SUPPLEMENTARY MATERIALS

Materials and Methods

Strains and plasmids.

Strains used in this study are listed in Table S1.

Bacterial growth conditions.

Agrobacterium tumefaciens strains were grown routinely at 28 C in LB medium supplemented with mannitol and glutamate (MG/L broth) (*S1*). For *vir* gene induction, cells were grown at 18 C in ABIM medium, supplemented with 200 μM acetosyringone (*S2*).

Transfer-ImmunoPrecipitation (TrIP) Assay.

Sample preparation **-** The Transfer ImmunoPrecipitation (TrIP) assay was adapted from the original yeast protocol of Chromatin ImmunoPrecipitation (ChIP) (Fig. S2) (*S3*). For information on the use of the ChIP assay for studies of protein – DNA interactions in bacteria, see references *S4* and *S5*. The TrIP protocol was performed as follows: 1.5 ml of exponential-growing cells in MG/L broth were pelleted and suspended in 6 ml of inducing medium (ABIM medium supplemented with 200 μM acetosyringone) and incubated for 14-16 h at 19 ºC with shaking. Induced cells were harvested, washed with 6 ml of 20 mM sodium phosphate buffer pH 6.8, and then suspended in the same buffer with formaldehyde (FA) at a final concentration of 0.1%. Cells were incubated for 20 min at 18 ºC with shaking. Formaldehyde was added in 0.2 % increments to reach a final concentration of 1% over a 15 min period, and the cells were incubated for 40 min at room temperature (RT) without shaking. Cells were pelleted, resuspended in 200 μl in TES buffer (50 mM Tris.Cl pH 6.8, 2 mM EDTA, 1 % β-mercaptoethanol, 1% SDS) and then incubated for 30 min at 37 C with shaking. Next, 900 μl of NP1 buffer (150 mM

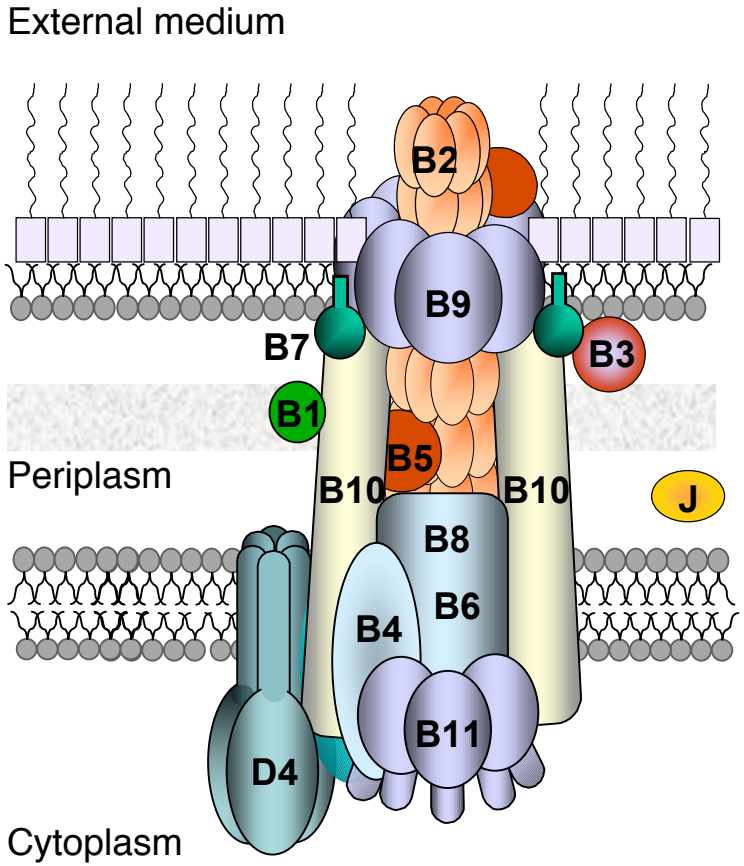
Tris.Cl pH 8.0, 0.5 M sucrose, 10 mM EDTA) supplemented with 1 mg/ml lysozyme was added, the mixture was incubated for 1-2 h on ice and then 30 min at 37 C with shaking. Triton X-100 was added to a 4 % final concentration and the mixture was incubated for 15 min at room temperature (RT) on a wheel. A 5X solution of protease inhibitors cocktail, EDTA-free (Complete, Boehringer-Mannheim) in 25 mM $MgCl_2$ was added and the mixture was incubated with rocking for 15 min at 37 ºC and then for 2-3 h at 4 C on a wheel. Then, 3.2 ml of NP1 buffer was added and the insoluble material was removed by centrifugation for 15 min at 14,000 *x g*. For recovery of the VirD2-T-strand with anti-VirD2 antibodies, whole cells were treated as described above in the absence of *in vivo* formaldehyde crosslinking.

