Recruitment of terminal protein to the ends of *Streptomyces* linear plasmids and chromosomes by a novel telomere-binding protein essential for linear DNA replication

Kai Bao[1] and Stanley N. Cohen[1,2,3]

[1]Department of Genetics and [2]Department of Medicine, Stanford University School of Medicine, Stanford, California 94305-5120, USA

Bidirectional replication of *Streptomyces* linear plasmids and chromosomes from a central origin produces unpaired 3′-leading-strand overhangs at the telomeres of replication intermediates. Filling in of these overhangs leaves a terminal protein attached covalently to the 5′ DNA ends of mature replicons. We report here the essential role of a novel 80-kD DNA-binding protein (telomere-associated protein, Tap) in this process. Biochemical studies, yeast two-hybrid analysis, and immunoprecipitation/immunodepletion experiments indicate that Tap binds tightly to specific sequences in 3′ overhangs and also interacts with Tpg, bringing Tpg to telomere termini. Using DNA microarrays to analyze the chromosomes of *tap* mutant bacteria, we demonstrate that survivors of Tap ablation undergo telomere deletion, chromosome circularization, and amplification of subtelomeric DNA. Microarray-based chromosome mapping at single-ORF resolution revealed common endpoints for independent deletions, identified amplified chromosomal ORFs adjacent to these endpoints, and quantified the copy number of these ORFs. Sequence analysis confirmed chromosome circularization and revealed the insertion of adventitious DNA between joined chromosome ends. Our results show that Tap is required for linear DNA replication in *Streptomyces* and suggest that it functions to recruit and position Tpg at the telomeres of replication intermediates. They also identify hotspots for the telomeric deletions and subtelomeric DNA amplifications that accompany chromosome circularization.

Streptomyces species have multiple biological properties that have made them important subjects for the study of mechanisms that regulate morphological and biochemical development in prokaryotes. Their complex life cycle, the high degree of cellular organization and morphological differentiation that exists within their colonies, and the genetic control mechanisms that regulate these events and processes have been of great biological interest (for review, see Champness and Chater 1994; Hopwood et al. 1995). *Streptomyces* synthesize a multitude of antimicrobial compounds and other agents used widely in medicine and agriculture (Chater 1992; Hopwood et al. 1995). Additionally, the presence in these organisms of plasmids and chromosomes that are linear, but which can circularize readily, has provided, and is

continuing to provide, an attractive experimental system for fundamental investigations of telomere function and replicon evolution (Shiffman and Cohen 1992; Chang and Cohen 1994; Chang et al. 1996; Chen 1996; Lin and Chen 1997; Volff et al. 1997; Huang et al. 1998; Qin and Cohen 1998, 2000; Volff and Altenbuchner 1998, 2000; Wang et al. 1999; Bao and Cohen 2001; Yang and Losick 2001; Chen et al. 2002; Yang et al. 2002).

The replication of *Streptomyces* linear plasmids has been shown to proceed divergently from a site located near the center of the molecule and to generate 3′-leading-strand overhangs at the telomeres (Chang and Cohen 1994). The recessed 5′ ends of the lagging strands produced by the joining together of Okazaki fragments (Kurosawa et al. 1975) are then extended (i.e., "patched") to produce full-length duplex DNA molecules (Chang and Cohen 1994). As *Streptomyces* linear chromosomes and linear plasmids have similar termini (Huang et al. 1998; Qin and Cohen 1998), and the linear chromosomes also replicate bidirectionally from an internal origin of repli-

cation (Musialowski et al. 1994), linear chromosome replication is presumed to also generate telomeric 3′ overhangs that require patching. Both linear plasmids and linear chromosomes of *Streptomyces* have a terminal protein attached covalently to their 5′ DNA ends (Hirochika and Sakaguchi 1982; Kinashi et al. 1987; Sakaguchi 1990; Lin et al. 1993; Bao and Cohen 2001; Yang et al. 2002). This protein is required for the propagation of these replicons in a linear form (Bao and Cohen 2001). Whereas purified terminal proteins of *Bacillus subtilis* phage ϕX29 and adenoviruses (Salas 1991; Yoo and Ito 1991; van der Vliet 1995; Hay 1996; Meijer et al. 2001) have been shown by biochemical studies to function in vitro to prime DNA synthesis, analogous data are not available for *Streptomyces* terminal proteins.

Sequence analysis indicates that the telomeres of *Streptomyces* linear replicons have similarities to, and differences with, the telomeres of eukaryotic chromosomes. The 3′ overhangs of both types of replicons contain multiple short repeats (Qin and Cohen 1998; Huang et al. 1998; for eukaryotes, see review, Greider 1996; Lingner and Cech 1998; McEachern et al. 2000; Blackburn 2001). However, whereas *Streptomyces* telomeres contain inverted repeats, eukaryote telomeres consist of a long series of tandem direct repeats. Moreover, the proteins that bind to telomeres of eukaryotes are not covalently attached to the terminus (Greider 1996; Bryan and Cech 1999; Blackburn 2001).

Earlier work has identified genes encoding the terminal proteins of *Streptomyces* spp. linear plasmids and chromosomes (i.e., terminal protein genes, *tpg*'s; Bao and Cohen 2001; Yang et al. 2002). During our investigations of the chromosomally encoded terminal protein gene of *Streptomyces lividans* (i.e., *tpgL*; Bao and Cohen 2001), we observed, immediately 5′ to *tpgL* on the *S. lividans* chromosome, a gene (herein named telomere-associated protein gene, *tap*) whose position and sequence we found to be highly conserved among multiple streptomycetes. We report here that the Tap protein is essential for the replication of *Streptomyces* chromosomes and plasmids in a linear form, and that Tap recruits Tpg to telomere termini by interacting with both Tpg and specific sequences on the 3′ overhang of telomeric DNA. Using sequence analysis, hybridization of *Streptomyces coelicolor* chromosomal DNA arrayed on glass slides, and a series of computer programs that apply knowledge-based algorithms to microarray analysis (Genetic Analysis By Rules Incorporating Expert Logic, GABRIEL; Pan et al. 2002), we show that telomere deletion, chromosome circularization, and amplification of subtelomeric DNA occur in bacteria mutated in the *tap* gene, map the deletion and amplification boundaries at single-gene resolution, and determine gene copy number in amplified DNA segments.

Results

A conserved putative ORF is located 5′ to tpg *genes in multiple* Streptomyces spp.

During experiments that identified and characterized terminal proteins (Tpg proteins; Bao and Cohen 2001)

linked to 5′ DNA ends of *Streptomyces* spp. linear replicons, we carried out Frame program analysis (Bibb et al. 1984) of genomic DNA sequences near the chromosomal *tpg* loci of *Streptomyces rochei* (cosmids pBC65, pBC66 and pBC67 of our *S. rochei* library) and *S. lividans* (cosmid pBC30 of our *S. lividans* library). This analysis identified conserved putative ORFs (Fig. 1, top) that are located immediately 5′ to the *tpg* genes of both species and are separated from *tpg* genes by 6 or 9 bp (Fig. 1A). Examination of the genomic DNA sequence of *S. coelicolor* (SC8D11.24; Bentley et al. 2002; http://www.sanger.ac.uk/Projects/S_coelicolor) revealed a similar ORF at a corresponding location. As the three *tpg*-proximal ORFs include a predicted helix–turn–helix DNA-binding motif (HTH_XRE family domain, overlined in Fig. 1, top), as indicated by Simple Modular Architecture Research Tool (SMART) analysis, they were speculatively designated as encoding telomere-associated DNA-binding proteins. Subsequent studies (see below) have established the correctness of this notion, and we have retained the telomere-associated protein (Tap) designation. The Tap ORFs of *S. lividans* (Tap$_L$) and *S. coelicolor* (Tap$_C$) are identical, as are two analogous ORFs located 5′ to the *tpgR1* and *tpgR3* genes of *S. rochei* linear replicons. The *S. rochei* and *S. lividans* Tap proteins show 56% sequence identity and 67% similarity. BLAST program analysis of GenBank databases showed no other homologies with Tap.
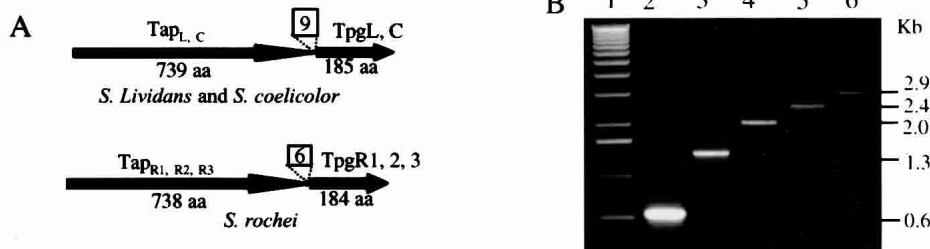
The positional relationship between *tap* and *tpg* genes, as conserved in several *Streptomyces* species, suggests that the *tap* and *tpg* genes may constitute a polycistronic operon. Amplification of *S. lividans* total RNA by RT–PCR using a 3′ primer complementary to *tpg* (i.e., primer RT–cDNA) and five 5′ primers complementary to different sequences within the *tap* gene yielded products having sizes consistent with this interpretation (Fig. 1B). The longest RT–PCR product, which was amplified by the most 5′ of the *tap*-specific primers (i.e., primer RT-2.9 kb) and primer RT–cDNA, yielded a 2.9-kb DNA band (Fig. 1B, lane 6) that included the full-length *tap* and *tpg* gene sequences (data not shown).

