Identification of an RcsA/RcsB Recognition Motif in the Promoters of Exopolysaccharide Biosynthetic Operons from Erwinia amylovora and Pantoea stewartii Subspecies stewartii* \\

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The regulation of capsule synthesis (Rcs) regulatory network is responsible for the induction of exopolysaccharide biosynthesis in many enterobacterial species. We have previously shown that two transcriptional regulators, RcsA and RcsB, do bind as a heterodimer to the promoter of amsG, the first reading frame in the operon for amylovoran biosynthesis in the plant pathogenic bacterium Erwinia amylovora. We now identified a 23-base pair fragment from position −555 to −333 upstream of the translational start site of amsG as sufficient for the specific binding of the Rcs proteins. In addition, we could detect an RcsA/RcsB-binding site in a corresponding region of the promoter of cpsA, the homologous counterpart to the E. amylovora amsG gene in the operon for stewartan biosynthesis of Pantoea stewartii. The specificity and characteristic parameters of the protein-DNA interaction were analyzed by DNA retardation, protein-DNA cross-linking, and directed mutagenesis. The central core motif TRVGAAWATSG of the amsG promoter was found to be most important for the specific interaction with RcsA/RcsB, as evaluated by mutational analysis and an in vitro selection approach. The wild type P. stewartii Rcs binding motif is degenerated in two positions and an up-mutation according to our consensus motif resulted in about a 5-fold increased affinity of the RcsA/RcsB proteins.

The ability to produce capsules or exopolysaccharides (EPS)1 is characteristic for most bacterial species. General benefits of encapsulation are the prevention of desiccation, advantages in the degradation of substrates by adherence, and the binding of toxins and nutrients (1). EPS is furthermore an essential determinant for the bacterial virulence in several host-pathogen interactions (2), e.g. during infections by the plant pathogenic bacteria Erwinia amylovora and Pantoea stewartii subsp. stewartii (Ref. 3; formerly Erwinia stewartii). The dense layer of EPS is supposed to shield invading microorganisms against host defense systems like the hypersensitive response reaction. It might further prevent cell aggregation by agglutinins, and it has been reported to accelerate the wilting of infected plants by plugging xylem vessels (4).

The EPS structure is highly variable, and different types have been classified by molecular weight and structural properties (5). The biosynthesis of the high molecular weight EPS type IA in several enterobacterial species is modulated by the Rcs (regulation of capsule synthesis) regulatory network (6). Prominent examples are the regulation of colanic acid and many K antigens in Escherichia coli (7, 8), Klebsiella aerogenes (9–11), and Salmonella typhi (12). In plant pathogenic bacteria, the regulation of amylovoran synthesis in E. amylovora (13–16) and stewartan synthesis in P. stewartii (17) by Rcs proteins has been reported. EPS biosynthesis is supposed to be induced after perception of external signals by membrane-located sensors like the RcsC protein. The signal might be subsequently transduced by phosphorylation of the response regulator RcsB, and the two proteins represent a typical bacterial two-component system (18). High levels of EPS biosynthesis require the coinduction by the unstable protein RcsA (7, 19, 20). RcsA and RcsB are grouped into the LuxR class of bacterial regulators based on the sequence of their C-terminal helix-turn-helix DNA binding motifs.

The structural genes for the biosynthesis of amylovoran in E. amylovora, stewartan in P. stewartii, and colanic acid in E. coli are clustered and regulated as operons (21–23).2 The essential regulatory region upstream of the first open reading frame of the ams operon covers an unusually large region of about 700 bp (21). The transcriptional start site of the homologous cps operon of E. coli was mapped at 340 bp upstream of the translational start site (23). The transcriptional units of EPS operons may therefore contain large leader regions of yet unknown function. We recently reported the binding of RcsA and RcsB proteins from E. amylovora and E. coli to a putative promoter region located between −578 and −501 relative to the translational start site of amsG, the first reading frame in the ams operon (24). We could demonstrate the binding of RcsA and RcsB as a heterodimer and that RcsB, but not RcsA, is able to bind alone at higher concentrations.

In this report, we could confine the essential Rcs-binding region, and we present the recognition motif for the RcsA/RcsB dimer at the amsG promoter of E. amylovora. We could furthermore identify an RcsA/RcsB-binding site at the corresponding location in the P. stewartii cpsA promoter. Our results give evidence that the binding of RcsA/RcsB to promoters in EPS operons could be a common principle for triggering the capsule synthesis in the Rcs regulatory pathway.

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§ The abbreviations used are: EPS, exopolysaccharide; RCS, regulatory system; ams, amylovoran synthesis; cps, colanic acid synthesis; RcsA, RcsB, regulators; EMSA, electrophoretic mobility shift assay; bp, base pair; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

2 D. L. Coplin, unpublished observations.
MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this work are described in Table I. Bacterial cells were routinely grown in LB broth at 37 °C, and ampicillin was added, if appropriate, to a final concentration of 100 μg/ml.

