Novel regulatory mechanism of establishment genes of conjugal plasmids

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ABSTRACT

The principal route for dissemination of antibiotic resistance genes is conjugation by which a conjugative DNA element is transferred from a donor to a recipient cell. Conjugal elements contain genes that are important for their establishment in the new host, for instance by counteracting the host defense mechanisms acting against incoming foreign DNA. Little is known about these establishment genes and how they are regulated. Here, we deciphered the regulation mechanism of possible establishment genes of plasmid p576 from the Gram-positive bacterium Bacillus pumilus. Unlike the ssDNA promoters described for some conjugal plasmids, the four promoters of these p576 genes are repressed by a repressor protein, which we named Reg576. Reg576 also regulates its own expression. After transfer of the DNA, these genes are de-repressed for a period of time until sufficient Reg576 is synthesized to repress the promoters again. Complementary in vivo and in vitro analyses showed that different operator configurations in the promoter regions of these genes lead to different responses to Reg576. Each operator is bound with extreme cooperativity by two Reg576 dimers. The X-ray structure revealed that Reg576 has a Ribbon-Helix-Helix core and provided important insights into the high cooperativity of DNA recognition.

INTRODUCTION

Horizontal gene transfer (HGT) is the process by which (large) DNA regions are transferred between bacterial cells (1–3). A single HGT event can therefore significantly alter the genetic content of a cell and, hence HGT plays an important role in the evolution of prokaryotes. In addition, HGT is also responsible for the rapid spread of antibiotic resistance genes and virulence factors in bacteria. HGT involves various routes. Of these, conjugation is majorly responsible for the spread of antibiotic resistance genes. Conjugation is the process by which a DNA element is transferred from a donor to a recipient cell via a connecting channel. A conjugative element can be located either on an autonomously replicating plasmid, which is named conjugative plasmid, or on a bacterial genome, which is then named integrated conjugative element (ICE). Conjugal elements are widespread in both Gram-negative (G−) and Gram-positive (G+) bacteria and the basic principles of the conjugation process are conserved. The initial steps of the conjugation process involve the formation of a mating pair in which a donor cell recognizes and interacts with a suitable recipient cell. Probably, this triggers a signal for processing the DNA of the conjugative element to generate the ssDNA, named T-strand, which is subsequently transferred...
into the recipient cell through the connecting channel. It has to be noted that although in most conjugation systems a single DNA strand is transferred into the recipient cell, double-stranded DNA is transferred in G+ mycelial Streptomyces bacteria (4,5). Transfer of the DNA into the recipient cell does not imply automatically a successful conjugation event. For instance, the transferred ssDNA must be circularized and converted into dsDNA, and, in the case of ICEs, become integrated into the bacterial chromosome. In addition, bacteria possess mechanisms that protect them against incoming foreign DNA. First-line defense mechanisms possessed by most bacteria recognize and inactivate foreign DNA by restriction-modification (RM) systems that encode a restriction endonuclease (REase) and a methyltransferase (MTase). The MTase methylates specific short DNA sequences and thereby protects these sequences from being digested by the cognate REase of the RM system. Foreign DNA entering a cell that is not properly methylated (including conjugative DNA) will be digested by the REase (for review see, 6,7). At least some conjugative elements encode anti-restriction proteins, named Ard (alleviation of restriction of DNA) proteins, which impair the REase activity. Once the entered DNA is properly methylated it is no longer recognized as foreign and inactivation of the REase can be relieved. In fact, expression of anti-restriction genes must be limited to a short period of time after the conjugative element has entered the cell because prolonged expression will make the cell vulnerable to the entry of other foreign DNA, like phage DNA. Thus, anti-restriction genes are important for stable establishment of the conjugative element in the new host, and hence they are referred to as establishment genes. Another establishment gene is psiB, present on at least several conjugative plasmids of G- origin. As explained above, during conjugation only a single DNA strand enters the recipient cell. ssDNA is a potent trigger of the host’s SOS response. The PsiB protein suppresses activation of the SOS response by binding the RecA protein (8). Like the anti-restriction genes, it is in the interest of the host that the SOS response is suppressed only for a limited time after entry of the conjugative DNA into the cell. In some conjugative elements of G- bacteria, like the F and ColIbP-9 plasmids, the anti-restriction gene ardA and the psiB gene are under the control of a special single-stranded promoter (ssDNA promoter) which ensures that they are only expressed for a short period of time after entry of the ssDNA into the recipient cell (9–12). The colIbP-9 plasmid contains three ssDNA promoters that control the expression of at least 10 genes (13). The function of most of these genes is unknown but because they are controlled by ssDNA promoters it is likely that they are functionally related, all playing a role in enhancing establishment of the conjugative element in the new host.

As a possible approach to combat the spreading of antibiotic resistance, drugs have been developed that affect the activity of some relaxase proteins, which are crucial for initiating the DNA processing step to generate the T-strand (14). Similarly, compromising the activity of establishment proteins and/or deregulating their expression may also be a fruitful strategy to impair conjugation. So far though, this approach has not attained much attention, and little is known about establishment genes and their regulation of conjugative elements present in G+ bacteria. In fact, only few conjugative plasmids of G+ bacteria have been studied so far. In our laboratory we study the related conjugative plasmids p576 from Bacillus pumilus and pLS20 from Bacillus subtilis (15–17). In this study we focused on the transcriptional regulation of the establishment genes of plasmid p576, which contains a putative ard gene and six other genes that may have a role in establishment of the plasmid in the recipient cell after transfer. We have identified the repressor of their promoters, named Reg576, and we show that expression of the p576 establishment genes is regulated in a fundamentally different way than the ssDNA promoters described for some conjugative plasmids of G- bacteria. The number and position of the operator sites is not the same for each promoter, and the different configurations control the sensitivity of a promoter to Reg576-mediated repression. Structure determination of the apo form revealed that Reg576 belongs to the Ribbon-Helix-Helix (RHH) family of DNA binding proteins. Reg576 binds DNA as a dimer-of-dimers, providing insights into the extremely high cooperative binding. The implications of these studies for regulation of the possible establishment genes on p576 and possibly other conjugative plasmids are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids and oligonucleotides

Bacterial strains were grown in Lysogeny Broth (LB) medium or on 1.5% agar plates of LB or Spizizen minimal medium (18). When appropriate, the following antibiotics were added: ampicillin (100 μg/ml) or kanamycin (30 μg/ml) for Escherichia coli; erythromycin (2 μg/ml) or spectinomycin (100 μg/ml) for B. subtilis. When indicated, LB agar plates were supplemented with isopropyl-β-D-1-thiogalactopyranoside (IPTG) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) to the final concentrations specified. Supplementary Table S1 lists the strains used. Plasmids and oligonucleotides used are listed in Supplementary Tables S2 and 3, respectively. All oligonucleotides were purchased from Isogen Life Science, The Netherlands.

Transformation

Escherichia coli cells were transformed by standard methods (19). Competent B. subtilis cells were prepared as described before (18). Transformants were selected on LB or Spizizen minimal agar medium plates supplemented with appropriate antibiotics and in the case of minimal agar medium plates complemented with tryptophan and/or threonine (20 mg/ml).

Construction of plasmids and strains

Detailed description of the construction of plasmids and strains is available in Supplementary Data.

β-Galactosidase activity assays

β-galactosidase activities were essentially determined as described previously (20). In short, overnight grown cultures
were diluted to an $OD_{600} \approx 0.025$ in fresh 37$^\circ$C LB. Growth was followed by measuring $OD_{600}$ at regular intervals. Samples were taken every 30 min from shaking cultures until $OD_{600} > 1$ and stored at $-80^\circ$C for later processing. Processing of the samples involved lysis of the cells, incubation at 28$^\circ$C of the lysate with the chromogenic substrate o-nitrophenyl-beta-D-galactopyranoside (ONPG) and determination of the $OD_{280}$ value of centrifuged sample after stopping the enzymatic reaction at the desired time.

