

Activation of the 2'-N-Acetyltransferase Gene [*aac(2')-Ia*] in *Providencia stuartii* by an Interaction of AarP with the Promoter Region

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The *aac(2')-Ia* gene in *Providencia stuartii* encodes a 2'-N-acetyltransferase capable of acetylating both peptidoglycan and certain aminoglycoside antibiotics. Regulation of the *aac(2')-Ia* gene is influenced in a positive manner by the product of the *aarP* gene, which encodes a small transcriptional activator of the AraC (XylS) family. In this study, we demonstrate the sequence requirements at the *aac(2')-Ia* promoter for AarP binding and activation.

Providencia stuartii is a gram-negative pathogen that is a leading cause of urinary-tract infections in patients undergoing chronic catheterization (16, 22, 28, 29). Eradication of *P. stuartii* infections can be extremely difficult due to the multiple antibiotic resistances that are often present in this bacterium (11, 15). An intrinsic, chromosomal acetyltransferase [AAC (2')-Ia] is present in *P. stuartii* and confers high-level aminoglycoside resistance when overexpressed (4, 24, 25, 31). In addition, this enzyme serves a housekeeping role in the O acetylation of peptidoglycan (21). Intrinsic chromosomal aminoglycoside acetyltransferases in other bacteria have been described previously, and a summary of these enzymes has been reported (26).

A central activator of *aac(2')-Ia* gene is AarP, a transcriptional activator similar to the MarA, SoxS, and Rob proteins (14). The MarA, SoxS, and Rob proteins have been shown to activate an intrinsic multiple-antibiotic resistance phenotype (Mar) and a defense regulon against superoxide-generating agents in *Escherichia coli* (1, 2, 5, 9, 10, 12, 13, 18–20, 30). In addition to activating the *aac(2')-Ia* gene, increased expression of AarP activates a Mar phenotype in both *P. stuartii* and *E. coli* (14). Presumably each of these proteins is capable of activating a common set of genes due to the extensive homology in their helix-turn-helix DNA binding motif (9).

Prior to this study, it was not clear whether AarP influenced expression of *aac(2')-Ia* by a direct or indirect mechanism. In this report, the *aac(2')-Ia* sequences required for the in vivo activation by AarP are identified. DNA binding studies using purified AarP protein suggest that activation of *aac(2')-Ia* by AarP is mediated by an interaction with the *aac(2')-Ia* promoter region.

Bacterial strains and growth conditions. *Escherichia coli* DH5 α (Gibco/BRL, Gaithersburg, Md.) and XL1-Blue (Stratagene, La Jolla, Calif.) were used as hosts for plasmids. *P. stuartii* PR50 has been described previously (24). Luria-Bertani broth was used for the growth of cells, and the following antibiotics were used at the indicated concentrations for selections: ampicillin, 150 μ g/ml for *E. coli* and 300 μ g/ml for *P. stuartii*; chloramphenicol, 100 μ g/ml for *P. stuartii*.

