3 METHODS

3.1 DNA techniques

3.1.1 Molecular cloning techniques

Plasmids were isolated using the QIAprep Spin Miniprep Kit or the Plasmid Maxi Kit. Single-stranded DNA (ssDNA) was isolated from phage M13 mp18 or from phage M13 mJF182 (Table 2.2), which were precipitated using PEG 6,000. Isolation of ssDNA was performed according to the method described by Messing (61). DNA fragments were purified from agarose gels using the QIAEX II gel extraction kit. Standard molecular cloning techniques were performed as described (80) or as recommended by the manufacturer.

3.1.2 Polymerase chain reaction (PCR) techniques

PCR fragments were generated with DeepVent$_R$ DNA polymerase (New England Biolabs) or, in the case of *his$_6$-hp0524Δ1* (fragment in pHY524Δ1), *pfu* DNA Polymerase (Stratagene). PCR reactions were performed with 50-100 ng template DNA, 40 pmol primers, 0.2 mM of each dNTP and 2 units of DNA polymerase in a total volume of 100 µl. In some reactions, a 50% mixture of 7-deaza-dGTP and dGTP was used instead of dGTP (60). Thermo cycling was carried out as described in Table 3.1.

Table 3.1. Cycling protocol applied to PCR reactions.

Step	Time [min]	Temperature [°C]	Number of cycles
Denaturing	1	95	1
Denaturing	1	95	
Primer annealing	1.5	58-65 [a]	25
Primer elongation	1-3 [b]	75	
Primer elongation	5	75	1

[a] the annealing temperature was adjusted to the calculated melting temperature of the according primers
[b] the elongation time was one minute per kb to be amplified with a minimum of one minute

3.1.3 DNA sequencing

Nucleotide sequences of PCR fragments were verified. The chain termination method by Sanger *et al.* (81) was applied for sequencing and was performed by the service group of the Max-Planck-Institut für Molekulare Genetik.

3.1.4 Construction of plasmids

Plasmids constructed in the present work are listed in Table 2.2. Constructions involving PCR reactions or DNA techniques other than the standard restriction/ligation technique are described in the following.

pGS002, a vector for construction and overexpression of *his*$_6$-tagged genes (Figure 3.1), was generated as follows: pMS470Δ8 was digested with *Nde*I. The resulting 5' overhangs were removed using mung bean nuclease. The plasmid was then digested with *Hin*dIII and ligated with a *his*$_6$-linker, which was prepared by annealing of oligonucleotides 1a and 1b (Table 3.2).

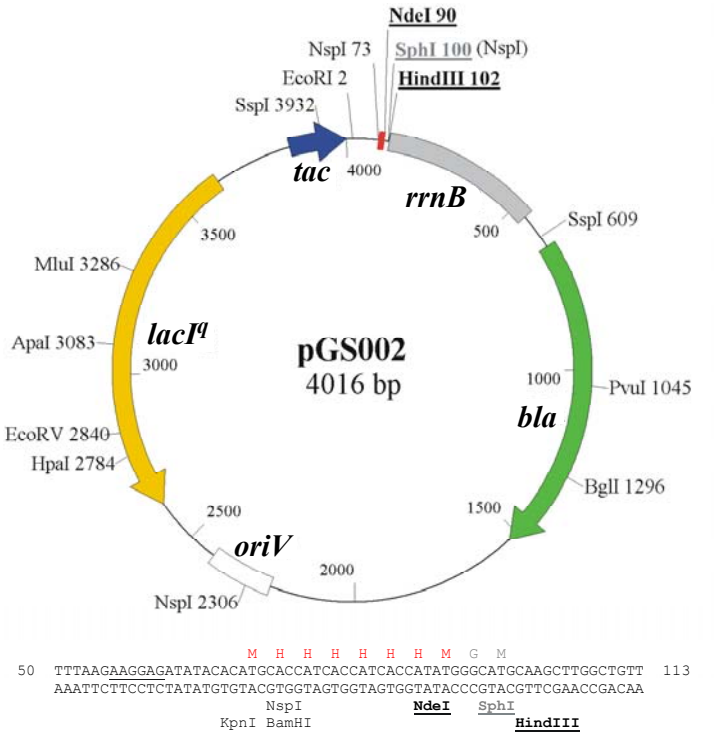


Figure 3.1. Physical and genetic map of pGS002, a vector for construction and overexpression of *his*$_6$-tagged genes. Restriction sites and their positions are indicated. Genes of interest were inserted as *Nde*I – *Hin*dIII fragments generated by PCR, with the *Nde*I site containing the original start codon (ATG) of the gene. The resulting constructs produced translational fusion to the *his*$_6$-tag sequence located upstream (sequence outlined in red). Alternatively, *Sph*I – *Hin*dIII fragments with the original start codon contained within the *Sph*I site were inserted for translational fusion to the *his*$_6$-tag (elongated by two additional amino acids, outlined in grey). The expression of inserted genes is regulated by the *tac* promoter / *lacI*^{q} system. The Shine Dalgarno sequence (SD, underlined) originates from the phage T7 gene *10*. Beta-lactamase (*bla*) confers ampicillin resistance. *oriV* marks the origin of vegetative pMMB1 replication. *rrnB*, the ribosomal RNA operon B, contains two highly efficient transcriptional terminator sequences (one on each strand).

pGS003Δ1 was constructed by insertion of a *Nde*I/*Nsi*I/*Bcl*I/*Hin*dIII/*Sac*I-linker (annealed oligonucleotides 2a and 2b) into pMS470Δ8. This was followed by deletion of an 18 bp *Eco*RI-*Sma*I fragment by consecutive restriction, removal of 5' overhangs (using mung bean nuclease) and religation.

Table 3.2. Forward (f) and reverse (r) primers or oligonucleotides used for manipulation of DNA.