**RNA isolation**

Total RNA was isolated from late exponentially growing cells using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s protocol. RNA protect solution (Qiagen, Germany) was used to ensure integrity of RNA during isolation and also to stop transcription at given time points.

**Protein purification**

*Escherichia coli* BL21 (DE3) cells carrying plasmid pJV9 (containing his$_{6}$/reg$_{576}$) were used to inoculate 10 l of fresh LB medium supplemented with kanamycin and grown at 37$^\circ$C with shaking. At an $OD_{600}$ of 0.4, expression of his$_{6}$/reg$_{576}$ was induced by adding IPTG to a final concentration of 1 mM and growth was continued for 2 h. After harvesting by centrifugation, the cells were lysed and processed as described before to purify the his-tagged Reg576 protein by affinity purification (21). Purified protein (>95% pure, 275 $\mu$M) was desalted with PD10 columns, equilibrated in buffer B (50 mM Tris (pH8), 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM $\beta$-mercaptoethanol, 50% glycerol) and stored in aliquots at $-80^\circ$C. In the case of fractions used for crystallization, the target buffer used for desalting was 20 mM Tris–HCl (pH8.0), 250 mM NaCl. Protein concentration was determined by $OD_{280}$ using an $\varepsilon_{0.1\%} = 0.411$ L g$^{-1}$ cm$^{-1}$.

**EMSA**

Electrophoretic mobility shift assay (EMSA) assays were essentially performed as described before (22). In short, 120 ng of the polymerase chain reaction fragment was incubated for 20 min at RT in Binding Buffer (20 mM Tris HCl [pH 8.0], 1 mM EDTA, 5 mM MgCl$_2$, 100 mM KCl, 10% (v/v) glycerol) without and with increasing amounts of purified his$_{6}$/Reg$_{576}$ in a total volume of 20 $\mu$L. Next, samples were incubated on ice for 10 min and were subsequently loaded onto a 2% agarose gel in 0.5-TBE. Electrophoresis was carried out in 0.5-TBE at 60 V at 4$^\circ$C. After electrophoresis, the gel was stained with ethidium bromide, de-stained in 0.5-TBE and photographed under UV illumination.

**Primer extension experiments (5’ RACE)**

5’ RACE analysis was carried out using the First Choice RLM-RACE kit (ThermoFisher Scientific). Briefly, total RNA was purified using the RNeasy Mini Kit (Qiagen, Germany), as indicated above, and treated with RNase-free DNase I (Turbo DNA-free kit, ThermoFisher Scientific) to remove any residual DNA. A total of 4 $\mu$g of this RNA was treated with terminator 5’-phosphate-dependent exonuclease (TEX). Reactions were terminated by extraction with acid phenol followed by ethanol precipitation. Samples were treated with tobacco acid pyrophosphatase, and the RNA oligonucleotide 5’ RACE Adapter was ligated to the RNA 5’-ends using the T4 RNA ligase. After obtaining cDNA using random primers and the Super Script III reverse transcriptase (Life Technologies), a specific amplification step was performed using the 5’ RACE Outer primer in combination with either the specific reverse primer 5’ RACE lacZ-rev-Outer (for promoter P$_{23c}$) or with primer 5’ RACE lacZ-rev-Inner (for promoters P$_{20c}$ and P$_{27c}$). The resulting products were cloned into vector pGEM-T easy (Promega) and transformed into *E. coli* NEB-5α competent cells (Biolabs). The 5’-end of the transcripts (corresponding to the point of ligation) was determined by DNA sequencing of several independent transformants.

**Ultracentrifugation**

**Sedimentation velocity assays (SV).** Samples of protein Reg$_{576}$ alone and mixed with the different DNA variants, in 20 mM Tris, 500 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA, 0.1 mM $\beta$-mercaptoethanol and 1% glycerol, pH 7.4, were loaded (320 $\mu$L) into 12 mm epon-charcoal standard double-sector centerpieces. The assays were performed at 48 000 rpm in a XL-I analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with both UV-VIS absorbance and Raleigh interference detection systems, using an An-50Ti rotor. Sedimentation profiles were recorded simultaneously by Raleigh interference and absorbance at 230 and 260 nm. Differential sedimentation coefficient distributions were calculated by least-squares boundary modeling of sedimentation velocity (SV) data using the continuous distribution $c(s)$ Lamm equation model as implemented by SEDFIT (23). These experimental $s$-values were corrected to standard conditions using the program SEDNTERP (24) to obtain the corresponding standard $s$-values ($s_{20,w}$). Multi-signal sedimentation velocity (MSSV) data were globally analyzed by SEDPHAT software (25) using the ‘multi-wavelength discrete/continuous distribution analysis’ model, to determine the spectral and diffusion-deconvoluted sedimentation coefficient distributions, $c_s(s)$, from which the number and stoichiometry of protein versus DNA molecules can be derived (26). Prediction of extinction coefficients for the different DNA fragments considering duplex hypochromism at 260 nm was done by means of the Microsoft Excel® application developed by A. Tautov (27).

**Sedimentation equilibrium assays (SE).** Short columns (95 $\mu$L) SE experiments of Reg$_{576}$ alone and mixed with the different DNA fragments were carried out at speeds ranging from 5500 to 22 000 rpm and at three different wavelengths (230, 260 and 280 nm), using the same experimental conditions and instrument as in the SV experiments. A last high-speed run (48 000 rpm) was done to deplete protein and DNA from the meniscus region to obtain the cor-
responding baseline offsets. Weight-average buoyant molecular weights of Reg576, DNA fragments and Reg576–DNA complexes were obtained by fitting a single-species model to the experimental data using the HeteroAnalysis program (28), once corrected for temperature and solvent composition with the program SEDNTERP (24). The equilibrium binding isotherms of Reg576 with the different DNA variants were built using a fixed DNA concentration of 0.1 μM titrated with increasing Reg576 concentrations (from 0.1 to 3 μM). The amount of Reg576 bound to the different DNA fragments was determined from the experimental apparent buoyant mass increments, using 0.7366 as partial specific volume for Reg576, calculated from its amino acid composition by SEDNTERP. The data were modeled with a three parameter Hill function, as implemented in SigmaPlot 11.0 software.

**Dynamic light scattering assays (DLS).** Dynamic light scattering (DLS) experiments were carried out in the same experimental conditions used in SV and SE, in a Protein Solutions DynaPro MS/X instrument (Protein Solutions, Piscataway, NJ, USA) using a 90° light scattering cuvette. Previous to measurements, samples were centrifuged during 10 min at 12 000 x g. Data were collected and analyzed with Dynamics V6 Software.

**Estimate of molar mass of Reg576 from hydrodynamic measurements.** The apparent molar mass of a single sedimenting solute species (M) was also calculated using measured values of the sedimentation coefficient s and the diffusion coefficient D according to the Svedberg equation (29),

$$M = \frac{RT_s}{(1 - \bar{v} \rho)} D$$

where T, R, \(\bar{v}\) and \(\rho\) stand for the absolute temperature, the universal gas constant, the partial specific volume of the protein, and the density of the solution, respectively.