DNA-binding properties of the Tap and Tpg proteins

As protein-primed initiation of DNA chains de novo leaves an amino acid of the primer linked to the first nucleotide of the nascent DNA chain (Salas 1991; Yoo and Ito 1991; van der Vliet 1995; Hay 1996; Meijer et al. 2001), a priming role for terminal proteins of *Streptomyces* linear replicons has been inferred from their covalent attachment to the 5′ ends of these DNAs (Hirochika and Sakaguchi 1982; Kinashi et al. 1987; Sakaguchi 1990; Lin et al. 1993; Bao and Cohen 2001; Yang et al. 2002). However, the mechanism that positions terminal proteins at telomere termini for their priming function has not been known. To investigate a possible role for Tap in this process, we expressed the Tpg and Tap proteins in *S. lividans* as His$_6$-tag fusion products, purified the fusion proteins as described in Materials and Methods, and used an electrophoretic mobility shift assay (EMSA) proce-

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                                                                    h-t-h
                                      _____
TapR1-R3    1  VATEDQ.LFSAVDALLEQVAQDA.LPSPAERKRLREAAGLSQAQVASALKTRRETVASWE
TapR2       1  VATEDQ.LFSAVDALLEQVAQDD.LPASAERKRLREAAGLSQAQVAQIAKALDARREAVGNWE
TapC-L      1  MTDQPQPLFAAVDALLAAVDDGGVLPVPDERVRLREAAGLTQAAVAQALGVRVASIQAWE

TapR1-R3   59  SGRNEPRPPQRAAYARLLEKLAERFPTP.DEAP.APEPESFPAPASVPLPVPAPAPVPAP
TapR2      59  TGKTEPRPPKRAAYARLLEGLAAARFPAPASDAPAAPPAPTVPEAFTGPAPVPAPA...AE
TapC-L     61  TGRAEPKAERIEAYRRLLEGLSRRFPASA.VPPGSSEAAPRPDP.GAPSP.SAPAAPESV

TapR1-R3  117  APVKTAPTSAVRPAPAARPATSSQRPGVQKA.AKGTAKGPATTAA..ADGRFENGPLAVV
TapR2     116  APAAPAPAAARPATAAKPATSSRQKA....VAK.KTTAAA..ADPRFENGPLAVI
TapC-L    118  LAAVPS.ASAAAPTAPPRPAAPGRQPAARRADGKQSAACLRTAAAGGTDPRFENGPLAVV

TapR1-R3  174  DVDDEGQVAAYCVGGLVLDVPAKTLPALVDWTLTEA.KLGQARLHWHGKDADPVLVLTSA
TapR2     169  DCED.GQVSAYCVGGLVLDVPAKTIPALVDWTLGRGPSRVRARLHRNGRDADPILVLTAS
TapC-L    177  DVED.GQVRAYCTGGLVLDVPAKSLAALVDWTLAEA.KLGQPKLSGPGKDADPLLVLTEA

TapR1-R3  233  ALERYGLPAELSPEDRRAGRLPADHKVIKQIARAGQQLTRRGFGAWARVFREPEGSRRRC
TapR2     228  VLERYGLPAALSPEERRAGRLAAGHKVVKQITKAGLQLTQRGFGPWARVYRAPEGSQRRC
TapC-L    235  ALDRYGLPTTLTDEERLAGRIPEGHKAVKQLARAHWKLTKRGFGPWARIYRPATGSERAC

TapR1-R3  293  VQECIPAWGALPIDEWDNKNDPRLPTMHAAELARFLGTYADRVMTPRGTTAVTGLELMTA
TapR2     288  VQLCILPWNALDAREWDKKDDPQLPTMHPADLARYLGLYAARVTTPRGSTATTGLELMTA
TapC-L    295  VQLCIPSWHALDTRHWGHAG..QLP...PAELARVLGVYASRVMTPRGSTAVTGLELMTA

TapR1-R3  353  LRPPTRAERDEATGEFKPAPNDDALTR.LYDVVECEIPDEHPKLRGRYARHHQRTPAEML
TapR2     348  LRPPTRAEKEPDTGEFKRAFNDDALTQ.LYPVVECEVPDEHPVLKGKFARHHLRTPAEML
TapC-L    350  LHPPTRASQPDAAGRRHSERNPGSLGQDPVDCAPCEAPDGHPLL.AALPRFHVRGPAEKL

TapR1-R3  412  MEEPYNWCRQLTDDECMRPYLVAIDVNMAFAAAANGLTVGLNGPTHLTGNPRFDPALPGS
TapR2     407  MEEPYDWCRPLTDEECANPYLVVVDLNMSFAAAANGLTVGLNGPTHLTGNPAFDPALPGS
TapC-L    409  FEEAYDWARPMTDDECMLRYLVGIDVNMAFAAGANGLTVGLGAPTRVE.RPAFDAKLPGS

TapR1-R3  472  WLVDLSHVDLSRVQVNGR..SVDADRLPSPFTPTGDRPDGPAWYATPTVAYAQELGFDVA
TapR2     467  WLVDLSHVDLSRVQVGGR..TLDADRLPSPFTPKGERPEGPAWYATPTVTYAVELGFDVA
TapC-L    468  WLVDLSHVDLSRVKVGKEWAELDASLLPSPFTPKGERPEGPAWYATPTVAYAKELGYEVR

TapR1-R3  530  PIEAYVRTRTGRYLDPWYKRLRDAYVTTMADLGVTTGMGGSEFLDAMARSRDADPALLLV
TapR2     525  PIEAYVRTQTGRYLDPWYKRLRDAYVVVDLGVVATMADMGVTTDLQQEFLETMARRKQVDPTMALL
TapC-L    528  PVEAWVRYENGRYLDGWYQRLRDAYLATMSDLGVDADRTPDDFLAAMDGYRSRDPELAIV

TapR1-R3  590  LKAIKATAKGGIGKLRQRNRGQ.VPYYEPWPALERPTWRPDIRAAVLAGVRISMHRKMMK
TapR2     585  ETAIKATAKGAIGKLRQRSRGQ.VPYYEPYPALNRETWRPDIRPAVLAAQRVGLHRKLMK
TapC-L    588  VSAMKATVKGGLGKLRERPRGEGWKPGQPWRALSRPTWRPDIRAAVISRTRINLHRKIVK

TapR1-R3  649  TAAAAGVYPVAIGTDALVYPSPGPSPLDFLPHTAEGKPAPGSFRLGVSPGMVKHQGTRTV
TapR2     644  TAAAADRYPVAIGTDAIVYPSPGPSPLDVLPYTPEGKPVPGTFRLGVSPGMVKHQGTQTV
TapC-L    648  HAAFTGQYPIAILSDCVVYAAAGPSPLDFLPYR.DGKPLPGGFKLGVNPGLVKHEGTQSV

TapR1-R3  709  LWAEQQFEE.HGGVFNIANLVKT.DPT.AGEGEZ
TapR2     704  LWAEQQFEE.RGEVFNIANLIKTADPT.AGEGEZ
TapC-L    707  LWGEEVRERFDAPELNLARYIKDGTVTDADSGEZ
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A — Tap$_{L, C}$ → 9 TpgL, C
739 aa — 185 aa
S. Lividans and *S. coelicolor*