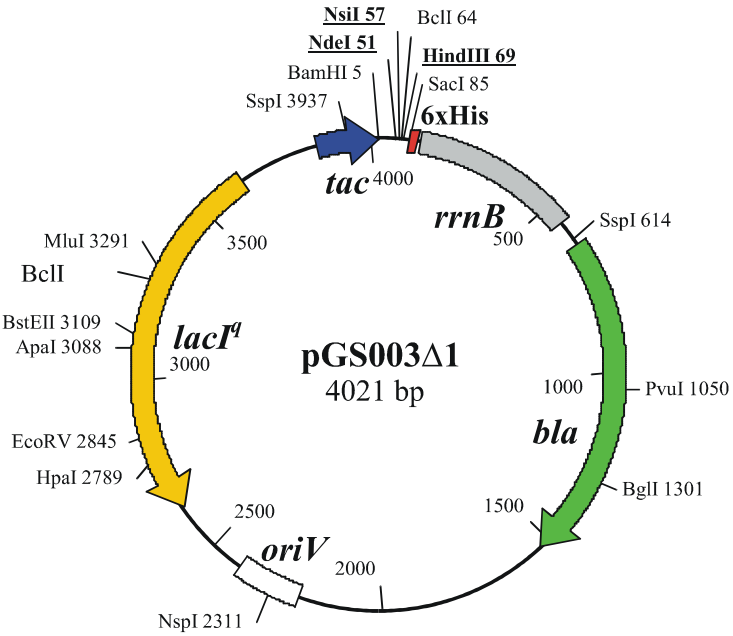
Name	Forward primer / Oligo a	Reverse primer / Oligo b
1	CATGCACCATCACCATCACCATATGGGCATGCA	AAGCTTGCATGCCCATATGGTGATGGTGATGGT GCATG
2	TATGCATCCGTCTGATCAAGCTTGTCCATGAGCTCA TCACCATCACCATCACTGAT	AGCTATCAGTGATGGTGATGGTGATGAGCTCAT GGACAAGCTTGATCAGACGGATGCA
3	CGACGACTCAT*ATGAAGAACCGAAACAACG* [a]	GCCTACGAAG*CTTGGTGAGGCGCTGGAAGC* [a]
4	GCATTCCCAT*ATG*CACCATCACCATCACCAT*AAGAA CCGAAACAACG* [a]	GCCTACGAAG*CTTGGTGAGGCGCTGGAAGC* [a]
5	CGACGACTCAT*ATGAAGAACCGAAACAACG* [a]	*GCTAATAAGCTTGCTCA*ATGGTGATGGTGATGGT G*TAT*CGTGATCCCCTCCC [a]
6	CGTTCGAGCATATG*ACCGCGACGCAATATTTCGCCC* [a]	CTGTTTTATCAGACCGCTTCTGCG
7	*GCCGTCACGCATATGGTCAAGGC* [a]	CTGTTTTATCAGACCGCTTCTGCG
8	GCATTCCCATATGCACCATCACCATCACCAT*AAGAA CCGAAACAACG* [a]	GCCTACGAAG*CTTGGTGAGGCGCTGGAAGC* [a]
9	CGACGACTCAT*ATGAGTTTTAACGC* [b]	GCCTACGAAGCTTCA*TCAGAAATCATC* [b]
10	GCATTCCCAT*ATG*CACCATCACCATCACCAT*AGTTTT AACGCAAAGG* [b]	GCCTACGAAGCTTCA*TCAGAAATCATC* [b]
11	CGACGACTCAT*ATGAGTTTTAACGC* [b]	GCTAATAAGCTTCA*TCA*ATGGTGATGGTGATGG TG*GAAATCATCTCCCGG* [b]
12	GGCCCCATATGC*GGACTAGAGATATAGGAGCG* [c]	CCGGATCC*CTCACAGTTCACTTGAACCCACAGG* [c]
13	*GTTGTTTGTCTGGCATATGAATAGCGTCG* [d]	CTGTTTTATCAGACCGCTTCTGCG

[a] nucleotides corresponding to the RP4 sequence are italicized, deviations from the original sequence are underlined

[b] nucleotides corresponding to the F sequence are italicized, deviations from the original sequence are underlined

[c] nucleotides corresponding to the *H. pylori* strain 26695 sequence are italicized

[d] nucleotides corresponding to the R388 sequence are italicized, deviations from the original sequence are underlined

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          NsiI        BclI
        NdeI              HindIII      SacI
40  GGAGATATACATATGCATCCGTCTGATCAAGCTTGTCCATGAGCTCATCAC  90
    CCTCTATATGTATACGTAGGCAGACTAGTTCGAACAGGTACTCGAGTAGTG
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Figure 3.2. Physical and genetic map of expression vector pGS003Δ1. Genes of interest may be inserted as *Nde*I or *Nsi*I – *Hin*dIII fragments, with the start codon (ATG) located within the *Nde*I/*Nsi*I site. The his_6 sequence located downstream of the *Hin*dIII site was designed for constructions of 3' terminal fusion genes to his_6 but has not been used for this purpose in plasmid constructions here described. The backbone of the plasmid is the same as in pGS002 (Figure 3.1).

RP4 *traG* (accession no.: X54459) in pSK470 (Table 2.2) was obtained as full-length gene by PCR, using pBS140 as a template and the primers 3f and 3r (Table 3.2). *his$_6$-traG* and *traG-his$_6$* in pFS241 and pFS141, were generated by PCR with pBS140 as the template and primer pair 4 and 5, respectively. Alternatively, *traG* deletion derivatives *his$_6$-traGΔ1* and *his$_6$-traGΔ2* in pGS006Δ1 and pGS006Δ2 were generated by PCR on pSK470 and the primer pairs 6 and 7, respectively. pFS241M was generated by PCR with pBS140(K187T) as the template and primers 8f and 8r. For construction of pSK470ΔB, the unique *Bam*HI site in pSK470 was disrupted by filling in its 5' overhangs, using T4 DNA polymerase.

Full length F *traD* (accession no. NC_002483) in pSK410 was obtained as a *Nde*I/*Hin*dIII PCR fragment, using pKI410 as template and the primers 9f and 9r. *his$_6$* fusions to *traD* were generated similarly, using the primer pair 10 (*his$_6$-traD* in pSK410NH) and 11 (*traD-his$_6$* in pSK410CH).