**Mathematical modeling**

Basal gene expression was neglected and linear degradations were considered for all species. Repression was modeled through effective Hill functions using the Hill constants given by the number of monomers of repressor binding the promoters: in our model Reg576 binds promoter P20o, which has two operator regions, with a Hill exponent of \(h_r = 8\) and Reg576 represses its own expression with a Hill exponent of \(h_r = 4\). Following experimental results, we consider the equilibrium constants \(K_r = 0.7\) for the repressor and \(K_r = 0.8\) for the establishment genes. Denoting time by t we obtain the following system of ordinary differential equations:

$$\frac{dm_r(t)}{dt} = \frac{a}{1 + \left(\frac{p_r(t)}{K_r}\right)^{h_r}} - d_m m_r(t)$$

$$\frac{dp_r(t)}{dt} = bm_r(t) - d_p p_r(t)$$

$$\frac{dm_e(t)}{dt} = \frac{3a}{1 + \left(\frac{p_r(t)}{K_r}\right)^{h_r}} - d_m m_e(t)$$

where \(m_r\) is the concentration of repressor mRNA, \(p_r\) is the concentration of repressor protein, \(m_e\) is the concentration of establishment gene mRNA, \(p_e\) is the concentration of establishment gene protein, \(a\) is the repressor’s maximum transcription rate, \(d_m\) its mRNA degradation rate, \(b\) is the repressor’s translation rate and \(d_p\) its protein degradation rate. For simplicity we consider the same degradation and translation rates for the establishment gene; the qualitative nature of our results does not depend on this choice or on the specific values of the parameters we use. According to our results, we use a maximum transcription rate for the establishment genes that is three times higher than that of the reg576. We have chosen the following dimensionless parameter values: \(a = 1\), \(d_m = 0.1\), \(b = 0.3\), \(d_p = 0.03\) and solved the equations using the ode45 solver from Matlab (30). We have no dynamical gene expression data to compare with the model predictions. Therefore, we have left units undefined, and use the model as a tool of qualitative exploration of the implications of the regulatory logic found in our experiments. Note however, that we used as equilibrium constants the results of our measurements in μM, and that the parameters which we have chosen for degradation rates are consistent with biological realistic rates in 1/min. A trivial scaling and fitting of parameter values allows us to fit the model to potential future experimental data to obtain the desired time scale for the pulses of expression observed in the model.

**Crystallization and structure determination**

Purified protein was concentrated to 25 mg/ml in [20 mM Tris (pH8.0), 250 mM NaCl]. Crystals of Reg576 were obtained by the sitting-drop vapor-diffusion method at 18°C, by equilibration of drops of 1 μl protein + 1 μl crystallization buffer (130 mM potassium bromide, 25% PEG 2000 monomethylether) against 50 μl of the crystallization buffer. Needle-shaped crystals appeared in less than a week. Cryo-cooling in liquid nitrogen was carried out using a cryo-protecting solution containing reservoir solution supplemented with 5% glycerol. Data collection was performed to the indicated resolutions at ALBA synchrotron Light Source on the BL13-Xaloc beamline (31). The crystals belonged to space group P4_12_2, with two protein molecules in the asymmetric unit. Data were processed with AutoPROC from Global Phasing (32). Data-collection statistics are listed in Supplementary Table S4.

**Structure refinement**

*In silico* phasing was performed using Arcimboldo Borges (33) Crystallographic refinement using reﬁmac 5.8.0222 (34) was interspersed with manual building in Coot (35), using the 2Fo–Fc and Fo–Fc electron-density maps from refinement. Molprobity (36) was used to validate and improve the final model. Refinement statistics are presented in Supplementary Table S4. Figures were prepared using PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) and PDBSum (37, Database issue D355-D359).
RESULTS

Identification of an anti-restriction gene and other putative establishment genes of p576, and detection of conserved features in their upstream regions

Plasmids p576 (43.4 kb) of B. pumilus NRS576 and pLS20 (64.8 kb) of B. subtilis natto UM3335 are related conjugative plasmids (16,38). Their replication regions, which share 72% identity, are flanked by conserved genes/operons: a bicistronic operon encoding a plasmid partitioning system is located upstream of each origin (16,39), and three regulatory genes followed by a large conjugation operon are located downstream of each origin (see Supplementary Figure S1, 16,40). However, the proteins encoded by the genes present on the remaining regions of both plasmids do not share significant similarity with proteins of known function, with one exception: gene 28c of p576 gene and gene 82c of pLS20cat ardCLS20. Anti-restriction genes are typical establishment genes that play important roles in the establishment of the plasmid in the recipient after its conjugative transfer. As explained in the introduction, establishment genes must be regulated such that they are expressed rapidly, but transiently, upon entry of the plasmid into the recipient. We analyzed the upstream sequences of ardC576 for clues of possible regulatory mechanisms. This analysis revealed a putative σA-dependent promoter (5′-TTtACt-n14-TGnTATAAT-3′) that we named PardC576, which is located 105 bp upstream of the start codon of ardC576 (see Figure 1B). Thus, (i) the 5′-TTtACt-3′ and the 5′-TATAAT-3′ hexamers are similar and identical to the consensus −35 (5′-TTGACA-3′) and −10 (5′-TATAAT-3′) boxes of σA type promoters; (ii) these putative boxes are separated by the optimal spacer length of 17 bp; and (iii) it contains a so-
called extended −10 motif (5′-TGn-3′) located upstream of the −10 box. Interestingly, three identical heptamer motifs (5′-TTATCC-3′) are located near this putative promoter (see Figure 1B). Two motifs, named ‘A1’ and ‘A2’, are located immediately upstream of the putative −35 box. These two motifs have the same orientation and are separated by 2 bps. The third copy of this motif, which is named ‘D1’ and has the opposite orientation with respect to the other two, is located 11 bps downstream of motif ‘A2’ and hence is located in the spacer region of the putative promoter (see Figure 1B). Intriguingly, further analysis revealed that p576 contains in total 18 copies of this heptameric sequence. Five of these are located inside the coding region of five different (putative) genes, while the remaining 13 copies are located upstream of gene 20c (four motifs), 23c (four motifs), 27c (two motifs) and ardC576 (three motifs). These four genes are clustered in a 7.9 kb region of p576 and are all transcribed in the opposite direction to that of the conjugation genes (see Figure 1A). Similar to that observed for PardC576, the motifs are located near putative αA-dependent promoters, which we named P20c, P23c and P27c. Whereas the putative promoters PardC576 and P23c are predicted to drive expression of one gene, putative promoters P27c and P20c are predicted to control the expression of two and three genes, respectively (Figure 1A). A schematic overview of the distribution of the conserved motifs with respect to their putative promoter is shown in Figure 1B. Strikingly, two of the mentioned heptameric motifs are located in a directed orientation immediately upstream of the −35 box of each of these four putative promoters. We will refer to such a pair of direct-repeated motifs as ‘dual motif’ (DM). In the case of putative promoters P20c and P23c, another DM is located just upstream of their predicted ribosomal binding site (RBS). The putative P27c promoter also contains an additional DM. However, in this case the motifs deviate slightly from the consensus sequence (5′-aTATCCC-3′ and TTATCCCT-3′), indicated as ‘C1’ and ‘C2’, respectively, in Figure 1B). In addition, this DM is located within the spacer region separating the −35 and −10 hexamers of the putative promoter and their orientation is opposite to that of the DM located upstream of the predicted −35 box. The various DMs are all separated by the dimer sequence 5′-AT-3′, except for the consensus motifs near putative P27c, which are separated by 5′-AC-3′.

Working model explaining transient expression of ardC576 and other putative establishment genes after transfer of plasmid p576 into a new host

As explained above, ardC576 has the features of a typical establishment gene. The direct repeated motifs near the putative promoters may be the binding sites for a regulatory protein. If correct, this indicates that the same transcriptional regulator controls the activities of promoters P20c, P23c, P27c, and PardC576. This, in turn, suggests that the genes controlled by these four promoters are members of the same regulatory network and that the encoded proteins would all play a role in plasmid establishment. The presence of identical or similar motifs near the putative promoters could hold the clue in achieving the temporal expression of these genes.

