Supplemental Data

Table S1. Strains, plasmids and cosmids used in this study

Strain/ Plasmid/ Cosmid	Relevant characteristics*	Reference or source
***Streptomyces* Strains**		
S. chartreusis	Wild-type of tunicamycin producing strain （NRRL3882）	NRRL
S. clavuligerus	A tunicamycin-like antibiotics (MM 19290) producing strain (ATCC 27064)	ATCC
S. lividans TK24	A derivative of *S. lividans* 1326	(Kieser, 2000)
S. avermitilis	Model strain for engineered production of tunicamycin (NRRL 8165)	NRRL
S. albus J1074	Model strain for engineered production of tunicamycin	(Wendt-Pienkowski et al., 2005)
***E. coli* strains**		
DH10B	F$^-$ *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) φ80d *lacZ*Δ M15 Δ *lac*X74 *deo*R *rec*A1*end*A1*ara*Δ139 D(*ara*, *leu*)1697 *gal*U *gal*Kλ $^-$ *rps*L *nup*G	GIBCOBRL
ET12567 (pUZ8002)	*dam dcm hsd*S pUZ8002	(Paget et al., 1999)
XLUZ	Host cell containing pUZ8002 for construction of genomic cosmid library	(Tao *et al.* unpublished)
BW25113/pIJ790	BW25113 strain bearing pIJ790 usded as host cell for PCR-targeting mutation of the target gene	(Gust et al., 2003)
BL21Gold(DE3)pLysE	F$^-$, *ompT, hsdS$_B$ (r_B^- m_B^-) , gal , dcm* (DE3), pLysE (Cm^R)	Stratagene
Bacillus subtilis	Indicator stain used for bioassay of tunicamycin	(Tsvetanova and Price, 2001)
Plasmids		
pIJ2925	*bla, lacZ*	(Kieser, 2000)
pBlueScriptII SK(+)	*bla, lacZ*, *orif*1	Stratagene
pSET152	*aa (3)I V*, *lacZ*, *rep*^{pMB1*} attΦC31, *ori*T	(Bierman et al., 1992)
pOJ446	*aa (3)I V,* SCP2, *rep*^{pMB1*}, attΦC31, *ori*T	(Bierman et al., 1992)
SuperCos1	*neo, bla, cos,ori*^{pUC}	Stratagene
pET28a	Kan, *rep* ^{pMB1}, T7 promoter	Novagen
pJTU2463	A derivative of pOJ446 with SCP2 replicon replaced by *int* and *attp* from pSET152	(Yao *et al.* unpublished)
pJTU2463b	A derivative of pJTU2463 with XbaI and SpeI sites blocked	this study
Cosmid		
11C3	A positive cosmid containing the complete tunicamycin biosynthetic gene cluster	this study

11D8	A positive cosmid containing the complete tunicamycin biosynthetic gene cluster	this study
pJTU5751a	Cosmid 11D8 derivative with XbaI site blocked	
pJTU5751	A derivative of 11C3 cosmid with XbaI and SpeI sites blocked	this study
12G1	A positive cosmid containing the complete tunicamycin biosynthetic gene cluster	this study
pJTU5751/-*1-neo*	A derivative of pJTU5751 with *orf-1* was replaced by *neo* cassette	this study
pJTU5751/*tunL-neo*	A derivative of pJTU5751 with *tunL* was replaced by *neo* cassette	this study
pJTU5751/+*1-neo*	A derivative of pJTU5751 with *orf1* was replaced by *neo* cassette	this study
pJTU5751/*tunA-neo*	A derivative of pJTU5751 with *tunA* was replaced by *neo* cassette	this study
pJTU5751/Δ*tunA*	A derivative of pJTU5751/*tunA-neo* with *neo* cassette removed	this study

*ori*T, origin of transfer of plasmid RK2; *aac(3)IV*, apramycin resistance gene; Cm^R, chloramphenicol resistance gene; *neo*, Neomycin resistance gene; Kan, kanamycin resistance gene; Center; rep^{pMB1*}, mutated rep^{pMB1}.