H. pylori hp0524 (accession no. AE000566) was generated as a truncated *his$_6$* fusion (*his$_6$-hp0524Δ1*) by PCR on *H. pylori* strain 26695 DNA as template, using the primers 12f and 12r. pHY524Δ1 was constructed by inserting this *Nde*I/*Bam*HI PCR fragment into pET-14b.

pGS007 was prepared by ligation of a 1617 bp *Nsi*I fragment of pSU4054 into pGS003Δ1. R388 *trwB* deletion derivative *his$_6$-trwBΔ1* in pGS012Δ1 was generated by PCR with pGS007 as template and primer pair 13.

3.1.5 ^{32}P-labelling of DNA fragments

dsDNA fragments were dephosphorylated using shrimp alkaline phosphatase. The dephosphorylated DNA was ^{32}P-labelled by 5' phosphorylation with [γ-^{32}P]-ATP as follows: 10 units of T4 polynucleotide kinase were incubated with 20 pmol of dsDNA fragments and 20 µCi (6.6 pmol) of [γ-^{32}P]-ATP at 37 °C for 45 min. After heat-inactivation of the kinase by incubation at 65 °C for 30 min, the labeled DNA was used in gel retardation experiments (3.8).

3.1.6 Generation and isolation of TraG insertion mutants TraGi31

In-frame insertion mutants of *traG* were constructed by transposon insertion mutagenesis with transposons Tn*lacZ*/in and Tn*phoA*/in (53) and subsequent *in vitro* DNA manipulation as illustrated in Figure 3.3.

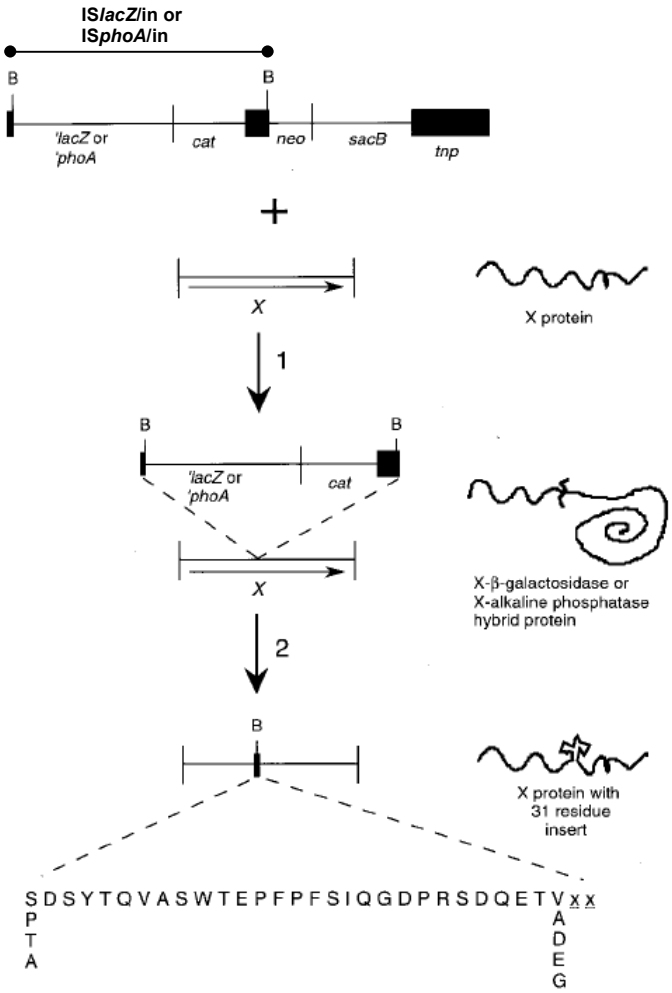

Figure 3.3. Generation of in-frame insertion mutants by use of Tn*lacZ*/in and Tn*phoA*/in. The structural arrangement of IS elements (black rectangles), genes and *Bam*HI restriction sites (B) is schematically represented (top). In the first step, a gene fusion between the target gene *X* and *lacZ* or *phoA* is generated by transposition of IS*lacZ*/in or IS*phoA*/in. In the second step, most of the transposed sequences are removed by consecutive *Bam*HI cleavage and religation. The remaining 31 amino acid in-frame insertion is shown (bottom), with x indicating residues determined solely by the insertion target site.

The transposons are derivatives of Tn*5* that carry *lacZ* or *phoA* sequences in addition to the chloramphenicol resistance determinant (*cat*) at their left end (referred to as IS*lacZ*/in and IS*phoA*/in). The central regions of the transposons contain a kanamycin resistance determinant (*neo*) and a gene conferring sucrose sensitivity (8) (*sacB*). Thus, cells carrying insertions of IS*lacZ*/in or IS*phoA*/in alone (i.e. insertions lacking both the Tn*5* central region and the right end) are isolated by selection for chloramphenicol resistance, sucrose resistance and kanamycin sensitivity. Insertion mutants that carry *lacZ* or *phoA* fusion genes also display a blue phenotype on media containing X-gal or X-phos, respectively.

Protocol for generation and isolation of TraGi31 mutants

E. coli strain CC191 carrying pSK470ΔB (*traG*$^{+}$) was infected with replication-deficient λ phages (λ28) containing either the Tn*lacZ*/in or Tn*phoA*/in transposons. Aliqots of 0.2 ml of stationary phase CC191 (pSK470ΔB) cells were mixed with λTn*lacZ*/in or λTn*phoA*/in at a multiplicity of infection of 0.1 to 0.3. After incubation for ten minutes at 37°C without aeration, 0.8 ml of LB medium was added and cells were grown for one to two hours with aeration. For selection of cells expressing plasmid-borne ampicillin resistance (Ap^{r}) and chloramphenicol resistance (Cm^{r}, aquired by transposition of the *cat* gene of Tn*lacZ*/in or Tn*phoA*/in into the plasmid), dilutions of the cultures were plated on TYE medium supplemented with ampicillin and chloramphenicol (100 μg/ml ; elevated choramphenicol concentration in this step reduced background growth). After one to two days of incubation at 37°C, plasmid DNA was prepared from pooled colonies (typically 2000/plate). The DNA was transformed into CC191, with plating on TYE (lacking NaCl) supplemented with sucrose (5%, w/v), chloramphenicol (10 or 40 μg/ml), ampicillin, IPTG and X-gal or X-phos, as appropriate (*lacZ* insertion mutants or *phoA* insertion mutants conferred a blue phenotype on media containing X-gal or X-phos, respectively). After overnight incubation at 37°C, transformant colonies were screened for kanamycin sensitivity (loss of the *neo* gene) by patching to TYE supplemented with kanamycin and TYE supplemented with chloramphenicol. Kanamycin-sensitive colonies were purified by streaking, and resulting single colonies were inoculated into LB medium supplemented with chloramphenicol. After overnight growth, plasmid DNA was isolated.