Tap$_{R1, R2, R3}$ → 6 TpgR1, 2, 3
738 aa — 184 aa
S. rochei

B — Lanes 1 2 3 4 5 6 — Kb
2.9
2.4
2.0
1.3
0.6

Figure 1. Tap ORFs in vicinity of terminal protein genes (*tpg*). (*Top*) Alignment of the amino acid sequences of Tap proteins in *Streptomyces coelicolor* (Tap$_C$), *Streptomyces lividans* (Tap$_L$), and *Streptomyces rochei* (Tap$_{R1}$, Tap$_{R2}$, and Tap$_{R3}$). TapR1-R3, Tap$_{R1}$ or Tap$_{R3}$; TapC-L, Tap$_C$ or Tap$_L$. (*A*) Schematic map of gene arrangements of *tpg* and *tap* in *S. rochei* (Tap$_{R1,R2,R3}$ and TpgR1, 2, 3), *S. lividans* (Tap$_L$ and TpgL), and *S. coelicolor* (Tap$_C$ and TpgC). The number of nucleotides between the stop codon of the *tap* gene and the start codon of the *tpg* gene are shown in the boxes. (*B*) Reverse transcriptase (RT)–PCR analysis of *tap* and *tpg* in *S. lividans* 1326. (Lane *1*) 1-kb DNA ladder. (Lane *2*) RT–PCR product of the primer pair of RT–cDNA and RT_0.6kb. (Lane *3*) RT–PCR product of the primer pair of RT–cDNA and RT_1.3kb. (Lane *4*) RT–PCR product of the primer pair of RT–cDNA and RT_2.0kb. (Lane *5*) RT–PCR product of the primer pair of RT–cDNA and RT_2.4kb. (Lane *6*) RT–PCR product of the primer pair of RT–cDNA and RT_2.9kb.

dure to test the ability of each purified protein to interact with telomeric DNA sequences. As seen in Figure 2A, migration of [α-^{32}P]-labeled single-stranded DNA corresponding to the 3′ overhang of plasmid pSLA2 telomeres was retarded by both Tpg and Tap. However, the binding of Tpg showed little specificity and was largely reversed by the addition of excess denatured DNA of *S. lividans* BKKO5 (Bao and Cohen 2001), which has a circular chromosome and lacks telomeres (Fig. 2A, lanes b–d). In contrast, binding of Tap to single-strand telomeric DNA was highly specific and was not affected detectably by even a 100-fold excess of denatured DNA from BKKO5 (Fig. 2A, lanes e–g). Quantitatively, Tap interacted with the [α-^{32}P]-labeled single-strand telomeric 3′ overhang DNA sequence 10 times more tightly than Tpg ($K_a = 1 \times 10^7$

M^{-1} vs. $K_a = 1 \times 10^6$ M^{-1}, respectively), as determined by binding studies that used unlabeled probe as competitor (Fig. 2B). Although Tpg interacted additionally with double-strand telomeric DNA, this binding also was nonspecific and was inhibited by an excess of native BKKO5 DNA (Fig. 2C).

DNase I footprinting using Tap protein identified two distinct sites of single-strand DNA on the telomeric 3′ overhang of pSLA2 that were protected by the binding of Tap protein (Fig. 2D). One Tap-protected locus consists of 18 nt (TGCGGGCTTCGCCCGCAG) beginning at a thymidine positioned 17 nt from the telomere end; the second region extends 26 nt (ACACGCCCCGCTGC GCGGGGGCGTGT) from an adenosine located 40 nt from the end. Both of these Tap-protected DNA se-
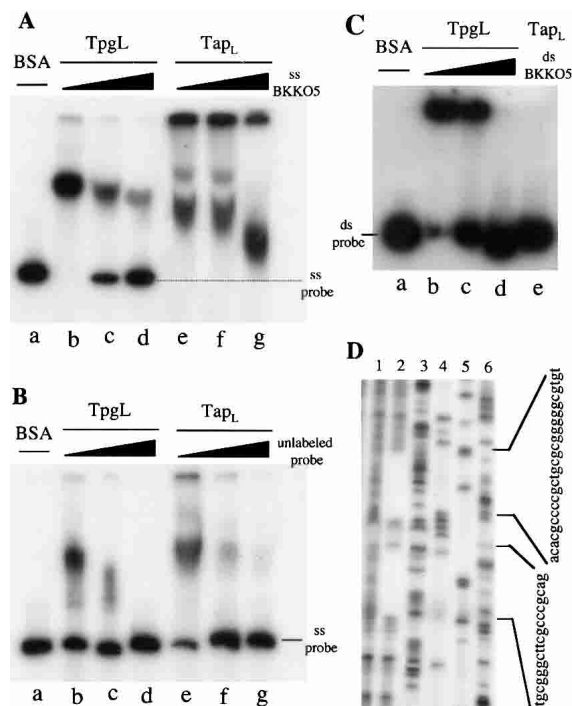
Figure 2. DNA binding activity and footprinting analysis. (*A*) EMSA using radioactively labeled single-stranded pSLA2 telomeric DNA. (Lane *a*) Probe + BSA as control. (Lanes *b,c,d*) TpgL protein with 5-fold, 20-fold, and 100-fold excesses of single-stranded BKKO5 DNA. (Lanes *e,g,f*) Same as lanes *b*, *c*, and *d* except Tap_L protein was used. (*B*) EMSA using radioactively labeled single-stranded pSLA2 telomeric DNA in the presence of unlabeled cold probe as competitor. (Lane *a*) Probe + BSA as control. (Lanes *b,c,d*) TpgL protein with 5-fold, 20-fold, and 100-fold excesses of single-stranded cold probe. (Lanes *e,g,f*) Same as lanes *b*, *c*, and *d* except Tap_L protein was used. (*C*) EMSA using radioactively labeled double-stranded pSLA2 telomeric DNA. (Lane *a*) Probe + BSA as control. (Lanes *b,c,d*) TpgL protein with 5-fold, 20-fold, and 100-fold excesses of circular chromosomal DNA of BKKO5. (Lane *e*) Tap_L protein alone. (*D*) DNase I footprinting analysis using single-stranded pSLA2 telomeric DNA and Tap_L protein was carried out as described in Materials and Methods. (Lanes *1,2*) Single-stranded telomeric DNA was incubated with 0 and 2 µg of Tap_L protein, respectively. (Lanes *3–6*) DNA sequencing ladder reactions with termination mix of ddG, ddA, ddT, and ddC. Brackets at the *right* show two regions of the protected DNA sequences.

quences previously were shown to be required for linear replication of pSLA2 derivatives (Qin and Cohen 1998), and both are highly conserved in all nine telomeric DNAs cloned from *Streptomyces* linear plasmids and chromosomes (Huang et al. 1998; Qin and Cohen 1998).

Tap and Tpg interact and form a telomere-associated complex

The ability of Tap to bind tightly and site-specifically to sequences in the telomeric 3′ overhangs of *Streptomyces* linear replication intermediates and the limited binding specificity of Tpg for this DNA region suggested that Tap

may position Tpg to prime the synthesis of 5′ ends of lagging-strand DNA. As seen in Figure 3, immunoprecipitation and immunodepletion experiments using anti-Tap or anti-Tpg antibodies (see Materials and Methods) indicated that the Tap and Tpg proteins interact with each other in vivo. Tpg was detected by Western blotting in pellets immunoprecipitated by anti-Tap antibody (Fig. 3A, lane 3), and conversely, Tap was found in pellets immunoprecipitated by anti-Tpg antibody (Fig. 3C, lane 2). As seen in Figure 3C, addition of purified Tap or Tpg protein resulted in depletion of the ability of the respective antibody to react with its intended target, confirming the specificity of the antibody and the identity of the bands designated in the figure as Tap or Tpg.

