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Characterization of *aarA*, a Pleiotrophic Negative Regulator of the 2′-*N*-Acetyltransferase in *Providencia stuartii*

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We have utilized transposon mutagenesis to obtain insertional mutations in *Providencia stuartii* that activate the chromosomal *aac(2′)-Ia* gene. Two closely linked mini-Tn*5*Cm insertions were obtained in a locus designated *aarA*, and a single insertion was obtained in a separate locus, *aarC*. Nucleotide sequence analysis, complementation studies, and localization of the sites of mini-Tn*5*Cm insertion have allowed the identification of the *aarA* coding region. The deduced AarA protein had a molecular mass of 31,086 kDa and displayed characteristics of an integral membrane protein. A strain deleted for the *aarA* gene by allelic exchange showed at least a fourfold increase in the accumulation of *aac(2′)-Ia* mRNA and an eightfold increase in aminoglycoside resistance. Mutations in *aarA* were pleiotrophic and also resulted in loss of pigmentation and a deficiency in cell separation during division.

Chromosomal antibiotic resistance genes intrinsic to a given organism are often subject to complex regulatory control. The inducible β-lactamase encoded by the *ampC* gene and found in members of the family *Enterobacteriaceae* is controlled by at least four gene products. In this system, *ampR* functions as a positive activator by binding to the *ampC* promoter region (2, 17). The *ampD* and *ampE* genes act as negative regulators of *ampC* expression (11, 16). Regulation by the *ampD* gene product may be indirect, since *ampD* mutants have an altered peptidoglycan composition, which may affect *ampC* expression (29). A fourth gene, *ampG*, which is necessary for *ampC* induction, encodes a putative *trans* membrane protein and may be involved in signal transduction (15, 18).

A second example of regulated antibiotic resistance occurs in the multiple antibiotic resistance locus (*mar*) in *Escherichia coli*. This operon, composed of three open reading frames, *marR*, *marA*, and *marB*, affects a regulon controlling resistance to a variety of unrelated antibiotics, such as tetracycline, chloramphenicol, and quinolones (4, 9). The *marA* gene product is a transcriptional activator and may serve to activate genes involved in the Mar phenotype (3, 7a). The *marR* gene appears to encode a repressor of the *marRAB* operon, and the function of the *marB* gene has yet to be determined (3). Expression of the Mar phenotype may result, in part, from the *marA*-dependent activation of *micF*. This decreases the amount of OmpF porin and subsequent antibiotic uptake (4, 5). It has also been demonstrated that the SoxRS system can also activate *micF*, resulting in increased resistance to some antibiotics (2a).

Expression of chromosomal aminoglycoside acetyltransferase genes in certain organisms is also subject to complex regulation. In *Serratia marcescens*, the *aac(6′)-Ic* gene is expressed at low levels in wild-type strains. Activation of this gene to confer high-level aminoglycoside resistance occurs at the transcriptional level (26).

We have recently characterized a chromosomally encoded aminoglycoside acetyltransferase, *aac(2′)-Ia*, in *Providencia stuartii* (25). Although, the *aac(6′)-Ic* gene in *S. marcescens* and the *aac(2′)-Ia* gene in *P. stuartii* appear to be universally present in each species (24, 26), the putative cellular function of these genes is unknown. Possibly, they have a housekeeping function and are not present for the sole purpose of protection against aminoglycosides. Expression of *aac(2′)-Ia* occurs at low levels in wild-type *P. stuartii* and is not inducible by aminoglycosides; however, a *trans*-acting negative regulator, defined by the *aar3* allele has been identified (25). Isolation of the *aar* gene by complementation has been hampered by the inability to introduce DNA into *P. stuartii* at high frequency. To circumvent this problem, we have utilized transposon mutagenesis to identify *trans*-acting negative regulators of *aac(2′)-Ia*.

