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TraG Encoded by the pIP501 Type IV Secretion System Is a Two-Domain Peptidoglycan-Degrading Enzyme Essential for Conjugative Transfer

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pIP501 is a conjugative broad-host-range plasmid frequently present in nosocomial *Enterococcus faecalis* and *Enterococcus faecium* isolates. We focus here on the functional analysis of the type IV secretion gene *traG*, which was found to be essential for pIP501 conjugative transfer between Gram-positive bacteria. The TraG protein, which localizes to the cell envelope of *E. faecalis* harboring pIP501, was expressed and purified without its N-terminal transmembrane helix (TraGΔTMH) and shown to possess peptidoglycan-degrading activity. TraGΔTMH was inhibited by specific lytic transglycosylase inhibitors hexa-*N*-acetylchitohexaose and bulgecin A. Analysis of the TraG sequence suggested the presence of two domains which both could contribute to the observed cell wall-degrading activity: an N-terminal soluble lytic transglycosylase domain (SLT) and a C-terminal cysteine-, histidine-dependent amidohydrolases/peptidases (CHAP) domain. The protein domains were expressed separately, and both degraded peptidoglycan. A change of the conserved glutamate residue in the putative catalytic center of the SLT domain (E87) to glycine resulted in almost complete inactivity, which is consistent with this part of TraG being a predicted lytic transglycosylase. Based on our findings, we propose that TraG locally opens the peptidoglycan to facilitate insertion of the Gram-positive bacterial type IV secretion machinery into the cell envelope.

Conjugation machineries of both Gram-negative (G−) and Gram-positive (G+) bacteria are classified as type IV secretion systems (T4SS), which function as translocators and mediate the transport of DNA and/or proteins across the cell envelope (1–3). Nevertheless, it is expected, due to the different architectures of the G− and G+ bacterial cell envelopes, that the mechanism of conjugation in G− and G+ bacterial systems may differ (2, 4–15).

The antibiotic resistance plasmid pIP501 from *Streptococcus agalactiae* is a model plasmid for conjugation in G+ bacteria and has a very broad host range for conjugative plasmid transfer and mobilization. Its host range includes virtually all tested G+ bacteria, including the multicellular filamentous streptomycetes and *Escherichia coli* (13, 16). The pIP501 transfer (*tra*) region encompasses 15 open reading frames (ORFs) that are organized in an operon negatively autoregulated by the first gene product TraA, a biochemically characterized conjugative relaxase (17, 18). The pIP501 *tra* gene products formerly named Orf1 to Orf15 have been renamed as TraA to TraO, as for the majority of the Tra proteins putative functions have been ascribed. $TraE_{pIP501}$ is a putative VirB4-like ATPase, $TraG_{pIP501}$ shows similarities to VirB1-like lytic transglycosylases (LTs), and $TraJ_{pIP501}$ is a putative VirD4-like coupling protein (2, 4, 19). Based on protein-protein interaction studies of the 15 pIP501 Tra proteins by yeast two-hybrid assays and *in vitro* pulldown assays, a first model of a T4SS-like system of G+ bacterial origin was proposed (4). Furthermore, *in vitro* ATP binding and hydrolysis were shown for both $TraE_{pIP501}$ and $TraJ_{pIP501}$ (M. Y. Abajy and E. Grohmann, unpublished data) as well as binding of single-stranded DNA for the putative coupling protein $TraJ_{pIP501}$ (K. Arends and E. Grohmann, unpublished data).

For the VirB1-like enzyme TraG, a modular architecture is anticipated (Fig. 1): at the N terminus of the protein, a putative transmembrane helix (TMH) is predicted which is followed by a soluble lytic transglycosylase (SLT) domain and an *N*-acetyl-D-glucosamine binding site (20, 21). LTs function as murein lyases by cleaving the β1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine (22–24). In addition to this, they also form a new glycosidic bond with the C_6 hydroxyl group of the same muramic acid residue. Members are found in phages, type II and type III secretion systems, and T4SS (25). At its C terminus, TraG contains a C-terminal cysteine-, histidine-dependent amidohydrolases/peptidases (CHAP) domain corresponding to an amidase function. Many proteins with CHAP domains are involved in cell wall metabolism of bacteria; often, the CHAP domain is found in association with other pepti-

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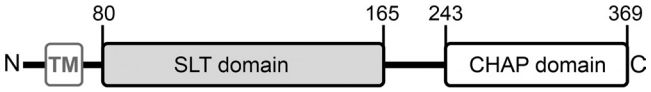

FIG 1 Domain composition of TraG. TraG shows a modular structure. The VirB1 ortholog $TraG_{pIP501}$ protein (GenBank sequence accession no. CAD44387.1) contains 369 amino acids. A transmembrane helix (TMH) is predicted at the N terminus (positions 20 to 36, HMMTOP), as well as a signal peptide with a putative cleavage site at positions 47 to 48 (SignalP 3.0). A specific lytic transglycosylase (SLT) domain is predicted at positions 80 to 165 (gray box); a cysteine-, histidine-dependent amidohydrolases/peptidases (CHAP) domain is putatively located at the C terminus (positions 243 to 369, white box). A peptidoglycan binding motif is predicted for the SLT domain (conserved domain search).

doglycan (PG)-cleaving domains. In these cases, the CHAP domain seems to have an endopeptidase specificity, thus opening the interlinks between several glycan strands (26, 27). The TraG CHAP domain also overlaps with an *N*-acetylmuramoyl-L-alanine amidase (PRK08581) domain (20, 21), indicating the putative cleavage of cross-bridges that interlink murein strands in PG (28).

The PG layer of the cell wall imposes structural constraints for the assembly of macromolecular secretion systems, such as the T4SS multiprotein complex. The structure of the PG must be rearranged to accommodate such structures, without compromising the integrity of the bacterial cell (29, 30). To overcome this barrier and to facilitate T4SS assembly, one or more PG lyases are generally encoded in the transfer regions of conjugative plasmids (29, 30). The PG lyases involved in locally opening the PG for the assembly of macromolecular secretion systems such as T4SS or type III secretion systems are denominated specialized LTs (25, 31).