DNA Techniques—Standard techniques such as DNA cloning, DNA analysis, and cell transformations were done as described (25). Sequencing grade plasmid DNA was isolated from strain XL1-Blue. DNA analysis, and cell transformations were done as described (25). The polymerase chain reaction (PCR) was performed with Vent polymerase after optimization for Mg²⁺ concentration. The P. stewartii rcsA gene was amplified from chromosomal DNA of the P. stewartii wild type strain DC283 by using the following primers: RcsA PS forward, GGGGAAAATTCATCTCCAGTAGGTTTG; RcsAPS reverse, GGAAGCTTACATTCAAGAAGACCTG.

Expression and Purification of Proteins—The bacteria were grown in a 10-liter fermenter at 28 °C with 90% O₂ saturation and at pH 7.0. LB broth was subcultured (1:200) into fresh medium from an overnight broth was subcultured (1:200) into fresh medium from an overnight culture, and 0.5 ml isopropyl-1-thio-β-D-galactopyranoside was added when the Aₕₕ reached 0.5. The cells were incubated for additional 3 h, pelleted by centrifugation, and stored at −25 °C. The RcsA proteins were expressed from plasmids pM-RcsAEA and pM-RcsAES in strain JB3034 as a C-terminal fusion to the maltose-binding protein. The RcsB proteins were expressed from plasmids pM-RcsBÆA until S-F contained the fragment F23 with the randomly substituted fragment M4/6 resulted in a decrease of retardation to about 75% of the wild type strain, Nal r 44.

RESULTS

Location of an RcsA/RcsB-binding Site in the amsG Promoter—The binding region for the RcsA/RcsB heterodimer was included the putative 35 region. The first reading frame of the ams gene

Electrophoretical Gel Mobility Shift Assay (EMSA)—DNA labeling with [α-³²P]dATP, DNA binding assays, and separation of protein-DNA complexes from unbound DNA by native PAGE were performed as described (24). Rearrangement of DNA fragments by Rcs proteins was monitored by gel electrophoresis and exposure of the dried gels to x-ray films. For quantitative assays, the developed x-ray films were aligned with the corresponding dried gel, the bands of retarded and free DNA were cut out, and the amount of labeled DNA in the gel slices was quantified in a scintillation counter.

In Vitro Selection of DNA Fragments—The 23-bp fragment F₂₃ representing the minimal RcsA/RcsB binding region, was fused to a restriction linker and to an approximately 0.2-kilobase pair DNA fragment from the vector pBluescript to PCR to facilitate the selection procedure. The complete sequence of the fragment F₂₃ was then permutated by generating six DNA pools in which three or four bases of the wild type sequence were replaced by randomized bases. The DNA pools were amplified by PCR using the previously proposed promoter region of the amsG gene, and plasmids used in this work are described in Table I. Bacterial cells were routinely grown in LB broth at 37 °C, and ampicillin was added, if necessary.

DNA-Protein Cross-link—Reactions were set up as described for the EMSA. After 20 min at 28 °C, the solution was irradiated 5 cm from the ultraviolet light source for various times (as specified) with 312 nm. The reactions were cooled on ice during illumination. Oligonucleotides labeled with the photoactive thymine analogue 5'-iododeoxyuracil were purchased from TIB-MolBiol/Berlin and reconstituted to double-stranded DNA. The proteins directly interacting with the template were photo-cross-linked to it and resolved by denaturing gel electrophoresis. Cross-links were carried out in combination with competitor λ-DNA and bovine serum albumin.

RESULTS

Location of an RcsA/RcsB-binding Site in the amsG Promoter—The binding region for the RcsA/RcsB heterodimer was located by making deletions from each terminus of the previously identified 183-bp fragment F₁₈₃ and testing the retardation of each in EMSAs. The smallest retarded fragment was 23 bp (F₂₃) (Fig. 1). Further deletions of 5 bp by either end did not completely abolish the binding of the E. amylovora RcsA/RcsB dimer (Fig. 1). The 23-bp Rcs binding region is localized from −555 to −533 upstream of the translational start site of amsG, the first reading frame of the ams operon (Fig. 2). The identified Rcs-binding site overlapped with the previously proposed promoter region of the ams operon and included the putative −35 region.

