Functional Characterization of *tlmK* Unveiling Unstable Carbinolamide Intermediates in the Tallysomycin Biosynthetic Pathway*

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EXPERIMENTAL PROCEDURES

Breaklight assay—Breaklight assay of the DNA cleavage activity of TLM A, TLM K-1(Cu-free) and TLM K-2 was carried out according to the literature procedure (1). A labeled oligonucleotide (i.e. breaklight), 5'-6-FAM-GGGTTAAGGGTTTTCCCTTAACCC-3'BHQ1 with a 5'-6-carboxyfluorescein (5'-6-FAM) and a 3'-Black Hole Quencher 1 (BHQ1), was purchased from Integrated DNA Technologies (Coralville, IA) and used as the DNA substrate. The assay mixture contains TLM A (200 nM), K-1 (Cu-free) (0.2 μM) or K-2 (0.2 μM), respectively, and 3.2 nM of the labeled oligonucleotide in 25 mM Tris-HCl buffer (pH7.5). The assay reaction was initiated by addition of $(NH_4)_2Fe(SO_4)_2$. All samples were filtered before analysis. The reactions were monitored over 5 min with a FluoroMax-3 spectrofluorometer equipped with DataMax for Windows (Instruments SA, Edison, NJ), and analyzed via a time base scan (λex =485 nm, λem=517 nm) in a Suprasil quartz cuvette (10-mm path) fitted with a magnetic stirring bar in a total volume of 2 mL. The temperature was controlled by a Haake Circulator DC10 set to 37 °C. Total cleavage of the breaklight was defined as the fluorescence emission under saturated cleaving conditions, 2 mg/mL DNase I, 37 °C, 4 hr. Emission units were converted to the amount of labeled oligonucleotide used within a procedure, thereby equating labeled oligonucleotide degradation as a function of the emission of fluorescence.

TABLE S1. Plasmids used in this study.

Plasmids	Description	Source/reference
SuperCos1	Cosmid vector containing a neomycin/kanamycin resistance gene (*neo*)	Stratagene
pIJ780	λ RED-mediated PCR-targeting vector containing a viomycin resistance gene (*vph*)	(2)
pBS8004	*ΦC31*-derived integrative vector containing an *aac(3)IV-tsr-oriT* cassette	(3)
pBS8008	Cosmid containing downstream part of the *tlm* cluster and an *aac(3)IV-tsr-oriT* cassette	(3)
pBS8010	λ RED-mediated PCR-targeting vector, derived from pBS8008, containing a neomycin-kanamycin resistance gene (*neo*)	This study
pBS8011	Cosmid containing downstream part of the *tlm* cluster, with *tlmK* replaced by *neo*	This study
pBS8012	Cosmid carrying the *ΔtlmK* in-frame deletion	This study
pBS8013	Integrative expression vector carrying *ErmE** promoter and *ΦC31* integration function	This study
pBS8014	pBS8013-derived construct for *ΔtlmK* complementation in which the expression of *tlmK* is under *ErmE**	This study

TABLE S2. Bacterial strains used in this study

Strains	Description	Source/reference
Escherichia coli		
DH5α		(4)
ET12567		(5)
BW25113/pIJ790		(2)
BT340		(2)
Streptoalloteichus hindustanus		
E465-94 (ATCC31158)	Wild type TLM producer	ATCC, Rockville, MD, USA
SB8003	*ΔtlmK*	This study
SB8004	*ΔtlmK* /pBS8014	This study

TABLE S3. Oligonucleotides as PCR primers used in this study

Oligonucleotides	Sequence
For replacement of *vph* in pIJ780 with *neo* from SuperCos1	
neo-FRT1	5’-GTTCCTATTCTCTAGAAAGTATAGGAACTTCGAAGTTCCCACGCTG CCGCAAGCACTCAG-3’
neo-FRT2	5’-CTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTGCTAGC TTGGTCGGTCATTTCGAACC-3’
For replacement of *tlmK* with FRT-*neo*	
tlmK-frt1	5’-ccctgatcccgccccccacaacggaaggaagccgccgatgATTCCGGGGATCCGTCGAC C-3’
tlmK-frt2	5’-ccggagcgggatggcggcctttctaccacttcccggctcaTGTAGGCTGGAGCTGCTTC-3’ (Low case letters represent DNA sequence originating from *S. hindustanus* E465-94 and upper case letter represent DNA sequence flanking the FRT-neo cassette from pBS8101)
For PCR and Southern blot verification of mutant strain	
tlmK-up	5’-CGCTGACAGCGCCGTCTGGG-3’
tlmK-down	5’-GGCGTTGAGCATCGGGGTGG-3’
For cloning of the *ErmE** promoter	
PermEI-f	5’-GGAATTCGTGATGCTAGTCGCGGTTGATC-3’
PermEI-r	5’-GGAATTCGTAATCATGCATTATCTCCTTCTCGCTGGATCCTACCAA CCGG-3’
For cloning of *tlmK*	
tlmK-NsiI	5’-TCCAATGCATGGGCCAGTCCTGGTGGTC-3’
tlmK-XbaI	5’-GCTCTAGATCAGGCCCCGGCGGGGAGAT-3’

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