Table S2. PCR primers used in this study

primers	sequence
Orf-1tgtF	5'-CTGCGACGCCCGGCACCCCGGCGGCGTGGTGCTCCGGCT TCTAGAGCTATTCCAGAAGT-3'
Orf-1tgtR	5'-GTCGATCCGTTCGTAGTCCGGTCGCCACTCGCGCGTGAT ACTAGTCTGGATGCCGACG-3'
Orf-1exF	5'-CCATATGTTCCGCCACACCCCGCAG-3'
Orf-1exR	5'-GGAATTCAGGAGGTAGTCGCCCA-3'
TunLtgtF	5'-CAGCGGTCCACGCCGGTCTGCTACTCGTCCTCGTCACCT TCTAGAGCTATTCCAGAAGT-3'
TunLtgtF	5'-ACGGGCCTCGCGTTGCAAGCGGCGGACTGACTGGCGGTG ACTAGTCTGGATGCCGACG-3’
TunLexF	5'-CCATATGATCCGAAACCACTCCGGC-3'
TunLexR	5'-GGAATTCATGACACGGGCTCGTC-3'
Orf1tgtF	5'-AAGGGCCCTTGAGGTGGGCTGCGGCACAGGGAGGTTTGC TCTAGAGCTATTCCAGAAGT-3’
Orf1tgtR	5'-AGGAGGCGCCGAATCCGCCTGCCTGGAAGATATTCAGCA ACTAGTCTGGATGCCGACG-3'
Orf1exF	5'-CCATATGTGCCGTTCAACCACAAC-3'
Orf1exR	5'-GGAATTCTAATCACTTCCCAGCCC-3'
TunAtgtF	5'-ATCGGTGGCCACCTTGCCCGCGCACTGGTGGCGTCCGGG

	TCTAGAGCTATTCCAGAAGT-3'
TunAtgtR	5'-ACGCTGCTCACCGTCGCCCTCGACAGGCAGTTCGTTCCT
	ACTAGTCTGGATGCCGACG-3'
TunAidF	5'-CCATATGCAAAGAAACACTTTGAA-3'
TunAidR	5'-GGAATTCTAGAGGATCCGGATGACG-3'

Table S3. Comparative analysis of the homologies of the *tun* genes with different origins

gene	aa (3882/27074/43827)*	*S. clavuligerus* ATCC 27074 (identity, positives, %)	*A. mirum* DSM 43827 (identity, positives, %)
A	321/323/322	226/312 (72%), 253/312 (81%)	164/300 (54%), 197/300 (65%),
B	338/338/340	305/338 (90%), 323/338 (95%)	267/340 (78%), 298/340 (87%),
C	318/322/318	194/323 (60%), 233/323 (72%)	140/323 (43%), 185/323 (57%)
D	472/461/451	296/461 (64%), 358/461 (77%)	217/457 (47%), 269/457 (58%)
E	234/236/230	171/221 (77%), 190/221 (85%)	146/229 (63%), 175/229 (76%)
F	327/327/332	249/327 (76%), 274/327 (83%)	146/229 (63%), 175/229 (76%)
G	203/208/223	120/184 (65%), 135/184 (73%)	94/185 (50%), 112/185 (60%)
H	515/518/510	339/511 (66%), 389/511 (76%)	270/504 (53%), 328/504 (65%)
I	304/302/302	235/306 (76%), 270/306 (88%)	180/296 (60%), 218/296 (73%)
J	262/261/253	197/259 (76%), 217/259 (83%)	148/242 (61%), 189/242 (78%)
K	81/81/79	52/79 (65%), 69/79 (87%)	without obvious homology
L	229/223	97/186 (52%), 126/186 (67%)	missing in DSM 43827

*(3882/27074/43827) means that proteins involved in confirmed/potential tunicamycin biosynthetic pathways were originated from *S. chartreusis* NRRL 3882, *S. clavuligerus* ATCC 27074, and *A. mirum* DSM 43827, respectively.