Fragment F₂₃ was analyzed by mutation for nucleotide positions responsible for the specific DNA-protein recognition. Sets of two or three nucleotides were mutated by nucleotide substitutions, and the binding of RcsA/RcsB to the fragments was quantified in EMSAs (Fig. 3). Most important for the recognition by RcsA/RcsB was the region from nucleotide positions 4–14. The A-C and T-G transitions at position 4 and 6 of fragment M₁₄ resulted in a decrease of retardation to about 18% compared with the wild type sequence. The nucleotide substitutions of positions 7–14 in the fragments M₇₋₉, M₉₋₁₁, CCACTGTTG; S-E, CGCTGCCGGGTATATTGAGAATAATCTTANNNNGGCGAGTTACATGATCCCCCATG-

TABLE I

| Strains and plasmids | Relevant genotype | Ref. |
|----------------------|-----------------|-----|
| XII-Blue             | recA δΔrcsAΔΔ1 (K mK') (F proAB lacI° ZΔM15 Tn10) | 42 |
| JB3034               | ΔrcsAΔΔ1 lon-100 cpaB10 Δlac (imm) recA | 43 |
| DC283                | P. stewartii wild type strain, Nal' | 44 |
| BL21                 | E. coli B F' dep onpT hsdS7(rK' m') gal | Stratagen |  
| pMalC2               | Ap', Expression vector | New England Biolabs |
| pQE30                | Ap', Expression vector | Qiagen |
| pBluescript KS+      | Ap' | Stratagen |
| pEA131               | 8.9-kb BamHI fragment with the ams promoter in pBluescript II KS+ | 21 |
| pM-RcsAÆA           | pBluescript II KS+, amsG-I | 24 |
| pM-RcsAES          | rcsAÆ in pMalC2, Ap' | 24 |
| pQ-RcsBÆA        | rcsBÆ in pQE30, Ap' | 24 |
| pM-RcsBÆA         | rcsBÆ in pMalC2, Ap' | 24 |

Strains and plasmids Relevant genotype Ref.

Rcs Motif of Erwinia spp.

[3301]
and M_{12-14} completely abolished a DNA shift by RcsA/RcsB. The terminal mutations at nucleotide positions 18/20 and 21/23 were tolerated by the Rcs proteins, and no or only minor decreases in the DNA retardation were detected. These results gave evidence that a specific recognition by the RcsA/RcsB dimer might be determined by the central region of fragment F_{23}, whereas the obvious essential contacts to nucleotides at positions 18–23 might not be sequence-specific and could occur with the phosphate backbone.

The protein-DNA interaction of RcsA/RcsB with nucleotides of the central region of fragment F_{23} was verified by UV cross-linking. The thymine bases at nucleotide positions 8, 10, 11, and 13 were substituted by 5'-iododeoxyuracil and the modified fragment F_{23} was incubated with RcsA/RcsB proteins at optimal DNA-binding conditions. The formation of cross-linked protein-DNA complexes was analyzed by SDS-PAGE after illumination of the DNA binding assay for 30 min at 312 nm (Fig. 4). A cross-linked band was clearly visible and demonstrated the close contact of the Rcs proteins with at least some of the labeled thymines.

**Characterization of the RcsA-RcsB-DNA Complex**—We estimated the affinity of the RcsA/RcsB heterodimer for fragment F_{183} with increasing concentrations of an equimolar solution of RcsA and RcsB. With this approach, the apparent binding constant of the RcsA/RcsB heterodimer was calculated at about 100 nM. The same result was obtained by using a constant amount of 30 nM RcsB or 2.5 μM RcsB with increasing amounts of RcsA starting from 19 nM up to 1.5 μM. The usage of different protein ratios did not change the DNA binding kinetics as the binding constant of a heterodimer to DNA should depend only on an equilibrium between the putative free RcsA/RcsB dimer and an RcsA-RcsB-DNA complex. The increase of one protein component will therefore affect the equilibrium between protein dimer and monomers in favor of the RcsA/RcsB heterodimer formation. However, the protein dimer might be rather unstable as we have not been able to detect any RcsA/ RcsB dimer formation neither in the yeast two-hybrid system nor by affinity chromatography of RcsA with the immobilized poly(His)$_6$-tagged RcsB (data not shown).

Relatively high protein concentrations of about 1.7 μM RcsA with 33 nM RcsB in the EMSA with fragment F_{183} of the amsG promoter resulted in an additional supershifted band (Fig. 5). We investigated whether this complex included additional copies of RcsB. The 24-kDa poly(His)$_6$-tagged RcsB protein in the protein-DNA complex was subsequently replaced by increasing concentrations of the 68-kDa RcsB protein modified with an N-terminal fusion of the maltose-binding protein. The resulting band pattern shown in Fig. 5 demonstrates that the supershifted protein-DNA complex obviously contains two copies of the RcsB protein. The appearance of the supershift was dependent on the concentration of RcsA and at least one additional copy of RcsA might also be included in the complex. We could not prove this possibility, as the expression of unmodified or poly(His)$_6$-tagged RcsA proteins results in the formation of inclusion bodies, and only the RcsA fusion to the maltose-binding protein was produced as soluble protein. Therefore it could not be completely ruled out that the supershifted complex does contain only one copy of RcsA together with two copies of RcsB. In that case, RcsB might be able to bind to the preexisting RcsA-RcsB heterodimer complex already formed with lower concentrations of RcsA. However, the lack of a supershifted band even at high concentrations of RcsA with smaller DNA targets like the fragments F_{28} or F_{23} of the amsG promoter make this assumption more unlikely. We propose that the supershifted complex represents an additional RcsA/RcsB heterodimer bound to a second less specific region in fragment F_{183}.