Immunoprecipitation – High-titer and high-specificity antiserum to each of the proteins under study was obtained by several rounds of adsorption to the purified cognate protein on a nitrocellulose blot. For immunoprecipitation, protein A-Sepharose CL4B (Pharmacia) (30 μl bed volume) was incubated with 1.1 ml of the detergent-solubilized material for 60 min at RT and centrifuged at 5,000 *x g* to remove to Protein A-Sepharose and non-specifically-bound proteins. The supernatant was incubated overnight at 4 ºC with antibody coupled to Protein A-Sepharose CL4B. The beads were pelleted by centrifugation and the remaining supernatant (labeled "S" in the Figs.) was analyzed in parallel with material eluted from the beads. The beads were washed twice with NP1 buffer supplemented with 1% Triton X-100 and once with NP1 buffer supplemented with 0.1% Triton X-100. Immunoprecipitates (labeled "IP" in the Figs.) were eluted by incubation for 20 min at 96 ºC in 20 μl of 10 mM Tris.Cl pH 6.8. For resolution of proteins by SDS-PAGE, material in the supernatant (S) fraction was precipitated with trichloro-acetic acid and washed with acetone, whereas immunoprecipitates (IP) were diluted 2-fold in 2X Laemmli's buffer.

Polymerase chain reaction - For the TrIP experiments, PCR was carried out in 25-μl volumes containing 1/20 of the immunoprecipitates or 1/100,000 of the soluble fraction and 50 pmoles of each of the four primers per reaction. The primers used for the amplification of the T-DNA region corresponding to gene 7 of the T_L-DNA are 5-gggcgattatggcatctcagaaagcc and 5-gtcggcggcccacttggcacacag. As a control, the *ophDC* locus (a region of the Ti plasmid ~25-kb from the transferred T-DNA; Fig. S2) was amplified using the primers 5- cctgcggatgtcagggctctcgt and 5-ctgtccgtgcttgccaatccccg. PCR products were separated by electrophoresis through 1.2% agarose gels and visualized by ethidium bromide staining.

Quantitative TrIP assay – To compare levels of T-strand, immunoprecipitates were subjected to 20 cycles of PCR amplification using the primers specific for gene 7 of the T-DNA. On the 21st cycle, a single round of PCR amplification was performed with addition of 1.0 μCi of [^{32}P]dGTP (Amersham Biosciences), as described previously (*S6*). PCR products were column-purified with the Qiaquick PCR purification kit (Qiagen) to remove unincorporated nucleotides. Aliquots of the eluted material were mixed with 3.5 ml of scintillation liquid (Ecolite, ICN) and counted used a Beckman Coulter. The entire TrIP protocol was repeated three times in triplicate and the average value from a single experiment was reported. Analyses of a given strain with a given antiserum yielded highly reproducible results with standard deviations of < 2 % within and between experiments. Quantitative data presented in Figs. 1 – 3 were obtained as described above. However, as shown in Fig. S3, for all samples subjected to quantitative analysis we also confirmed that the 21st cycle was in the logarithmic phase of PCR amplification by measuring radionucleotide incorporation minimally at the 18th, 21st, and 24th cycles of amplification.