Following this protocol, about 85,000 choramphenicol resistant colonies were screened, of which 20 (eleven from the screen for *lacZ* fusions and nine from the screen for *phoA* fusions) were identified as different in-frame translational fusions to *traG* after DNA sequence analysis (Table 4.1, page 43). The IS elements were removed from the *traG* sequence by *in vitro* manipulation of the plasmids with *Bam*HI digestion and religation with T4 DNA ligase. This resulted in a 31-codon insertion left in the *traG* gene at the site of the original transposition event (Figure 3.3). For all of these mutations, 27 of the 31 inserted codons are the same, with variation at each end caused by duplication of target sequences during the transposition event. The plasmids generated were named p*traG::i31*X and the

resulting TraGi31 proteins TraGiX, with 'X' designating the last unaltered residue of TraG before the insertion sequence (Table 4.1, page 43).

3.2 Characterization of TraGi31 mutant proteins

The stablity and steady-state expression levels of the TraG insertion mutant proteins (3.1.6) was examined by Western blot analysis (3.10), using an antiserum specific for the insertion epitope (59). Of the 20 different mutant proteins, only 3 did not accumulate to significant levels in the cells. The ability of the TraGi31 mutant proteins to participate in RP4-mediated conjugation was examined in conjugation experiments (3.3). The membrane topology of TraG was characterized by a trypsinization assay of spheroplasted cells (53) expressing stable TraGi31 mutants:
Cells carrying *traG::i31*-expression plasmids were grown overnight in LB medium supplemented with ampicillin (100 µg/ml). Cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in fresh medium and grown at 37°C to an OD_{600} of 0.9. IPTG (2 mM, final concentration) was added for induction of *traG::i31* expression, and cells were incubated at 37°C for an additional 45 to 60 min. Equal amounts of cells were pelleted by centrifugation at 4°C for 10 min at 5,000 × g, washed in sucrose buffer (18% sucrose, 100 mM Tris HCl [pH 8.0]), and centrifuged as before. After resuspension in 500 µl of sucrose buffer containing lysozyme (207 µg/ml) and EDTA (17 mM), cells were incubated for 30 min on ice. The resulting spheroplast preparations were divided into two 290-µl aliquots. One aliquot was incubated with trypsin (30 µg/ml, final concentration), and the second was incubated with inactivated trypsin (30 µg/ml, final concentration). Inactivated trypsin was prepared by adding 32 µl of soybean trypsin inhibitor (STI; 1 mg/ml) and 20 µl of phenylmethylsulfonyl fluoride (PMSF; 20 mg/ml) to 32 µl of trypsin (300 µg/ml). After 30 min of incubation at room temperature, the reactions with active trypsin were terminated by the addition of STI (90 µg/ml, final concentration) and PMSF (1.1 mg/ml, final concentration). The spheroplasts were then centrifuged for 3 min at 16,000 × g at 4°C. The resulting pellets were resuspended in SDS-PAGE sample buffer (3.9.2), heated to 65°C for 20 min and subjected to Western blot analysis (3.10) using antibodies directed against the target protein or the 31-residue inserted sequence (25). Trypsin cleavage of the OmpA protein periplasmic domain was monitored to determine the efficiency of spheroplast formation and proteolysis. Trypsin cleavage of an abundant

45-kDa cytoplasmic protein, tentatively identified as elongation factor Tu, was monitored to control for cell lysis before or during proteolysis.

3.3 Conjugations

The transfer frequencies of conjugations mediated by RP4 derivatives (pDB127 plus a plasmid encoding a *traG* derivative) were determined in mating experiments on filters (4). Donor cells (0.7 ml, 1×10^8 cells/ml) and HB101 Nx^r recipient cells (7 ml, 3-4×10^8 cells/ml) were mixed and filtered on nitrocellulose filters (0.45 μm pore size, 25 mm diameter). The filters were incubated for one hour at 37°C on YT+ agar plates without antibiotics. Cells were then suspended in 5.5 ml of 10 mM $MgSO_4$ and aliquots of serial dilutions were plated on YT+ agar supplemented with (a) nalidixic acid and chloramphenicol to select for transconjugants or (b) ampicillin and chloramphenicol to select for donors.

Conjugation of F plasmid derivatives (pOX38*traD411* plus a *traD*-encoding plasmid) was carried out as follows: overnight cultures of *E. coli* XK1200 donor strains were diluted 1:25 and growth was allowed to continue at 37°C without shaking until an OD_{600} of 0.2 to 0.3 was reached. A 100-fold excess of recipient SG13109 cells over donor cells was added directly from overnight culture to the growing donor culture. Mating cultures were incubated at 37°C without shaking for 45 minutes. Transfer was stopped by vigorous mixing for one minute and rapid cooling to 0°C. Transconjugants were selected using tetracycline and kanamycin; selection for donors was by kanamycin and epicillin resistance. The conjugation frequency was expressed as the number of transconjugants per donor cell.

3.4 Protein purification

RP4 TraI protein was purified as described (70). RP4 TraG, R388 TrwB, F TraD and *H. pylori* HP0524 and were purified as N-terminally hexahistidine (His_6) tagged or truncated (Δ) derivatives (Table 3.3).

These derivatives were used in all assays and for simplicity were referred to as TraG, TraGK187T, TraGΔ2, TrwBΔ1, TraD and HP0524Δ1.