An important factor for the binding efficiency of the RcsA/ RcsB dimer is the length of the offered target DNA. We determined the retardation of different DNA fragments containing the mapped Rcs recognition site at identical assay conditions in EMSAs by using 7.6 μM RcsA and 1.6 μM RcsB protein. Relatively high protein concentrations had to be used to obtain substantial amounts of retarded DNA even with the smallest fragment F_{23}. The largest target was fragment F_{183}, and the amount of retarded DNA was calculated at 52 ± 6%. The retardation of the smaller 28- and 23-bp fragments F_{28} and F_{23} were clearly decreased to 14.2 ± 8.8 and 10.1 ± 5.4%, respectively. The results demonstrate that additional and nonspecific nucleotides might stabilize the protein-DNA interactions.

Once formed, the stability of the RcsA-RcsB-DNA complex might be one major determinant for the induction of ams expression. We determined the half-life of the complex in a competition experiment with the labeled fragment F_{183} as a target and about 30 nM RcsB and 550 nM RcsA, respectively. These conditions were found to be ideal for the retardation of fragment F_{183}. The binding assays were first incubated at standard conditions for 10 min to receive an equilibrium between free ligands and the RcsA-RcsB-DNA complex. The assays were then supplemented at varying time intervals starting from 2 s to 1 h with about 30-fold excess of unlabeled fragment F_{183}. Finally, all samples were analyzed by native PAGE, and the amounts of protein-DNA complex and unbound DNA were quantified. The results were plotted, and the half-life of the RcsA-RcsB-F_{183} complex could be calculated at about 42 s. The Rcs-DNA complex can be considered to be of only low stability if compared with known half-lives of transcriptional repressors to their target DNAs. However, this result is expected for a transcriptional inducer, as the EPS biosynthesis is controlled by environmental signals and the relative low stability of the inductive Rcs-DNA complex might be essential for a fast response to changing conditions.

**Determination of the RcsA/RcsB Recognition Motif in the amsG Promoter**—The sequence of the identified Rcs binding region in the amsG promoter shows some palindrome elements, which could be important for the recognition by the proteins. However, the binding of a protein heterodimer does not require a palindromic binding motif. To identify nucleotide positions, which are responsible for the specificity of the RcsA/RcsB binding, we analyzed the sequence of fragment F_{23} by an in vitro
selection approach. The sequence of fragment \( F_{23} \) was permuted by substitution of 3- or 4-bp-long stretches of nucleotides by randomized nucleotides. The resulting six probes contained random positions in different regions throughout the fragment \( F_{23} \) and represented a pool of 64 and 256 DNA fragments, respectively. Each mixture was used as target DNA in EMSAs with 1.89 \( \mu M \) RcsA and 0.17 \( \mu M \) RcsB protein as described under “Materials and Methods.” The higher protein concentrations had to be used to obtain sufficient retarded DNA for the visualization by ethidium bromide staining. The retarded DNA fragments were isolated, and the selection was repeated twice. The retarded DNA fraction after the third selection by the RcsA/RcsB proteins was quantified and compared with the retardation efficiencies of the unselected DNA mixtures (Table II). The retardation of each DNA mixture was enhanced after the selection for at least 200%. The sequence of the nucleotide positions 9–11 of fragment \( F_{23} \) was most important for the binding of the RcsA/RcsB proteins as almost no retardation of the corresponding unselected DNA mixture was notable. This result corresponds to our previous findings in the mutation analysis and is also in agreement with the observed UV cross-link of the Rcs proteins to that DNA region.

The sequence of the terminal nucleotide positions of fragment \( F_{23} \) seems to be of minor importance for a specific binding of RcsA/RcsB, as already the unselected pools showed a considerable retardation in the EMSA.

The DNA fragments of the selected pools were cloned into pBluescript KS\(^+\), and at least 27 clones of each pool were sequenced (Table III). Corresponding to our previous results, a stringent selection was found at the nucleotide positions 9–11, and a purine was absolutely required for the positions 10 and 11. Only 5 of 64 possible codons were obtained after selection of positions 9–11 of fragment \( F_{23} \) was most important for the binding of the RcsA/RcsB proteins as almost no retardation of the corresponding unselected DNA mixture was notable. This result corresponds to our previous findings in the mutation analysis and is also in agreement with the observed UV cross-link of the Rcs proteins to that DNA region. The sequence of the terminal nucleotide positions of fragment \( F_{23} \) seems to be of minor importance for a specific binding of RcsA/RcsB, as already the unselected pools showed a considerable retardation in the EMSA.

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the random positions 9–11 with a clear preference for the wild type sequence GAA. Further conserved bases were a thymine at position 6 and the sequence WAWT from positions 12–15. The center of the Rcs binding region from positions 6–15 had a high A/T content of about 80%.