Interaction between Tap and Tpg proteins was further confirmed by yeast two-hybrid analysis (Fig. 4). In the latter studies, a Tap fusion to the GAL-4 DNA-binding domain was constructed (construct pBC191) and tested for interaction with Tpg fused to the GAL-4 activation domain (construct pBC193). The cotransformants of pBC191 and pBC193 (Fig. 4, row 5) and the positive control (Fig. 4, row 2) all showed induced expression of the three reporter genes used in the analysis, whereas reporter gene expression was not detected in cells cotransformed with the negative control [vectors pDBLeu + pDEST22 (Fig. 4, row 1), pBC191 + pDEST22 (Fig. 4, row 3), and pBC193 + pDBLeu (Fig. 4, row 4)].

PCR analysis of proteinase K-treated anti-Tpg or anti-Tap immunoprecipitates using the telomere-specific primers P-end and P300 (see Materials and Methods) indicated the presence of telomeric DNA in both types of immunoprecipitates (Fig. 3E, lanes 3,4), whereas control PCR analyses using primers corresponding to two separate regions of nontelomeric DNA failed to generate the appropriate-sized PCR amplicons (data not shown).

Tap is essential for propagation of Streptomyces chromosomes and plasmids in linear form

Is Tap essential for linear DNA replication in *Streptomyces*? To investigate the effects of inactivation of *tap*, we used a gene-replacement/disruption procedure that introduced a spectinomycin-resistance (*Spc^r*) gene into the chromosomal *tap* gene (Fig. 5). Because the *tap* and *tpg* genes are cotranscribed, and we wished to obtain an *S. lividans* isolate defective only in *tap*, we performed the *tap* disruption in a strain that contained a plasmid expressing *tpg* from an adventitious promoter. Southern blot analysis confirmed replacement of the native *tap* locus with an allele containing the *Spc^r* insert (BKKO17; Fig. 5A,B). In analogous experiments, we used a similar gene-replacement strategy to disrupt both the *tap* and *tpg* genes, using plasmid pBC141, which contains fragments of *tap* and *tpg* that are colinear with the chromosome but separated by *Spc^r* (Fig. 5, top). The double knockout of *tap* and *tpg* was confirmed by Southern blot analysis (BKKO19, Fig. 5A,B).

Southern blots of DNA isolated from the *tap* insertion mutant BKKO17 or from the doubly mutated *tap^- tpg^-*
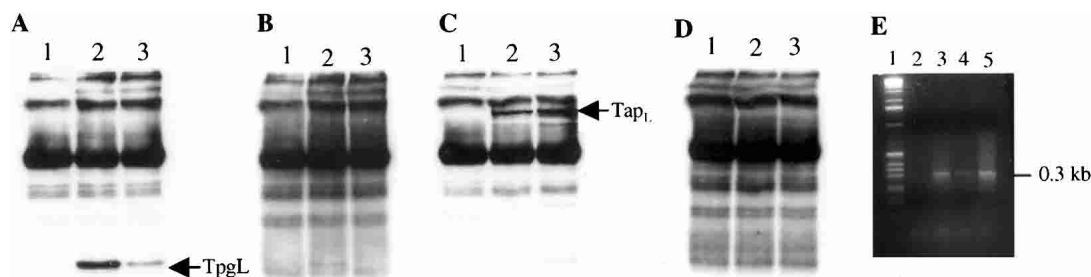
Figure 3. Tap coimmunoprecipitates with Tpg. (*A–D*) *Streptomyces lividans* 1326 cell extracts were incubated with prebleeding sera (lane *1*), anti-TpgL antibodies (lane *2*), and anti-Tap$_L$ antibodies (lane *3*). (*A*) Western blotting using anti-TpgL antibodies. (*B*) Immunodepletion analysis of lanes shown in *A* using anti-TpgL antibodies. (*C*) Western blotting using anti-Tap$_L$ antibodies. (*D*) Immunodepletion analysis of lanes shown in *C* using anti-Tap$_L$ antibodies. (*E*) Agarose gel electrophoresis of the products of IP–PCR using different immunoprecipitates as templates and the telomere-specific primers P-end and P300 (Materials and Methods). (Lane *1*) 1-kb DNA ladder. (Lane *2*) IP with prebleeding anti-sera as negative control. (Lane *3*) IP with anti-TpgL. (Lane *4*) IP with anti-Tap$_L$. (Lane *5*) Template of total DNA of *S. lividans* 1326 as positive control.

strain BKKO19 using telomeric DNA as a probe (data not shown) suggested that the clones surviving *tap* mutation had undergone telomere deletion. Telomere deletion was confirmed, and the endpoints of deletions in BKKO17 and BKKO19 were mapped at single-gene resolution by using whole-genome DNA microarrays from *S. coelicolor* (Fig. 6; Huang et al. 2001), a close relative of *S. lividans*. In these experiments, preparations of total DNA from mutant and wild-type *S. lividans* strains were differentially labeled with fluorescent dyes as described in Materials and Methods and hybridized concurrently to microarray slides. The relative hybridization of mutant and wild-type probes was assessed as described for *Streptomyces* RNAs (Huang et al. 2001). Data were analyzed by the GABRIEL program's continuity/gap algorithm (Huang et al. 2001; Pan et al. 2002) using the *S. coelicolor* genomic DNA sequence reported by Bentley et al. (2002). This analysis (Fig. 6) showed DNA deletion (depicted by green) at both chromosomal ends for each strain, identified the ORFs that were deleted in BKKO17 and BKKO19, and defined the deletion endpoints at single-ORF resolution. In both strains, the endpoint of the deletion that removed the telomeres occurred within a 500-bp DNA region located between ORF SCF1.13 and ORF SCF1.14 (indicated by jcnL in Fig. 6A,B) at the "left" end of the chromosome [according to the orientation of Redenbach et al. (1996) as reported by Bentley et al. (2002)] and within a 380-bp segment between ORF SC8D11.26 and ORF SC8D11.27c (indicated by jcnR) at the "right" end.

Streptomyces plasmids and chromosomes require telomeres to replicate as linear molecules, but can replicate in a circular form following telomere loss (Shiffman and Cohen 1992; Chang and Cohen 1994; Chang et al. 1996; Lin et al. 1993; Lin and Chen 1997; Volff et al. 1997; Qin and Cohen 1998, 2002; Volff and Altenbuchner 2000). The above results thus imply that the telomere-deleted chromosomes of the Tap knockout isolates BKKO17 and BKKO19 had circularized, as observed previously in cells that survive mutation of the *tpg* gene (Bao and Cohen 2001). Chromosome circularization in these isolates was

confirmed by sequencing cloned PCR-generated DNA fragments containing the junction between the two deletion endpoints. The cloned 2.1-kb junction fragment of BKKO17 showed at one end a 627-bp DNA sequence matching the ORF SCF1.13 sequence. At the other end was a 589-bp sequence matching ORF SC8D11.26, a 35-bp intergenic region, and a 342-bp sequence corresponding to the C-terminal sequence of ORF SC8D11.27c. The DNA sequences of ORFs SCF1.13 and SC8D11.26, which normally are located at separate ends of the linear chromosome, were found also on the cloned ~2.0-kb junction fragment of BKKO19. An adventitious 935-bp DNA segment containing two incomplete consecutive ORFs (SC5H4.15, putative secreted sugar hydrolase; and SC5H4.16c, putative transcriptional regulator), that map to the central portion of the linear *S. lividans* chromosome was inserted between the joined chromosomal DNA ends of BKKO17. BKKO19 contained a 445-bp sequence of pBC141 vector DNA inserted between the joined chromosome ends. Analogous insertions of adventitious DNA previously have been discovered between the ends of plasmids circularized by nonhomologous end joining (NHEJ) in *Streptomyces* (Qin and Cohen 2002), and at the junctions of recombined nonhomologous linear DNAs of other organisms (e.g., Moore and Haber 1996; Gorbunova and Levy 1997; Yu and Gabriel 1999).

