Induction of Plasmid Conjugation in Bacillus subtilis Is Bistable and Driven by a Direct Interaction of a Rap/Phr Quorum-sensing System with a Master Repressor

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Background: Plasmid pLS20 conjugates only during growth of cells. Results: DNA binding activity of repressor protein Rco is repressed through binding of Rap protein, which is counteracted by a Phr peptide.

Conclusion: Plasmid-encoded Rap and Phr directly restrict the timing of conjugation to exponential growth, leading to bistable expression of conjugation genes.

Significance: A Rap/Phr/repressor system mediates the unusual timing of plasmid conjugation.

Conjugation of plasmid pLS20 from Bacillus subtilis is limited to a time window between early and late exponential growth. Genetic evidence has suggested that pLS20-encoded protein RcoLS20 represses expression of a large conjugation operon, whereas Rap protein RapLS20 relieves repression. We show that RapLS20 is a true antirepressor protein that forms dimers in vivo and in vitro and that it directly binds to the repressor protein RcoLS20 in a 1:1 stoichiometry. We provide evidence that RapLS20 binds to the helix-turn-helix-containing domain of RcoLS20 in vivo, probably obstructing DNA binding of RcoLS20 as seen in competitive DNA binding experiments. The activity of RapLS20 is in turn counteracted by the addition of the cognate PhrLS20 peptide, which directly binds to the Rap protein and presumably induces a conformational change of the antirepressor. Thus, a Rap protein acts directly as an antirepressor protein during regulation of plasmid conjugation, turning on conjugation, and is counteracted by the PhrLS20 Peptide, which, by analogy to known Rap/Phr systems, is secreted and taken back up into the cells, mediating cell density-driven regulation. Finally, we show that this switchlike process establishes a population heterogeneity, where up to 30% of the cells induce transcription of the conjugation operon.

Conjugative elements are either carried on plasmids or maintained in genomes as integrative conjugative elements (ICEs)2 (2). Transfer of ssDNA occurs via type IV secretion systems (3) and needs to be tightly regulated to minimize the metabolic burden on the host caused by transcription and synthesis of conjugation genes/proteins and the conjugal transfer itself. Therefore, conjugation genes are either kept in a default OFF state and need to be induced by signaling molecules to be switched ON, or conjugation genes are constitutively expressed but limited in their expression in order to not increase the fitness costs of the host. Constitutive systems, such as those of the IncF plasmid family, integrate transcriptional cues of plasmid and host factors as well as environmental stimuli to control the expression of their transfer region. Inducible conjugation systems either rely on sensing of phenolic compounds like the Ti-plasmids from Agrobacterium tumefaciens or on quorum-sensing systems using homoserine lactones as signaling molecules in Gram-negative bacteria or signaling peptides in Gram-positive bacteria (4). Among the peptide-regulated systems, regulation of conjugation of the Enterococci plasmids pCF10 and pAD1 has been extensively studied. Conjugative transfer of these plasmids is controlled through the ratio of two peptides; a plasmid-encoded peptide (iCF10/iAD1) promotes the inhibitory function of the master regulator (TraA/PrgX) by keeping the conjugation operon in an OFF state, and a chromosomally encoded peptide (cCF10/iAD1) found in many Enterococci strains relieves transcriptional repression of the conjugation operon through competitive binding to the master regulator at high concentrations. Thus, in the presence of plasmid-free cells, the conjugation operon is turned ON, whereas at high donor cell densities, the operon is turned OFF. In Bacillus subtilis, the quorum-sensing Rapl-Phrl module regulates the transfer of the mobile genetic element ICEBs1, which can also be activated by the RecA-dependent SOS response. In contrast

Horizontal gene transfer is a central driving force of bacterial genome evolution and plasticity. Beside transformation and transduction, conjugation is the major determinant in the spread of genetic information among bacteria, endowing recipient cells with new traits, such as catabolic pathways, antibiotic resistance, or even virulence (1, 2).

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The abbreviations used are: ICE, integrative conjugative element; Bicine, N,N-bis(2-hydroxyethyl)glycine; qRT-PCR, quantitative RT-PCR.
to PrgX, RapI activates excision and conjugation of ICEBs1 through induction of cleavage of the master regulator ImmR, which is inhibited at high concentrations of PhrI (5).