The results of the in vitro selection were verified by two different approaches using the EMSA. First we quantified the retardation of representative DNA fragments isolated from the selection procedure (Table IV). The selected DNA fragments (SF) were approximately 200 bp in length and contained fragment F23 from the amsG promoter with mutations relative to the wild type sequence. As expected, the nucleotides at both ends of fragment F23 did not show high sequence specificity in the in vitro selection. However, the increase in DNA retardation of the optimized fragment SF (C1C2C3T4) indicates that some selectivity does exist. Substitution of a thymine and an adenine residue in fragment SF (G1C8) by guanine and cytosine resulted in an about 2-fold increase in retardation. Fragments SF (G2A7C8) and SF (G1C7C8) demonstrate the requirement for a purine in position 7. The thymine at position 6 was shown to be essential in fragment SF (G1C6C7C8). The positions 9–16 were already optimal in the amsG wild type sequence.

positions 9 and 10 tolerated only purines, whereas guanine was almost strictly required at position 9 and an adenine at position 10 enhanced the retardation about 2-fold. The adenine at position 11 could be replaced with pyrimidines with some decrease of the retardation efficiencies. Substitution of the thymine residue at position 16 by cytosine resulted in only about 25% residual retardation. Replacement of the thymine at position 18 with guanine in fragment SF (G18) did not increase the retardation as expected from the in vitro selection. Position 19 required a pyrimidine, and an adenine considerably diminished the retardation as shown with fragments SF (C17G18) and SF (A17G18). Position 19 might be specific for purines as the replacement of adenine by thymine in fragment SF (G18T19) only yielded about 25% residual retardation.

In a second approach, we analyzed the retardation of 23-bp DNA fragments reconstituted from oligonucleotides and designed according to our consensus motif (Table V). Fragment F23 (A1C2T4G5C8) contained optimized substitutions at the 5′-end, and the retardation in EMSAs was enhanced about 2-fold. This result does completely agree with our observations with fragments SF (C1C2C3T4) and SF (G1C8) (Table IV). The optimization of positions 20–23 in fragment F23 (G20G21C22A23) resulted in about a 4-fold enhancement of retardation. Interestingly, the replacement of the wild type thymine at position 18 with guanine in fragment F23 (G18) increased the retardation about 3-fold, whereas the same mutation in the context of the −200-bp fragment SF (G18) did not show notable effects on the retardation efficiency (Table IV). Substitution of the thymine at position 17 with adenine completely abolished the retardation of fragment F23 (A17G18). This in agreement with the observed drastically reduced retardation of the 200-bp fragment SF (A17G18). Fragment F23 (consensus) combined all optimized positions previously analyzed in the fragments F23 (A1C2T4G5C8), F23 (G20G21C22A23), and F23 (G18). However, the retardation was not enhanced as expected but diminished to about 50% of the wild type sequence. This result gave evidence that the recognition of the distinct nucleotide positions does not take place independently and/or secondary structures of the DNA target might be important for the recognition by RcsA/RcsB proteins.

Identification of an RcsA/RcsB-binding Site in the P. stewartii cpsA Promoter—The cps operon for stewartan biosynthesis in P. stewartii is homologous to the ams operon of E. amylovora, and both are regulated by Rcs proteins. The sequence of an about 600-bp region containing the promoter of cpsA, the

3304  

**Rcs Motif of Erwinia spp.**

![Image](image-url)

**FIG. 4. Cross-linking of RcsA/RcsB with the 5-iododeoxyuracil-labeled fragment F23.** The 23-bp fragment with the Rcs-binding site of the amsG promoter was incubated with about 1.9 μM RcsA and 0.2 μM RcsB protein at standard conditions and illuminated at 312 nm for (lane 1) 15 min, (lane 2) 30 min, and (lane 3) 60 min. The arrow indicates the cross-linked DNA fragments after separation by SDS-PAGE.

![Image](image-url)