Based on these assumptions we devised the following working model. Plasmid p576 encodes a regulatory protein that has affinity for the 5′-TTATCC-3′ motif, and upon binding the regulatory protein would interfere with transcription from the nearby promoters and hence impair expression of the downstream-located establishment genes in cells harboring plasmid p576. During conjugation an ssDNA strand of the plasmid, but not the presumed regulatory protein, will be transferred into the new host. Once the ssDNA is converted into double-stranded plasmid DNA, the four promoters will be active resulting in expression of the establishment genes. Simultaneously, the gene encoding the repression protein will be expressed. Once sufficient level of the repressor protein has been synthesized the promoters will be shut off again. Consequently, the promoters will be expressed during a short window of time soon after entry of the DNA into the recipient. Experiments were designed to test this model and their results are presented below.

The upstream regions of p576 genes 20c, 23c, 27c and ardC576 encompassing the heptamer sequences contain active promoters

To study whether the p576 genes 20c, 23c, 27c and ardC576 are preceded by a promoter their upstream regions were each cloned in front of the lacZ reporter gene, and a single copy of each resulting fusion was placed at the B. subtilis chromosomal thrC locus. As a control, strain PKS7 was constructed, which contains a copy of a promoter-less lacZ at thrC (see ‘Materials and Methods’ section). As a first qualitative approach, colonies of the constructed strains were tooth picked onto LB agar plates supplemented with the chromogenic substrate Xgal. After overnight growth, all the colonies were blue except that of the negative control strain PKS7. Similar levels of the chloramphenicol resistance test this model and their results are presented below.

We next determined the apparent strength of these four promoters by determining the transcription start sites of promoters P20c, P23c, P27c, and PardC576, while that of promoter P27c was slightly lower. To localize the positions of the promoters we determined the transcription start sites of promoters P20c, P23c and P27c, and PardC576, while that of promoter P27c was slightly lower. By RNA ligase-mediated rapid amplification of 5′ cDNA ends (5′-RACE, see ‘Materials and Methods’ section), the obtained transcription start sites are indicated in Figure 1B and confirmed that the promoter positions predicted above are correct.

We next determined the apparent strength of these four promoters by determining the β-galactosidase activity (βGA) of late exponentially growing cultures of the strains (Figure 2B). As expected, very low levels of βGA were obtained for the negative control strain PKS7. Similar levels of βGA, in the range of 270–325 Miller units, were obtained for strains containing lacZ fused to promoters P20c, P23c and PardC576. The βGA of strain DG127c containing the P27c-lacZ fusion was about three times lower, though. The sequences of promoters P20c and P23c are alike; their −10 boxes are identical, while their −35 boxes are very similar to the consensus αA-type promoter boxes, (see Figure 1B). Hence, it was not surprising that they displayed a similar high promoter activity. However, the relatively high activity of promoter PardC576 was surprising considering that its −35
p576 gene 27c encodes a repressor of promoters P_{20c}, P_{23c}, and P_{ardC576} and regulates its own expression

To study if p576 encodes a repressor of promoters P_{20c}, P_{23c}, P_{26c}, and P_{ardC576} we aimed to introduce the plasmid into the above lacZ-reporter strains for these promoters. However, p576 does not contain an antibiotic resistance or other markers for its selection in B. subtilis. Despite several attempts and using different approaches we did not succeed in labeling the p576 plasmid with an antibiotic resistance marker. We did obtain, though, a derivative of p576 in which the largest of the three EcoRI fragments, corresponding to approximately the 3’ half of the conjugation operon, was replaced by a chloramphenicol resistance gene. We introduced this derivative, which we named pBCM1, into the B. subtilis strain harboring the P_{20c}-lacZ fusion, resulting in strain BCM1. Colonies of strain BCM1 (P_{20c}-lacZ, pBCM1) were white when grown on LB plates containing Xgal (not shown). This result strongly indicates that p576 encodes a repressor of promoter P_{20c}.

The conjugation operons and the surrounding regions of p576 and pLS20 are similarly organized. The conjugation operon of pLS20 is repressed by default, and only under appropriate conditions the conjugation genes are activated during a rather short window of time (40). Based on this, we reasoned that it was unlikely that the repressor gene would be located within the conjugation operon of p576. We also discarded genes located on the large EcoRI fragment that is absent in pBCM1, and genes to which a function not involving DNA binding could be attributed based on similarity with known genes. The deduced proteins of the remaining genes were subjected to in silico analyses to identify the presence of putative DNA binding domains. Using this approach genes 20c, 27c, 29, and 30c were identified as candidate repressor genes. To test whether any of these genes encodes the repressor of promoters P_{20c}, P_{23c}, P_{26c}, and P_{ardC576} we placed each of these four genes at the chromosomal amyE locus under the control of the IPTG-inducible P_{spank} promoter. Gene 27c was an exception in the sense that this gene was cloned behind the P_{spank} promoter together with its transcriptionally coupled downstream gene 26c. We then introduced the P_{spank}-lacZ reporter fusion into each of these four strains. In parallel, we also introduced into each of the four strains a cassette in which the lacZ gene was fused to the upstream region of p576 gene 16, predicted to contain a promoter but without a 5’-TATCCG-3’ motif and so would serve as a repressor-insensitive control. As shown in Figure 3A, colonies of all the eight strains, including those carrying the lacZ reporter for the upstream region of gene 16, were blue when grown on agar plates supplemented with only Xgal. In the presence of IPTG colonies were also blue except for strain JV5 harboring the P_{spank}-27c-26c cassette. These results demonstrate that gene 27c and/or gene 26c encodes a regulator that represses P_{spank} but not promoter P_{16}. We next tested whether gene 27c or 26c alone was sufficient to repress promoter P_{26c} using cassettes containing either P_{spank}-27c or P_{spank}-26c. Results presented in Figure 3B show that induction of gene 27c, but not 26c, resulted in repression of the P_{26c} promoter. To study whether expression of 27c also repressed the activities of promoters P_{23c}, P_{27c} and P_{ardC576}, we introduced the respective tran-
and 26c a regulatory protein that represses the promoters P_{20c} and P_{27c}. We fused a regulatory protein that represses P_{20c} promoter P_{27c}. As shown in Figure 4A, Reg576 clearly bound to the promoter site of Reg576. To facilitate the purification of Reg576, we grew overnight at 37 °C on plates supplemented with X-gal, or with Xgal and IPTG.

Figure 3. Gene 27c (reg576) encodes a regulator that represses promoters P_{20c}, P_{27c} and P_{ardC576}, as well as its own promoter P_{reg576} (A). Strains containing a copy of lacZ fused to each of the wild-type promoter P_{20c} or P_{27c} in combination with another cassette containing one of the candidate repressor genes 26c, 27c, 26c, 29 and 30c under the control of the IPTG-inducible promoter P_{pungk} (see Supplementary Table S1) were grown overnight at 37°C on plates supplemented with X-gal, or with Xgal and IPTG. (B) Strains containing a copy of lacZ fused to each of the promoters P_{20c}, P_{27c}, P_{ardC576}, P_{pungk} in combination with another cassette containing both genes 27c and 26c, or only gene 27c (reg576) or gene 26c under the control of the inducible promoter P_{pungk} were grown overnight at 37°C on plates supplemented with X-gal, or with Xgal and IPTG.