In both BKKO17 and BKKO19, amplification of a segment of contiguous genes located immediately adjacent to the point of breakage was observed by microarray analysis (Fig. 6A,B, indicated by red color), consistent with earlier Southern blot data (Volff et al. 1997) showing gene amplification in an *S. lividans* strain having a circular chromosome. The region of amplified ORFs in both BKKO17 and BKKO19 (Fig. 6A,B, genes shown in red) extends for ~9500 bp (from ORFs SCF1.14–SCF85.08c) from the deletion endpoint at the left chromosome end, and shows an estimated five to six copies of each gene based on the relative intensity of fluorescence of mutant strain versus wild-type DNA in microarray spots. The amplified ORFs at the right-end chromosome junctions of BKKO17 and BKKO19 are different:
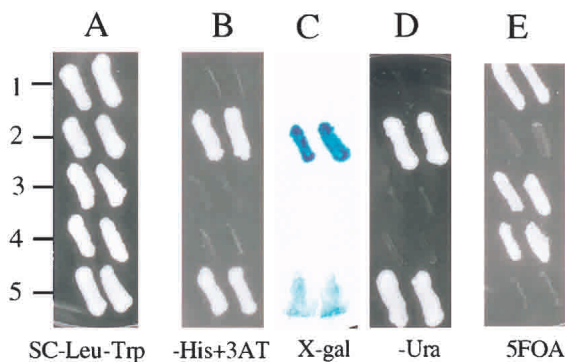
Figure 4. Yeast two-hybrid analysis of Tap$_L$ and TpgL. Selection plates of SC-Leu-Trp (*A*), SC-Leu-Trp-His + 3AT (*B*), X-gal (*C*), SC-Leu-Trp-Ura (*D*), and SC-Leu-Trp + 0.2% 5FOA (*E*) were used to examine reporter gene phenotypes of yeast strain MaV203. (Row *1*) Cotransformants of pDBLeu and pDEST22 as negative control. (Row *2*) Cotransformants of pPC97-Fos and pPC86-Jun as positive control. (Row *3*) Cotransformants of pBC191 and pDEST22. (Row *4*) Cotransformants of pBC193 and pDBLeu. (Row *5*) Cotransformants of pBC191 and pBC193.

~5200 bp from ORFs SC8D11.17 to SC8D11.23** were amplified in BKKO17 and ~8400 bp from ORFs SC8D11.17 to SC8D11.26 were amplified in BKKO19. In both cases, amplified genes are estimated to exist at two to three copies per chromosome. ORFs located internal to the amplified segment showed similar hybridization with differentially labeled DNA from BKKO17 or BKKO19 when compared with DNA from the parental (linear chromosome) strain (Fig. 6A,B, ORFs represented by black).

The above experiments strongly support the argument that Tap is required for the propagation of *Streptomyces* chromosomes in a linear form. To learn whether the same is true for plasmid replicons, pSLA2-derived plasmids that include the *Escherichia coli* plasmid pSP72 replicon and also contain the *S. lividans tpg* gene (pBC167), the *tap* gene (pBC178), or both genes (pBC181) were constructed as described in Materials and Methods and cloned in *E. coli*. In Figure 7, lanes 2, 5, and 8 contain DNA of plasmids pBC167, pBC178, and pBC181, respectively, isolated from *E. coli* and linearized by cleavage at the *Ssp*I site of pSP72. Introduction of these *Ssp*I-cleaved DNAs into *S. lividans* 1326, which contains intact chromosomal *tpg* and *tap* genes, yielded viable transformants at a frequency of 5×10^2/µg of DNA. These transformants all contained linear plasmids (Fig. 7; shown in lanes 3,6,9 for typical transformants receiving each plasmid), in agreement with previous evidence that such plasmid DNAs can generate linear replicons when introduced into *S. lividans* by transformation (Shiffman and Cohen 1992; Qin and Cohen 1998, 2000; Bao and Cohen 2001). The linearity of the replicons isolated from transformants was confirmed by alkaline lysis (Qin and Cohen 1998, 2000), which removes both linear plasmid DNA and fragments of chromosomal DNA (Fig. 7, cf. lanes 4,7,10 and 3,6,9). Only plasmid pBC181, which car-

ries cloned *tap* and *tpg* genes, yielded transformants after *Ssp*I cleavage and introduction by transformation into BKKO19—which lacks the chromosomal *tap* and *tpg* genes. Linearity of the plasmids propagated in these transformants was shown by their sensitivity to alkaline lysis (Fig. 7, cf. lanes 11 and 12). We conclude that both *tap* and *tpg* are essential for replication of linear plasmid DNA as well as chromosomal DNA in a linear form in *S. lividans*.

Discussion

Because of the 5'-to-3' polarity of DNA replication, the propagation of all linear replicons requires a solution to the "end replication problem," that is, the need to provide both a template and primer for DNA synthesis at telomeres (Watson 1972; Kornberg and Baker 1992). Eukaryotes have evolved a strategy that uses an RNA template and the riboenzyme telomerase for telomeric DNA synthesis (for review, see McEachern et al. 2000; Blackburn 2001). For *B. subtilis* bacteriophage φ29, *E. coli* phage PRD1, and the adenoviruses of mammalian cells, the end replication problem is solved by a mechanism of strand-displacing protein-primed synthesis of the full-length genome (Salas 1991; Yoo and Ito 1991; van der Vliet 1995; Hay 1996; Meijer et al. 2001). Still other linear replicons have evolved mechanisms of "turn-around" replication to accomplish lagging-strand DNA
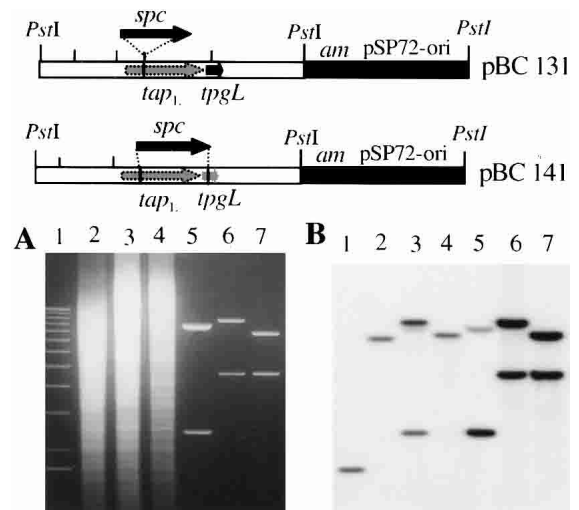
Figure 5. Analysis of *Streptomyces lividans* 1326 chromosomal DNA showing disruption of *tap$_L$* gene and both *tap$_L$* and *tpgL*. (*Top*) Schematic representation of gene disruption. *tap$_L$* was disrupted by an in-frame insertion of *spc* on pBC131; both *tap$_L$* and *tpgL* were disrupted by the replacement of *spc* on pBC141. *am*, apramycin-resistance gene; *spc*, spectinomycin-resistance gene. (*A*) Ethidium bromide-stained agarose gel. (Lane *1*) 1-kb DNA ladder. (Lane *2*) 1326 total DNA. (Lane *3*) BKKO17 total DNA. (Lane *4*) BKKO19 total DNA. (Lane *5*) pBC117 plasmid DNA. (Lane *6*) pBC141 plasmid DNA. (Lane *7*) pBC129 plasmid DNA. (*B*) Southern blot of same gel probed with ^{32}P-labeled pBC141. All DNAs were digested with *Pst*I.
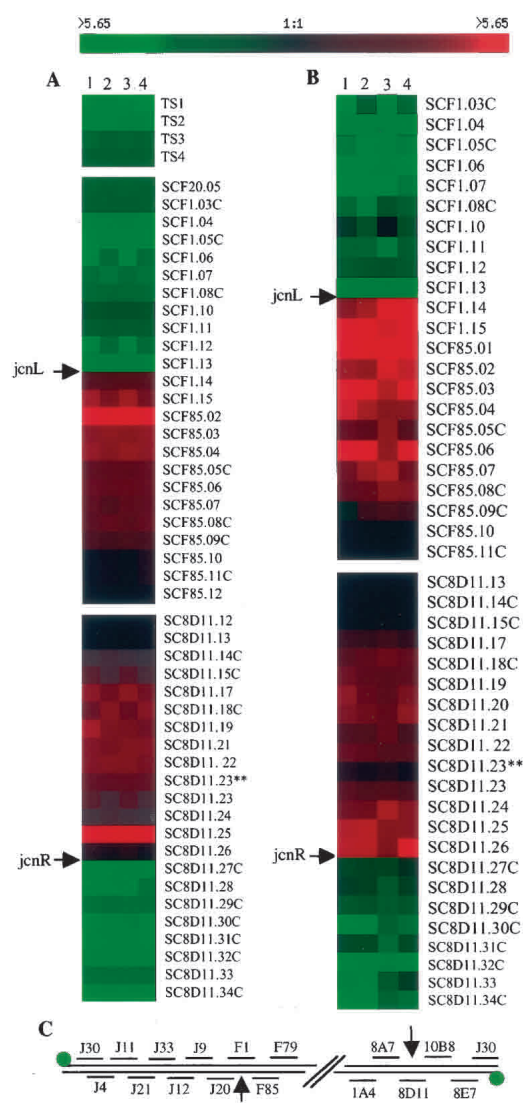
Figure 6. Whole-genome comparison of knockout strain BKKO17 (*tap_L^-*) or BKKO19 (*tap_L^- tpgL^-*) with wild-type *Streptomyces lividans* 1326. Profiles indicate gene deletion and amplification in knockout strains. Genes were analyzed and clustered by GABRIEL analysis (Pan et al. 2002). Consistency in the amount of DNA used for analysis at different repeats was inferred from our finding that 97% of the genes analyzed showed no change in the compared strains. DNA ratios are represented in tabular form according to the color scale shown at the *top*; rows correspond to individual genes, and columns correspond to different repeats. Green shades represent gene deletion in knockout strain, and red shades represent gene amplification in knockout. Black indicates unchanged ORFs in the comparing DNA. jncL, junction of the left chromosome end; jncR, junction of the right chromosome end. Lanes *1–4* represent 4 repeats. (*A*) Profile of the comparison of BKKO17 and *S. lividans* 1326. TS1, 2, 3, and 4, telomere sequences 1, 2, 3, and 4. Chromosomal telomere DNA was PCR-amplified and printed on the DNA microarray slides for detecting telomere loss. (*B*) Profile of the comparison of BKKO19 and *S. lividans* 1326. (*C*) The ordered cosmids at two chromosomal ends (Redenbach et al. 1996). Green dots represent terminal protein covalently bound to chromosome end. Black arrows point to the chromosomal breakpoints.