For G− bacterial systems, it was shown that VirB1-like proteins encoded by T4S genes are important, but some were found not to be essential for the T4SS to be functional, indicating that their function might be partially complemented by chromosomally encoded LTs (32–35). Transfer frequencies of the R1 plasmid were reduced between 5- and 10-fold in a gene *19* (*virB1* homolog) deletion variant (31, 32, 35). *virB1* mutants caused attenuated tumor formation in *Agrobacterium tumefaciens* (33), whereas HP0523 (Cag-gamma), the LT encoded by the *cag* pathogenicity island (PAI) from *Helicobacter pylori*, was found to be essential for CagA translocation into human host cells (36). Because of the thick multilayered PG sacculus in G+ bacteria and because of only a limited number of known muramidases encoded on the G+ bacterial chromosome, it was speculated that VirB1-like proteins could have an essential role in DNA/protein transport via T4SS (4). For G+ bacterial systems, Bantwal et al. (30) showed that the peptidoglycan hydrolase TcpG encoded by the *tcp* transfer locus of plasmid pCW3 is required but not essential for conjugative transfer in *Clostridium perfringens*. For *Lactococcus lactis*, a cell wall synthesis inhibitor with a CHAP domain has been shown to be essential for conjugative transfer of the chromosomally located sex factor (37).

Here, we report our results on the functional characterization of *traG*. TraG exhibits a modular structure. At its N terminus, TraG contains an SLT domain and an *N*-acetyl-D-glucosamine binding site (20, 21). SLTs catalyze the cleavage of the β1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine, as do "goose-type" lysozymes. However, in addition to this, they also form a new glycosidic bond with the C_6 hydroxyl group of the same muramic acid residue. Members are found in phages, type II and type III secretion systems, and T4SS (25). At its C terminus, TraG contains a CHAP domain corresponding to an amidase function. Many proteins with CHAP domains are involved in cell wall metabolism of bacteria; often, the CHAP domain is found in association with other peptidoglycan-cleaving domains. In these cases, the CHAP domain seems to have an endopeptidase specificity, thus opening the interlinks between several glycan strands (26, 27). The TraG CHAP domain also overlaps with an *N*-acetylmuramoyl-L-alanine amidase (PRK08581) domain (20, 21), indicating the putative cleavage of cross-bridges that interlink murein strands in PG (28).

A *traG*-knockout mutant showed that the protein is indispensable for pIP501 transfer between enterococci. *In vivo* localization of the TraG protein revealed an association of the protein with the *Enterococcus* cell envelope. TraG was expressed and purified without its TMH, and TraGΔTMH was demonstrated to possess PG cleavage activity on PG isolated from *Enterococcus* spp. and *E. coli*. Furthermore, we could demonstrate inhibition of TraG's activity by bulgecin A and hexa-*N*-acetylchitohexaose, known inhibitors of LTs, suggesting that the proposed SLT domain was inhibited. There was a residual activity observed with the LT inhibitors which is expected and consistent with the presence of a functionally independent CHAP domain in TraG. In accordance with the proposed two-domain structure of TraG, both domains, when assayed independently, demonstrated PG-degrading activity.

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains used in this work are listed in Table 1. If not stated otherwise, all *E. faecalis* strains were grown in brain heart infusion (BHI) medium (Condalab, Madrid, Spain) at 37°C. BHI medium was supplemented, when required, with the following antibiotics: 50 μg fusidic acid (Fus)/ml and 20 μg chloramphenicol (Cm)/ml for *E. faecalis* JH2-2(pIP501); 1 mg spectinomycin (Spec)/ml, 10 μg tetracycline (Tet)/ml, and 20 μg erythromycin (Em)/ml for *E. faecalis* CK111(pCF10-101, pKAΔ*traG*); 50 μg Fus/ml, 20 μg Cm/ml, and 100 μg gentamicin (Gent)/ml for *E. faecalis* OG1RF(pIP501, pKAΔ*traG*); and 1.5 mg streptomycin (Sm)/ml for *E. faecalis* OG1X. In the case of *E. faecalis* OG1RF(pIP501, pKAΔ*traG*), BHI medium was amended with 200 μg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/ml. *E. coli* XL10 (Stratagene) and BL21-CodonPlus(DE3)-RIL (Stratagene) harboring derivatives of pMAL-c2x (New England BioLabs), pQTEV (38), or pBlueScript SK^- (Stratagene) were grown in LB medium supplemented with 100 μg ampicillin (Ap)/ml and 50 μg Cm/ml at 37°C. *E. coli* DH5α (Invitrogen) harboring pEU327 or derivatives thereof was grown in LB medium amended with 100 μg Spec/ml, *E. coli* EC1000(pCJK47) was grown in BHI medium with 500 μg Em/ml, and *E. coli* EC1000(pKAΔ*traG*) was grown in BHI medium supplemented with 20 μg Gent/ml.

Construction of a *traG* in-frame knockout mutant. A pIP501 *traG* in-frame deletion mutant was obtained using an allelic exchange method described by Kristich et al. (39) with minor modifications. First, the pSK41 Gent resistance gene *aacA-aphD* encompassing both the promoter and terminator sequence (40) was amplified by PCR using primer pairs listed in Table S1 in the supplemental material. The PCR product was cut with BglII and cloned into plasmid pCJK47/BglII, thus delivering the Gent resistance-encoding vector pKA.