Mutation of the targeted genes for the boundaries determination of the tunicamycin gene cluster

For *orf-1* disruption, a ca.1.5-kb *neo* cassette as selectable marker was amplified by rTaq DNA polymerase (Takara) with primers Orf-1tgtF and Orf-1tgtR. Subsequently, the cassette was recombined into the *orf-1* to give pJTU5751/*-1-neo via* PCR-targeting technology (Gust et al., 2003), and the primers orf-1exFand orf-1exR

were used for identification of the *orf-1* mutation. Similarly, *tunL* and *orf1* mutant cosmids were individually constructed as pJTU5751/*tunL-neo* and pJTU5751/*1-neo.*

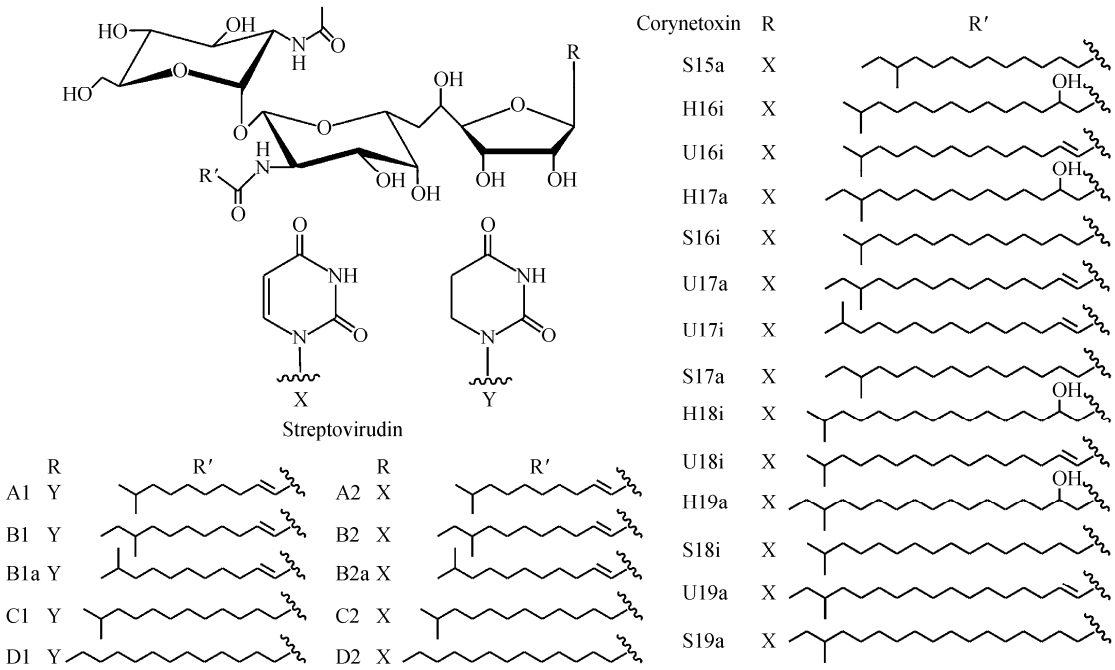

Figure S1. Chemical structures of streptovirudin and corynetoxin. All components of corynetoxin and streptovirudin differ only in N-acyl chain and/or substitution of 5, 6-dihrouracil for uracil group.

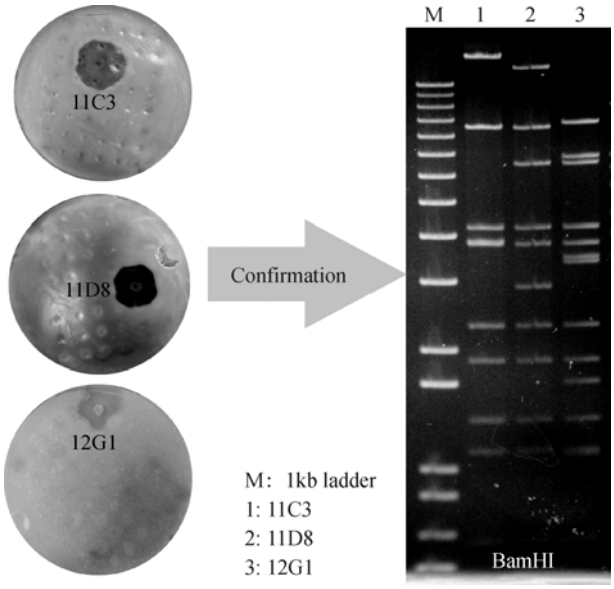

Figure S2. Screening of the positive cosmids via high-throughput heterologous expression (HHE) strategy. Left: Screening of the positive *S. lividans* TK24 recombinants combining HHE strategy and Bioassay. Right: Confirmation of the correlations among the three positive cosmid. The three cosmids were digested by *Bam*HI.