Rap proteins have been best characterized for their role in the regulation of developmental processes, such as sporulation and competence in B. subtilis (6). Phr peptides are secreted and are generally modified; their gene is located downstream of the gene encoding for their cognate Rap protein, which in most cases is a phosphatase. Dephosphorylation of aspartate response regulators prevents the activation of the master regulator of sporulation, Spo0A. Spo0A receives a phosphoryl group through a relay including the response regulator Spo0F, which the Rap protein acts upon. Increasing levels of processed Phr peptides are taken back up into the cells as cell density rises, and during the transition to stationary phase, enough peptide has accumulated to efficiently bind to the Rap protein, displacing it from the response regulator, such that the phosphorelay can proceed toward initiation of sporulation. During competence development, ComP phosphorylates the response regulator ComA in response to high cell density. Phosphorylation of ComA triggers its DNA binding activity, which leads to the expression of several genes, ultimately resulting in the expression of late competence genes. Unlike sporulation-inhibiting Rap proteins, Rap proteins RapF and RapC act on ComA not via inhibition of its function through dephosphorylation, but through allosterically blocking its DNA-binding domain (7–9) and thereby delaying activation of competence genes until a high concentration of the corresponding Phr is reached.

Recently, it was found that a Rap/Phr-like module also plays a role in the regulation of transfer of the conjugative plasmid pLS20 from B. subtilis natto (10), which shows an unusual expression of several genes, ultimately resulting in the expression of late competence genes. Unlike sporulation-inhibiting Rap proteins, Rap proteins RapF and RapC act on ComA not via inhibition of its function through dephosphorylation, but through allosterically blocking its DNA-binding domain (7–9) and thereby delaying activation of competence genes until a high concentration of the corresponding Phr is reached.

Materials and Methods

Bacterial Strains, Plasmids, and Strain Construction—Bacterial strains, plasmid, and primers used in this study are listed in Table 1. Escherichia coli strain DB3.1 was used to propagate ccdB-containing plasmids. Mach1 cells were used for construction of plasmids, and Rosetta (DE3) 2 or BL21 (DE3) 2 cells were used for expression of proteins in E. coli. E. coli and B. subtilis cells were grown in LB medium at 30 or 37 °C, supplemented with the appropriate antibiotics at the following final concentrations: 100 μg ml⁻¹ ampicillin, 5 or 15 μg ml⁻¹ chloramphenicol, (B. subtilis or E. coli), 10 or 50 μg ml⁻¹ kanamycin (B. subtilis or E. coli), 25 μg ml⁻¹ lincomycin, 1 μg ml⁻¹ erythromycin, 100 μg ml⁻¹ spectinomycin, and 5 μg ml⁻¹ phleomycin.

To transfer the deletion of sigW, rsIW, and the overexpression construct of rsiW in the background of the PY79 pLS20neo strain, PY79 pLS20neo cells were transformed with 0.1–1 μg of chromosomal DNA from the cognate strains listed in Table 1. To generate a Gateway-accessible vector for B. subtilis, the dest42 cassette containing a ccdB gene, a chloramphenicol resistance gene, and the required attachment (att) sites were introduced into plasmid pSG1193 (14). The dest42 cassette was amplified by PCR using primer pair DEST42_KpnI/DEST42_XhoI and plasmid pCGFP (15) as template. The PCR product was digested with KpnI and Xhol and cloned into correspondingly digested pSG1193, creating plasmid pDESTBs1.

All expression plasmids were generated using the Gateway cloning system (Invitrogen). To generate entry clones of rco, rap, ΔC-rco, and ΔN-rco, the genes were amplified by PCR using pLS20neo as DNA template and primer pairs Rco_FW/Rco_STOP_RV, Rap_FW/Rap_RV, ΔC-Rco_FW/ΔC-Rco_STOP_RV, and ΔN-Rco_FW/ΔN-Rco_STOP_RV. All PCR products were cloned in pENTR-D-TOPO according to the protocol of the manufacturer. To generate the expression plasmids of the individual genes, we used the Gateway LR Clonase II enzyme mix (Invitrogen). All plasmids were sequenced to verify that the genes were integrated in the right orientation.

To generate a transcriptional fusion between the promoter of the conjugation operon (Pc) and a fluorescent reporter gene, the promoter region and the mcherry gene were PCR-amplified using primer pairs Pc_FW/Pc_RV and mcherry_FW/mcherry_RV and the templates pLS20 and pSG1164-mcherry. PCR products were digested using BamHI/HindIII (Pc) and NheI/EcoRI (mcherry) and ligated into the similarly digested pDG1664 plasmid.

Conjugation Assays—Mating experiments were performed as described previously (16) using strain PG300 as a recipient. For induction of rco gene expression, xylose was added to the medium at a final concentration of 0.5%. Accordingly, the addition of 1 mm isopropyl-1-thio-β-d-galactopyranoside to the growth medium induced expression of rsiW.