Second, fragments of the pIP501 *traG* up- and downstream (1,017-bp and 1,038-bp, respectively) regions were amplified by PCR using primer pairs listed in Table S1 in the supplemental material and subcloned into plasmid pBlueScript SK^- (Stratagene) via EcoRI/BamHI and BamHI/XbaI, respectively. The fused up- and downstream regions were cut with

TABLE 1 Bacterial strains and plasmids used

Strain or plasmid	Genotype or description[a]	Reference or source
Strains		
E. coli		
XL10	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lac* I^qZΔM15 Tn*10*(Tet^r) Amy Cm^r]	Stratagene
BL21-CodonPlus(DE3)-RIL	F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ (DE3) *endA* Hte [*argU ileY leuW* Cm^r]	Stratagene
EC1000	F^- $RepA^+$ *araD139* (*araABC-leu*)*7679 galU galK lacX74 rspL thi* Km^r	61
BL21 Star-pLysS	F^- *omp T hsdSB*(r_B^- m_B^-) *gal dcm rne 131* (DE3) pLysS (Cam^r)	Invitrogen
Top10	F^- *mcr A* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZ YA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* $λ^-$	Invitrogen
One Shot Mach1-$T1^R$	F^- ϕ80(*lacZ*)ΔM15 Δ*lac X74 hsdR*(r_K^- m_K^+) Δ*recA1398 endA1 tonA*	Invitrogen
E. faecalis		
JH2-2	Rif^r Fus^r	62
OG1RF	Rif^r Fus^r	63
CK111	OG1Sp *upp4*::P_{23}*repA4* $Spec^r$	39
OG1X	Sm^r	64
Plasmids		
pIP501	*tra*$^+$ Cm^r MLS^r	65
pMAL-c2x	P_{tac} $lacI^q$ *malE lacZ* Ap^r	New England Biolabs
pQTEV	P_{t4} $lacI^q$ His_7 Ap^r	38
pBlueScript SK^-	pUC *ori lacZ* Ap^r	Stratagene
pCJK47	$oriT_{pCF10}$ P-*pheS** pORI280 derivative Em^r	39
pCF10-101	pCF10Δ*oriT2*	39
pKA	pCJK47 *aacA-aphD* at BglII site; Em^r $Gent^r$	This study
pKAΔ*traG*	pKA with *traG* up- and downstream regions at EcoRI/XbaI sites	This study
pIP501Δ*traG*	pIP501 *traG* in-frame deletion	This study
pEU327	*E. coli*/G+ bacterial shuttle plasmid, $Spec^r$ *xylA* promoter	42
pEU327-*traG*ΔTMH	pEU327 with *traG*ΔTMH	This study

[a] Tet^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Rif^r, rifampin resistance; Fus^r, fusidic acid resistance; $Spec^r$, spectinomycin resistance; Sm^r, streptomycin resistance; Ap^r, ampicillin resistance; $Gent^r$, gentamicin resistance; MLS^r, macrolide-lincosamide-streptogramin B resistance; *tra*$^+$, transfer proficient.

EcoRI/XbaI and inserted into EcoRI/XbaI-cut plasmid pKA, thus delivering pKAΔ*traG*. Subsequently, the conjugative strain *E. faecalis* CK111(pCF10-101) (39) was transformed with the suicidal vector pKAΔ*traG* by electroporation (41). pKAΔ*traG* was maintained in *E. faecalis* CK111 by P_{23}*repA4* encoded in *trans* on the chromosome.

To transfer pKAΔ*traG* from *E. faecalis* CK111(pCF10-101, pKAΔ*traG*) to the recipient *E. faecalis* OG1RF(pIP501), a biparental mating was performed. Transconjugants were selected on BHI medium supplemented with 50 μg Fus/ml, 20 μg Cm/ml, 100 μg Gent/ml, and 200 μg X-Gal/ml and screened for the integration of plasmid pKAΔ*traG* into pIP501 at homologous sites with primer pairs listed in Table S1 in the supplemental material. Transconjugants in which pKAΔ*traG* had integrated up- or downstream of *traG* were grown on MM9YEG agar supplemented with 10 mM DL-*p*-chlorophenylalanine (39) (Sigma-Aldrich) and subsequently screened for *traG* in-frame deletion by PCR. The *traG* in-frame deletion (pIP501Δ*traG*) was verified by PCR with flanking primer pairs binding outside the cloned region (see Table S1) and sequencing of the PCR product.

Western blot analysis of pIP501 T4SS proteins. *E. faecalis* OG1RF, OG1RF(pIP501), and OG1RF(pIP501Δ*traG*) were grown at 37°C in BHI medium overnight. The cultures were centrifuged (4,000 × *g*, 10 min, 4°C), and the pellet was washed in 5 ml of potassium phosphate buffer (50 mM, pH 7.0) and resuspended in 0.75 ml lysis buffer (50 mM KH_2PO_4/K_2HPO_4 [pH 7.0], 1 mM EDTA, 1 mM $MgCl_2$, 10 μl of 1-mg/ml DNase I). The cells were broken by sonication (30% intensity, 30 s with 0.5-s pulses; Sonopuls HD2070; Bandelin). The lysate was kept on ice for 30 min and centrifuged (4,000 × *g*, 5 min, 4°C) to remove unlysed cells. Fifteen microliters of the supernatant was mixed with 6 μl of 4× SDS loading buffer. The samples were loaded onto 18% SDS-polyacrylamide gels, separated by electrophoresis, and blotted onto nitrocellulose membranes (Bio-Rad) using liquid transfer for 90 min at 90 mA (Mini Protean III system; Bio-Rad). The membranes were blocked in RotiBlock blocking solution (Carl Roth GmbH, Karlsruhe, Germany). The pIP501 T4SS proteins, TraH, TraK, and TraM, were detected through immunodetection with primary polyclonal anti-TraHΔTMH, anti-TraKΔTMH, and anti-TraMΔTMH antibodies and a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Promega).