**FIG. 5. Identification of two RcsB copies in the supershifted DNA fragment F183 from the amsG promoter.** Only retarded DNA fragments are shown. The labeled fragment F183 was incubated in standard retardation assays with about 1.9 μM RcsA, 100 nM H-RcsB (lanes 1–4), or 40 nM M-RcsB (lanes 5–8). The M-RcsB protein was added in concentrations of 40 nM (lane 2), 110 nM (lane 3), and 180 nM (lane 4). The H-RcsB protein was added in concentrations of 100 nM (lane 6), 300 nM (lane 7), and 600 nM (lane 8). I and II, RcsA-RcsB heterodimer complex with fragment F183; III–V, supershifted protein-DNA complexes. The proposed compositions of the protein-DNA complexes are shown in the scheme. M-RcsB, fusion with the 44-kDa maltose-binding protein; H-RcsB, fusion with a poly(His)6-tag.
first reading frame of the cps operon, was aligned with the sequence of the amgs promoter (Fig. 2). The nucleotide positions –538 to –516 correspond to the Rcs binding region of the amgs promoter, and they were analyzed as a 28-bp fragment in EMSAs with the RcsB protein of E. amylovora and the RcsA protein of E. amylovora (RcsA_EA) or P. stewartii (RcsA_PS). The fragment was clearly retarded with both protein combinations (Fig. 6), giving evidence that the binding of an RcsA/RcsB heterodimer to a region from about –510 to –540 bp upstream of the translational start site might be a common principle in the regulation of EPS biosynthesis in Erwinia. The retardation with RcsA_EA compared with RcsA_PS seems to be somewhat better with the fragment F_{23} of the homologous cpsA promoter. Vice versa, RcsA_PS seems to be more effective in the retardation of the fragment F_{23} from the amgs promoter. This gives evidence that the RcsA proteins are involved in the specific DNA recognition. According to the consensus motif of the amgs promoter, the RcsA/RcsB-binding site of the cpsA promoter was degenerated in two essential positions (Table III) and did not contain palindromic elements. The replacement of the degenerated adenine by the conserved guanine in fragment F_{cpsA-(G9)} resulted in about a 5-fold enhanced retardation in the EMSA (Table V). Despite the degenerated positions, the analyzed 28-bp fragment from the cpsA promoter showed a similar retardation compared with fragment F_{23} from the amgs promoter (Table V). The degeneration might therefore be compensated by optimized nucleotides at other positions of the cpsA Rcs binding motif.

**DISCUSSION**

Genetic data gave evidence that RcsA/RcsB heterodimers play a major role in mediating transcriptional activation on positively regulated genes involved in EPS biosynthesis. The principal aim of this study was to identify essential nucleotide positions implicated in Rcs protein recognition within promoters of EPS biosynthetic operons. We previously described a synergistic binding of RcsA and RcsB to the promoter of amgs, the first reading frame of the E. amylovora operon for amylovoran biosynthesis. The minimal DNA fragment retaining substantial RcsA/RcsB binding has been confined to a 23-bp region now. Both gel mobility assays and protein-DNA cross-linking studies indicate the interaction of purified RcsA/RcsB proteins with that region. The RcsA/RcsB binding region covers the putative –35 consensus of the amgs promoter (21). Its relatively poor homology to E. coli σ70 promoters (27) is consistent with RcsA/RcsB acting as activators for the amgs promoter. The confined minimal RcsA/RcsB-binding site is small compared with those covered from transcriptional regulators of other two-component systems. Footprinting revealed protected DNA fragments of about 80 bp for VirG and VanR (28, 29). Additional sequences might also be bound by the RcsA/RcsB proteins as longer DNA fragments stabilized the protein-DNA complex more than 5-fold, but the analyzed 23-bp fragment appears to contain the nucleotide positions responsible for the RcsA/RcsB binding specificity.

The affinity of the RcsA/RcsB heterodimer to its target sequence in the amgs promoter was determined with an apparent KD value of 100 nM. It has to be considered that the determination of the KD values is based on the assumption that the activities of the protein fractions are 100%. In other well-characterized bacterial two-component systems, e.g. NtrB/NtrC (30), EnvZ/OmpR (31), PhoR/PhoB (32), ComP/ComA (33), and VanS/VanR (29), phosphorylation of the response regulators increased affinity, presumably due to the formation of higher oligomeric protein states and cooperative binding (34, 35). RcsB contains a well-conserved phosphorylation motif, and its activity might be modulated upon phosphorylation by RscC (36). The percentage of phosphorylation in our RcsB preparations was unknown, but it might be rather low due to the kinetic lability of aspartyl-phosphate linkages. It is therefore possible that the KD values could be considerably increased after an increased phosphorylation of RcsB. An apparent KD value of 40 nM was reported for the transcriptional regulator VanR after increasing the percentage of phosphorylated protein with acetyl phosphate to a total of about 8%, whereas the KD value of unphosphorylated VanR was found to be about 500-fold lower (29). Both forms of VanR bind to identical DNA regions, but the phosphorylated VanR covers a larger part of DNA. Additionally, the KD value of 14 nM for the unphosphorylated form of the transcriptional regulator NtrC could be increased to a KD value of 1 nM upon phosphorylation (37). However, the affinities of the phosphorylated procaryotic transcriptional activators ComA and OmpR to their DNA targets with KD values of 1 μM (33) and 1.5 μM (31), respectively, are significantly lower than the affinity of RcsB to its target. The RcsA-RcsB-DNA complex shows a relative short half-life of 42 s, and there

**Table II**

| Selected region | Retardation | Retardation after selection |
|----------------|-------------|-----------------------------|
| NNNNTGAGAATACTTAATTT | 13.9 ± 1.2 | 35.6 ± 1.5 |
| TATANNNNGAATACTTAATTT | 3.9 ± 0.7 | 16.3 ± 3.1 |
| TATATTGAGANTAATTAATTT | 0.9 ± 0.1 | 23.9 ± 6.5 |
| TATATTGAGANTAATTAATTT | 1.6 ± 0.3 | 10.9 ± 2.6 |
| TATATTGAGANTAATTAATTTN | 3.6 ± 0.6 | 44.2 ± 2.8 |
| TATATTGAGANTAATTAATTTNN | 9.6 ± 1.6 | 37.7 ± 1.9 |