Qualitative and Quantitative RT-PCR—Isolation of total RNA, DNase I treatment, and reverse transcription were performed as described previously (16). For the qualitative RT-PCR analysis of the conjugation operon, 1.5 μl of the cDNA reactions, 1.5 μl of the cDNA reactions omitting reverse transcriptase, and 1.5 μl of pLS20neo DNA were used in a 30-μl
PCR performed with Q5 High Fidelity DNA polymerase and the primers indicated in Fig. 1A and listed in Table 2. Quantitative RT-PCRs of rco and virB11 in the corresponding background strains harvested during exponential growth were conducted as indicated previously (16).

**Protein Expression and Purification**—Rco and RapLS20 were overexpressed as N-terminal His6 tag fusion proteins. Single colonies of freshly transformed Rosetta (DE3) 2 cells were inoculated in LB medium containing ampicillin and grown overnight at 30 °C. The next day, cells were diluted 1:100 in LB medium containing ampicillin and grown until an optical density of 0.4 at 30 °C. Overexpression of Rco and RapLS20 was induced by the addition of 1 and 0.25 mM isopropyl 1-thio-β-D-galactopyranoside, and incubation of cells continued for 3–4 h before cells were harvested by centrifugation (4,000 rpm, 20 min). Cells were washed with LEW buffer (50 mM NaH2PO4, 300 mM NaCl; 4 °C, 4,000 rpm, 20 min) and frozen at −20 °C.

Frozen cells were resuspended in LEW buffer containing Complete Protease Inhibitor Mixture (Roche Applied Science), 1 µg of DNase, and 0.1 mg/ml lysozyme. Cells were homogenized by three passages through a French press cell and centrifuged at 15,000 rpm for 30 min at 4 °C. The lysates were cleared through a 0.45-µm syringe filter (Filtropur S, Sarstedt) and applied to a Ni-TED 2000 column (Macherey-Nagel). The column was washed with 20 column volumes of LEW, and the proteins were eluted in increasing steps of 25 mM imidazole. Fractions containing His6-Rco were pooled and concentrated by ultrafiltration (Vivaspin 20, 10,000 molecular weight cut-off; Sartorius Stedim). The concentrate was further polished by size exclusion chromatography equilibrated in LEW buffer. Fractions containing Rco were concentrated by ultrafiltration. Glycerol was added to a final concentration of 50%, and aliquots were stored at −20 °C.

His6-Rap was identically purified by affinity chromatography as His6-Rco. Fractions containing His6-Rap were concentrated by ultrafiltration (Vivaspin 20, 10,000 molecular weight cut-off; Sartorius Stedim) and further polished by size exclusion chromatography. For EMSA experiments, the size exclusion chromatography column was equilibrated in Buffer A (12 mM HEPES, 4 mM Tris-HCl, 60 mM KCl, 1 mM EDTA, 1 mM EGTA, pH 8.0), and for all other experiments, the column was equilibrated in LEW100 buffer (50 mM NaH2PO4, 100 mM NaCl).

**Chemical Cross-linking of Rco**—Aliquots of 10 µg of Rco were incubated in Buffer B (100 mM Bicine (pH 8), 300 mM NaCl, 1

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TABLE 2
Oligonucleotides used in this study
Restriction sites are underlined.

| RT-PCR analysis          | Oligonucleotide sequence (5’–3’)                                      |
|--------------------------|-----------------------------------------------------------------------|
| 1_FW                     | AAGACCTCCATCACTCTAAACAT                                               |
| 1_RV                     | TAAATGATCTTCTTCTAACAAGATTTGAGAGATCAA                                  |
| 2_FW                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 2_RV                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 3_FW                     | CAACATGACTGAAAAGGATTGTTTGAAGATAT                                       |
| 3_RV                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 4_FW                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 4_RV                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 5_FW                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 5_RV                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 6_FW                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 6_RV                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 7_FW                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 7_RV                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 8_FW                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 8_RV                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 9_FW                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 9_RV                     | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 10_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 10_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 11_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 11_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 12_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 12_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 13_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 13_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 14_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 14_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 15_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 15_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 16_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 16_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 17_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 17_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 18_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 18_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 19_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 19_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 20_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 20_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 21_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 21_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 22_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 22_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 23_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 23_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 24_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| 24_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| Amplification of genes   |                                                                 |
| Rco_FW                   | CACCCTGCGCCATAGAGAGCAAT                                               |
| Rco + STOP_RV            | CTACTTCTTCTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| ΔC-Reo_FW                | CACCCTGCGCCATAGAGAGCAAT                                               |
| ΔC-Reo_RV                | CTACTTCTTCTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| ΔN-Reo_FW                | CACCCTGCGCCATAGAGAGCAAT                                               |
| ΔN-Reo_RV                | CTACTTCTTCTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| Rap_FW                   | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| Rap_RV                   | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| DEST42_KpnI              | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| DEST42_XhoI              | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| Pc_FW                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| Pc_RV                    | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| mcherry_FW               | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| mcherry_RV               | TAAGCTTCTACTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| PCR fragments used for electrophoretic mobility shift assays |                                                                 |
| P_Lnstr_FW               | CACCCTGCGCCATAGAGAGCAAT                                               |
| P_Lnstr_RV               | CTACTTCTTCTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
| P_Gs57_FW                | CACCCTGCGCCATAGAGAGCAAT                                               |
| P_Gs57_RV                | CTACTTCTTCTTCTTCTTCTAAAGATTTGAGAGATCAA                               |
mm DTT) with increasing concentrations of glutaraldehyde. After 15 min at room temperature, the reactions were stopped through the addition of glycine (pH 8) to a final concentration of 140 mM and incubated for 5 min at room temperature. Aliquots were precipitated by acetone and resuspended in 1 × SDS loading buffer. Samples were applied to a Tris-glycine PAGE (Nusep) and visualized by silver staining.