Complementation of pIP501Δ*traG*. To complement the markerless *traG* deletion in *trans*, the expression vector pEU327 (42) was selected. *traG* was amplified from pIP501 with TraG_SalI-fw and TraG_SalI-rev primers. To amplify *traG* with its own ribosomal binding site (RBS), TraG_RBS_SalI-fw and TraG_Sal1-rev primers were used (see Table S1 in the supplemental material). The 1,109- and 1,132-bp PCR products were cut with SalI and inserted into pEU327/SalI. The ligation mixtures were transformed into *E. coli* DH5α, Top10 (Life Technologies), XL10 Gold (Promega), and BL21 Star-pLysS (Life Technologies), respectively, and incubated at 37°C. With all the *E. coli* strains tested, only a few tiny colonies were obtained on selective agar plates (100 μg Spec/ml for pEU327 and 34 μg Cm/ml for *E. coli* BL21 Star-pLysS). No growth of putative transformants was obtained after inoculation into fresh selective solid or liquid medium, respectively, not even after incubation for up to 7 days. The cloning experiment with the *traG* wild-type gene cloned into pEU327 was repeated applying *E. coli* DH5α and One Shot Mach1-$T1^R$ chemically competent *E. coli* cells (Invitrogen) as host and testing distinct incubation temperatures for the selective plates, namely, room temperature, 30°C, and 37°C. Transformants were tested for the insertion of *traG*. None of them contained *traG*. Cloning of *traG* into the expression vector pMSP3535VA (43) also failed. Finally, *traG* without the putative TMH

was amplified from pIP501 with TraG_Δtmh_SalI-fw and TraG_SalI-rev primers. The 1,013-bp PCR product was cut with SalI and inserted into pEU327/SalI. The ligation mixture was transformed into *E. coli* DH5α cells. Transformants obtained after 2 days of growth at 30°C were tested for the insertion of the *traG*ΔTMH fragment by sequencing with pEU327_Test_SalI_fw and pEU327_Test_SalI_rev primers (see Table S1). Plasmid DNA of pEU327-*traG*ΔTMH was electroporated into *E. faecalis* OG1RF(pIP501Δ*traG*).

Filter matings. Overnight cultures were diluted 1:10 in fresh BHI medium and incubated until an optical density at 600 nm (OD_{600}) of 0.5 was reached. A 1:10 mixture of donor *E. faecalis* OG1RF(pIP501) and *E. faecalis* OG1RF(pIP501Δ*traG*), respectively, and recipient (*E. faecalis* OG1X) cells were collected on a sterile nitrocellulose membrane filter (0.45 μm; Millipore). The membrane was incubated overnight cell-side-up on BHI agar at 37°C. Serial dilutions of the cells recovered by suspension in 1 ml phosphate-buffered saline (PBS) were plated on BHI agar supplemented with 1 mg Sm/ml to enumerate recipients and 1 mg Sm/ml and 20 μg Cm/ml to enumerate transconjugants, respectively. To analyze complementation of the pIP501Δ*traG* in-frame deletion in *trans*, matings were performed with *E. faecalis* OG1RF(pIP501Δ*traG*), *E. faecalis* OG1RF(pIP501Δ*traG*, pEU327-*traG*ΔTMH), *E. faecalis* OG1RF(pIP501Δ*traG*, pEU327), and *E. faecalis* OG1RF(pIP501) as donor, respectively, and *E. faecalis* OG1X as recipient. Transconjugants were selected on BHI agar supplemented with 1.5 mg Sm/ml, 20 μg Em/ml, and 20 μg Cm/ml. Recipients were selected on BHI agar amended with 1.5 mg Sm/ml. The matings were repeated three times.

Expression and purification of TraGΔTMH, SLT_{TraG}, $SLT(E87G)_{TraG}$, and $CHAP_{TraG}$ for biochemical studies. All proteins were expressed as maltose binding protein (MBP) fusion proteins and purified as described in the supplemental material (see Fig. S1). Prior to application in the muramidase assay, they were concentrated to 0.7 mg/ml (TraGΔTMH) and to 5 mg/ml for the protein domains.

L-[^{3}H]lysine labeling of PG from *E. faecalis*. Five milliliters of an *E. faecalis* JH2-2 overnight (o/n) culture was transferred to 100 ml Todd-Hewitt broth (THB) (supplemented with 25 μg rifampin [Rif]/ml). One milliliter L-[^{3}H]lysine (1 mCi) was added, and the culture was grown at 37°C and 225 rpm to an OD_{540} of 0.5. Cells were harvested (7,000 × g, 15 min, 4°C) and washed with 8 ml 25 mM Tris-HCl (pH 8.0) at 4°C. The pellet was vacuum dried and suspended in 8 ml 4% SDS. The following steps were performed as described under "PG isolation and purification" in the supplemental material. The labeled PG was lyophilized and stored at −80°C.

2,6-[^{3}H]diaminopimelic acid labeling of PG from *E. coli*. Five milliliters of an *E. coli* DH5α o/n culture was transferred to 100 ml M9 minimal broth, and 1 ml 2,6-[^{3}H]diaminopimelic acid (1 mCi) was added. Incubation at 37°C continued until stationary phase was reached (OD_{600} of ca. 1.0). The protocol continued as described under "PG isolation and purification from *E. coli* DH5α" in the supplemental material.

Muramidase assay. The muramidase assay is based on the measurement of the solubilization of L-[^{3}H]lysine-labeled PG from *E. faecalis* and 2,6-[^{3}H]diaminopimelic acid-labeled PG from *E. coli* (31). Labeled PG (approximately 5,000 cpm) was incubated with 3 μM respective protein in a total volume of 100 μl 20 mM Bis-Tris buffer (pH 5.3) for 30 min at 37°C. One percent cetyltrimethylammonium bromide (CTAB) was added to precipitate the insoluble substrate. Samples were kept on ice for 30 min. After centrifugation (4 min at 17,000 × *g* and 4°C), 160 μl of the supernatant was added to 7 ml scintillation cocktail (Ionophor Gold scintillation cocktail; Fuji, Tokyo, Japan). Radioactivity was measured using the Wallac 1409 liquid scintillation counter (PerkinElmer). Purified MBP and lysozyme from hen egg white were used as negative and positive controls, respectively.

Cy3 labeling of PG. Cy3 labeling of PG was performed according to the method of Zahrl et al. (31) with modifications. Five hundred microliters PG from *E. faecalis* JH2-2 (1 mg PG in 500 μl distilled water) was incubated with 50 μl 1 M borate buffer (pH 9.6) and 5 μl Cy3 *N*-hydroxysuccinimide (NHS) ester (1 mg/ml in dimethyl formamide) for 60 min at room temperature. Excess Cy3 was removed by centrifugation (5,000 × *g*, 5 min at 4°C). Cy3-labeled PG was stored at −20°C.