synthesis and consequently contain a single-strand loop located between inverted repeat sequences at the ends of duplex DNA (for review, see Kornberg and Baker 1992). As *Streptomyces* linear plasmids and chromosomes are known to initiate replication bidirectionally from a internal site near the center of the molecule (Shiffman and Cohen 1992; Chang and Cohen 1994; Musialowski et al. 1994), producing replication intermediates that contain a 3′ overhang of leading-strand DNA (Chang and Cohen 1994), their telomeres must undergo patching to generate blunt-ended postreplicative DNA molecules. Because deletion of widely spaced palindromic DNA sequences in the telomeres of *Streptomyces* linear plasmids precludes plasmid DNA replication in a linear form, it has been proposed that pairing of sequences of the 3′-leading-strand overhang with internal sequences near the base of the overhang provides a recognition site for DNA-binding proteins involved in telomere patching (Qin and Cohen 1998, 2000). The data reported here indicate that the function of Tap is central to telomere function in *Streptomyces*, and further suggest that Tap's dual interactions with Tpg and specific sequence of the 3′-leading-strand overhang can position Tpg to prime synthesis of the lagging-strand 5′ terminus. *S. lividans* bacteria require a functional *tap* gene to propagate chromosomal and plasmid DNA in a linear form, and cells that survive after disruption of *tap* show telomere deletion and recombinational circularization of the chromosome.

The Tpg protein, which is attached covalently to 5′ DNA ends of *Streptomyces* linear replicons, protects these ends from degradation by exonucleases (Hirochika and Sakaguchi 1982; Hirochika et al. 1984; Lin et al. 1993; Chang and Cohen 1994). The telomere-binding properties of Tap parallel those of eukaryotic proteins that interact with 3′ overhangs of replication intermediates and protect DNA ends from degradation (e.g., Garvik et al. 1995; Baumann and Cech 2001; Blackburn 2001; Pennock et al. 2001; Baumann et al. 2002). It has not been determined whether Tap also has a role in protecting *Streptomyces* linear chromosomes and linear plasmids from initiating an SOS-like response (Volff et al. 1993a,b; Zakrzewska-Czerwinska et al. 1994; Sutton et al. 2000) or in interfering with degradation of the 3′ termini of replicative intermediates.

It is well recognized that the chromosomes of *Streptomyces* spp. show extensive genetic instability (for review, see Volff and Altenbuchner 1998, 2000; Chen et al. 2002), and in particular that very large deletions can be accompanied by high-copy-number tandem amplification of flanking DNA (Redenbach et al. 1993; for reviews, see Leblond and Decaris 1994; Dary et al. 2000; Chen et al. 2002). Experimentally induced circularization of the *S. lividans* chromosome by a targeted recombination procedure can enhance genetic instability and genome rearrangement (Lin and Chen 1997; Volff et al. 1997). Commonly, DNA cloning, PCR, and Southern blot hybridization have been used to identify endpoints of deletions in genomic DNA as well as the extent of
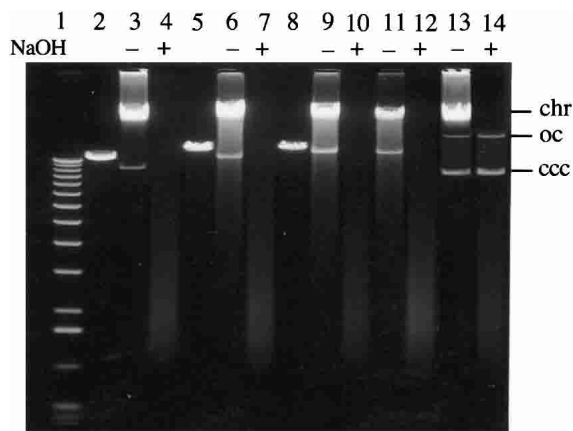
Figure 7. Analysis of plasmid DNA replication of pSLA2 derivatives (pBC167, pBC178, and pBC181) in *Streptomyces lividans* 1326 and in the tap_L and $tpgL$ double-knockout strain BKKO19 (with a circular chromosome). pBC167 contains a functional $tpgL$ gene, pBC178 contains a functional tap_L gene, and pBC181 contains both $tpgL$ and tap_L. *Streptomyces* linear plasmid DNA was isolated from 1326 and BKKO19 transformants by treatment with proteinase K and SDS (Qin and Cohen 1998, 2000) and was electrophoresed for 12 h at 30 V in a 0.6% agarose gel; linear samples indicated by + were isolated by an alkaline lysis procedure (incubation in 0.2 N NaOH at 37°C for 30 min) followed by phenol/chloroform extraction to remove or degrade linear plasmids and chromosomal DNA fragments (Qin and Cohen 1998, 2000). (Lane *1*) 1-kb DNA ladder. (Lane *2*) *Ssp*I-digested pBC167 plasmid DNA isolated from *Escherichia coli*. (Lane *3*) DNA isolated from a 1326 transformant receiving *Ssp*I-cleaved pBC167. (Lane *4*) Same DNA as in lane *3*, but following NaOH treatment. (Lane *5*) *Ssp*I-digested pBC178 plasmid DNA. (Lane *6*) DNA isolated from a 1326 transformant receiving *Ssp*I-cleaved pBC178. (Lane *7*) Same DNA as in lane *6*, but following NaOH treatment. (Lane *8*) *Ssp*I-digested pBC181 plasmid DNA. (Lane *9*) DNA isolated from a 1326 transformant receiving *Ssp*I-cleaved pBC181. (Lane *10*) Same DNA as in lane *9*, but following NaOH treatment. (Lane *11*) DNA isolated from a BKKO19 transformant receiving *Ssp*I-cleaved pBC181. (Lane *12*) Same DNA as in lane *11*, but following NaOH treatment. (Lane *13*) The banding positions of chromosomal (chr) and plasmid (ccc, covalently closed circular; OC, open circular) DNAs isolated from BKKO19 transformants receiving uncleaved pBC178 DNA. (Lane *14*) The bands that were recovered from lane *13* DNA following NaOH treatment.

gene amplification (Lin and Chen 1997; Volff et al. 1997). In the investigations reported here, we have instead used a map of the genomic sequence of *S. coelicolor* (Bentley et al. 2002; http://www.sanger.ac.uk/Projects/S_coelicolor) together with DNA microarray analysis and algorithms of the GABRIEL knowledge-based program (Pan et al. 2002; http://gabriel.stanford.edu) to more readily investigate these parameters. This approach has identified hotspots that define endpoints for telomere deletion and DNA amplification at single-ORF resolution in independent circularization events and—consistent with earlier work using more traditional methods of analysis (Lin and Chen 1997; Volff et al. 1997)—has demonstrated that that amplification of subtelomeric DNA at both ends occurs in a region immediately proximate to the

deletion endpoint. Whether such amplification has a role in maintaining chromosome function or is simply a consequence of telomere loss and chromosome circularization is not known.