**EMSA**—Fragments P<sub>c</sub> and P<sub>UTR</sub> as well as the upstream regions of c<sub>ds57</sub>, c<sub>ds80</sub>, and c<sub>ds82</sub> were PCR-amplified using Q5 high-fidelity DNA polymerase, pLS20<sub>neo</sub> as template, and the primers indicated in Table 2. For DNA binding reactions, Rco was diluted in Binding Buffer A (12 mM HEPES (pH 8), 4 mM Tris-HCl (pH 8), 60 mM KCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM DTT, and 100 μg/ml BSA) and incubated in Binding Buffer A for 20 min at 37 °C with a 50 μm concentration of the PCR fragments. For competitive DNA binding reactions, the repressor was incubated with the antirepressor Rap<sub>L520</sub> and the Phr<sub>L520</sub> peptide for 20 min at room temperature prior to the addition of a 50 μm concentrations of fragments X. All reactions were stopped through the addition of DNA loading buffer (TBE, 3% (w/v) glycerol and bromphenol blue) and loaded on 1% agarose gels prepared with 0.5 μl TBE as running buffer.

**Gradient Sedimentation Analysis**—Aliquots of 10, 20, and 30 μM Rco were incubated for 30 min on ice in LEW buffer, and then centrifuged at 10,000 × g for 60–90 min after induction. For single-cell gene expression assays shown in Fig. 5 and the time course experiment shown in Fig. 7A, we performed using a Zeiss Axio Imager AX10 microscope equipped with a 100× objective (Zeiss Plan Fluor 100×, numerical aperture = 1.45) and a CCD camera (CoolSnap HQ<sup>2</sup>, Photometrics). Pictures shown in Fig. 6A were acquired using a Nikon Eclipse Ti equipped with a 100× objective (CFI Plan Apo Lambda DM 100× oil, numerical aperture = 1.45, Ph3, Nikon) and a CCD camera (DS-Qi1, Nikon). For time lapse microscopy, cells were first grown to OD 0.4 in LB medium, diluted to an OD of 0.01 in minimal medium, and seeded on an agarose pad made with S750 medium containing 0.1% (w/v) glucose, which was placed into a small imaging dish (µDish 35 mm, Ibidi). Growth of cells was followed at 37 °C using a Nikon Eclipse Ti equipped with a motorized stage, a 100× objective (CFI Apo TIRF 100× oil, numerical aperture = 1.49), a thermostat (Tokai Hit), and an EM-CCD camera (ImagEM X2, Hamamatsu). Fluorescence microscopy pictures were processed using ImageJ version 2.0. Single-cell gene expression was measured either by using MicrobeTracker (17), where phase-contrast images were available, or by manually extracting fluorescence values from each cell in ImageJ version 2.0.

**Results**

**Mapping of the Conjugation Operon Encoded by pLS20**—To define the transcriptional organization of the conjugation operon present on pLS20, we performed RT-PCR analysis of total RNA from exponentially growing cultures containing pLS20<sub>neo</sub>, using primer pairs to link adjacent genes (Table 2). For every RT-PCR, we included a cDNA synthesis reaction omitting reverse transcriptase to confirm the absence of DNA contamination and a DNA control reaction to verify the specificity of the reactions. In accordance with our previous study (16), we confirmed co-transcription of genes c<sub>ds12</sub> (designated as pLS20_014 in the NCBI database) to c<sub>ds35</sub> (pLS20_039) and extended the analysis to the regions located downstream. Fig. 1B shows that the operon starts at open reading frame c<sub>ds3</sub> (pLS20_005), which corresponds to the second RT-
PCR product of the analysis (Fig. 1A), and ends at open reading frame \textit{cds43} (pLS20\_049), which corresponds to the second to last RT-PCR product (Fig. 1A). Thus, the conjugation operon spans in total more than 33 kbp and also includes genes presumably not involved in the conjugative transfer itself, like \textit{roK} (pLS20\_046), the repressor of competence (18), or a putative DNA primase encoded by \textit{cds36} (pLS20\_040), which may be required for the establishment of pLS20 in the recipient cell. Our analysis reveals all genes that are up-regulated after cells have been diluted into fresh medium and suggests that their expression is driven by a single promoter region.