Upstream *aac(2')-Ia* sequences required for activation by AarP. We previously reported that the presence of *aarP* on a multicopy pACYC184 derivative (pSF2) was capable of 10-fold activation of an *aac(2')-lacZ* fusion contained on a separate compatible plasmid, pR401 (14). To determine the sequences required for this activation, a series of progressive 5' deletions of the full-length *aac(2')-Ia* promoter were constructed. As shown in Fig. 1, plasmid pR402 contains a 456-bp *XhoI-HindIII* fragment inserted into pQF50 digested with *SalI* and *HindIII*. pR403 contains a 357-bp *XmnI-HindIII* fragment cloned into pQF50 digested with *SmaI* and *HindIII*. pR404 contains a 292-bp *AluI-HindIII* fragment cloned into pQF50 digested with *SmaI* and *HindIII*. To construct pR405, plasmid pBC.KS-*aac(2')* was first digested with *ApaI* followed by Klenow treatment to create blunt ends. This was followed by digestion with *HindIII*, which released a 273-bp fragment that was cloned into pQF50 digested with *SmaI* and *HindIII*. Plasmid pR406 was constructed in two steps. First the *aac(2')-Ia* promoter region from pR401 was cloned into pACYC184 as a *BamHI-HindIII* fragment. This plasmid was then linearized with *ApaI*, which cuts at position -49 in the *aac(2')-Ia* promoter region end filled with Klenow treatment in the presence of deoxynucleoside triphosphates. Religation then created a 4-bp insertion of CATG at position -47. This promoter fragment was then recloned into pQF50 as a *BamHI-HindIII* fragment to create pR406. Each of these derivatives was electroporated into PR50, and then either pSF2 or pACYC184 was introduced into each strain. The ability of AarP to activate each promoter derivative was determined by analysis of β -galactosidase accumulation in sodium dodecyl sulfate (SDS)-chloroform-treated cells by the method of Miller (17). Plasmid pR401 was activated 9- to 10-fold in the presence of pSF2 (Fig. 1). Deletion derivatives extending to -233 (pR402), -134 (pR403), or -67 (pR404) relative to the start of transcription still supported a seven- to ninefold activation by pSF2. However, pR405 containing a deletion extending to an *ApaI* site at -49 failed to support activation by AarP. This *ApaI* site was centered within a region of dyad symmetry that represented a potential sequence required for activation by AarP. To examine this possibility, pR406 containing a 4-base insertion of CATG at -47 was assayed for activation. The presence of this insertion in the context of the full-length promoter resulted in a severe reduction in the activation conferred by pSF2, 1.5-fold compared to the 10-fold increase seen in pR401.

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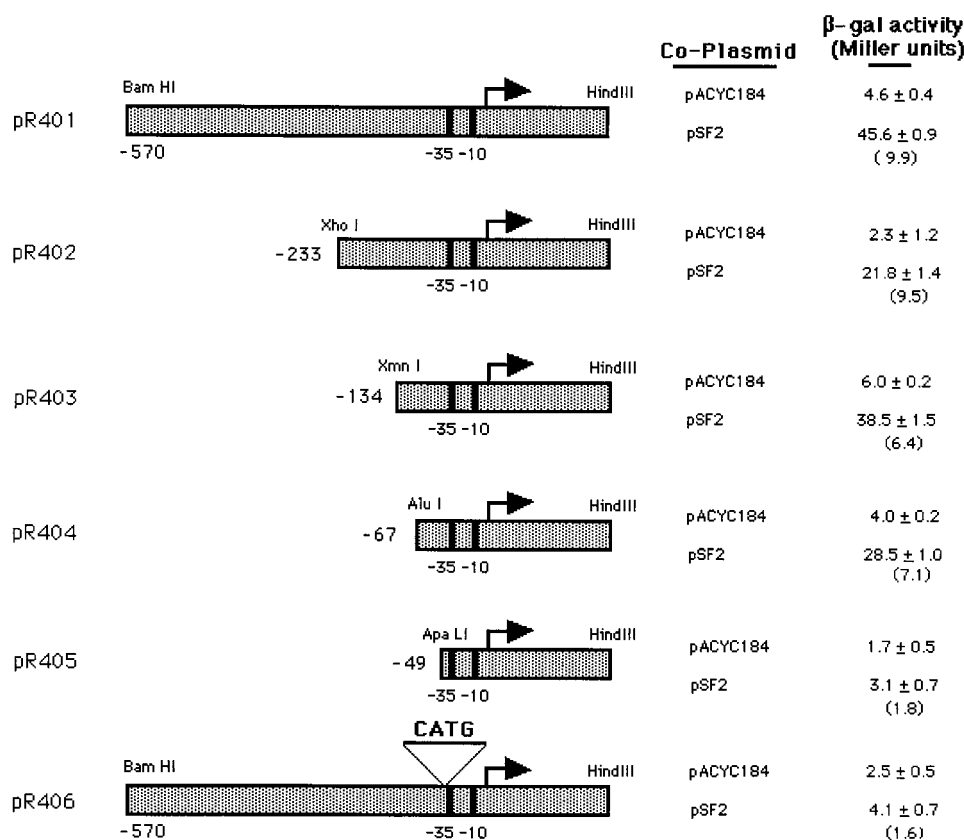


FIG. 1. Deletion analysis of *aac(2')-Ia* sequences required for AarP-mediated activation. A series of 5' deletion derivatives of the *aac(2')-Ia* promoter were constructed and fused to the *lacZ* gene in pQF50 (7). *P. stuartii* PR50 containing each deletion derivative fused to *lacZ* was then transformed with a compatible plasmid containing *aarP* (pSF2) or with a pACYC184 control. The ability of each *aac(2')-Ia* promoter derivative to support activation by AarP was determined by analysis of β -galactosidase (β -gal) accumulation according to the method of Miller (17). A representative experiment is shown, with the values in parentheses representing the fold induction conferred by the presence of pSF2 containing the *aarP* gene, relative to pACYC184. Standard deviations are also given, and repeat experiments gave similar induction values to those shown.