A


External medium
B2
B9
B7
B3
B1
B5
Periplasm
B10
B10
J
B8
B6
B4
B11
D4
Cytoplasm

B

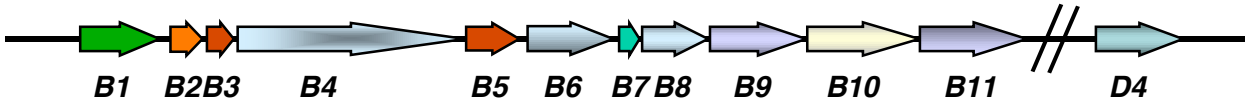

B1
B2B3
B4
B5
B6
B7B8
B9
B10
B11
D4

Fig. S1

Transfer DNA immunoprecipitation
TrIP

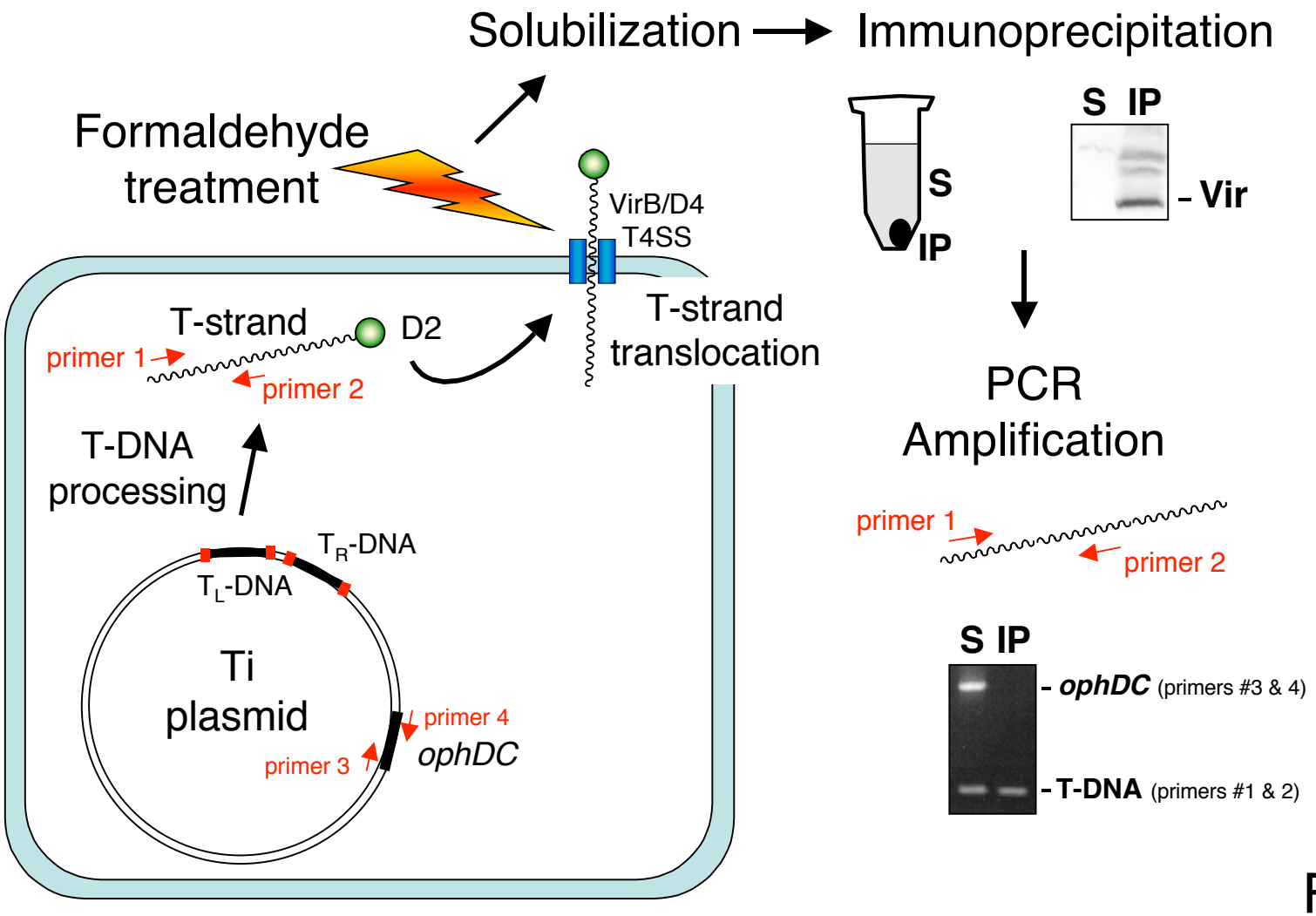


Fig. S2

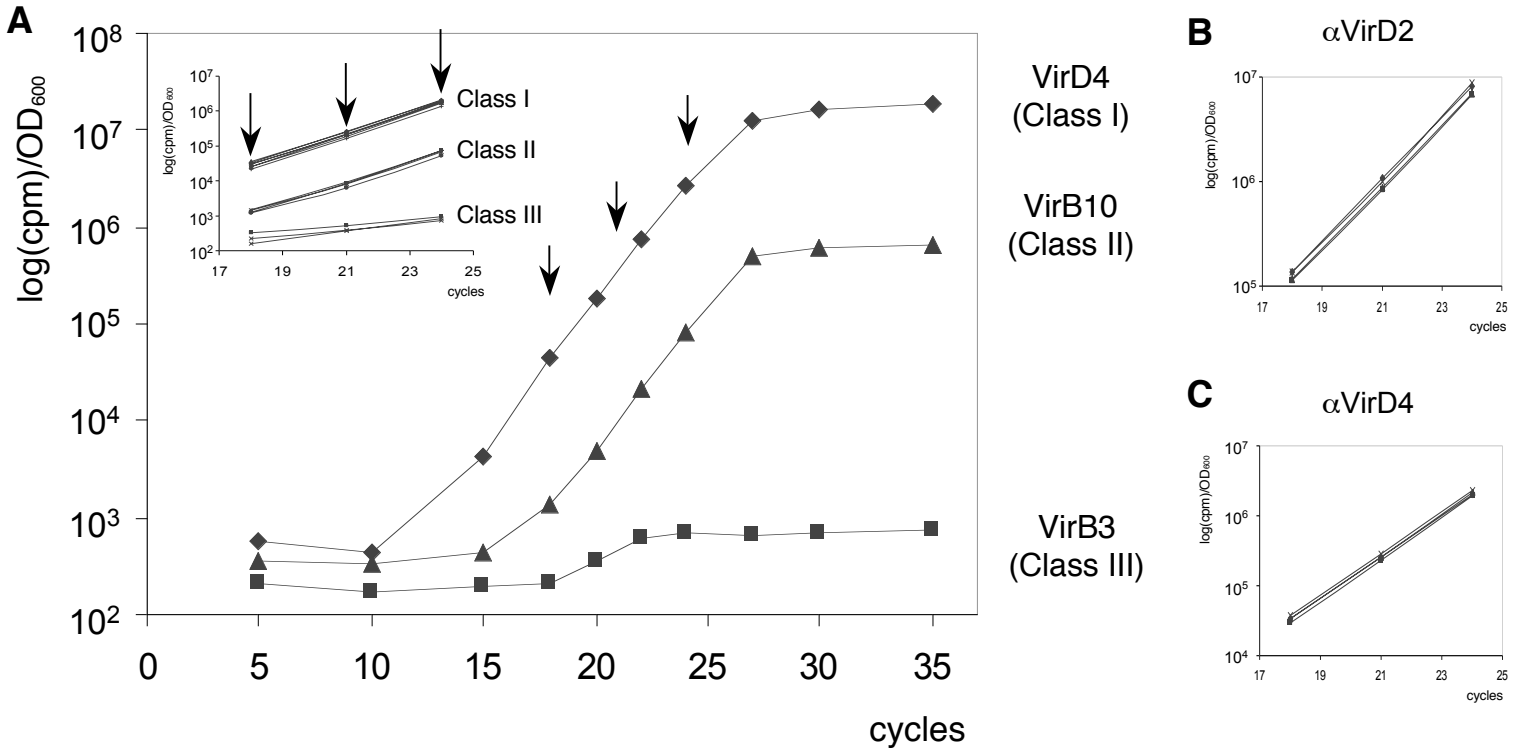

A
log(cpm)/OD600
cycles
Class I
Class II
Class III
VirD4
(Class I)
VirB10
(Class II)
VirB3
(Class III)
B
αVirD2
C
αVirD4