**Conjugation Is Affected by Enhanced SigW Activity**—Rco has been shown to negatively regulate conjugation in \textit{B. subtilis} strain 168 (10). We have found that this is also the case for strain PY79 (data not shown), confirming that the protein acts as a repressor independent of strain background. Ramachandran \textit{et al.} (19) identified two convergent and overlapping promoters that drive the expression of the repressor gene and the conjugation operon. \textit{In silico} analysis of the intergenic region between the first gene of the conjugation operon and the repressor gene using the DBTBS database (20) revealed a \textit{sigW} promoter-like sequence 27 bp upstream of the \textit{rco} gene (5'-cGAAAa-N16'-CGTATA). SigW is an extracytoplasmic \textit{σ}-factor, which controls expression of several membrane proteins and which is fully activated upon cell envelope stress (21). It has been shown that the transition state regulator AbrB regulates expression of \textit{sigW} and that transcription of \textit{sigW}-dependent genes increases in a growth phase-dependent manner, with highest expression during stationary phase (21). To test whether the regulatory region of the \textit{rco} gene is a target of SigW, we performed conjugation assays with a \textit{sigW} deletion strain and strains modulating the activity of SigW. Fig. 1C shows that the deletion of \textit{sigW} or overexpression of \textit{rsiW}, its cognate anti-\textit{σ} factor, resulted in slightly higher transfer rates of pLS20. Interestingly, the transfer rate seen in the \textit{rsiW} deletion strain was 2 orders of magnitude lower than that of the wild type strain. Quantitative RT-PCR showed that the relative transcript levels of \textit{rco} and \textit{virB11} as a marker for the conjugation operon were not affected in the background of \textit{sigW} strain but that deletion of \textit{rsiW} resulted in a lower abundance of \textit{virB11} transcripts (Fig. 1D). These experiments reveal a link between SigW activity and transcription of the conjugation operon, which
Calculation of the repressor was also found to be in trimeric and tetrameric forms. The observation that Rco mainly exists in a dimeric conformation (Fig. 2A) suggests that the newly identified binding region may act as a secondary repressor binding site.

We wondered if Rco might regulate the expression of other pLS20-encoded genes, and we performed EMSA experiments with the regulatory regions of genes, which were shown to be differentially regulated in the background of Rco- or RapLS20-overexpressing strains, as indicated by RNA sequencing analysis of Meijer and co-workers (10). We found that Rco bound to the promoter region of cds80 (pLS20_102; Fig. 3, second panel), a protein of unknown function with a predicted signal peptide sequence, but not to regions preceding gene cds81 and cds82 (pLS20_105 and pLS20_106). Similar to fragment P_{UTR}, the region upstream of cds80 contains a conserved consensus sequence, which differs only in 1 base (5’-CAGTGAAA-3’) from the sequence shown to be the consensus binding site of Rco. Nevertheless, it seems that additional purine residues at the 3’-end of the consensus sequence increase the binding affinity of the repressor. These experiments show that Rco binds to several sites in the conjugation promoter region and to at least one additional site on the plasmid.

Rco and Rap Are Homodimers in Solution, and Rco Binds to Several Sites on the Plasmid—To determine the native weight of the transcriptional regulator and its putative modulator protein, Rap_{LS20}, we performed size exclusion chromatography of affinity-purified His_{6}-Rco and His_{6}-Rap_{LS20}. Rco eluted at a column volume of 52.6 ml (Fig. 2A, solid line), which corresponds to a hexameric state of the protein, whereas Rap_{LS20} eluted after 55 ml (Fig. 2A, dashed line), which corresponds to a dimeric state. Due to the large Stokes radius of Rco (R_s = 4.6) we subsequently performed a sucrose gradient sedimentation analysis of Rco in varying concentrations to further evaluate its oligomeric state. SDS-PAGE analysis and application of the Monte-Siegel formula showed that the repressor mainly exists as a dimer in solution (Fig. 2B). At higher concentrations, the repressor was also found to be in trimeric and tetrameric forms. Calculation of the S_{max}/S ratio shows that the monomeric conformation probably represents the most elongated shape, whereas the oligomeric conformations have moderately elongated shapes compared with proteins of known structure. Finally, glutaraldehyde cross-linking experiments confirmed the observation that Rco mainly exists in a dimeric conformation (Fig. 2C). Dimer formation was even present without the addition of the cross-linking agent. In its presence, the protein was also seen as trimer, tetramer, and hexamer.

