacO* mutant complemented by *pacO*.

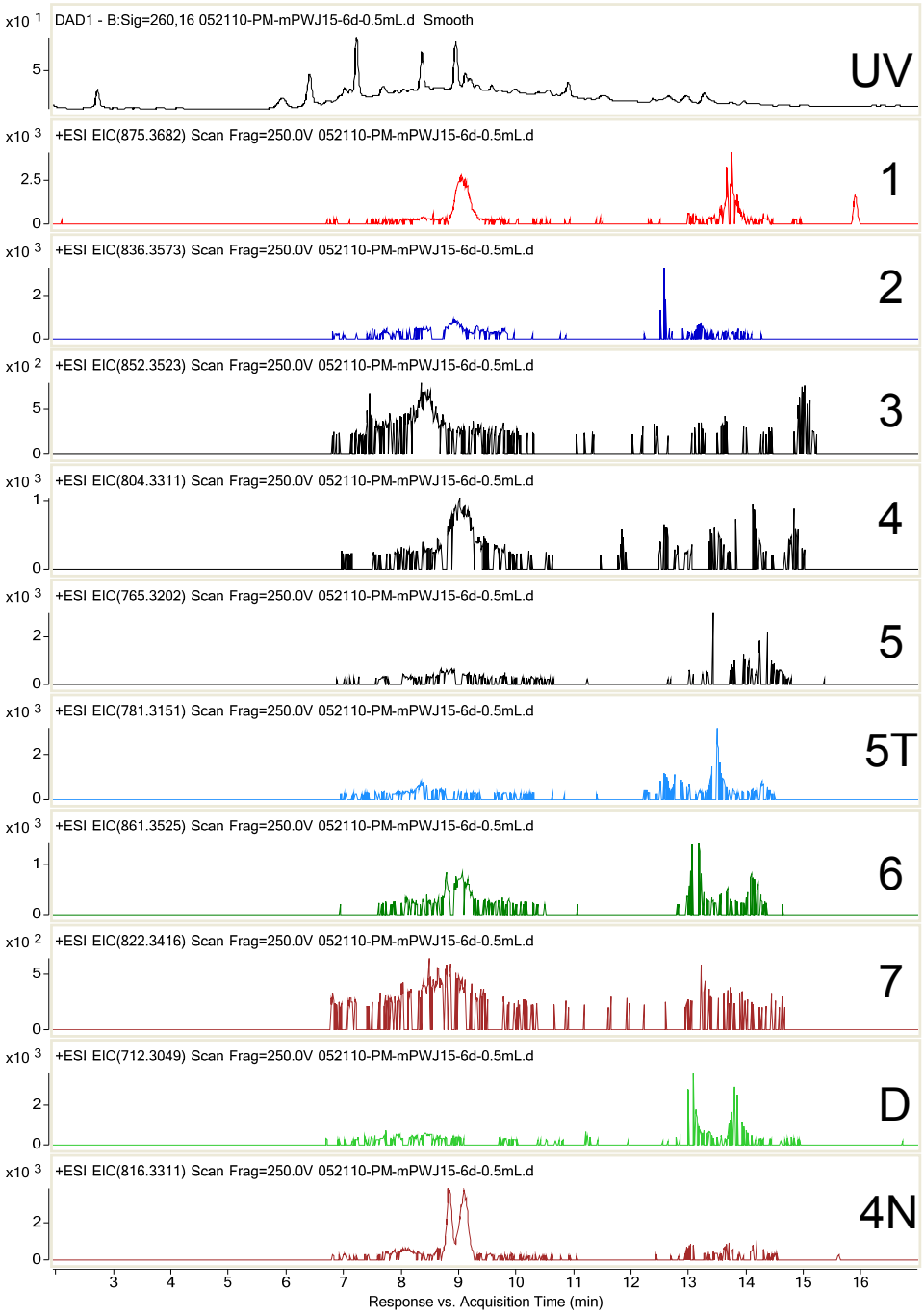


Fig. S10. Extracted ion chromatograms of pacidamycins extracted from culture of Δ*pacQ* mutant. The change in the y-axis of the mass signal scale (compared to Fig. S6) led to the appearance of mass noise.