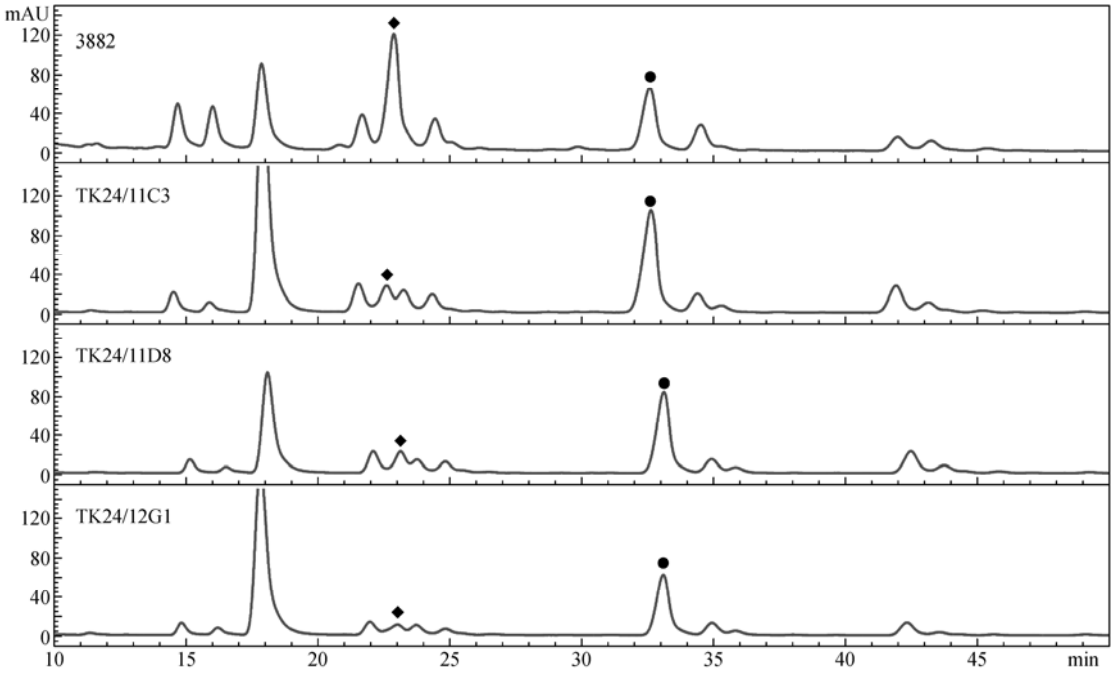

Figure S3. HPLC analysis of the metabolites produced *S. lividans* TK24 recombinants. The sample of *S. charteusis* wild type (3882) was used as positive control, and the samples of *S. lividans* recombinants carrying the positive cosmids were correspondingly indicated as TK24/11C3, TK24/11D8 and TK2/12G1. (♦): Tun 15:1$_B$, (●): Tun 16:1$_A$.