Fig. S3

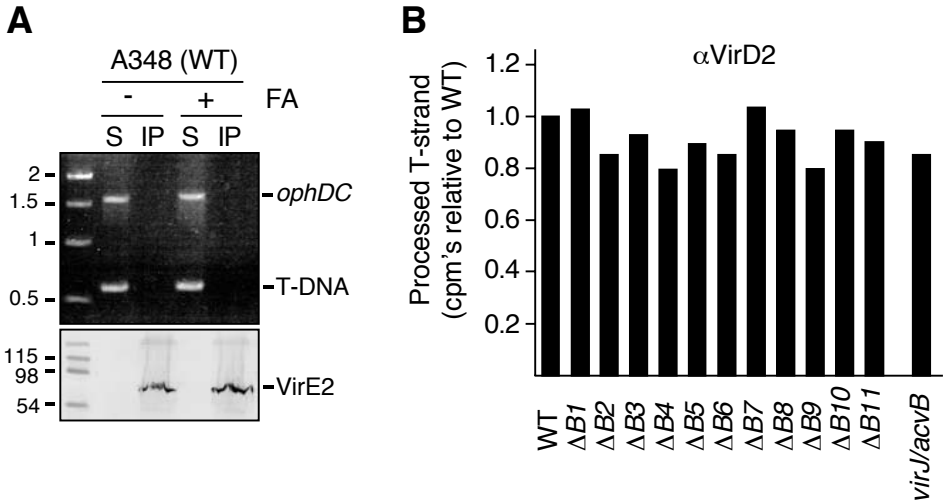

A
A348 (WT)
-
+
FA
S IP S IP
2
1.5
1
0.5
ophDC
T-DNA
115
98
54
VirE2
B
αVirD2
Processed T-strand
(cpm's relative to WT)
1.2
1.0
0.8
0.6
0.4
0.2
WT
ΔB1
ΔB2
ΔB3
ΔB4
ΔB5
ΔB6
ΔB7
ΔB8
ΔB9
ΔB10
ΔB11
virJ/acvB

Fig. S4

Figure Legends

Fig. S1. A model depicting the subcellular locations and interactions of the VirB and VirD4 subunits of the *A. tumefaciens* VirB/D4 T4SS. The VirD4 coupling protein assembles as a homohexameric, F1-ATPase-like structure (*S7*) juxtaposed to the VirB channel complex. VirB11, a hexameric ATPase structurally similar to members of the AAA ATPase superfamily (*S8*), is positioned at the cytoplasmic face of the channel entrance, possibly directing substrate transfer through a VirB6/VirB8 inner membrane (IM) channel. The VirB2 pilin and VirB9 comprise channel subunits to mediate substrate transfer to and across the outer membrane (OM). VirB10 regulates substrate transfer by linking IM and OM VirB subcomplexes.

Fig. S2. Schematic of Transfer DNA Immunoprecipitation (TrIP) The protocol, adapted from the ChIP technique (*S3*), monitors the dynamics of DNA translocation through a secretory channel, in this case, the type IV secretion channel of the bacterium *Agrobacterium tumefaciens*. The steps include: *in vivo* formaldehyde crosslinking, detergent-solubilization, immunoprecipitation with antibodies to a T4SS subunit of interest, and PCR amplification to detect the T-DNA transfer intermediate and a nontransferred region (*ophDC*) of the pTi plasmid in the immunoprecipitate (IP) or the supernatant fraction (S).

Fig. S3. Quantitative TrIP measures T-strand levels during the logarithmic phase of PCR amplification. Panel A: Antibodies to VirD4 (Class I protein), VirB10 (Class II), and VirB3 (Class III) were used to precipitate the cognate Vir protein from extracts of formaldehyde-treated A348 (WT) cells. At the following cycles of PCR amplification - 5, 10, 15, 18, 20, 22, 24, 27, 30, and 35 - [^{32}P]dGTP was added and amplification was allowed to proceed for one cycle. Free nucleotides were removed with a Qiaquick PCR purification column and radionucleotide incorporation into the amplification product was determined with a Beckman Coulter. Results,

reported as log cpm's per 1.0 OD_{600} of starting cells, show that this method detects an exponential increase in radionucleotide incorporation between the ~15 – 27th amplification cycles for Class I and II immunoprecipitates, whereas radionucleotide incorporation remained at background levels throughout 35 amplification cycles for Class III immunoprecipitates (due to the absence of T-strand). For the Class I and II samples, radionucleotide incorporation also likely increased exponentially below the 15th cycle, but the amount of incorporation was below the threshold of detection with this assay. The inset shows results of parallel studies in which radionucleotide was added to the 18th, 21st, or 24th cycle of amplification using as starting material the immunoprecipitates recovered with antibodies to each of the remaining Class I (top lines), Class II (middle), and Class III (bottom) proteins; in all cases, the amount of incorporated radionucleotide increased linearly. At each of the three amplification cycles, standard deviations for samples from a given Class were less than 2 %. With all samples analyzed in this study, the 21st cycle was in the middle of the logarithmic phase of PCR amplification. **(B)** Corresponding studies with immunoprecipitates recovered from extracts of the following strains with the anti-VirD2 antibodies: A348 (WT), *virD4*, *virE2*, Δ*virB*, Δ*B1* through Δ*B11*, *acvB/virJ*. **(C)** Corresponding studies with immunoprecipitates recovered from extracts of the following strains with the anti-VirD4 antibodies: A348, Δ*virB*, *virE2*.