All together, these results show that p576 gene 27c encodes a regulatory protein that represses the promoters P_{20c}, P_{27c} and P_{ardC576} as well as its own promoter P_{reg576}. Based on these results we denominated p576 gene 27c reg576 (repressor of establishment genes).

The operator site of Reg576 is constituted by a dual 5′-TTATCCC-3′ motif

In vitro and in vivo approaches were used to establish the operator site of Reg576. To facilitate the purification of Reg576 we fused reg576 in frame to a region encoding a 6-histidine tag at either the C- or N-terminus of reg576 (see ‘Materials and Methods’ section). Both recombinant genes, reg576 his(6) and his(6)reg576, were functional as, when induced, they were able to repress the expression of the P_{20c}-lacZ fusion in B. subtilis (not shown). Recombinant gene his(6)reg576 was chosen for further studies and the purified protein, which we referred to as Reg576, was used in EMSAs to identify the operator site. As shown in Figure 4A, Reg576 clearly bound to each of the four promoter-containing DNA fragments at concentration as low as 42 nM, and all the DNA fragments were shifted at 380 nM, independent of the promoter analyzed. However, there was a fundamental difference between the retardation patterns of promoters P_{20c}/P_{27c} and P_{27c}/P_{ardC576}. Only a single shift was observed for the latter two promoters whereas, depending on the protein concentration applied, up to two shifts were observed for promoters P_{20c} and P_{27c}. These results indicate that promoters P_{20c}/P_{27c} and P_{27c}/P_{ardC576} contain two and one Reg576 operator sites, respectively.

We then selected promoters P_{20c} and P_{27c} to study the binding of Reg576 in more detail by analyzing the effects of mutations in one or more of the repeated heptamer motifs. A summary of the DNA fragments used with the specific mutations and the results of EMSAs is given in Figure 4B; representative results of the corresponding EMSAs are shown in Supplementary Figure S3. As shown above, the wild-type P_{20c} promoter (fragment F-20c) gave up to two retardation species (see also Figure 4A), but the fragments containing mutations in motifs [‘A1’ and ‘A’] or [‘B1’ and ‘B2’] gave only a single shift. Furthermore, no retarded species was observed for fragment F-mut1C having mutations in all four motifs ‘A1’, ‘A2’, ‘B1’ and ‘B2’. These results provide firm evidence that (i) Reg576 binds specifically to the 5′-TTATCCC-3′ motifs and (ii) that motifs [‘A1’ and ‘A’] and [‘B1’ and ‘B2’] each constitute an operator site. Based on these results we named the DM located upstream of the −35 box operator 1, and the DM upstream of the RBS operator 2. We next analyzed whether mutations in only one of the two motifs of an operator site affected Reg576 binding. Only a single retarded species was observed for DNA fragments F-mut1D, F-mut1E, F-mut1F and F-mut1G demonstrating that a functional Reg576 operator requires a dual 5′-TTATCCC-3′ motif.

Similar results were obtained for promoter P_{27c}. Only one retarded species was observed for fragment F-27c containing the wild-type P_{27c} promoter, but no retardation was observed for fragment F-mut2A containing mutations in motifs ‘A1’ and ‘A2’. These results showed (i) that motifs [‘A1’ and ‘A’] and [‘B1’ and ‘B2’] constitute a functional Reg576 operator of promoter P_{27c}, and (ii) that the motifs [‘C1’ and ‘C2’], which contain two and one deviations with respect to the consensus sequence, respectively, do not constitute a functional operator. The observation that fragments F-mut2B and F-mut2C gave one and no retarded species, respectively, further substantiates these conclusions.

We next studied how the mutations in the 5′-TTATCCC-3′ boxes analyzed by EMSA affected Reg576-mediated repression of the P_{20c} and P_{27c} promoters in vivo. For this, lacZ fused to each of the wild-type or the mutated DNA regions were introduced into strain JV23 containing a copy of reg576 under the control of the inducible P_{pungk} promoter, and the resulting strains were used to determine the strength of the P_{27c} and P_{20c} promoter without or with different levels of reg576 induction. An overview of the transcriptional fusions and a graphical representation of the results obtained are given in Figure 5. As shown above as well as below, the motifs ‘C1’ and ‘C2’ of P_{27c} do not constitute a functional operator site. Mutations in these motifs, which are located in between the −35 and −10 boxes of promoter P_{27c}, drastically affected its promoter activity and hence the response of these mutant derivatives to reg576 induction could not be tested. Mutations in boxes ‘A1’, ‘A2’, ‘B1’ and/or ‘B2’ mod-
Figure 4. Determination of the operator site of Reg576 by EMSA. (A) Indications that promoters P\textsubscript{20c}/P\textsubscript{23c} and P\textsubscript{reg576}/P\textsubscript{ardC576} contain two and one operator, respectively. EMSA results obtained using ∼400 bp DNA fragments (150 ng) encompassing promoters P\textsubscript{20c} (F-20c), P\textsubscript{23c} (F-23c), P\textsubscript{reg576} (F-27c) or P\textsubscript{ardC576} (F-28c). −, loaded without protein (lanes ‘a’). Increasing concentrations of Reg576 were prepared using a 3-fold dilution method, and ranged from 4.7 (lanes ‘b’) to 1150 nM (lanes ‘f’). (B) Schematic representation of the DNA fragments of variants of promoter P\textsubscript{20c} and P\textsubscript{27c} (P\textsubscript{reg576}) used as probes and summary of the EMSA results. −35 and −10 promoter boxes are highlighted in blue. 5′-TTATCCC-3′ motifs [A1, A2, B1, B2] and [C1, C2] are highlighted against a red and pink background, respectively. Deviations with respect to the consensus sequence are given in green. Introduced mutations are shown in purple.


erately improved the promoter strength (up to a maximum of 1.8- and 1.5-fold for promoter P\textsubscript{27c} and P\textsubscript{20c}, respectively, see Supplementary Figure S4). In agreement with the results presented in Figure 3, in the presence of IPTG the activities of the wild-type promoters P\textsubscript{20c} and P\textsubscript{27c} were affected although they responded differentially to the increasing levels of reg576 induction. When grown in the presence of 4 μM IPTG, a clear reduction in promoter activity was observed for promoter P\textsubscript{20c} but not P\textsubscript{27c}. In the presence of 8 μM IPTG the activities of promoter P\textsubscript{20c} and P\textsubscript{27c} dropped about 5- and 2-fold, respectively; and at 32 μM of IPTG the activities were reduced by about 20-fold for P\textsubscript{20c} and 7-fold for promoter P\textsubscript{27c}. This indicates that P\textsubscript{20c} is repressed more strictly and at lower Reg576 concentrations than the P\textsubscript{27c} promoter. P\textsubscript{20c} and P\textsubscript{27c} derivatives containing mutations in operator 1 (mut1A and mut2A, respectively) also responded differently to reg576 induction. Whereas the activity of promoter P\textsubscript{27c} derivative mut2A was not affected upon reg576 induction, the activity of P\textsubscript{20c} derivative mut1A was lower when reg576 was induced. Interestingly, promoter
Figure 5. Effects of operator mutations on Reg576-mediated repression of the p576 promoters P\textsubscript{20c} and P\textsubscript{27c} (P\textsubscript{reg576}) \textit{in vivo}. Strains containing transcriptional lacZ fusions with the wild-type sequences encompassing promoters P\textsubscript{20c} or P\textsubscript{reg576} (P\textsubscript{27c}) orderivatives having mutations in one or more of the 5′-TTATCCC-3′ boxes were subjected to βGAs to determine their relative promoter strength. Cell samples were withdrawn from late exponential growing cultures (OD\textsubscript{600} = 1). (A) Schematic overview of the features of the strains used, and cartoons showing which heptamer boxes are mutated. Mutations are identical to those shown in Figure 4B. (B) Sedimentation coefficient distribution profile obtained by SV showing a single sedimenting species. (B) Concentration gradient obtained by SE. Data (empty circles) are shown together with best-fit analysis assuming a protein dimer (solid line), monomer (dashed line) and tetramer (dotted line). The lower plot shows the difference between experimental data and estimated values for the protein dimer model (residuals).