Materials and methods

Bacterial strains, plasmids, and general methods

S. coelicolor M145 and *S. lividans* 1326 (Hopwood et al. 1983) were kindly provided by D.A. Hopwood (John Innes Centre, Norwich, UK). *S. rochei* 7434-AN₄ (Hirochika and Sakaguchi 1982) was kindly provided by K. Sakaguchi (Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan). *Streptomyces–E. coli* shuttle plasmid pHZ1272, which was used for gene expression in *Streptomyces*, was kindly provided by Z. Deng, Shanghai Jiaotong University, Shanghai, P.R. China (pers. comm.). *E. coli* DH5α (Invitrogen Life Technologies) and pSP72 (Promega) were used as the *E. coli* host and cloning vector, respectively. Standard methods were used for culturing cells, DNA cloning, and so on in *E. coli* (Sambrook and Russell 2001), *Streptomyces* (Kieser et al. 2000), and yeast (Kaiser et al. 1994). Restriction-enzyme-digested DNA fragments were extracted from agarose gel by Qiaquick Gel Extraction Kit (QIAGEN). Southern blot hybridization used the procedure of Church and Gilbert (1984). DNA sequencing was performed using an Applied Biosystems (ABI) Prism 310 Genetic Analyzer and ABI dye terminator sequencing kit. DNA sequencing was carried out for both strands. Linear plasmid DNA was isolated as described by Qin and Cohen (1998).

Reverse transcriptase (RT)–polymerase chain reaction (PCR) experiments

RT–PCR was used to check cotranscription of tap_L and $tpgL$. Total RNAs were prepared from 36-h mycelium of *S. lividans* 1326 by modified Kirby mix, phenol/chloroform extraction (Kieser et al. 2000), and DNase I (Worthington, DPRFS grade) treatment. The same amount of total RNA was then used for each reaction in a first-strand cDNA synthesis reaction using primer RT–cDNA: AACTCCAGGTGCTCGATGTCCGTGAA and Superscript II reverse transcriptase (Invitrogen Life Technologies). The cDNAs were then PCR-amplified by HotStar Taq DNA polymerase (QIAGEN) using five specific pairs of primers as follows: RT–cDNA and RT_0.6kb, ACGCCGACAGCGGAG AGTAGGA; RT–cDNA and RT_1.3kb, GTGAAGGTGGGCA AGGAGTGGG; RT–cDNA and RT_2.0kb, GCGCACTGGA AGCTGACGAAGC; RT–cDNA and RT_2.4kb, GTACTTGC CGCCGTGCCTTCCG; and RT–cDNA and RT_2.9kb, ATCA GGGCCTGAATTCCTCCCA. To control for genomic DNA contamination, cDNA synthesis reactions were also done in the absence of the RT. No PCR product was observed with the first pair of primers: RT–cDNA and RT_0.6kb, ACGCCGACAGC GGAGAGTAGGA in the absence of RT.

Purification of fusion proteins and generation of antibodies

The tap_L gene sequence was amplified from cosmid pBC30 of genomic DNA library of *S. lividans* ZX7 (Bao and Cohen 2001) by PCR using a HotStar Taq DNA polymerase (QIAGEN) and a pair of primers, 5′-AAACATATGCATCATCATCATCATCA TGTGTCCGGTAGAGGAGCGCAG-3′ and 5′-GCCGTTGCC GAACAGGCTCAT-3′, and was introduced into TA cloning vector pCR2.1 (Invitrogen Life Technologies) as plasmid pBC171. The correctness of the construct was confirmed by DNA se-

quence analysis. The tap_L gene was further cloned into *Nde*I–*Eco*RI-digested pHZ1272 from pBC171 (as plasmid pBC172), which enabled production of a fusion protein containing a histidine tag at the N-terminal end in *S. lividans* 1326. The $tpgL$ was similarly cloned into *Nde*I–*Eco*RI-digested pHZ1272 (as plasmid pBC156) using the primer pair 5′-AATTCATATGCAT CATCATCATCATCATATGAGCCTGTTCGGCAACGGC-3′ and 5′-GCCTGGAACGTCACCGTCCTACAG-3′ for overexpression of TpgL in *S. lividans* 1326. After induction (10 µg/mL thiostrepton) at 30°C for 12 h, cells of *S. lividans* 1326 containing pBC172 or pBC156 were harvested and resuspended in 50 mM NaH$_2$PO$_4$ (pH 7.5), 300 mM NaCl, 10 mM imidazole, 10% glycerol, 0.2% Triton X-100, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for lysis by French Press (pressure at 1000 kg/cm^2). DNA in the lysis mixture was sheared by sonication, and cell debris was removed by centrifugation (39,000*g* for 20 min). The Tap$_L$/TpgL fusion protein, whose components were confirmed by Western blot analysis using His-tag gel antibody, was further purified by Ni^{2+}-column chromatography (QIAGEN). Tap$_L$ was eluted around 100 mm imidazole and TpgL was eluted around 150 mM imidazole. The fusion protein-containing fractions were dialyzed against H buffer (50 mM HEPES at pH 7.5, 10% glycerol, 50 mM KCl, 1 mM EDTA, 1 mM PMSF, and 1 mM dithiothreitol) and were further purified on a Heparin column (Amersham Pharmacia Biotech) using a linear gradient of KCl (0.1–1.0 M) at 4°C. Tap$_L$ was eluted at 0.6 M KCl, and TpgL was eluted at 0.4 M KCl. The proteins were dialyzed against H buffer plus 100 mM KCl and used for electrophoretic mobility shift assays and for the production of rabbit anti-Tap$_L$ and anti-TpgL polyclonal antibodies (Covance Research Products), respectively. Antisera from the rabbit were first screened for protein binding using an ELISA assay; positive antisera were then tested for specificity on Western analysis and further purified by a protein A column (Pierce).

Electrophoretic mobility shift assays (EMSA) and footprinting analysis

Double-stranded 365-bp telomeric DNA of pSLA2 was gel-purified from *Bgl*II–*Xba*I-digested pQC5 (Qin and Cohen 1998) and end-labeled with [α-^{32}P]dCTP and DNA polymerase Klenow fragment (Invitrogen Life Technologies). Labeled DNA was separated from free [α-^{32}P]dCTP by filtration through a MicroSpin S-400 HR column (Amersham Pharmacia Biotech Inc). Labeled double-stranded DNA probe was denatured at 100°C for 10 min and then immediately put into an ice-cold waterbath and used as a single-stranded DNA probe in EMSA. The DNA-binding reaction was performed at 25°C for 1 h using 0.5 nM radiolabeled DNA probe, 100 nM purified TpgL or Tap$_L$ and the binding buffer (20 mM Tris at pH 8.0, 1 mM EDTA, 100 mM NaCl, 5% glycerol, 2 mM DTT) supplemented with 50 µg/mL double- or single-stranded salmon sperm DNA in a final volume of 20 µL. Competition was accomplished by adding 5-fold, 20-fold, and 100-fold excess of sonicated circular chromosomal DNA of BKKO5 (Bao and Cohen 2001) or 5-fold, 20-fold, and 100-fold excess of cold probe to the binding reaction. The DNA–protein complexes were separated on prerun 5% Tris-borate-EDTA (TBE) native acrylamide gels in 0.5× TBE buffer at 200 V for 3 h. Gels were dried and exposed to X-ray film at –80°C. The DNase I footprinting method for identifying protein-binding sites on DNA (Galas and Schmitz 1978) was modified as follows: DNA–protein-binding reactions were carried out as described as above. CaCl$_2$ and MgCl$_2$ were added to final concentrations of 2.5 and 5 mM, respectively, and 1 µL DNase I (10 µg/mL) was added. After incubation at room temperature for 1 min, an equal volume of stop buffer (1% SDS, 20 mM EDTA,

300 mM NaCl, and 2 mg/mL yeast tRNA) was added. The mixtures were extracted with phenol/chloroform, ethanol-precipitated, and separated on an 8% denaturing polyacrylamide gel. A DNA ladder showing the sequence of the region footprinted was prepared using a chain-terminating reaction (USB, Sequenase 7-deaza-dGTP sequencing kit) and an oligonucleotide primer complementary to the 3′ end of the probe fragment. The reaction products were separated by electrophoresis in a gel alongside of the footprinting reaction mixture to identify nucleotide bonds protected by the binding of Tap.