Table 3.3. Proteins purified in this study.

Origin	Protein	Encoding plasmid
RP4	TraI	pDB173
	His_6-TraG	pFS241
	His_6-TraGK187T	pFS241M
	His_6-TraGΔ2	pGS006Δ2
	His_6-TraGΔ2K187T	pGS011
R388	His_6-TrwBΔ1	pGS012Δ1
F	His_6-TraD	pSK410NH
H. pylori	His_6-HP0524Δ1	pHY524Δ1

For overproduction, overnight cultures of the indicated *E. coli* strains were diluted 1:30 in medium (1.2 liters in 5-liter flasks) and growth was allowed to continue at 30 °C with shaking until an OD_{600} of 0.5 to 0.6 was reached. IPTG was added to a final concentration of 1 mM, and growth was continued for four to five hours. After harvesting by centrifugation at 5,000 × g for 15 min at 20 °C, cells were resuspended in 5.0-5.5 ml of spermidine buffer per g (wet weight) of cells.

spermidine buffer: 20 mM spermidine-HCl
200 mM NaCl
2 mM EDTA

Cell suspensions were shock-frozen in liquid nitrogen and were stored at -78°C. Further steps were carried out at 4 °C or on ice unless noted otherwise. Protein purity and concentrations were determined by laser-densitometric quantification of Coomassie-Blue stained SDS-polyacrylamide gels (3.9.2) with serial concentrations of BSA as a reference. Quantification of protein contents was performed according to the method described by Lowry *et al.* (57). Immunoblot analysis (3.10) of RP4 TraG and its derivatives was performed with TraG-specific antiserum (116). Purified proteins were stored at -20 °C in buffer containing 50 % glycerol, except HP0524Δ1, which was stored at 4°C.

SDS crude cell extracts (SDS-CCE) were prepared as follows: cells (1-5 ml of cell cultures) were centrifuged and the resulting cell pellets were resuspended in 30 µl SDS-lysis buffer per OD_{600} of cells.

SDS-lysis buffer: 100 mM Tris-HCl (pH 6.8)
100 mM NaCl
5 % SDS
1 M mercaptoethanol
15 % glycerol

Cell suspensions were incubated for each 5 min at 60 °C and at 95 °C. After centrifugation at 100,000 × g for 30 min at 4 °C, supernatants (= SDS-CCE) were analyzed by SDS-PAGE (3.9.2).

3.4.1 Purification of RP4 TraG and TraGK187T

Full-length TraG and point mutation derivative TraGK187T were purified from SCS1 cells carrying pFS241 and pFS241M, respectively. Since the purification procedure was identical for both proteins, only the purification of TraG is described.

SCS1 (pFS241) cells (35 g, resuspended in 200 ml spermidine buffer) were thawed, supplemented with 400 ml of buffer A and stirred for 90 min at room temperature. After centrifugation at 100,000 × g for 60 min, the supernatant was kept and the pellet was resuspended in 100 ml of 50 mM Tris-HCl (pH 7.6), 1 M NaCl and 0.25 % Brij-58, using a Dounce homogenizer. The suspension was centrifuged as before and the supernatants of both centrifugation steps were combined. Proteins were precipitated by addition of $(NH_4)_2SO_4$ (60% saturation (23)), collected by centrifugation at 25,000 × g for 60 min and resuspended in 100 ml of buffer B (fraction I, 100 ml). Fraction I was dialyzed against buffer B and applied to a Ni-NTA column. The column was washed with buffer B and buffer B / 20 mM imidazole (pH 7.6). Proteins were eluted with buffer B / 250 mM imidazole. Fractions containing TraG were pooled (fraction II, 85 ml) and concentrated by dialysis against buffer B / 20% polyethylene glycol (PEG) 20,000.

3.4.2 Purification of RP4 TraGΔ2 and TraGΔ2K187T and R388 TrwBΔ1

TraGΔ2, TraGΔ2K187T and TrwBΔ1 were purified following the same protocol, thus only the purification of TraGΔ2 is described in detail.

SCS1 (pGS006Δ2) cells (19.7 g, resuspended in 100 ml spermidine buffer) were thawed, supplemented with 200 ml of buffer A and stirred for 90 min at room temperature. After centrifugation at 100,000 × g for 45 min, the supernatant was kept and the pellet was resuspended in 100 ml of buffer C, using a Dounce homogenizer.

The suspension was centrifuged as before and the supernatants of both centrifugation steps were combined. Proteins were precipitated by addition of $(NH_4)_2SO_4$ (60% saturation), collected by centrifugation at 25,000 × g for 30 min and resuspended in 35 ml of buffer C (fraction I, 35 ml). Fraction I was dialyzed against buffer D and applied to a Ni-NTA column. The column was washed with buffer D and buffer D containing 10% (v/v) glycerol and 20 mM imidazole (pH 7.6). Proteins were eluted with buffer D containing 250 mM imidazole. Fractions containing TraGΔ2 were pooled (fraction II, 97 ml), dialyzed against buffer E and applied to a phosphocellulose P11 column. Adsorbed proteins were eluted with a linear gradient (40 mM to 600 mM NaCl) in buffer E. TraGΔ2 containing fractions were pooled and concentrated by dialysis against buffer F containing 20% polyethylene glycol (PEG) 20,000 (fraction III, 17.5 ml).

TraGΔ2K187T was purified from SCS1 (pGS011), following the same protocol as for TraGΔ2, starting with 19.1 g cells in 100 ml spermidine buffer. Purification of TrwBΔ1 from SCS1 (pGS012Δ1) was done similarly with 16.4 g cells in 80 ml spermidine buffer, except that NaCl concentration in buffer A and buffer C was 1 M. Purified TraGΔ2, TraGΔ2K187T and TrwBΔ1 were stored at -20 °C in buffer F containing 50 % (w/v) glycerol.