Cy3 spot assay. The Cy3 spot assay was performed according to the method of Zahrl et al. (31) with modifications. Eight-well glass slides were coated with poly-L-lysine (Sigma-Aldrich) for 30 min at room temperature, rinsed with deionized water, and dried. Ten microliters deionized water and 1 μl Cy3-labeled PG (containing approximately 2 μg PG) were spotted onto the poly-L-lysine-coated wells and incubated at room temperature for 45 min in the dark. The slides were rinsed with deionized water for 2 min, dried at room temperature in the dark, and scanned with a GenePix 4000B microarray scanner with 532-nm excitation (GenePix Pro 4.1 software). Ten microliters Bis-Tris buffer (pH 5.3) was loaded onto the PG, and approximately 6 pmol of the respective protein, lysozyme, TraGΔTMH, and MBP, was added. The slides were incubated at 37°C for 2 h 30 min. To remove the digested material, the slides were rinsed with deionized water for 2 min and dried at room temperature. To quantitate the fluorescence signals, dry slides were scanned before and after incubation with the enzyme using an array scanner (Axon GenePix 4000B; photomultiplier tube [PMT] setting, 320; scan power, 33%), and images were obtained and analyzed using the GenePix Pro 4.1 program. Line scans from the images were produced using a 50-pixel (2-mm) window across the center of the PG spots. Relative PG-degrading activity of TraGΔTMH was calculated using the following formula: 100 − [(Fi532b/Fi532a) × 100], where Fi532a is the mean fluorescence intensity of Cy3-labeled PG, corresponding to a total of 460 pixels in a circle with a diameter of 1 mm, before incubation and Fi532b is the same value after incubation, respectively.

To test inhibitory effects of the specific lytic transglycosylase blockers bulgecin A (Hoffman-La Roche) and hexa-*N*-acetylchitohexaose (Seikagaku, Japan) on the PG hydrolysis activity of TraGΔTMH, the Cy3 spot assay was repeated with the exception that prior to addition of the respective protein the lytic transglycosylase blockers were added in concentrations ranging from 0.5 to 4 mM for hexa-*N*-acetylchitohexaose and 1 to 20 mM for bulgecin A. Data analysis and calculation of relative PG-degrading activities were done as described above.

Subcellular fractionation of *E. faecalis* JH2-2(pIP501) and immunolocalization of TraG. Subcellular fractionation of *E. faecalis* JH2-2(pIP501) was performed according to the method of Buttaro et al. (44) with modifications. Briefly, *E. faecalis* JH2-2(pIP501) was grown at 37°C in BHI medium to an OD_{600} of 0.5. The culture was chilled on ice for 15 min, washed twice in an equal volume of potassium phosphate buffer (50 mM, pH 7.0), and resuspended (1:50 [vol/vol]) in lysis buffer (50 mM KH_2PO_4/K_2HPO_4 [pH 7.0], 1 mM EDTA, 1 mM $MgCl_2$, 100 μg/ml DNase, 100 μg/ml RNase). The cells were broken by FastPrep-24 (MP Biomedicals) using lysing matrix E (1.4-mm ceramic spheres, 0.1-mm silica spheres, and 4-mm glass beads; MP Biomedicals). The lysate was centrifuged (1,500 × *g*, 20 min, 4°C) to remove unlysed cells. The supernatant was transferred and centrifuged at 17,000 × *g* for 20 min at 4°C to collect the cell wall fraction. Membranes were harvested by ultracentrifugation of the supernatant at 163,000 × *g* for 2 h at 4°C (50 Ti rotor; OTD Combi ultracentrifuge; Thermo Fisher Scientific GmbH) and resuspended in 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 1% Triton X-100. The remaining supernatant contained the cytoplasmic fraction of proteins.

Subsequently, equal amounts of the cell wall, membrane, and cytoplasmic fraction were applied onto SDS-polyacrylamide gels, separated by electrophoresis, and blotted onto nitrocellulose membranes (Bio-Rad) using liquid transfer for 90 min at 90 mA (Mini Protean III system; Bio-Rad). The membrane containing the transferred proteins was initially blocked in RotiBlock blocking solution (Carl Roth GmbH, Karlsruhe, Germany). TraG was then localized in the fractions (cell wall, membrane, and cytoplasm) by immunostaining of TraG with primary polyclonal anti-TraGΔTMH antibodies and a secondary HRP-conjugated anti-rabbit IgG antibody (Promega).

RESULTS

TraG is an essential T4SS protein. The indispensability of VirB1 and homologous proteins could not be demonstrated for T4SS originating from G− bacteria (31–35). However, due to the multilayered and cross-linked PG meshwork in G+ bacteria, we raised the question whether a specific PG degradation caused by the putative lytic transglycosylase TraG might be a crucial step during conjugative pIP501 DNA transfer in G+ bacteria. To investigate the potential indispensability of TraG in conjugative transfer, we constructed a pIP501Δ*traG* in-frame deletion mutant using the PheS counterselection markerless exchange system (39). Ninety-seven percent of the TraG coding region was thereby deleted, except for the first 5 N-terminal codons and the last 6 C-terminal codons, to not alter transcription and translation of downstream *tra* genes.

We assessed the influence of the *traG* deletion on conjugative transfer of pIP501 by biparental matings. Assuming the indispensability of TraG, no transfer should occur in the *traG*-knockout mutant in a biparental mating in G+ bacteria, whereas transfer rates with an isogenic, *traG*-proficient pIP501 plasmid should be in the expected range of approximately 10^{-5} transconjugants per recipient. The biparental matings were performed with donor strains *E. faecalis* OG1RF(pIP501Δ*traG*) and isogenic *E. faecalis* OG1RF(pIP501), respectively, and *E. faecalis* OG1X as recipient.

The isogenic *E. faecalis* OG1RF(pIP501) originated from the same *E. faecalis* OG1RF strain that harbored the merodiploid pIP501-pKAΔ*traG* complex. Both the *traG*-knockout plasmid pIP501Δ*traG* and pIP501 used for the biparental mating assay were obtained after excision and segregation of the suicide vector pKAΔ*traG* from the pIP501-pKAΔ*traG* complex.