*Only the sequence of the upper strand of fragment F_{23} is shown; random positions are in bold.

| Retarded DNA was analyzed by EMSA as described under “Materials and Methods” with 1.89 μM RcsA and 0.17 μM RcsB. The retardation was calculated as percent from the total amount of labeled DNA in each experiment.

| Determined after three rounds of selection. |

**Table III**

| Evaluation of the RcsA/RcsB recognition motif at the amgs promoter |

| T | A | T | A | T | A | T | G | A | T | A | T | C | T | A | T | T | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | N | B | D | T | R | V | G | A | W | A | W | T | S | Y | G | R | G | R | N | N |
| C | A | B | C | A | T | G | A | A | L | A | A | A | C | T | G | A | T | T | T |

A 8 6 6 3 7 1 7 8 1 21 19 11 21 11 7 4 9 10 7 7 4 8
C 9 8 10 7 3 4 6 12 -- -- -- 2 3 2 3 3 13 10 3 7 3 6 12 8
G 5 7 4 7 12 -- 16 7 28 8 -- 3 2 5 3 8 3 14 8 18 13 9 6
T 6 7 8 11 7 24 -- 2 -- -- 4 14 4 11 17 2 11 1 2 2 4 5 8

Degenerated positions are classified after IUPAC nomenclature. The most frequent bases in the analyzed sequences are in bold. Lines indicate conserved positions in the E. amylovora (EA) and P. stewartii (PS) wild type (wt) sequence, less conserved positions are marked with colon, and degenerated positions are in lowercase. Palindromic elements in the wild type sequences are underlined. The in vitro selection was carried out with 1.89 μM RcsA and 0.17 μM RcsB protein. Top line of boxhead is wt (EA); middle line is consensus (consensus determined after in vitro selection and analysis of mutated fragments in the EMSA); bottom line is wt (PS). Bases not found by sequencing are indicated by dashes.
might be a continuous association/dissociation. In general, transcriptional activator-DNA complexes tend to have a much shorter half-life than transcriptional repressor-DNA complexes, most probably to ensure continuous gene activation only upon the ongoing stimulation of the activator protein. It is further possible that additional factors might bind \textit{in vitro} to stabilize the complex. \textit{As in vitro} transcription data are not available so far, it is not known whether the formation of the DNA bound RcsA/RcsB heterodimer alone is sufficient to dictate transcriptional activity.

RcsA and RcsB are grouped into the LuxR family of transcriptional regulators (7), but the determined RcsA/RcsB recognition motif at the \textit{amsG} promoter did not show extensive similarities to the \textit{lux} box, a 20-bp palindromic similar to the LexA repressor recognition motif (38). Palindromic elements were not necessary in the recognition motif derived by \textit{in vitro} selection, and they are absent in the RcsA/RcsB-binding site at the \textit{P. stewartii} \textit{cpsA} promoter. The \textit{in vitro} selection revealed a region of about 13 bp as most important for the RcsA/RcsB binding specificity. The cross-link of 5-iododeoxyuracil-substituted thymines within this area demonstrated its close contact to the Rcs proteins. The binding mechanism of RcsA/RcsB to their target DNA seems to include a complex combination of recognition of specific bases in concert with local structural features. Proposed local single or multiple “up” mutations according to our consensus motif within the 23-bp fragment always increased the binding of RcsA/RcsB. However, the binding was decreased upon combination of up mutations from more distantly located areas, indicating that the tight binding of one Rcs protein at the 23-bp fragment requires some flexibility of the second Rcs protein.

Heterodimerization of transcriptional regulators is quite uncommon in procaryotic systems but prevalent in eucaryotic regulation mechanisms (39), and one advantage might be the recognition of additional DNA targets. RcsB, but not RcsA, is essential for the stimulation of EPS biosynthesis, and it is involved in the regulation of further RcsA-independent pathways like the stimulation of Vi polymer synthesis in \textit{Salmonella typhimurium} (12) and \textit{ftsZ} expression in \textit{E. coli} (40). It might therefore be speculated that RcsB is responsible for the initiation of transcription, possibly by interacting with RNA polymerase, and the alternative association with specific coinducers like RcsA might enable RcsB to bind and regulate different promoters in the cell. It is yet uncertain if RcsB first binds weakly to DNA and the complex is then recognized by RcsA, or if a preformed RcsA/RcsB heterodimer binds to the DNA.