3.4.3 Purification of F TraD

SCS1 (pSK410NH) cells (18 g, resuspended in 90 ml spermidine buffer) were thawed, supplemented with 180 ml of buffer A and stirred and centrifuged as described for RP4 TraG. The supernatant was discarded and the pellet was resuspended with a homogenizer in 28 ml of 50 mM CHES-NaOH (pH 9.5), 1 M NaCl, 5 mM $MgCl_2$ and 1% (v/v) Triton X-100. The suspension was centrifuged as before and the supernatant was collected (fraction I, 28 ml). Fraction I was applied to a Ni-NTA column equilibrated with buffer G. The column was washed stepwise with buffer G and with buffer G / 35 mM imidazole (pH 7.6). TraD protein was eluted with buffer G / 250 mM imidazole. Fractions containing TraD were pooled (fraction II, 38 ml) and concentrated by $(NH_4)_2SO_4$ precipitation (40% saturation). The precipitate was resuspended in 13 ml of buffer G and dialyzed against the same buffer.

3.4.4 Purification of HP0524Δ1

BL21DE3(pLysS) cells carrying pHY524Δ1 (6 g, resuspended in 30 ml spermidine buffer) were thawed and supplemented with 30 ml of lysis buffer (0.5 % Brij-58, 1.8 M NaCl, 0.1 M Tris-HCl [pH 7.6], 1.34 mg/ml lysozyme, 10% sucrose). The mixture was stirred for 80 min and centrifuged for 45 min at 81,000 x g. The supernatant was discarded and the pellet was washed twice by thorough resuspension with a homogenizer in 38 ml of wash buffer 1 (0.25% Brij-58, 1 M NaCl, 50 mM Tris-HCl [pH 7.6], 10 mM spermidine · 3HCl, 1 mM EDTA) and 38 ml of wash buffer 2 (10 mM Zwittergent 3-16, 1 M NaCl, 50 mM Tris-HCl [pH 8.7]) and centrifuging as before. The remaining pellet was resuspended in 250 ml of wash buffer 2 containing Zwittergent 3-14 instead of Zwittergent 3-16. The suspension was sonicated (Branson Sonifier), warmed briefly to 25 °C and centrifuged as before. The supernatant was concentrated by $(NH_4)_2SO_4$ precipitation (40% saturation) and resuspension of the precipitate in 18 ml of buffer H (fraction I, 18 ml). Fraction I was dialyzed against buffer H and applied to gel filtration on a HiLoad 16/60 Superdex 200 pg column. Fractions with purest protein were pooled and concentrated by dialysis against buffer H / 20% PEG 20,000 (fraction II, 12 ml).

3.5 Gel filtration

A Superdex 200 HR 10/30 column was calibrated with a gel filtration standard (Bio-Rad) consisting of 4 globular proteins of 670, 158, 44 and 17 kDa and Vitamin B-12 (1.4 kDa). The column was run with buffer H or with buffer I at a flow rate of 0.4 ml/min. Protein elution, which was monitored at λ= 280 nm, was identical in both buffer systems. TraG, TraGΔ2, TraD and HP0524Δ1 were applied to gel filtration under the same conditions, using buffer H for HP0524Δ1 and buffer I for TraG, TraGΔ2 and TraD. A trendline correlating the elution volumes of the gel filtration standards to the corresponding M_r was used to obtain an estimate of the M_r of TraG, TraGΔ2, TraD and HP0524Δ1.

3.6 Glycerol gradient centrifugation

Purified TraG (630 µg) or TraD (372 µg) in 150 µl 20 mM Tris-HCl (pH 7.6), 500 mM NaCl, 2 mM DTT, 0.1% Brij-58 and 0.1 mM EDTA was layered on a 3.7 ml, 15-35% linear glycerol gradient. Centrifugation was at 270,000 × g for 38 h at 3 °C.

Fractions of 250 µl were collected, analyzed by SDS-PAGE (3.9.2) and tested for NTPase activity (3.7). Three proteins were centrifuged in a parallel experiment for reference: aldolase (158 kDa, $S_{20,w}$ = 7.8), BSA (67 kDa, $S_{20,w}$ = 4.4) and ovalbumin (43.5 kDa, $S_{20,w}$ = 3.6).

3.7 Nucleoside triphosphate hydrolase (NTPase) assay

NTPase activity was determined by monitoring hydrolysis of [γ-^{32}P] NTPs. NTPases catalyze one of the following reactions:

$$NTP \rightarrow NDP + P_i \quad \text{(Eq. 1)}$$

or

$$NTP \rightarrow NMP + PP_i \quad \text{(Eq. 2)}$$

Thus, hydrolysis of [γ-^{32}P] NTPs deliberates ^{32}P-labelled phosphate (P_i) or pyrophosphate (PP_i). NTP hydrolysis reactions were performed with 0.1 to 4 µM protein, 0.1 µCi of [γ-^{32}P]NTP and 0.2 mM unlabeled NTP at 30 °C for 20 min in a total volume of 20 µl of NTPase buffer.

NTPase buffer: 50 mM Tris-HCl (pH 7.6)
2 mM $MgCl_2$
50 mM KCl
1 mM DTT
50 µg/ml BSA

When appropriate, 50 ng/µl of ssDNA (phage M13 mJF182) was added. Reactions were quenched by addition of EDTA (10 mM final concentration) and chilling on ice. Reaction products were separated by thin-layer chromatography on cellulose MN300 in a buffer containing 1 M LiCl and 1M acetic acid. Radioactive NTP and P_i or PP_i were quantified using storage phosphor technology (41) with the PhosphorImager scanning device and the ImageQuant software. P4α protein (0.25 µM) and HP0525 (0.25 µM) with known NTPase activity (117), (45) served as positive controls in the experiments. The measured ATPase activity was expressed as the conversion rate of ATP (in µmol/min) in the presence of 1 µM protein and 50 ng/µl ssDNA.

3.8 Fragment retardation assay

DNA binding activity was analysed in fragment retardation assays with either ^{32}P-labelled DNA fragments (3.8.1) or with non-labeled DNA fragments (3.8.2).