Transfer rates of the *traG* deletion mutant were below the detection limit of the assay ($<3.5 \times 10^{-9} \pm 2.4 \times 10^{-9}$, mean value of three independent assays), whereas transfer rates with the isogenic strain *E. faecalis* OG1RF(pIP501) were at least 20,000 times higher and in the expected range for pIP501 of $7.6 \times 10^{-5} \pm 5.7 \times 10^{-5}$ transconjugants per recipient cell (mean value of three independent assays). As transfer frequencies were in the expected range for pIP501, we can exclude polar effects due to unwarranted suicide vector integration into pIP501. Unfortunately, we were not able to complement the *traG* knockout in *E. faecalis* pIP501Δ*traG* by supplying the *traG* wild-type (wt) gene on an expression plasmid in *trans*. Despite several attempts, it was not possible to clone *traG* in *E. coli*/G+ bacterial shuttle vectors. A possible explanation is that TraG is toxic in *E. coli* even if basal expression is very low. In the natural plasmid context, expression of all pIP501 *tra* genes is tightly controlled by the first gene product of the *tra* operon, TraA (18). Furthermore, we tried to complement the *traG* deletion by supplying *traG*ΔTMH in *trans*. We obtained transformants of pEU327-*traG*ΔTMH in *E. coli*, and the plasmid DNA was subsequently electroporated into *E. faecalis* OG1RF(pIP501Δ*traG*). However, transfer activity of pIP501 could not be restored by supplying *traG*ΔTMH in *trans*.

A complete transfer deficiency was observed for the *traG*-knockout strain (*E. faecalis* pIP501Δ*traG*) and the complementation strain (*E. faecalis* pIP501Δ*traG*, pEU-327-*traG*ΔTMH), as well as for the negative control (*E. faecalis* pIP501Δ*traG*, pEU-327), in three independently performed assays. Transfer frequencies of pIP501 were in the expected range of approximately 5×10^{-5} transconjugants/recipient under the same conditions.

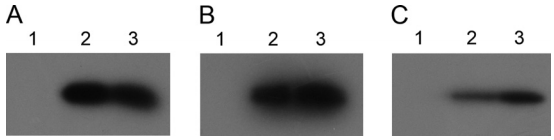

FIG 2 Expression of *traG* downstream genes in the pIP501 *tra* operon is not affected by *traG* deletion. Immunodetection of proteins TraH (A), TraK (B), and TraM (C) using polyclonal antibodies against the respective proteins showed comparable expression levels for all three proteins in lysates from *E. faecalis* OG1RF(pIP501 wt) and *E. faecalis* OG1RF(pIP501Δ*traG*). The plasmid-free *E. faecalis* OG1RF strain was used as a negative control. Lanes 1, *E. faecalis* OG1RF lysate; lanes 2, *E. faecalis* OG1RF(pIP501 wt) lysate; lanes 3, *E. faecalis* OG1RF(pIP501Δ*traG*) lysate.

To ensure that the excision of *traG* by allelic exchange did not alter transcription and translation of downstream *tra* genes in pIP501Δ*traG* that could have caused the failure of complementation, we performed immunoblot assays with protein lysates obtained from *E. faecalis* OG1RF(pIP501) and *E. faecalis* OG1RF(pIP501Δ*traG*). Three genes located downstream of *traG* in the pIP501 *tra* operon were selected for immunodetection with the respective polyclonal anti-Tra antibodies, namely, *traH*, *traK*, and *traM*. The expression level of all three genes was unaltered in the *traG*-knockout mutant (Fig. 2), proving that deletion of 97% of the *traG* coding region exerts no negative effect on expression of the downstream genes in the pIP501 *tra* operon.

Therefore, the inability of TraGΔTMH to complement the *traG* knockout could most probably be caused by an incorrect folding of TraGΔTMH due to the missing putative TMH or by a misled location of TraGΔTMH within the enterococcal cell or both.

TraG localizes to the *E. faecalis* cell envelope. T4SS of G− bacteria are multiprotein complexes that span the cell envelope (6, 7). It is likely that, due to its indispensability for conjugative pIP501 transfer and its predicted PG-degrading activity, TraG localizes within the cell envelope. *In silico* predictions postulated TraG being predominantly localized in the cell wall fraction (PSORTb v.3.0.2 [45]) with a TMH (amino acid [aa] positions 17 to 36, CAD44387, HMMTOP [46]) and a possible signal peptide (putative cleavage site between aa 47 and 48, CAD44387, SignalP3.0 [47]). To localize TraG *in vivo*, an exponentially growing culture of *E. faecalis* JH2-2(pIP501) was fractionated into cell wall, membrane, and cytoplasmic fractions according to the method of Buttaro et al. (44) with modifications. As expected, TraG was exclusively found in the cell envelope fractions (cell wall and membrane, Fig. 3A). Other results from our lab confirmed that the cell wall fractions were not contaminated with cytoplasmic proteins, since the pIP501 Tra protein TraN, under the same conditions, was exclusively found in the cytoplasmic fraction (Fig. 3B). This is in agreement with the *in silico* prediction for the cytoplasmic localization of TraN in G+ bacteria (N. Goessweiner-Mohr, K. Arends, E. Grohmann, and W. Keller, unpublished data).