Previous work has shown that Rco regulates transcription of the conjugation operon through binding to two operator sites within the intergenic region of rco and the first gene of the conjugation operon, possibly by forming a DNA loop of 75 bp (19). Although we did not observe a preference of the repressor to form tetramers as stated before, we found a DNA binding behavior comparable with that described in the literature (Fig. 2D). Specifically, Rco binds with high affinity to sequences containing 5’-CAGTGAAA-3’, which we confirmed using EMSAs and a 125-bp fragment containing an identical sequence, which is located 175 bp upstream of the first gene of the conjugation operon (named P_{UTR}; Fig. 3, first panel). However, binding affinity was lower in this region compared with binding to the previously described operator binding sites (19) (Fig. 2D), suggesting that the newly identified binding region may act as a secondary repressor binding site.

Rap_{LS20} Binds Directly to Rco to Inhibit Its DNA Binding Activity, whereas the Addition of Phr_{LS20} Relieves the Inhibitory Function of Rap_{LS20} on Rco—Genetic evidence has shown that an endogenous Rap-Phr module on pLS20 (Rap_{LS20} and Phr_{LS20}) affects the activity of the repressor of conjugation (10). To determine whether Rap_{LS20} directly regulates the activity of the repressor protein through protein-protein interactions, we performed analytical size exclusion chromatography of Rap_{LS20}-Rco, and a mixture of both proteins. Fig. 4A shows that Rap_{LS20} eluted much earlier from the column upon the addition of Rco than alone, providing evidence for complex formation of these proteins. The complex migrated at a molecular mass of 154 kDa, suggesting the formation of a heterotetrameric complex (2 × 46.6 kDa) + (2 × 21.8 kDa) with a 1:1 stoichiometry, as determined by densitometric measurements of the corresponding bands.

It is known that the function of Rap proteins is controlled by their cognate Phr peptides through protein-peptide interactions (6, 22). Meijer and co-workers (10) showed that the 5 C-terminal residues of the Phr_{LS20} prepeptide (corresponding to QKGMY) efficiently repressed conjugation of plasmid pLS20...
and that repression could be relieved by deletion of the oligopeptide permease \textit{opp}.

To verify that Phr\textsubscript{LS20} acts on Rap\textsubscript{LS20}, thereby possibly interfering with complex formation of Rap\textsubscript{LS20}-Rco, we added the synthetic pentapeptide to Rap\textsubscript{LS20} and to Rap\textsubscript{LS20}-Rco complexes. Indeed, synthetic Phr\textsubscript{LS20} bound to Rap\textsubscript{LS20}, causing Rap\textsubscript{LS20} to elute earlier from the column (Fig. 4B) than when in complex with Rco. Additionally, co-incubation of Phr\textsubscript{LS20} with Rap\textsubscript{LS20} and Rco prevented complex formation between the two proteins (Fig. 4C), at the expense of the Rap\textsubscript{LS20}-Phr\textsubscript{LS20} complex.

To test whether an interaction between Rap\textsubscript{LS20} and Rco results in an inhibition of the DNA binding activity of the repressor protein, we conducted EMSA experiments in the presence of increasing amounts of Rap\textsubscript{LS20}. As indicated in Fig. 4D, Rap\textsubscript{LS20} showed no DNA binding activity by itself but diminished the binding activity of Rco when the molar ratio reached a 1:1 stoichiometry and completely repressed DNA binding at an 8-fold molar excess (Fig. 4D, top). To see whether Phr\textsubscript{LS20} relieves the inhibitory function of Rap\textsubscript{LS20} on Rco, we added increasing amounts of Phr\textsubscript{LS20} to the (8-fold) molar ratio of Rap\textsubscript{LS20} and Rco at which no more DNA binding activity occurred. Interestingly, the addition of the peptide restored the DNA binding activity of Rco, although not to the same extent as without the addition of Rap\textsubscript{LS20} (Fig. 4D). These results show that the Rco/Rap\textsubscript{LS20}/Phr\textsubscript{LS20} module on pLS20 operates via direct protein-protein and protein-peptide interactions to regulate the conjugative activity of the plasmid.