3.8.1 Fragment retardation with γ-^{32}P-labeled DNA fragments

pJF143 (Table 2) was digested with *Eco*RI and *Bam*HI, yielding an *oriT*-containing fragment of 287 bp and additional fragments of 608 and 3,543 bp. After 5' labeling with [γ-^{32}P]ATP (3.1.5), the fragments (0.75 nM each) were incubated for 30 min at 37 °C with increasing amounts of TraG, TraD or HP0524Δ1 in a total volume of 20 µl of buffer J. In competition experiments, the previously incubated protein/DNA mixture was supplemented with increasing amounts of ssDNA and incubated for 30 min at 37 °C. Samples were electrophoresed on non-denaturing polyacrylamide gels (3.9.1). The ^{32}P-labeled DNA bands were visualized by the storage phosphor method (41), using the PhosphorImager scanning device, and were analyzed with the ImageQuant software.

For determination of dissociation constants, 36 fmol of a ^{32}P-labeled 773 bp *Acc*I-*Ava*I DNA fragment of pBR329 (Table 1) was incubated as before with different amounts of TraGΔ2 or TrwBΔ1. Complex formation was determined by monitoring the decrease of free DNA. The amount of free DNA in each lane was quantified with reference to the free DNA present in the absence of protein.

Competition/inhibition of DNA-binding of TraGΔ2 or TrwBΔ1 by ssDNA, Mg^{2+}, ATP or ADP was analyzed by quantifying the displacement of bound dsDNA fragments from dsDNA-protein complexes. 75 fMol of ^{32}P-labeled DNA fragments obtained by *Dra*I/*Acc*I restriction of pJF143 (Table 1) were incubated as before with 10 pMol TraGΔ2 or 3 pMol TrwBΔ1. After 10 min, $MgCl_2$, ATP, ADP or ssDNA were added as appropriate, and mixtures were incubated for another 20 min at 37 °C. Samples were electrophoresed and fragments were visualized and quantified as before. The fraction of free DNA vs total DNA was calculated to determine the percentage of complex resolution.

3.8.2 Fragment retardation with non-labeled DNA fragments

pJF143 (Table 1) was digested with *Eco*RI and *Bam*HI. The fragments (12 nM each) were incubated for 30 min at 37 °C with increasing amounts of TraG, TraGΔ2 or TraGΔ2K187T in a total volume of 20 µl of buffer J. Samples were electrophoresed on non-denaturing polyacrylamide gels (3.9.1). After staining with ethidium bromide, gels were analyzed with the FluorImager 575 scanning device and the ImageQuant software.

3.9 Electrophoresis techniques

3.9.1 DNA electrophoresis

For analysis or purification of DNA, samples were electrophoresed on horizontal agarose gels (0.7-1.5 %). TBE buffer was used as diluent and electrophoresis buffer.

TBE buffer: 90 mM Tris-borate (pH 8.0)
2 mM EDTA

Samples were prepared for electrophoresis by addition of 1/3 volume of DNA sample buffer.

DNA sample buffer: 20 % Ficoll-400
1 % SDS
0.3 % bromphenol blue

Electrophoresis was performed at 6-10 V/cm. Gels were stained with ethidium bromide by incubation of the gels in 2.5 µg/ml ethidium bromide solution after electrophoresis.
Vertical 3.5 % polyacrylamide gels were used in fragment retardation experiments (3.8). Gels were composed of 3.5 % acrylamide and 0.175 % bisacrylamide in TAE buffer.

TAE buffer: 40 mM Tris-acetate (pH 8.0)
1 mM EDTA

0.1 % $(NH_4)_2S_2O_8$ and 0.1 % TEMED were added shortly before casting. Electrophoresis was performed at 6-10 V/cm in TAE buffer.

3.9.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analyzed on vertical, discontinuous SDS polyacrylamide gels (51). Gels were composed of:

15 % acrylamide
0.087 % bisacrylamide
0.1 % SDS
375 mM Tris-HCl (pH 8.7)

0.03 % $(NH_4)_2S_2O_8$ and 0.03 % TEMED were added shortly before casting. After

complete polymerization, stacking gels were layered on top of the gels. Stacking gels were composed of:

5 % acrylamide
0.13 % bisacrylamide
0.1 % SDS
125 mM Tris-HCl (pH 6.8)

0.05 % $(NH_4)_2S_2O_8$ and 0.05 % TEMED were added shortly before casting.
Samples were prepared for electrophoresis by addition of 15 % (v/v) SDS sample buffer and heating at 95 °C for 3 minutes.

SDS sample buffer: 625 mM Tris-HCl (pH 6.8)
20 % SDS
333 mM DTT
50 % (v/v) glycerol
1 % bromphenol blue

Electrophoresis was performed at 17 V/cm in 25 mM Tris, 190 mM glycine and 0.1 % SDS. Gels were stained by incubation in Coomassie solution for 20 min at room temperature.

Coomassie solution: 0.25 % Coomassie Brilliant Blue R250
50 % (v/v) methanol
7 % (v/v) acetic acid

After washing in 20 % (v/v) methanol / 7 % (v/v) acetic acid for 4-6 hours, gels were scanned and analysed using the Personal Densitometer scanning device and the ImageQuant software.

3.10 Immunoblot analysis (Western blot)

For immunological detection of TraG and TraG derivatives, proteins were subjected to Immunoblot analysis. Samples were first electrophoresed by SDS-PAGE (3.9.2). Proteins were then transferred to nitrocellulose or PVDF membranes by electroblotting, using the “semi-dry” technique described by Kyhse-Anderson (49). Membranes were incubated in BSA buffer for 20 minutes.

BSA buffer: 3 % BSA
150 mM NaCl
10 mM Tris-HCl (pH 7.6)

Membranes were next incubated for 45 min with primary antibodies (rabbit anti-TraG IgG serum), which were diluted 1:500 in BSA buffer. After washing three times for 5 min in buffer W, membranes were incubated for 30 min with fluorescence-labelled secondary antibody solution (FITC-labelled goat-anti-rabbit antibodies, diluted 1:100 in buffer W).

buffer W: 150 mM NaCl
10 mM Tris-HCl (pH 7.6)

After washing in buffer W as before, membranes were analyzed by fluorescence scanning, using the FluorImager scanning device and the ImageQuant software.