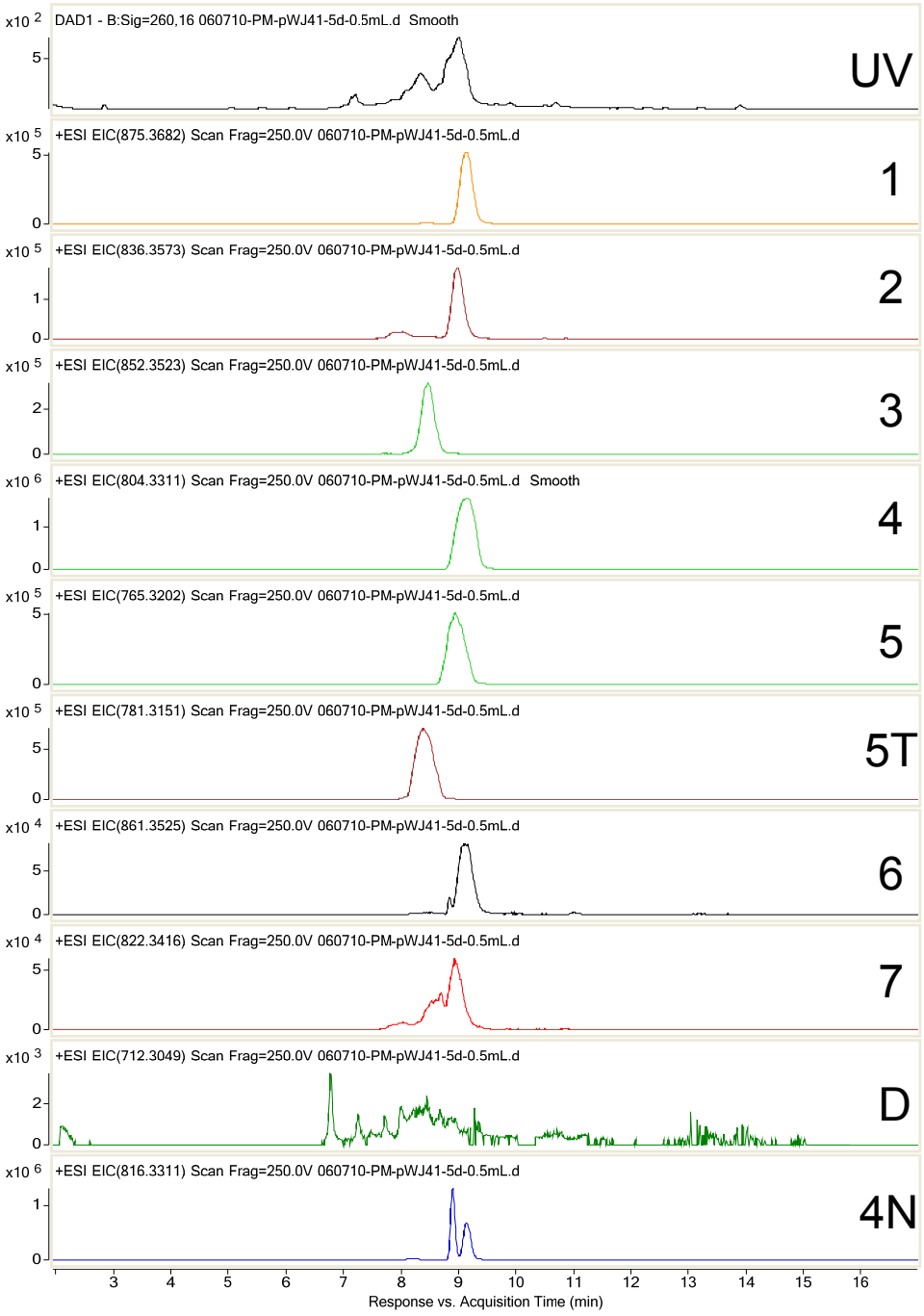


Fig. S11. Extracted ion chromatograms of pacidamycins extracted from culture of Δ*pacU* mutant.