Protein Reg576 is a dimer in solution

To determine its oligomerization state \textit{in vitro} we subjected purified Reg576 to analytical ultracentrifugation sedimentation velocity (SV, Figure 6A) and dynamic light scattering (DLS) analyses in parallel. In the SV assays, Reg576 at concentrations ranging from 3 to 124 μM was observed always as a single species with an experimental sedimentation coefficient of 1.6 S. This value, corrected to standard conditions (s\textsubscript{20,w} = 1.8 S), was compatible with the theoretical mass of the nearly globular Reg576 dimer (Figure 6A). DLS analysis of Reg576 yielded a translational diffusion coefficient (D) of 7.16 × 10\textsuperscript{-7} ± 0.04 cm\textsuperscript{2}/s, which introduced with the obtained s-value of 1.6 S into the Svedberg equation, resulted in an apparent molar mass of 22 093 Da, very close to the molecular mass of Reg576 dimers (21 779 Da). Finally, sedimentation equilibrium (SE) assays with Reg576 at concentrations ranging from 3 to 30 μM showed a buoyant mass of 5398 Da, corresponding to a molar mass of 21 840 ±
180 Da, matching the molecular weight of the protein dimer (Figure 6B). Together, these data show that Reg576 forms dimers in solution.

**Two Reg576 dimers bind to one operator**

Results presented above show that (i) a functional operator in vivo is constituted by a dual 5′-TTATCCC–3′ motif; (ii) Reg576 is unable to bind in vitro to an operator containing mutations in one of the two motifs and (iii) Reg576 is a dimer in solution. Based on these results it seemed likely that one protomer of a Reg576 dimer would bind to one 5′-TTATCCC–3′ motif, and the other protomer to the flanking motif. To test this prediction we performed multi-signal sedimentation velocity (MSSV) experiments using samples containing a DNA fragment without Reg576 or with a >100-fold excess of Reg576 (0.1 versus 11 μM). The fragments used were the 196 bp DNA F-P20c encompassing promoter P20c and its two flanking Reg576 operators, or derivatives in which 5′-TTATCCC–3′ motifs have been mutated ([‘A1’ and ‘A2’ (mut1A)], [‘A1’, ‘A2’, ‘B1’, ‘B2’ (mut1C)] or [‘A1’ (mut1D)], see Figure 7A for a schematic view of the DNA fragments used). Simple absorbance plots for the DNA fragments without or with Reg576 (OD260 nM) are shown in Figure 7B. As expected, the different DNA fragments in the absence of Reg576 showed the same s-value of 5.0 S. The presence of Reg576 did not increase the s-value of fragment F-mut1C, which has mutated all the four motifs, but it did result in a major increment (from 5.0 S to 6.3 S) for F-P20c (having both operators intact) and moderate (from 5.0 S to 5.6 S) increments for fragments F-mut1A (mutations in ‘A1’ and ‘A2’) and F-mut1D (mutations in only ‘A1’). These results show that Reg576 did not bind to fragment mut1C, but that it bound to the other three DNA fragments. Based on the increase in s-value it seemed that fragments F-mut1A and F-mut1D bound the same or similar amounts of Reg576, and that fragment F-P20c bound more Reg576 dimers than mut1A and mut1D. However, care has to be taken with this interpretation since the s-value is not only determined by the molecular weight of the complex but also by its size and shape. To fully extract the maximum information enclosed in the SV data, besides the hydrodynamic separation of the complexes, we took advantage of the simultaneous absorbance data acquisition at 230 and 260 nm and globally analyzed them through SEDPHAT to get the diffusion-deconvoluted sedimentation coefficient distributions with spectral deconvolution of the absorbance signals, c(s). Further improvement of the molar ratio resolution was achieved by using both, mass conservation constraint and multi-segmented model restriction, using our prior knowledge that only free Reg576 sediments in the low-s region and no free DNA can be found from 1 S to 3 S. The MSSV analysis of Reg576-F-P20c complex indicated that the areas under the peaks corresponded to a stoichiometry of 7.9 moles of Reg576 bound per mol of F-P20c. The ratios of Reg576 moles with respect to DNA fragments F-mut1A and F-mut1D were 4.3 and 3.7, respectively (see Figure 7C). These results are in line with those obtained in the EMSAs using the maximum amounts of Reg576. Thus, they confirm that (i) Reg576 binding is abolished when all four motifs are mutated; (ii) operator 1 and 2 are both binding sites for Reg576; and (iii) mutation of one motif of an operator abolishes binding of Reg576 to that operator even at a >100-fold excess of Reg576 over DNA.

Contrary to our expectations though, these results indicated that one 5′-TTATCCC–3′ motif is bound by two Reg576 monomers (i.e. one 5′-TTATCCC–3′ motif is bound by a Reg576 dimer). To assure that the calculated stoichiometries determined by MSSV were correct we performed SE analytical ultracentrifugation experiments. This technique permits determining the exact molecular weight of the complexes and, since the molecular weight of the DNA and protein components are known, the obtained values can be used directly to calculate the number of protein molecules bound to a DNA molecule. We selected DNA fragments F-P20c, F-mut1A and F-mut1D for this analysis. The sedimentation behavior of the different DNA fragments alone fitted well with a single sedimenting species model with apparent buoyant mass of 57 000 ± 380 Da, in good agreement with the expected molecular weight for these DNA fragments (data not shown). As explained in material and methods, a fixed DNA concentration of 0.1 μM was titrated with increasing Reg576 concentrations (from 0.1 to 3 μM). Figure 7D shows the binding isotherms built from the experimental buoyant mass increments obtained at low speed and 260 nm, through an empirical three parameters Hill plot (Equation 6):

\[ y = \frac{ax^b}{K_d + x^b} \]  

Where y stands for the number of proteins bound per DNA, a denotes the maximum number of proteins bound at saturation, x is the total concentration of protein, K_d is the concentration of half-maximal binding and b is an empirical cooperativity parameter.

Taking into account the complex stoichiometry experimentally determined for Reg576-F-P20c, an apparent cooperative model (b = 7.8) can explain the experimental binding isotherm, with a macroscopic K_d of 0.8 ± 0.1 μM. Analogously, for Reg576-F-Mut1A and Reg576-F-Mut1D complexes, an apparent cooperative model (b = 4.1 and 3.2, respectively) can account for the binding isotherm with a macroscopic K_d of 0.7 ± 0.1 μM. These results, together with those obtained by MSSV technique and taking into account that Reg576 is a dimer in solution, demonstrate unequivocally that two Reg576 dimers bind to one functional operator. In addition, these results confirm that mutation of only one of the two motifs of an operator abolishes binding of both Reg576 dimers to the operator. Altogether, the results obtained confirmed that Reg576 binds its operator as a dimer of dimers.