3.11 Transmission electron microscopy

dsDNA (25 fmol of pJF143 digested with *Eco*RI and *Bam*HI) and/or ssDNA (25 fmol M13 mp18) was incubated for 10 min at room temperature with TraG or TraGΔ2 (0.48 pmol each). Following fixation with 0.2 % glutaraldehyde for 10 min, the samples were prepared for electron microscopy (Philips EM400) by adsorption to mica as described previously (93).

3.12 Protein interaction analysis by surface plasmon resonance (SPR)

Interactions between TraI and TraG, TraGK187T or TraGΔ2 were studied using the Biacore 2000 optical biosensor system with pioneer sensor chip B1. Proteins were covalently coupled to chip surfaces of flow cells (FC) using the BIAcore amine coupling kit. TraI protein (571 resonance units (RU)) was coupled to FC3 of sensor chip #1. A second and a third flow cell served as controls and were loaded with 231 RU of BSA (FC2) or without protein (FC1, saturated with ethanolamine). Sensor chip #2 was loaded with 480 RU of BSA (FC2), 498 RU of TraI (FC3), 460 RU of TraGΔ2 (FC4) and without protein (FC1). HBS-EP buffer (Biacore) was used as eluent and dilution buffer.

For determination of the association rate constant (k_a) of TraG-TraI interactions, increasing concentrations of TraG (10-200 nM) were injected into sensor chip #1 at a flow rate of 100 µl/min for 2 min. Dissociation was monitored for 5 min. Between injections, flow cells were washed with 10 µl of 0.2 M Na_2CO_3 and 10 µl of HBS-EP / 0.01% SDS at 10 µl/min.

Interactions between TraI, TraGK187T and TraGΔ2 were analyzed with sensor chip #2. 100 nM solutions of TraGK187T, TraGΔ2 or TraI were injected into the chip (FC1-2-3-4) at a flow rate of 50 µl/min. Signals were corrected for non-specific binding by subtracting curves for BSA interaction from each curve. Binding constants were determined with the BIAevaluation software, applying the Langmuir binding model (1:1 interaction) for computational fitting.

3.13 Nucleotide binding assay

Fluorescent nucleotide analogues TNP-ATP and TNP-ADP were used to study nucleotide binding activity of TraG and TrwB derivatives. Proteins and nucleotides were diluted in buffer H in a total volume of 400 µl. Final concentrations of NaCl were adjusted to 36 mM (TraGΔ2, TraGΔ2K187T) or 50 mM (TrwBΔ1). Mixtures were incubated for 20 seconds before measurement. Fluorescence spectra were taken at room temperature using a Shimadzu RF-5000 spectrofluorometer with excitation at 410 nm and emission scanning in the range of 470-620 nm. The fluorescence maxima were determined graphically. For determination of K_d, 7 µM protein solutions were titrated with TNP-ATP or TNP-ADP. The K_d values of non-labeled ATP and ADP, as well as the inhibition constant of Mg^{2+} were determined by displacement of protein-bound TNP-nucleotides. 7 µM protein solutions were incubated for 20 sec with 50 µM TNP-ATP or 70 µM TNP-ADP (50 µM TNP-ADP in the case of TrwBΔ1). ATP, ADP or $MgCl_2$ (from 0.5 M or 0.1 M stock solutions in buffer H) were added and fluorescence was measured after incubation for 20 sec.

Enhanced fluorescence (ΔF) was calculated as the difference between total fluorescence (F_t) and the intrinsic fluorescence of TNP-nucleotides, buffer and proteins:

$$\Delta F = F_t - F_{TNP\text{-}N} - F_{buffer} - F_{protein} \qquad \text{(Eq. 3)}$$

ΔF represents the amount of receptor-ligand complexes (RL) that are formed throughout the titration. ΔF reaches a maximum (ΔF_{max}) as the receptor becomes saturated, i.e. when RL equals the total receptor concentration (R_t):

$$\Delta F = \Delta F_{max} \cdot \frac{RL}{R_t} \qquad \text{(Eq. 4)}$$

RL / R_t represents the fractional saturation of receptor. Substitution of RL by an expression that relates RL, R_t, L and K_d and takes ligand depletion into account (40) results in equation 5:

$$\Delta F = \Delta F_{max} \cdot \frac{(R_t + L + K_d) - \sqrt{(R_t + L + K_d)^2 - 4R_t L}}{2R_t} \qquad \text{(Eq. 5)}$$

Displacement of TNP-nucleotides by non-labeled nucleotides or by Mg^{2+} is described by equation 6:

$$\Delta F = \Delta F_{max} - (\Delta F_{max} - \Delta F_{min}) \cdot \frac{(R_t + L + I_{50}) - \sqrt{(R_t + L + I_{50})^2 - 4R_t L}}{2R_t} \qquad \text{(Eq. 6)}$$

ΔF_{max} and ΔF_{min} are the fluorescence at start and at end of titration and L is the concentration of competitor (ATP, ADP or Mg^{2+}). I_{50} represents the concentration of competitor necessary to displace 50 % of bound TNP-nucleotides. It is related to the inhibition constant (K_i) of the competitor (17):

$$K_i = \frac{I_{50}}{1 + (L_t / K_d^{TNP-N})} \qquad \text{(Eq. 7)}$$

L_t is the total concentration of TNP-nucleotide at start of titration and K_d^{TNP-N} is the dissociation constant of the respective TNP-nucleotide. In case of displacement by ATP and ADP, K_i corresponds to K_d^{ATP} and K_d^{ADP}, respectively. The coefficients of independent values in equations 5 and 6 were fitted to the data using Sigma Plot version 2.0 (Copyright ©1986-1994, Jandel Corp.).

3.14 Sequence alignment of TraG-like proteins

A BLAST search (1) for TraG-like proteins was performed at NCBI (http://www.ncbi.nlm.nih.gov/blast/), with the RP4 TraG sequence as the query. From the search results, 19 representative proteins with significant similarity to RP4 TraG were chosen. The sequences were aligned using the Clustal W software (98) with the BLOSUM series as protein weight matrix. Profile alignments were used for alignments of more distantly related sequences to a set of aligned TraG-like sequences.