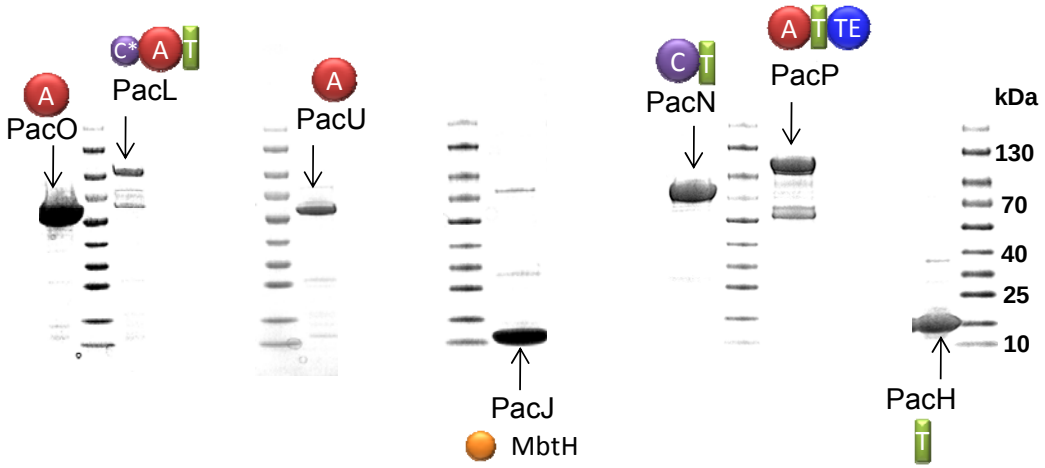
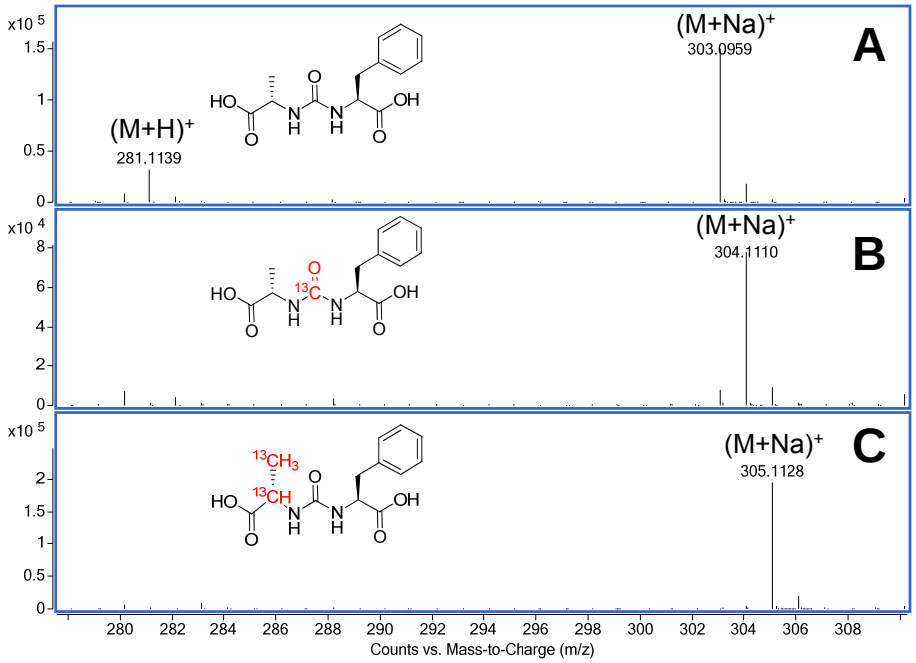
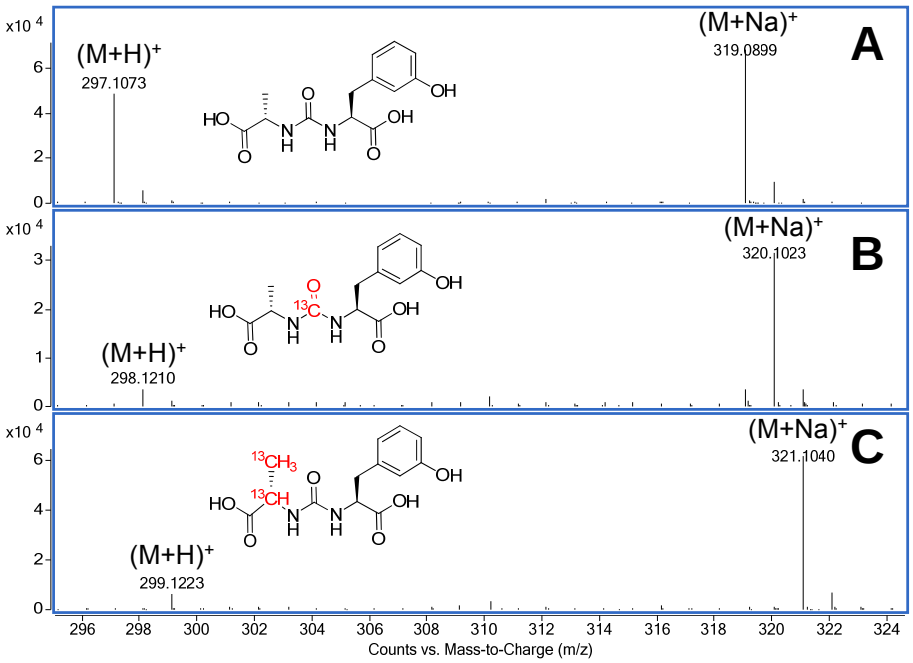


Fig. S12. SDS-PAGE analysis of the *E. coli* purified proteins. Most proteins have *N*-terminal hexahistidine tag except that PacJ has a *C*-terminal hexahistidine tag.



Panel	t_R	Ion	Exp *m/z*	Obz *m/z*	Δ mmu
A	14.34	M+H	281.1132	281.1139	0.7
		M+Na	303.0951	303.0959	0.8
B	14.43	M+H		*not found*	
$NaH^{13}CO_3$		M+Na	304.0985	304.1110	12.5
C	14.38	M+H		*not found*	
Ala (2,3-^{13}C2)		M+Na	305.1019	305.1128	10.9

Fig. S13. HRMS of the unlabeled and [^{13}C]-labeled Ala-CO-Phe ureido dipeptide.



Panel	t_R	Ion	Exp *m/z*	Obz *m/z*	Δ mmu
A	11.76	M+H	297.1081	297.1073	-0.8
		M+Na	319.0901	319.0899	-0.2
B	11.79	M+H	298.1115	298.1210	9.5
$NaH^{13}CO_3$		M+Na	320.0934	320.1023	8.9
C	11.82	M+H	299.1148	299.1223	7.5
Ala (2,3-^{13}C2)		M+Na	321.0968	321.1040	7.2

Fig. S14. HRMS of the unlabeled and [^{13}C]-labeled Ala-CO-*m*-Tyr ureido dipeptide.