**Mathematical modeling shows that high cooperativity and differential repression are key for tight temporal regulation of establishment genes**

To explore the qualitative dynamics of gene expression that our experimental findings imply, we designed a minimal mathematical model for the regulation of the putative p576 establishment genes based on modeling the dynamics of the mRNA and protein of the repressor Reg576, and those of
Figure 7. Evidence that Reg576 binds its operator as a dimer of dimers. (A) Cartoons illustrating with asterisks which of the 5′-TTATCCC-3′ motifs are mutated in the 196 bp DNA fragments used. Mutations in the motifs are identical as those shown in Figure 4B. (B) Sedimentation coefficient distributions, c(s), obtained from SV assays at 260 nm with the different DNA fragments in the presence or absence of Reg576, showing the shift in the s-value of Reg576–DNA complexes (dashed trace) relative to the corresponding DNA alone (solid trace). To facilitate comparison of the peak positions only the s-range encompassing the DNA and Reg576–DNA complexes is shown. (C) Global multi-wavelength analysis of Reg576–DNA complexes and decomposition into component sedimentation coefficient distributions, c_k(s), for Reg576 (dashed trace) and the different DNA fragments (solid trace). For clarity, when comparing the areas under the peaks ascribed to the complexes the low-s-range where only Reg576 sediments is not shown. (D) Binding isotherms for the interaction of Reg576 with F-P20c (black circles), F-mut1A (white triangles) and F-mut1D (black triangles). The solid curves represent the best fit of the three-parameter Hill equation to the SE experimental data.

the putative establishment gene 20c. Since our data indicate that gene 23c is similarly regulated, we only selected gene 20c for modeling.

Our model is constructed with a repressor that represses itself, and also represses its targets with a tighter repression than itself (see ‘Materials and Methods’ section for details). Experimentally, these conditions on the regulation are justified by the high cooperativity with which Reg576 binds the wild-type P20c promoter that is flanked by two functional operators, and which is higher than the cooperativity obtained for mutated derivatives, like mut1A, containing only one functional operator. The promoter region of reg576 also contains only one functional operator (see above), and results presented in Figure 5 show that promoters P27c and the P20c derivative mut1A respond similarly to Reg576. We therefore assume that Reg576 binds its own promoter with a cooperativity that is similar to that determined for the P20c derivative containing one functional operator. Under these conditions, in our model establishment genes have a transient time interval of expression where they are strongly expressed before being shut down by the repressor (see Figure 8). The long time repression of establishment genes is not efficient if there is not enough cooperativity in the binding of the repressor to its targets (Supplementary Figure S5) or if the cooperativity with which the establishment genes are repressed is not significantly higher than the repression of the repressor itself (Supplementary Figure S6), showing that these conditions, consistent with our experimental findings, are key properties of the mechanism regulating the temporal expression of establishment genes. Our model not only shows that the regulatory logic found in our experiments is consistent with transient pulse-like expression of target genes, but more generally shows that an auto-inhibitory motif can play this role. Negative feedback loops have been associated with keeping homeostasis, to speed up response times, taming fluctuations or the emergence of oscillations (44). Multiple binding sites for transcriptional repressors can produce regular bursting and enhance noise suppression (44), but here we show that under the appropriate conditions of high cooperativity and differential repression of targets, they can also produce pulses of expression. Pulses have usually been associated to more complicated regulatory motifs like feed-forward loops (45).
The crystal structure of Reg576 reveals that it is a dimeric Ribbon Helix Helix protein and provides a model of the tetramer bound to a double DNA heptamer

To gain insights into the structural basis of DNA recognition of Reg576, we embarked on structure determination of the protein. The overall structure of Reg576, determined at 2 Å, revealed that the N-terminal R7-L47 residues or Reg576 form a Ribbon-Helix-Helix (RHH, see Figure 9). As is the case for other RHH proteins, the RHH core consists of a dimeric structure in which a double stranded anti-parallel β-sheet is formed by the two β-strands from the monomers (46). Beyond this core structure, Reg576 contains a third α-helix (H3, residues 49–65). An additional C-terminal helix is also present in the RHH proteins Mnt (1MNT) and SSV-RH (4AAI) for which structural data are available. However, the placement of this third helix with respect to the core is quite different in Reg576 compared to those in Mnt and SSV-RH. In Reg576 helix H3 contacts the outward flank of α-helix 1 (H1) of the opposite monomer. In Mnt, the third helix folds back on the H2′, but on the opposite side compared to Reg576, interacting with H1′. In the case of the SSV-RH protein, H3 folds back over the outward flank of H2′. The presence of an additional α-helix and its particular position with respect to the RHH core distinguishes Reg576 from other RHH proteins. Indeed, we have not found this geometry, with the exception of the structure of a protein from *Nitrosomonas europaea* of unknown function (PDB code: 1ZX3).

To gain insight into the recognition of DNA by Reg576, we compared the binding site length and separation of the Reg576 recognition sites shown in Figure 1 with RHH proteins for which structures of the protein–DNA complex are available. The length of the Reg576 DNA sequence is 7 bps, with a 9 bp separation between the start of the two repeats. This binding mode resembles that of the RHH proteins Arc (e.g. 1PAR), CopG (e.g. 1EA4) and AmrZ (3QOQ), which all bind to DNA repeats that are separated by approximately one turn of the DNA double helix. When we superpose two dimers of Reg576 on the protein chains of the structure of the Arc repressor complexed to DNA (PDB code: 1PAR), the interactions formed between the Reg576 dimers are feasible and chemically sensible, with no strong clashes between residues (Figure 9). To identify the different chains, we follow here the naming scheme as shown in Figure 9, where chains denoted by A and B are from one dimer, and A′ and B′ are the chains from the other dimer, where the prime indicates that the chains are related by C2 symmetry between the dimers of the tetramer, A and A′ being the chains with the β-strands closest to each other in the tetramer. The putative interactions between consecutive Reg576 dimers are formed between the H1–H2 loops of monomers from the two different dimers. More precisely, residues V29 and V29′ and residues E33 and D28′, all from...
chains B and B′, interact. Residues K27 from chain B interact with Y42 from A′ and with I37 from B′. In addition, residues from the H2-H3 loop also form an interaction in the tetrameric model. Residues K46 from chain A and E51 from A′ form a salt bridge, and K46 interacts with the backbone residues from K46-S48 from chain A′ through hydrophobic interactions. Additionally, N45 from chain A comes close to the N-terminal residues of helix H3 (S48, E49 and K50). Residues from K46 to E49 constitute the loop that connects helices H2 and H3 of Reg576. Together the model indicates that two Reg576 dimers involve more interactions than two Arc dimers. This is also reflected by an increase in the buried accessible surface area, as calculated by the program PISA (47). Whereas the buried surface between two Arc dimers (1PAR structure) is 297.8 Å², that of two Reg576 dimers is 547.6 Å² according our model.

**DISCUSSION**

Establishment genes contribute to the stable establishment of a conjugative element in the new host. Deregulating expression of these genes or inactivating the activity of the encoded proteins are potential strategies to inhibit conjugation and thereby restraining conjugation-mediated spread of antibiotic resistance. Our knowledge on establishment genes and how they are regulated is scarce, especially for those present on conjugative elements of G+ bacteria. The few establishment genes for which their function has been established include anti-restriction (ard) genes, and genes encoding proteins that bind ssDNA (SSB) or suppress the SOS response (PsiB). While the SSB protein is required to coat the single-stranded form of the transferred conjugative element and are important for DNA replication, the anti-restriction (Ard) and anti-SOS (PsiB) proteins improve the success rate of establishment in the recipient cell by inhibiting the restriction enzyme and SOS response, respectively. For proper functioning, these genes must be expressed rapidly upon entry of the conjugative element into the new host on the one hand, and on the other hand their expression must be silenced after a relatively short time. This is because prolonged expression may be harmful for the cell: lengthy inactivation of the restriction proteins makes the cell vulnerable to the entry of other foreign DNA like phage DNA, and extended suppression of the SOS system prevents the cell to respond appropriately to for example DNA damage. Hence, particular regulatory mechanisms must ensure that the genes are expressed rapidly but transiently after transfer of the conjugative element into the recipient cell. Most knowledge on the expression of establishment genes is based on studies of a few conjugative E. coli plasmids, especially F and ColIB-P9. The sSB, ard and psiB genes, which are present on enterobacterial plasmids of different incompatibility groups (48, 49), are indeed expressed rapidly and transiently upon entry of the plasmid into the recipient cell (9,50,51). Special promoters that are only active when the DNA is in its single-stranded form (11) control these three genes, as well as other genes of unknown function. Consequently, these genes are expressed when the ssDNA strand enters the recipient but their expression is turned off when the immigrating ssDNA has been converted into double stranded DNA.