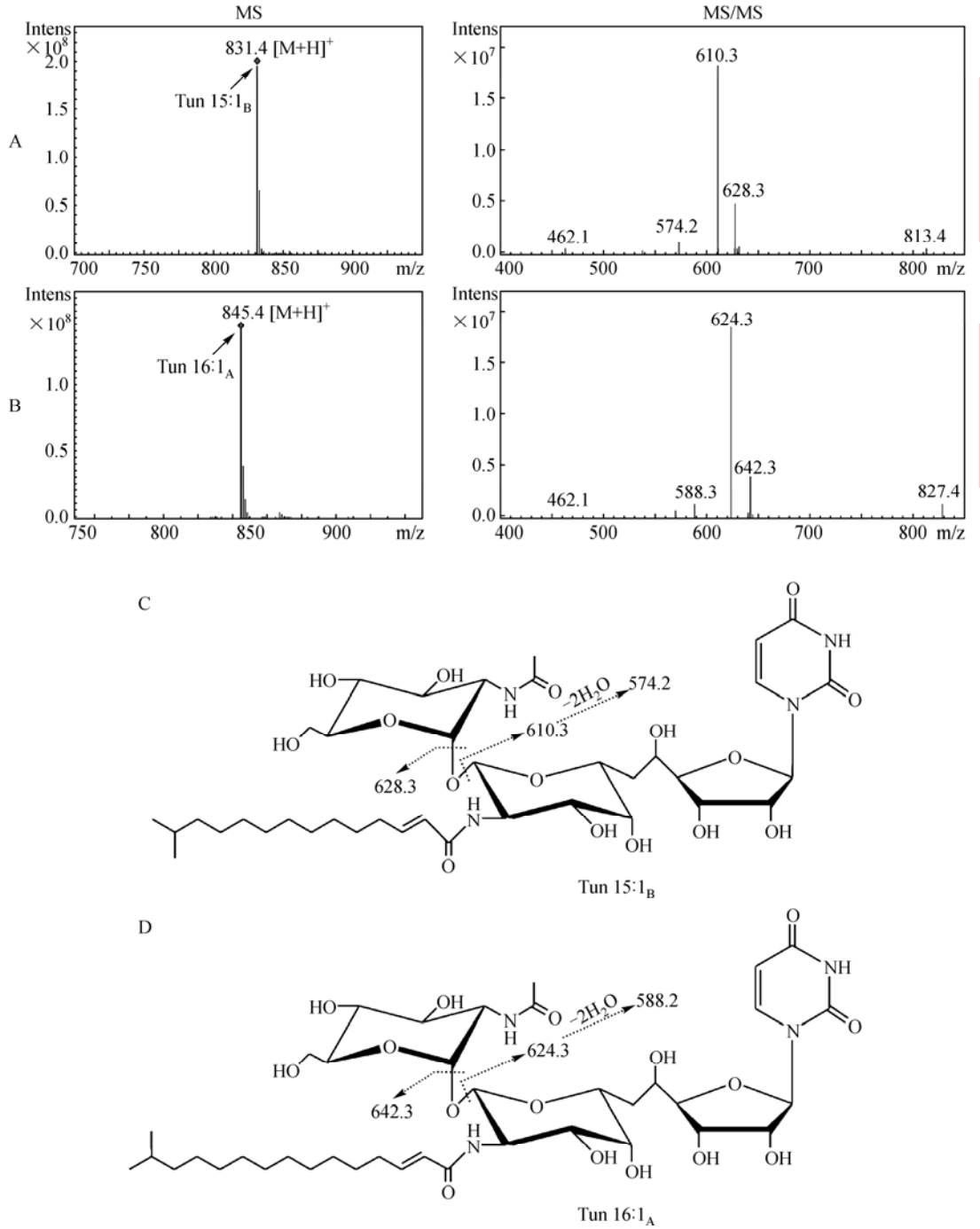

Figure S4. MS and MS/MS analysis of the Tun 15:1$_B$ and Tun16:1$_A$ components of tunicamyin standard. (A) MS and MS/MS analysis of the Tun 15:1$_B$ component. The component generated a $[M+H]^+$ ion at m/z 831.4, which was fragmented into main ions of 610.3, 628.3, 574.2, *etc*. (B) MS and MS/MS analysis of the Tun 16:1$_A$ component. The component generated a $[M+H]^+$ ion at m/z 845.4, which was fragmented into main ions of 624.3, 642.3, 588.3, *etc*. (C) MS/MS fragmentation pattern of the Tun 15:1$_B$. (D) MS/MS fragmentation pattern of the Tun 16:1$_A$.