Purification of AarP. To determine if the observed activation by AarP was mediated by a direct interaction with the *aac(2')-Ia* promoter region, a His₆-AarP protein was constructed by PCR amplification with primers to introduce the His₆ tag at the amino terminus. DNA sequence analysis confirmed that no mutations were introduced from the PCR amplification. The His₆-AarP fusion protein was shown to function *in vivo* as indicated by the 48-fold activation of an *aac(2')-lacZ* fusion observed in *P. stuartii* when His₆-AarP was introduced (data not shown). To express the fusion protein via the T7 RNA polymerase, an *EcoRI-SalI* fragment containing the His₆-*aarP* construct and the ribosomal binding site from pDS56/RBSII (2a) was cloned into pET24(+) (Novagen) digested with the same enzymes to create pET24.His(6)-aarP.

The His₆-AarP protein was purified by affinity chromatography using Talon resin (Clontech, Palo Alto, Calif.). Initial attempts to overexpress and purify AarP from *E. coli* BL21 (DE3) gave reproducibly poor yields. Examination of codon usage for AarP revealed a number of rare codons for *E. coli*; therefore, purification of AarP was subsequently done in *P. stuartii* PR50 harboring pET24.His(6)-aarP. To introduce the T7 RNA polymerase gene into *P. stuartii*, a 4.4-kb *Bam*HI fragment from pAR1219 (6) containing both gene I from phage T7 driven by the *lac* UV5 promoter and the *E. coli lacI* gene was cloned into pACYC184 (3) linearized with *Bam*HI to create p184.T7. Cells were grown at 37°C in 400 ml of Luria-

Bertani broth to an optical density at 600 nm of \sim 0.6. IPTG (isopropyl- β -D-thiogalactopyranoside) was added to a final concentration of 2 mM and cultures were returned to 37°C for an additional 3 h. Cells were disrupted by passing twice through a French pressure cell at 10,000 lb/in², and cellular debris was removed by centrifuging twice at 35,000 \times *g* for 30 min at 4°C. The His₆-AarP protein was purified with Talon affinity resin by a batch purification method as described by the manufacturer (Clontech). Protein concentration was determined by the method of Bradford. The purified His₆-AarP fusion protein was visualized on SDS-12% polyacrylamide gels after Coomassie blue staining and was greater than 90% pure as shown in Fig. 2.

Binding of AarP to the *aac(2')-Ia* promoter. Mobility gel shift analysis (8) was performed with the purified His₆-AarP protein and fragments of the *aac(2')-Ia* promoter region generated by PCR amplification and end labeled with digoxigenin-11-2'-deoxy-uridine-5'-triphosphate followed by gel purification.

DNA binding reactions contained 50 mM KCl, 10 mM Tris (pH 8.0), 2 mM dithiothreitol, bovine serum albumin (0.4 mg/ml), 10% (vol/vol) glycerol, and 4 fmol of labeled DNA fragment and purified His₆-AarP protein as described in the text. Binding reaction mixtures were incubated for 20 min at room temperature and then loaded onto 5% polyacrylamide gels. Gels were electrophoresed at 200 V, washed twice for 20

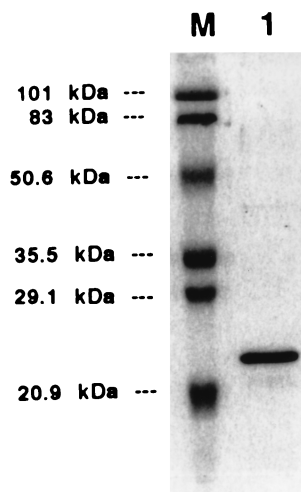


FIG. 2. Purification of AarP protein. A His₆-AarP fusion protein was purified from an induced culture of *P. stuartii* PR50 as described in Materials and Methods. The purity of the final preparation was examined on an SDS-12% polyacrylamide gel stained with Coomassie blue. Prestained molecular mass standards (Bio-Rad) are in lane M, and the estimated molecular masses are indicated.