Here, we found that the p576 promoters P_{20c}, P_{26c}, P_{27c} and P_{ardC576} contain in their vicinity (dual) boxes of the heptamer sequence 5′-TTATCCC-3′. We show that these promoters are similarly regulated due to binding of the Reg576 protein to the dual heptamer sequences. These and other results support the view that the Reg576-controlled promoters are derepressed upon transfer of the plasmid into the recipient until sufficient Reg576 is produced to silence them again. One of the genes regulated in this way is ardC576, whose encoded protein shows about 50% similarity with type C anti-restriction proteins encoded by conjugative plasmids of Gram-negative bacteria that play a role in establishment of plasmids in the recipient cell after conjugative transfer (9,13,16). Based on this, we propose that ardC576 encodes an anti-restriction protein that may enhance the establishment of p576 in a recipient cell. It seems reasonable to assume that the other Reg576-controlled genes encode proteins that are functionally related and that they play a role very soon after transfer of the plasmid in the recipient cell, which may include enhancing establishment of the plasmid in the new host.

The mechanism in which a promoter is derepressed until sufficient repressor is synthesized to silence its activity is very similar to zygotic induction, whose term was first used to describe the phenomenon that conjugation-mediated transfer of the E. coli prophage λ, but not the repressor of the lytic genes, into a recipient cell lacking the λ prophage resulted in immediate induction of the prophage and subsequent lysis of the ‘zygote’ (52,53). Establishment genes of the G- broad host-range plasmid pKM101 may be controlled by a zygotic induction-like system. The pKM101 establishment genes are located in seven transcriptional units that are all preceded by >400 bp long conserved DNA regions that may be binding sites for two repressor proteins, ArdR and ArdK (54). However, the regulatory function of these repeated sequences and the putative regulatory proteins have not been studied in detail. As far as we know, this is the first detailed study of a mechanism that ensures transient expression of establishment genes without using ssDNA promoters. Intriguingly, it appears that the putative establishment genes of the related plasmid pLS20 are regulated in a fundamentally different way. This assumption is based on the observation that sequences highly similar to those that precede its putative anti-restriction gene are present upstream of several other pLS20 genes/operons, suggesting that they are co-ordinately regulated. However, these sequences do not share similarity to any of the upstream sequences of the p576 promoters studied here. It will be interesting to unravel the way these pLS20 genes are regulated.

Members of the superfamily of RHH transcription factors are involved in the regulation of diverse bacterial processes such as amino-acid biosynthesis, cell division, control of plasmid copy number and lytic cycle of bacteriophages (for review see, 46). Recently, we have shown that many auxiliary relaxosome proteins encoded by conjugative plasmids of G+ bacteria are also RHH type proteins (22). Here we show that Reg576 too is a RHH type protein, thereby expanding even further the range of processes in which this family of proteins is involved. To bind DNA in a sequence-specific manner, RHH proteins use a relatively short con-
erved 3D structural domain (generally 36 residues) that is formed by intertwining of the RHH motif of two monomers referred to as RHH$_2$. Hence, most RHH proteins are dimers having a 2-fold symmetry. Reg$_{576}$ is no exception. In many cases the operators to which RHH proteins bind are composed of two or multiple binding sites arranged as inverted or tandem repeats to which higher order RHH oligomers bind. We show that this is also the case for Reg$_{576}$: its operator is composed of two subsites that are arranged in a direct repeated orientation spaced by 2 bps and that this operator is bound by two Reg$_{576}$ dimers. However, compared to other RHH proteins, Reg$_{576}$ stands out with its extremely high level of cooperativity for binding its operator. For comparison, the well-studied 53 residue RHH Arc protein of bacteriophage P22 binds its two subsite operator (total of 21 bp) in a highly cooperative manner with a calculated Hill constant of 3.5 (55). Nevertheless, binding of individual Arc dimers to a DNA fragment containing only one subsite was observed by EMSA (56). Using EMSA and analytical ultracentrifugation techniques we were unable to detect binding of Reg$_{576}$ to DNA fragments containing only one intact subsite, indicating that it binds the operator with extreme high cooperativity. This is supported by the high calculated Hill coefficient of 7.8. Based on the crystal structure presented here and the DNA-bound structure of the Arc repressor (PDB code: 1PAR), we constructed a model of two Reg$_{576}$ dimers bound to two consecutive DNA repeats. The results suggest that the additional Reg$_{576}$ helix H3 and the preceding H2-H3 loop, both at the C-terminus of the RHH core, are able to form contacts between the two dimers of the tetramer. These contacts are not observed in the Arc structure or structures of other RHH proteins bound to two DNA repeats. These additional interactions of Reg$_{576}$ may explain the high cooperativity of the Reg$_{576}$ tetramer binding to its operator DNA site.

Another interesting finding of this work is that Reg$_{576}$ regulates expression of its own relatively weak promoter P$_{27c}$. Interestingly, the P$_{27c}$ promoter has a different configuration compared to the strong P$_{23c}$ and P$_{25c}$ promoters: the latter two promoters both contain two Reg$_{576}$ operators, whereas P$_{27c}$ contains only one. Consequently, Reg$_{576}$ represses P$_{27c}$ less strictly than P$_{23c}$ and P$_{25c}$. Modeling suggests that this organization results in a regulatory system in which the P$_{23c}$ and P$_{25c}$ promoters will be efficiently repressed by the relatively low levels of Reg$_{576}$.

The P$_{ardC576}$ promoter driving the expression of the putative anti-restriction gene ardC$_{576}$ appeared to be a strong promoter with a strength that is similar to that of promoters P$_{23c}$ and P$_{25c}$. However, we showed that the strength of P$_{ardC576}$ is similar to that of P$_{27c}$, and that the apparent high promoter activity is due to the presence of inverted repeated sequences in the long untranslated region upstream of the ardC$_{576}$ gene that are predicted to form secondary structures when transcribed into RNA and which probably increase the stability of the transcripts. Promoter P$_{ardC576}$ contains only one Reg$_{576}$ operator and therefore it is expected that it responds less sensitive to Reg$_{576}$ than promoters P$_{23c}$ and P$_{25c}$. Whether this serves a biological purpose requires further research.

In summary, in this work we have provided evidence that the expression of several possible establishment genes of the conjugative plasmid p576, located in different transcriptional units, are regulated by the dimeric Reg$_{576}$ RHH-type repressor protein by a zygotic induction mechanism, which ensures that these genes are transiently expressed when the plasmid enters the recipient cell. Two Reg$_{576}$ dimers bind their operator that is composed of a dual 5'-TTATCCC-3' motif with extremely high cooperativity, and the crystal structure of the Reg$_{576}$ dimer provides an explanation for this high cooperative binding. These studies have determined for the first time in detail how establishment genes are regulated in a manner different from that of the ssDNA promoters. It would be very interesting to understand the functions of the putative p576 establishment genes other than ardC$_{576}$.

**DATA AVAILABILITY**

Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data bank under accession number 6GYG.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**REFERENCES**