Western, immunoprecipitation (IP), immunodepletion, and IP-PCR analysis

Western and immunoprecipitation (IP) analysis were performed according to the procedures of Harlow and Lane (1988). *S. lividans* 1326 cell extracts were made for IP analysis in Nonidet P-40 (0.5%) buffer (Harlow and Lane 1988) using a French Press (1000 kg/cm^2) and then sonication. The immunoprecipitate of TpgL antibody was treated with DNase I (Worthington, DPRFS grade) for Western blotting against TpgL antisera. Chemiluminescence Reagent Plus (PerkinElmer NEN Life Sciences) was used for detection in immunoblotting. The immunoblotted membrane was stripped by Restore Western Blot Stripping Buffer (Pierce) and then used for immunodepletion analysis. In immunodepletion experiments, the primary antibody (anti-TpgL or anti-Tap$_L$ antisera, 1:2000 dilution) was incubated with the relevant antigen (purified TpgL or Tap$_L$ from *S. lividans*; i.e., Tap$_L$) in PBST buffer with 5% milk at 4°C for 12 h and then was used as the primary antibody for the second round of immunoblotting chemiluminescence detection. For IP-PCR analysis, a pair of primers (P-end, 5′-CCCGCGGAGCGGG TACCCTATCGCT-3′ and P-300, 5′-CGAGCCCCGGTCCCT GTAGGCGCTC-3′) was designed to amplify the 300-bp chromosome end of *S. lividans* 1326. Template DNA was isolated from immunoprecipitate after being treated with proteinase K and was then extracted with phenol/chloroform.

Yeast two-hybrid analysis

The yeast two-hybrid assay was carried out according to ProQuest Two-Hybrid System with Gateway Technology (Invitrogen Life Technologies). Two primers containing *att*B1 and *att*B2 were designed to amplify the $tpgL$ gene as follows: *att*B1_$tpgL$_5′, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGATGAGC CTGTTCGGCAACGGC-3′ and *att*B2_$tpgL$_3′, 5′-GGGGACA CTTTGTACAAGAAAGCTGGGTCCTACAGGTCGAACTC CAGGTG-3′. The PCR product of the $tpgL$ gene was cloned into the Entry Vector and then transferred into pDEST22 containing GAL4-AD (prey vector) as plasmid pBC193 by Gateway Cloning Technology (Invitrogen Life Technologies). GAL4-DB-tap_L was constructed by subcloning an *Nco*I and *Not*I fragment of tap_L from pBC162 (tap_L on pET28a) into the same enzyme-digested GAL4-DB plasmid pDBLeu (bait vector), as plasmid pBC191. All constructs were verified by DNA sequencing to confirm identity and reading frame. The expression of GAL4 fusion proteins was confirmed by Western blotting analysis against anti-TpgL or anti-Tap$_L$ antisera. Plasmids pBC191 and pBC193, pBC191 and pDEST22, and pBC193 and pDBLeu were cotransformed into host yeast strain MaV203. Cotransformants were screened and selected on yeast minimal medium SC-Leu-Trp plates and used for testing interactors for the induction of reporter genes on SC-Leu-Trp-His plates containing 20 mM 3-AT (3-aminotriazole, Sigma), SC-Leu-trp-Ura plates, SC-Leu-trp + 0.2% 5FOA (5-fluoroorotic acid, Sigma) plates, and X-gal assay plates.

Gene disruption and complementation

A *tap$_L$* gene-disruption plasmid (pBC131, see Fig. 5) was constructed by inserting a spectinomycin-resistance gene (*spc^r*) at the *Eco*47III site of the *tap$_L$* gene following partial digestion of the *tap$_L$* fragment cloned in *E. coli*. Another gene-disruption plasmid (pBC141, see Fig. 5) was also constructed for the replacement of *tap$_L$* and *tpgL* with a spectinomycin-resistance gene. Because *tap$_L$* and *tpgL* are cotranscribed (see RT–PCR result in Fig. 1), in order to mutate the *tap$_L$* gene of *S. lividans* 1326, plasmid pBC117 carrying the *tpgL* gene at *Nde*I–*Bam*HI sites of pHZ1272 (Zixin Deng, pers. comm.) was used to express TpgL in *S. lividans* 1326. Protoplasts of 1326(pBC117) were transformed with pBC131, which lacks the ability to replicate in *Streptomyces* and contains an apramycin-resistance gene, and transformants (*spc^r am^r*) were selected on R5 media containing spectinomycin. Clones in which a double crossover led to replacement of *tap$_L$* by the insertionally mutated gene were isolated by testing spores for sensitivity to apramycin; this was done using replica plating of colonies selected in the presence of spectinomycin and in the absence of apramycin (*spc^r am^s*). Replacement of wild-type *tap$_L$* by the insertionally mutated gene in *spc^r tsr^s* bacteria was confirmed by Southern blot hybridization. Replacement of both *tap$_L$* and *tpgL* by *spc^r* was accomplished by transforming pBC141 into *S. lividans* 1326. For gene complementation, *tpgL*, *tap$_L$*, and *tap$_L$* + *tpgL* were cloned into the *Nde*I–*Eco*RI sites of pHZ1272 as pBC166, pBC177, and pBC180, respectively. The *Bgl*II fragments of these three plasmids containing a functional *Streptomyces* promoter and each insert were then cloned into the pQC18-derived replicon pBC104 (Bao and Cohen 2001) to generate pBC167, pBC178, and pBC181, respectively. *S. lividans* 1326 and its derivative BKKO19 (*tpgL$^-$ tap$_L^-$*, see above) were transformed with the linearized DNA of pBC104, pBC167, pBC178, and pBC181. Transformants were analyzed for linear plasmid replication.

DNA microarray analysis

Construction of *S. coelicolor* M145 DNA microarrays was done as previously described (Huang et al. 2001). The complete genomic DNA sequence consisting of 7825 known and putative ORFs (ftp://ftp.sanger.ac.uk/pub/S_coelicolor/sequences) and a 30-kb chromosome end fragment (http://www.sanger.ac.uk/Projects/S_coelicolor) were used to design prime pairs for PCR amplification (protocol is available at http://sncohenlab.stanford.edu/streptomyces). Total DNA from *S. lividans* 1326 or from knockout strains BKKO17 (*tap$_L^-$*) or BKKO19 (*tap$_L^-$ tpgL$^-$*) was used as template, and incorporation of fluorescent nucleotide analogs Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia Biotech, Inc.) in DNA was accomplished by a randomly primed DNA polymerase reaction. The labeling reaction (50 μL) contained 2 μg of template DNA, 5 μL of 10× buffer, 5 μL of dNTP mix (4 mM dATP, 4 mM dTTP, 10 mM dGTP, and 1 mM dCTP), 10 μg of 72% (G + C) content hexamers, 3 μL of Cy3-dCTP or Cy5-dCTP, and 2 μL of DNA polymerase Klenow fragment (Invitrogen Life Technologies). Labeled DNAs from the strains being compared were mixed together and purified through a GFX-column (Amersham Pharmacia Biotech, Inc.). Microarray hybridization and slide washing and scanning were performed as described previously (DeRisi et al. 1997; Behr et al. 1999). Microarray data analysis was performed with the software available at http://genome-www5.stanford.edu/MicroArray/SMD/restech.html and by GABRIEL, a knowledge-based machine learning computer program (Pan et al. 2002; http://gabriel.stanford.edu). PCR amplification of the junction fragments formed by chromosome circularization used two primers, JncL-

SCF1.14 (5′-AGCAGCGGCCACAGGTAGTC-3′) and JncR-SC8D11.26 (5′-TGCTTGAGCCGACCGATGAG-3′), which are identical, respectively, to the right and left primers used for amplifying the PCR products of ORF SCF1.14 and ORF SC8D11.26 for DNA microarray analysis. The PCR products were then introduced into the TA cloning vector pCR2.1 (Invitrogen Life Technologies), and the inserted DNA was sequenced.

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