Fig. S4. (A) VirE2 SSB does not interact with VirD2-T-strand in *A. tumefaciens*. Anti-VirE2 antibodies precipitated VirE2 (lower panel) but no detectable T-strand (upper panel) from extracts of FA-treated (+) and untreated (-) wild-type A348. **(B)** Levels of VirD2-T-strand recovered from extracts of the nonpolar *virB* gene deletion mutants and other strains shown by immunoprecipitation with anti-VirD2 antibodies, as determined by quantitative TrIP without formaldehyde treatment of whole cells. Data are presented as cpm's of incorporated radionucleotide during one cycle of PCR amplification. Values for the mutant strains are presented as a fraction of WT, normalized to 1.0.

Table S1

Strain as in text	Original name	Description	Reference
WT	A348	A136 bearing the octopine-type pTiANC plasmid	S9
Δ*Ti*	A136	C58 cured of the nopaline-type pTiC58 plasmid	S9
virD2	Mx311	A348 *virD2*::Tn3*HoHo*1	S10
virD4	Mx355	A348 *virD4*::Tn3*HoHo*1	S10
virE2	Mx358	A348 *virE2*::Tn3*HoHo*1	S10
*virD2**	Mx311(pKAB9)	A348 *virD2*::Tn3*HoHo*1 expressing *virD4* from IncP	S11
Δ*virB*	PC1000	A348 Δ*virB* operon	S1
Δ*B1*	PC1001	A348 Δ*virB1* nonpolar	S2
Δ*B2*	PC1002	A348 Δ*virB2* nonpolar	S2
Δ*B3*	PC1003	A348 Δ*virB3* nonpolar	S2
Δ*B4*	PC1004	A348 Δ*virB4* nonpolar	S2
Δ*B5*	PC1005	A348 Δ*virB5* nonpolar	S2
Δ*B6*	PC1006	A348 Δ*virB6* nonpolar	S2
Δ*B7*	PC1007	A348 Δ*virB7* nonpolar	S2
Δ*B8*	PC1008	A348 Δ*virB8* nonpolar	S2
Δ*B9*	PC1009	A348 Δ*virB9* nonpolar	S2
Δ*B10*	PC1010	A348 Δ*virB10* nonpolar	S2
Δ*B11*	PC1011	A348 Δ*virB11* nonpolar	S2
virJ/acvB		A348 *virJ, acvB*	V. Kalogeraki and S. Winans

References

S1. D. Fernandez *et al.*, *J. Bacteriol.* **178**, 3156 (1996).

S2. B. R. Berger, P. J. Christie, *J. Bacteriol.* **176**, 3646 (1994).

S3. S. Strahl-Bolsinger, A. Hecht, K. Luo, M. Grunstein, *Genes Dev.* **11**, 83 (1997).

S4. S. Ben-Yehuda, D. Z. Rudner, R. Losick, *Science* **299**, 532 (2003).

S5. V. Molle et al., *J. Bacteriol.* **185**, 1911 (2003).

S6. D. F. Purcell, M. A. Martin, *J. Virol.* **67**, 6365 (1993).

S7. F. X. Gomis-Ruth *et al.*, *Nature* **409**, 637 (2001).

S8. H.-J. Yeo, S. N. Savvides, A. B. Herr, E. Lanka, G. Waksman, *Mol. Cell* **6**, 1461 (2000).

S9. J. Zhu *et al.*, *J. Bacteriol.* **182**, 3885 (2000).

S10. S. E. Stachel, E. W. Nester, *EMBO J.* **5**, 1445 (1986).

S11. K. Atmakuri, Z. Ding, P. J. Christie, *Mol. Microbiol.* **49**, 1699 (2003).