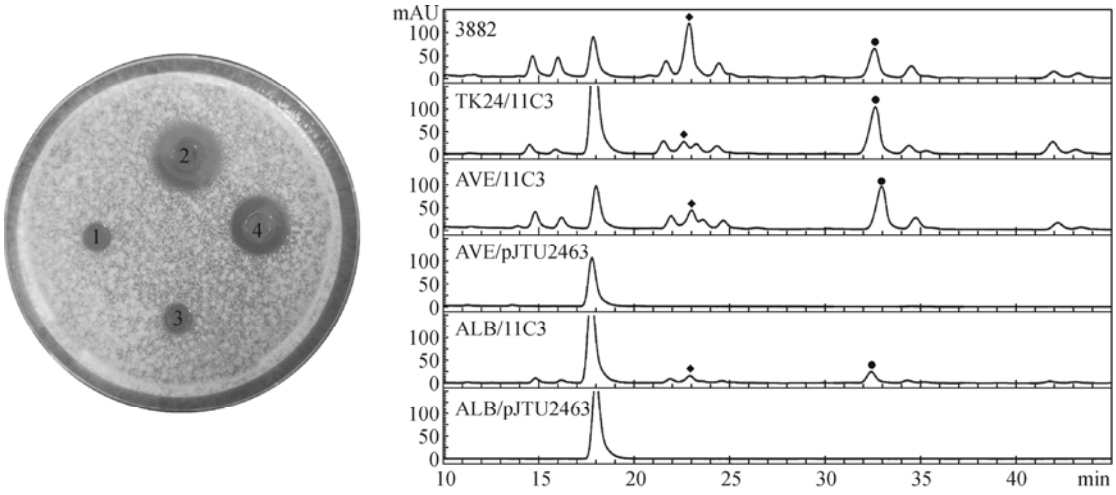

Figure S5. Analysis of the metabolites produced related *Strepomyces* recombinants containing *tun* cluster. Left: Bioassay of the metabolites produced by recombinants of *S. avermitilis*/11C3 (2) and *S. albus/*11C3 (4). The samples of *S. avermitilis*/pJTU2463 (1) and *S. albus/*pJTU2463 (2) were used as negative control. Right: HPLC analysis of the metabolites produced by related recombinants. Samples of *S. charteusis* (3882) and *S. lividans* TK24/11C3 (TK24/11C3), *S. albus/*pJTU2463 (ALB/pJTU2463) and *S. avermitilis*/pJTU2463 (AVE/pJTU2463), and *S. avermitilis*/11C3 (AVE/11C3) as well as *S. albus/*11C3 (ALB/11C3) were compared. (♦): Tun 15:1$_B$, (●): Tun 16:1$_A$.