TraGΔTMH degrades peptidoglycan and is inhibited by hexa-*N*-acetylchitohexaose and bulgecin A. To demonstrate the sequence-inferred PG-degrading activity of TraG *in vitro*, we performed an assay as described by Zahrl et al. (31) using Cy3-labeled PG from *E. faecalis* JH2-2. PG-hydrolyzing activity was calculated by comparing fluorescence levels of Cy3 PG spots on a glass surface before and after enzyme treatment (for details, see Materials and Methods). To visualize PG degradation, line

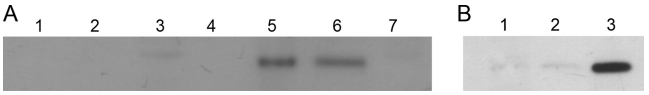

FIG 3 (A) TraG subcellular localization. TraG localizes to the cell envelope of *E. faecalis* JH2-2 harboring pIP501. The localization of TraG in the cell fractions was detected by immunoblotting with polyclonal anti-TraG antibodies. Plasmid-free *E. faecalis* JH2-2 was applied as a negative control. Lane 1, cell wall fraction; lane 2, membrane fraction; lane 3, cytoplasmic fraction; lane 4, empty well; lane 5, cell wall fraction; lane 6, membrane fraction; lane 7, cytoplasmic fraction; lanes 1 to 3 represent samples from *E. faecalis* JH2-2 cells without pIP501; lanes 5 to 7 contain samples from pIP501-harboring cells. (B) TraN subcellular localization. TraN localizes to the cytoplasmic fraction and thus serves as a negative control for the localization of TraG to the cell envelope. Lane 1, cell wall fraction; lane 2, membrane fraction; lane 3, cytoplasmic fraction.

scans through the PG spots before and after treatment were superimposed (Fig. 4). PG degradation was observed in the case of TraGΔTMH and lysozyme (99%), whereas for purified MBP, which was used as a negative control in all of the performed assays, no or only minimal background activity (as in this case, 15%) was seen (Fig. 4A).

To investigate a possible lytic transglycosylase activity of TraGΔTMH, we repeated the Cy3 spot assay in the presence of the specific lytic transglycosylase blockers hexa-*N*-acetylchitohexaose and bulgecin A, respectively. For hexa-*N*-acetylchitohexaose, which binds to the active center of LTs but is not a substrate for these enzymes (48–50), concentrations from 0.5 mM to 4 mM were tested: the addition of 4 mM inhibitor reduced TraGΔTMH activity, resulting in a residual activity of 27% (Fig. 4B). For bulgecin A, concentrations between 1 mM and 20 mM were added (Fig. 4C). Concentrations of 5 to 20 mM bulgecin A resulted in 21 to 25% residual TraGΔTMH activity (no PG degradation occurred with the MBP control in this experiment; data not shown). Since bulgecin A, a specific glycopeptide inhibitor of LTs (51), is known to bind to the active site of LTs and is frequently used as a ligand in crystallographic studies revealing the structure and function of LTs (52, 53), our data suggest binding (at 5 mM bulgecin A) of the inhibitor to the predicted SLT domain, leaving the CHAP domain uninhibited. To accurately quantify PG degradation activity of TraGΔTMH and its domains, a radioactive muramidase assay was performed.

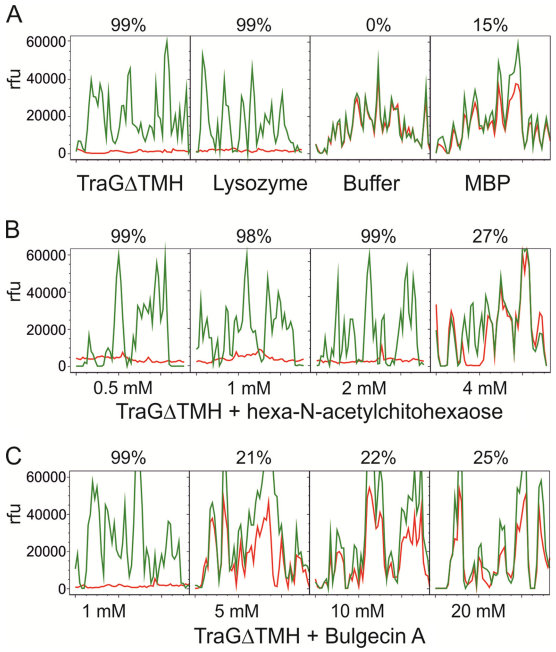

FIG 4 Cy3 PG degradation by TraGΔTMH. Cy3-labeled PG was measured before (green lines) and after (red lines) digestion. Line scans are shown for a window of 50 pixels (2 mm). rfu, relative fluorescence units. Values above each box indicate the reduction of the fluorescence in each Cy3 PG spot and therefore reflect enzymatic activity (for details, see Materials and Methods). (A) Cy3 PG was digested with the indicated proteins or incubated with buffer alone. (B and C) Inhibition of TraGΔTMH with indicated concentrations of hexa-*N*-acetylchitohexaose and bulgecin A, respectively.

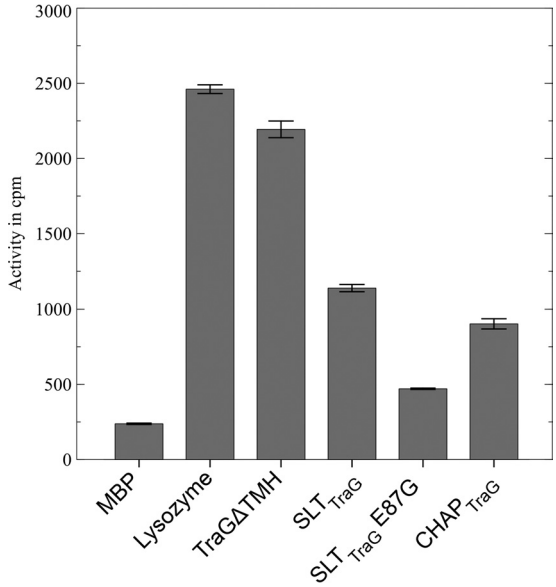

FIG 5 TraGΔTMH and mutant protein muramidase activity on L-[^{3}H]lysine-labeled PG from *E. faecalis* JH2-2. Activities (in cpm) are given as average values of three independent measurements with standard deviations. MBP and lysozyme (from hen egg white) were applied as negative and positive controls, respectively.