\textit{Rap/Phr-regulated Repressor System on a Conjugative Plasmid}

![FIGURE 3. Identification of additional binding sites of the Rco repressor.](image)

![FIGURE 4. Rap\textsubscript{LS20} binds to Rco and inhibits the DNA binding activity of Rco, whereas Phr\textsubscript{LS20} relieves the inhibitory effect of Rap\textsubscript{LS20}.](image)
based on the intrinsic property of DivIVA to localize at negative membrane curvatures (23, 24). Therefore, we fused RapLS20 to GFP and co-expressed it pairwise with DivIVA fusions to full-length Rco or with DivIVA fusions to the C- or N-terminal ends of Rco (ΔC-Rco and ΔN-Rco; Fig. 5A) in *E. coli* BL21 (DE3) cells. In all cases, we observed strong GFP signals even in the absence of the inducing agent (isopropyl 1-thio-β-D-galactopyranoside); thus, we split the cultures before induction of the DivIVA fusion proteins to clearly distinguish DivIVA-like localization from polar localization caused by overexpression of the GFP fusion proteins.

We found that GFP-RapLS20 localized in a half-moon-like manner to the cell pole when co-expressed with a DivIVA fusion of RapLS20 of full-length Rco, or of ΔC-Rco, but not when it was co-expressed with a DivIVA fusion of ΔN-Rco (Fig. 5B, top). To test whether these proteins indeed interact via the N terminus of Rco, we also assayed the localization of GFP-ΔC-Rco only containing the helix-turn-helix motif upon co-expression with DivIVA-RapLS20. As shown in Fig. 5B (bottom), DivIVA-RapLS20 targeted GFP-ΔC-Rco to the cell pole and to a certain extent also DivIVA-ΔN-Rco, but no translocation was observed when GFP-ΔC-Rco was co-expressed with DivIVA-Rco or with DivIVA-ΔC-Rco. Taken together, we provide evidence that RapLS20 recognizes the N-terminal part of Rco containing the helix-turn-helix motif.

**Expression of the Conjugation Operon is Heterogeneous**—To test whether, during growth, all cells or just a subset induce the conjugation operon, as is often characteristic of developmental pathways in bacteria, we monitored the activity of the promoter driving expression of the large conjugation operon at the single cell level. Therefore, we generated a transcriptional fusion of the promoter (Pc) with the fluorescent mCherry protein and integrated the reporter gene fusion into the *thrC* locus in the chromosome of *B. subtilis* PY79. First, we analyzed the expression during growth in cells devoid of the plasmid and its regulatory elements. Signal intensities of the fluorescent reporter were homogeneously distributed during all growth stages in cells lacking the plasmid (Fig. 6, A and B), although the mean fluorescence intensity varied between the time points (Fig. 6B).

Contrary to plasmid-free cells, cells containing the plasmid exhibited a phenotypic heterogeneity in the expression pattern of the fluorescent reporter (Fig. 7A, red and white arrows). To quantitatively analyze expression of the promoter of conjugation, we measured the mCherry fluorescence signal of individual cells at each time point during growth and plotted the log-transformed fluorescence intensities in histograms. Fig. 7B shows that the overall signal intensity increases with ongoing growth and that the fluorescence intensity shifts from a rather unimodal distribution to a bimodal distribution upon entry into mid-exponential growth (first and second panel from the top). Similarly, after 8 h of growth, the fluorescence intensity shifted back to a unimodal distribution. To determine the number of cells switching on the conjugation operon, we set a threshold that separated the two populations from each other and calculated the number of cells exceeding the intensity threshold (Fig. 7B). Fig. 7C shows that under our conditions, a maximum of about 30% of the cells switched the operon to the ON state during the transition from the lag phase to exponential phase of growth. To further analyze the phenotypic heterogeneity of the promoter of conjugation at the single cell level, we performed time lapse microscopy. In accordance with the previous time course experiment, we observed two distinct populations. Additionally, we found that once induced, expression of the conjugation promoter was stably inherited in dividing cells and slowly disappeared when cells ceased to divide (Fig. 7D and supplemental Movie S1). These experiments reveal that the Rap/Phr/repressor system on pLS20 operates in a bistable manner, similar to the ComS/MecA/ComK system in competence and to the phosphorelay in sporulation. Our findings extend the previous observation that only a subset of cells assemble the conserved type IV secretion proteins at the membrane (13) and provide evidence that the assembly of the type IV secretion is already regulated at the transcriptional level.