### Table IV

| Fragment | Sequence | Retardation |
|----------|----------|-------------|
| Wild type | TATATTGAGAATAATCTTTTTT | 24.7 ± 7.6 |
| SF (C1C2C3T4) | CCCTTTGAGAATAATCTTTTT | 41.5 ± 1.8 |
| SF (G5C7C8) | TATATTGAGAATAATCTTTTTTT | 55.2 ± 4.0 |
| SF (G5A7C8) | TATATTGAGAATAATCTTTTTTTT | 20.0 ± 0.7 |
| SF (G5C8) | TATATTGAGAATAATCTTTTTTTTT | 6.4 ± 1.8 |
| SF (G5A6C7C8) | TATATTGAGAATAATCTTTTTTTTTT | 3.3 ± 0.9 |
| SF (G5A6C7) | TATATTGAGAATAATCTTTTTTTTTTT | 12.6 ± 0.6 |
| SF (G10) | TATATTGAGAATAATCTTTTTTTTTTTT | 11.0 ± 1.1 |
| SF (G11) | TATATTGAGAATAATCTTTTTTTTTTTTT | 19.2 ± 0.3 |
| SF (G12) | TATATTGAGAATAATCTTTTTTTTTTTTTT | 2.3 ± 0.2 |
| SF (A1C2T4G5C8) | TATATTGAGAATAATCTTTTTTTTTTTTTTT | 16.2 ± 1.0 |
| SF (A1C2T4G5) | TATATTGAGAATAATCTTTTTTTTTTTTTTTT | 5.3 ± 1.3 |
| SF (A1C2T4G5) | TATATTGAGAATAATCTTTTTTTTTTTTTTTTTT | 13.1 ± 0.2 |
| SF (A1G5) | TATATTGAGAATAATCTTTTTTTTTTTTTTTTTTTT | 3.5 ± 0.9 |
| SF (G10) | TATATTGAGAATAATCTTTTTTTTTTTTTTTTTTTT | 22.6 ± 2.5 |
| SF (G12) | TATATTGAGAATAATCTTTTTTTTTTTTTTTTTTTTT | 14.6 ± 1.9 |
| SF (G12G13) | TATATTGAGAATAATCTTTTTTTTTTTTTTTTTTTTTT | 36.3 ± 0.2 |
| SF (G12G13) | TATATTGAGAATAATCTTTTTTTTTTTTTTTTTTTTTTTT | 6.8 ± 0.8 |
| SF (G12G13G14) | TATATTGAGAATAATCTTTTTTTTTTTTTTTTTTTTTTTTTT | 16.5 ± 5.5 |

* Down mutations with respect to the wild type fragment are underlined; up mutations are underlined twice.

### Table V

| Fragment | Sequence | Retardation |
|----------|----------|-------------|
| \textit{E. amylovora} | TATATTGAGAATAATCTTTTTT | 4.8 ± 1.1 |
| F23 (wt) | TATATTGAGAATAATCTTTTTTT | 9.6 ± 1.0 |
| F23 (A1C2T4G5C8) | TATATTGAGAATAATCTTTTTTTTTT | 17.6 ± 0.8 |
| F23 (G10) | TATATTGAGAATAATCTTTTTTTTTTTT | 14.6 ± 2.5 |
| F23 (G12) | TATATTGAGAATAATCTTTTTTTTTTTTTT | 2.4 ± 1.2 |
| F23 (consensus) | TATATTGAGAATAATCTTTTTTTTTTTTTTTT | 4.7 ± 0.5 |
| \textit{P. stewartii} | TATATTGAGAATAATCTTTTTTTTTTTTTTTTTT | 26.1 ± 0.1 |

* The upper strand of the DNA fragment is shown. Altered bases with respect to the wild type sequences are underlined (down mutations) or underlined twice (up mutations).
recognition site. Evidence for the latter possibility could be the appearance of the supershifted band, notable after increasing the concentration of RcsA to about 40-fold excess. The increased RcsA concentration could enhance the formation of RcsA/RcsB heterodimers, resulting in the binding of secondary sites with lower affinity. However, the supershifted band would also be consistent with the formation of a nucleoprotein complex and a secondary binding of RcsB and possibly RcsA to the already bound RcsA/RcsB heterodimer (41). The presence of a free heterodimer is further supported by the observation that overexpression of the C-terminal deleted RcsB protein inhibits EPS biosynthesis in E. coli, possibly by competition with the wild type ResB for the RcsA protein, 3 resulting in the formation of a nonproductive heterodimer. However, the heterodimers of RcsA/RcsB might associate only weakly, as we could not detect an interaction in the yeast two-hybrid system or by affinity chromatography of RcsA with immobilized RcsB. 3

In summary, the identification of RcsA/RcsB-binding sites at corresponding locations in the main promoters of the EPS operons of E. amylovora and P. stewartii gave evidence for a conserved regulation mechanism for type IA polysaccharide biosynthesis. The description of critical nucleotides in the RcsA-RcsB-DNA complex should help to analyze further the molecular interactions in the regulation of capsule biosynthesis by Rcs proteins.

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