TraG SLT and CHAP domains degrade PG independently of each other. To further analyze whether the residual TraGΔTMH activity correlates with a putative PG-degrading activity of the CHAP domain, we expressed and purified both domains independently. PG hydrolase activity of TraGΔTMH and its N-terminal SLT and C-terminal CHAP domain was investigated by a radioactive muramidase assay in which solubilized cell wall components were measured with two different substrates (PG from G+ and G− bacteria, respectively). TraGΔTMH showed high muramidase activity (1,955.6 cpm after subtracting background activity of 215.5 cpm) when using L-[^{3}H]lysine-labeled PG from *E. faecalis* JH2-2 and was comparable to the activity of lysozyme from hen egg white (2,438 cpm) under the same conditions. SLT_{TraG} still exhibited 46% (900.07 cpm) of TraGΔTMH enzyme activity (1,955.6 cpm), and $CHAP_{TraG}$ exhibited 34% of TraGΔTMH activity. A single-amino-acid change in the putative catalytic center of $TraG_{SLT}$ (E87G) resulted in a 74% reduction of muramidase activity as measured for the $SLT(E87G)_{TraG}$ domain in comparison to SLT_{TraG} (Fig. 5).

DISCUSSION

In this work, we demonstrated the essential function of the VirB1-like PG hydrolase TraG in conjugative plasmid transfer of broad-host-range plasmid pIP501. To the best of our knowledge, this is the first time that indispensability of a VirB1-like PG hydrolase could be shown for T4SS-mediated DNA/protein transport. Bantwal and coworkers constructed deletion mutants of the pCW3 *virB1* homolog, *tcpG*. The pCW3Δ*tcpG* mutants showed ca.-1,000-fold-reduced conjugative plasmid transfer capacity (30) whereas the pIP501Δ*traG* variant did not show any residual transfer activity in intraspecies transfer between different *E. faecalis* strains. Bantwal et al. (30) explained the residual pCW3 transfer activity by putative functional redundancy between PG hydrolases produced by *C. perfringens*, with chromosomally encoded enzymes able to catalyze the PG degradation required for conjugative transfer, albeit at lower efficiency. For *E. faecalis*, only scarce information on genomic PG hydrolases is available; most literature deals with the two autolysins, AtlA and Atn, and their role in autolysis of *E. faecalis* cells as well as in DNA-dependent *Enterococcus* biofilm development (54–56). No putative role in controlled local opening of the PG has been proposed for these enzymes.

Both TraG domains, the SLT_{TraG} and the $CHAP_{TraG}$ domains, have been expressed separately and shown to possess PG degradation activity on PG from *E. faecalis* (Fig. 5). PG degradation activity of the SLT_{TraG} domain is in agreement with lytic transglycosylase activity of several putative lytic transglycosylases encoded by G− bacterial T4SS, P19 from the conjugative resistance plasmid R1, VirB1 encoded by the Ti plasmid of *A. tumefaciens*, VirB1 from the small chromosome of *Brucella suis*, and HP0523 encoded by the *cag* pathogenicity island of *H. pylori* (31); AtlA from the *Neisseria gonorrhoeae* T4SS-secreting DNA (57); and TcpG encoded by the G+ bacterial T4SS from *C. perfringens* plasmid pCW3 (30).

We mutated the putative catalytic residue of SLT_{TraG}, the glutamate at position 87 (GenBank sequence accession no. CAD44387.1), by replacement with glycine, generating $SLT(E87G)_{TraG}$ and introducing a BamHI restriction site to facilitate screening for mutants. Surprisingly, the mutant protein retained about 26% of its LT activity compared to SLT_{TraG}. Supposedly, the mutated catalytic active glutamate at position 87 (GenBank CAD44387.1) in TraG could be partially complemented by a second putative ES motif at positions 100 to 101 (GenBank CAD44387.1) (positions 48 to 49, HMM logo, http://pfam.sanger.ac.uk/family/PF13702.1#tabview=tab4 [58]), thus explaining the strongly reduced, but not completely abolished, activity of $SLT(E87G)_{TraG}$.

TraGΔTMH PG-degrading activity was significantly reduced by the specific lytic transglycosylase inhibitors bulgecin A and hexa-*N*-acetylchitohexaose (48, 49, 51), suggesting a possible lytic transglycosylase activity for TraG. For IpgF, the lytic transglycosylase from the plasmid-encoded type III secretion system of *Shigella sonnei* (59, 60), a similar observation was made with hexa-*N*-acetylchitohexaose. It completely inhibited IpgF at a concentration of 4 mM. Zahrl et al. (31) showed that, in contrast, lysozyme was not affected by the presence of hexa-*N*-acetylchitohexaose, which is in accordance with the observation that this substance is not a substrate for lysozyme and solely inhibits lytic transglycosylases (48, 49). Zahrl et al. (31) also tested bulgecin A, which has also been described as an inhibitor of lytic transglycosylases (51), and found that a concentration of 20 mM was sufficient to completely inhibit IpgF. TraGΔTMH, however, was not totally inhibited by bulgecin A. Its activity was reduced to around 21 to 25% residual activity at 5 mM and higher (up to 20 mM) bulgecin A concentrations, which could be due to binding of bulgecin A to the SLT domain but not to the CHAP domain. This observation is consistent with the SLT and CHAP domains of TraG representing independent domains, with both displaying PG-degrading activity.

TraGΔTMH was also able to degrade 2,6-[^{3}H]diaminopimelic acid-labeled PG from *E. coli* DH5α, albeit with less activity. For TraGΔTMH, 2,398 cpm of soluble PG fragments was measured; for lysozyme, we obtained 6,712 cpm, thus resulting in 38% activity for TraGΔTMH compared to lysozyme. These data are in agreement with the broad host range of pIP501, which was, in addition to self-transfer to virtually all Gram-positive bacteria, shown to transfer in and be stably maintained in *E. coli* (16). The reduced TraGΔTMH PG degradation activity observed for *E. coli* PG might be compensated for by other LTs encoded on the *E. coli* chromosome.

Our data strongly suggest that TraG is a PG-degrading protein that is indispensable for the intraspecies conjugative transfer of pIP501 in *E. faecalis*. To the best of our knowledge, it is the first time that indispensability of a VirB1-homologous protein has been shown for conjugative plasmid transfer.

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