**Discussion**

Rap-Phr modules play a pivotal role in determining the fate of individual cells in bacterial communities and are best characterized in *B. subtilis* (22, 25). So far, several modules in the
The genome of *B. subtilis* have been discovered that affect sporulation, competence development, secretion of extracellular proteases, or the horizontal transfer of the mobile genetic element ICEBs1. In this work, we provide *in vitro* and *in vivo* evidence that the Rap/Phr module on conjugative plasmid pLS20 directly regulates conjugation via binding to the Rco master repressor and that induction of conjugation occurs in a bistable manner, similar to the developmental processes of competence development and sporulation, leading to heterogeneous expression of the type IV secretion machinery encoded on pLS20. We show that RapLS20 acts as a direct antirepressor of the Rco protein and that its activity is regulated by the cognate PhrLS20 peptide. We show that the repressor and its antirepressor preferentially form dimers in solution and that complex formation occurs in a 1:1 stoichiometry that disrupts DNA binding of the repressor protein through obscuring the DNA-binding domain of the repressor. Unlike the plasmid-encoded Rap60, which presumably inactivates ComA activity by occluding its interaction with the RNA polymerase (26), inactivation of the Rco repressor by RapLS20 resembles that of RapC, -F, and -H, which prevent DNA binding of ComA, which, however, is an activator, rather than a repressor, like Rco. Structural analysis of the RapF-ComA interaction showed that the N-terminal 3-helix bundle of RapF captures the helix-turn-helix-containing domain of ComA and thereby prevents binding of ComA to its target promoters (8). Concurrent with its different binding partner, RapLS20 shows substitutions in all amino acid residues shown to be essential for the interaction of RapF and ComA (8).

Recent work showed that high levels of Phr peptide inhibit horizontal transfer of the plasmid, whereas at lower levels, the Rap protein becomes active and switches on the expression of the conjugation operon (10). Therefore, the known *in vivo* data nicely corroborate our *in vitro* findings.
Interestingly, several plasmids, such as pBS32 of the undomesticated *B. subtilis* strain NCIB3610, pX01 of *Bacillus anthracis*, and pTA1060 of *B. subtilis* were found to carry Rap/Phr modules known to affect the developmental fate of their host (27–29), showing that the use of Rap/Phr modules is widespread.

The interaction of RapF-ComA is antagonized through binding of PhrF to the C-terminal tetratricopeptide repeat domain of RapF. In the PhrF-bound state, RapF constricts and exerts a conformational change that leads to dissociation of the RapF-ComA complex (30, 31). PhrFLS20 seems to induce a conformational change in RapPLS20 as well, but our size exclusion chromatography data indicate that RapPLS20 rather elongates or expands upon binding to the peptide, because the protein-peptide complex is much larger than RapPLS20 alone. In fact, although Rap proteins share a high degree of overall sequence homology, it was not possible to model the structure of RapPLS20 based on the previously solved structures. Thus, it would be interesting to reveal how the structures of RapPLS20 and RapPLS20-PhrFLS20 differ from already known structures.

Furthermore, we show that Rco also binds to a second promoter region on the plasmid, suggesting that it regulates at least two transcriptional units. Interestingly, we found that increased activity of SigW through deletion of its antagonist RsiW resulted in a diminished plasmid transfer rate, whereas expression of the *rco* gene was not significantly affected in the background of these strains. Nevertheless, we found that expression of *virB11* was slightly but significantly changed in the *rsiW* strain. Due to the only moderate effect, we think that the reduced plasmid transfer rate seen in the *rsiW* strain is probably due to secondary effects caused by enhanced SigW activity and not by direct transcriptional regulation of the repressor gene or the conjugation operon. In any event, it is intriguing to note that changes in the activity of a host-encoded σ factor affect conjugation activity of a plasmid, which may be important for the plasmid’s decision to prevent conjugation in case of existing cell wall stress.

Last, we provide evidence that expression of the conjugation operon occurs in a heterogeneous but stable manner. Whereas in the absence of the plasmid, expression of the ectopic conjugation promoter showed a unimodal distribution, the presence of the plasmid elicited a bimodal distribution and thus a mutually exclusive expression pattern of the conjugation promoter in the population. Interestingly, time lapse microscopy revealed that once the ON state is established, expression of the conjugation promoter is propagated to the next generation of cells and vanishes with additional cell divisions. Interestingly, the Meijer group (19) recently showed that repressor protein RcoPLS20 autoregulates itself and keeps the conjugation operon in the OFF state through binding to two operator sites, presumably forming a DNA loop. Loop formation may contribute to the relatively tight on/off regulation seen in our expression studies.

Our work shows that pLS20 harbors a classical Rap/Phr system, which modulates the activity of a repressor protein, but its output *in vivo* differs from known Rap/Phr systems, in that the partner switch shows an offset *in vivo* relative to known Rap/Phr systems as it occurs during a time window, when cells actively divide rather than arrest their cell cycle. Thus, the Rco/Rap/Phr regulatory circuit encoded by plasmid pLS20 could be employed for the rational design of expression systems that are limited to the cell’s growth phase. It will be interesting to further analyze which factor(s) governs the heterogeneous expression of the conjugation operon.

**Author Contributions**—T. C. R. and P. L. G. conceived of and coordinated the study and wrote the paper. T. C. R. designed, performed, and analyzed the experiments. All authors reviewed the results and approved the final version of the manuscript.

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