min in 1× Tris-borate-EDTA buffer, and transferred to nylon membranes with a Bio-Rad (Hercules, Calif.) Semi-Dry blotting system. Filters were developed with LumiPhos 530 (Boehringer Mannheim, Indianapolis, Ind.) and exposed to X-ray film.

The ability of AarP to bind the *aac(2')-Ia* promoter is demonstrated by the presence of a DNA fragment with decreased mobility in the presence of AarP (Fig. 3) relative to that ob-

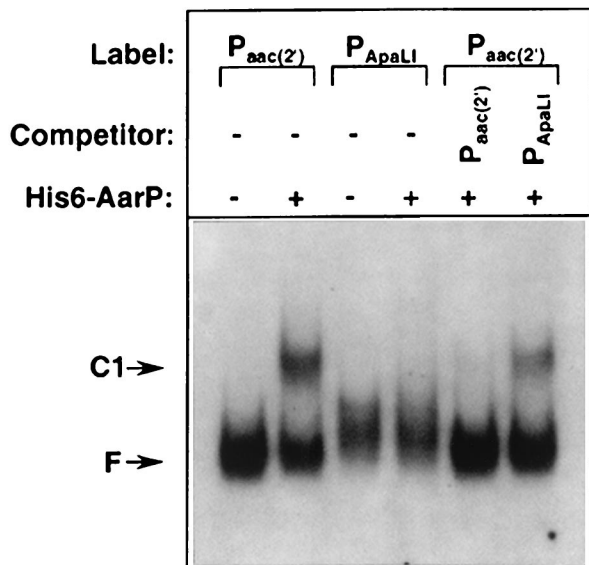


FIG. 3. Binding of purified His₆-AarP to *aac(2')-Ia* promoter sequences. Binding reactions were set up, subjected to electrophoresis on polyacrylamide gels, and visualized as described in the text. Approximately 2.5 fmol of labeled wild-type *aac(2')-Ia* promoter fragment [P_{aac(2')}] or the mutant promoter with a 4-bp insertion at -47 (P_{ApaLI}) was included in the reaction mixtures. Purified His₆-AarP (20 pmol) and unlabeled competitor DNA fragments (1.25 pmol) were added to the binding reactions where indicated. The designation F denotes free unbound fragment, and C1 indicates an AarP complex with the *aac(2')-Ia* promoter.

served when no AarP protein is present (Fig. 3). In the presence of a DNA fragment containing the 4-base insertion centered at position -47, there was no observable binding by AarP, a result in agreement with the *in vivo* analysis (Fig. 1). Control experiments using an excess of unlabeled specific and nonspecific competitor DNAs indicated that the observed binding was specific for the *aac(2')-Ia* promoter (Fig. 3).

Concluding remarks. The results of this study indicate that the AarP protein directly interacts with the *aac(2')-Ia* promoter region to mediate transcriptional activation. The *in vivo* activation of deletion derivatives of the *aac(2')-Ia* promoter and a 4-bp insertional mutation have indicated that bases at position -47 are important for activation. The purified AarP protein has been shown to bind the wild-type *aac(2')-Ia* promoter region but was unable to bind a mutant derivative containing a 4-bp insertion at position -47. The sequences at position -47 include a GCA motif centered at position -47, which may be equivalent to the GCAY motif proposed to be important in SoxS binding (13a).

The AarP protein is a member of the AraC (XylS) family (23) and like the SoxS and MarA proteins is missing the portion involved in ligand binding (5, 9, 23, 30). Therefore, like that of SoxS and MarA, the regulation of gene expression mediated by AarP appears to be the result of changes in expression (5, 9, 23, 30). Studies will now focus on the mechanisms that regulate *aarP* expression and result in the subsequent activation of *aac(2')-Ia* and genes involved in the multiple-antibiotic resistance phenotype in *P. stuartii*.

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