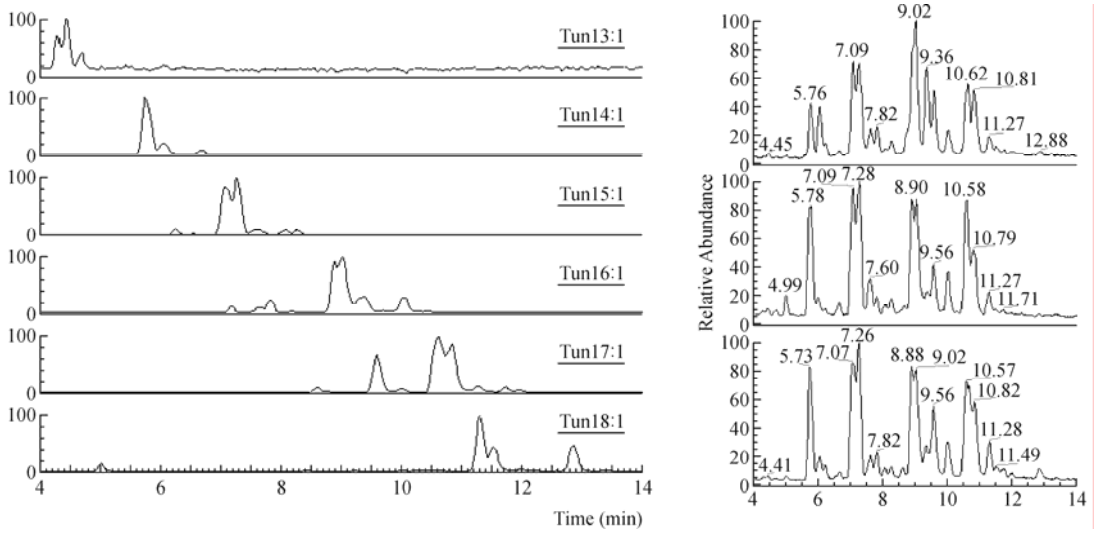

Figure S6. Tunicamyin MS profiles for related *Streptomyces* recombinants. Left: Pile-up of selective ion monitored (SIM) LC-MS chromatographs of *S. lividans* TK24:11C3. Right: LC-SIM-MS chromatographs of transconjugate strains: (A) *S. albus*:11C3; (B) *S. avermitilis*:11C3; TK24:11C3. Ion extractions are as follows: *m/z* 825-826 (Tun13:1), *m/z* 839-840 (Tun14:1), *m/z* 853-854 (Tun15:1), *m/z* 866-867 (Tun16:1), *m/z* 881-882 (Tun17:1), *m/z* 895-896 (Tun18:1). The retention times are shown in minutes (min).

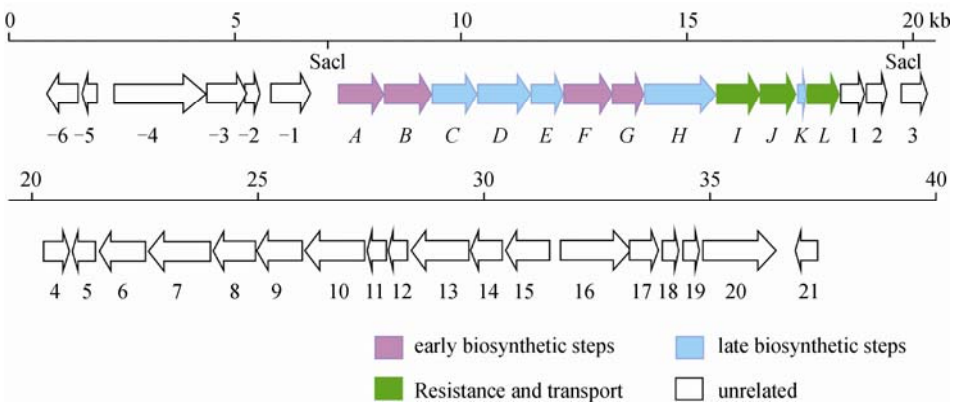

Figure S7. Genetic organization of the sequenced cosmid 11C3 containing the *tun* gene cluster. The sequenced region was revealed to contain 39 ORFs, 12 of which constituted the minimal gene cluster of tunicamycin.

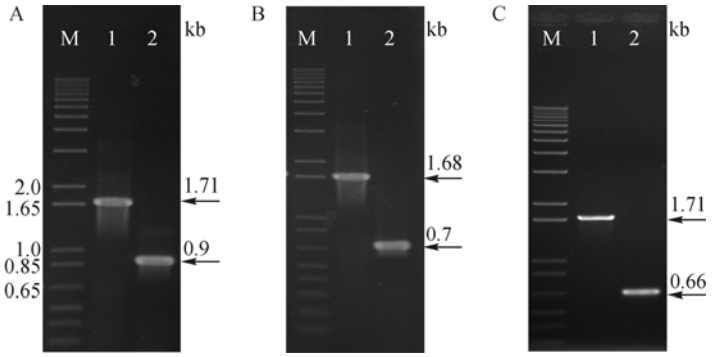

Figure S8. PCR identification of the mutant pJTU5751 cosmids for boundaries determination. (A) PCR identification of *orf-1* mutation in pJTU5751, as ca. 0.7-kb of *orf-1* gene was replaced by ca. 1.5-kb *neo* cassette, the resultant *orf-1* mutant cosmid produces 1.71-kb PCR product, and the intact pJTU5751 gives a PCR product of 0.9-kb. (B) PCR confirmation of *tunL* mutation in pJTU5751, as ca. 0.5-kb of *orf-1* gene was replaced by ca. 1.5-kb *neo* cassette, the resultant *tunL* mutant cosmid produces 1.68-kb PCR product, while the intact pJTU5751 gives a PCR product of 0.7-kb. (C) PCR identification of *orf1* mutation in pJTU5751, ca. 0.45-kb of *orf1* gene was replaced by ca. 1.5-kb *neo* cassette, accordingly, the *orf1* mutant cosmid produces 1.71-kb PCR product, and the intact pJTU5751 gives a PCR product of 0.66-kb.

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