Identification of *aarA*. All strains and plasmids used in this study are listed in Table 1. To identify genes that regulate *aac(2′)-Ia* in *trans*, a genetic screen was utilized to identify transposon (mini-Tn*5*Cm) insertions that increased *aac(2′)-Ia* expression both from the chromosomal copy and from a plasmid-encoded *aac(2′)-lacZ* transcriptional fusion. Introduction of mini-Tn*5*Cm into the *P. stuartii* chromosome was achieved by a conjugal mating with *E. coli* S17.1 λ*pir*/pUT:: mini-Tn*5*Cm (6). Wild-type *P. stuartii* PR50 containing a plasmid-encoded *aac(2′)-lacZ* fusion (pSCH4500.lac) (Table 1) forms white colonies on media containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) because of the low levels of *aac(2′)-Ia* transcription. Three independent mini-Tn*5*Cm insertional libraries were constructed in PR50 (pSCH4500.lac) and screened for blue colonies on Luria-Bertani (LB) plates containing X-Gal. A total of 9 blue colonies of approximately 30,000 colonies screened were identified. These blue colonies were then tested for gentamicin resistance at 20 μg/ml, which represents a fivefold increase over the MIC for wild-type PR50 and would indicate increased expression of the chromosomal *aac(2′)-Ia* gene. Three strains with this phenotype were analyzed and designated PR50.A1, PR50.A6, and PR50.B3, each with gentamicin MICs that were 32 μg/ml, an eightfold increase over that of the wild type. To determine the site of mini-Tn*5*Cm insertion in these mutants, chromosomal DNA was prepared as previously described (1) and digested with *Cla*I, which does not cut within mini-Tn*5*Cm. These DNAs were then probed with a 3.6-kb *Eco*RI fragment

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and relevant markers	Source or reference
Strains		
E. coli		
XL1-Blue	*recA1 endA1 hyrA96 thi-1 hsdR17 supE44 relA* Δ*lac-pro* [F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*	Stratagene
S17.1 λ*pir*	*thi pro hsdR recA* λ*pir*	28
SM10 λ*pir*	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu Km^r λ*pir*	20
P. stuartii		
PR50	Wild type	25
PR50.A1	PR50 *aarC1*::mini-Tn*5*Cm	This study
PR50.A6	PR50 *aarA1*::mini-Tn*5*Cm	This study
PR50.B3	PR50 *aarA2*::mini-Tn*5*Cm	This study
PR51	PR50 Δ*aarA3*	This study
Plasmids		
pBluescript SK(−)	High-copy-number vector Ap^r	Stratagene
pSCH4500	pBluescript derivative with 1.3-kb *Sau*3A fragment containing *aac(2′)-Ia*	25
pKNG101	R6K-derived suicide plasmid containing Str^r and *sacB*	12
pSCH4500.lac	pSCH4500 with promoterless *lacZ* cassette inserted at *Nde*I site within *aac(2′)-Ia*	This study
pQF50	Promoter probe vector for construction of *lacZ* transcriptional fusions	7
pR400	Derivative of pQF50 containing the *aac(2′)-la* promoter region from −233 to +223 fused to *lacZ*	25
pR401	Derivative of pQF50 containing the *aac(2′)-la* promoter region from −233 to approximately +570 fused to *lacZ*	This study
pSK.aarA	pBluescript SK(−)::3.0-kb *Cla*I fragment containing *aarA*	This study
pSK.aarA ΔH/X	A *Hpa*I/*Xho*I deletion derivative of pSK.aarA	This study
pSK.aarA ΔE/N	pSK.aarA containing an in-frame *Eco*47III-*Nar*I deletion within *aarA*	This study
pADV1	pKNG101::*Sal*I-*Xba*I fragment from pSK.aarA ΔE/N	This study

internal to mini-Tn*5*Cm (6). Two of the mutants, designated PR50.A6 and PR50.B3, contained a single hybridizing fragment of 6.6 kb, suggesting that mini-Tn*5*Cm had inserted into the same *Cla*I fragment in these isolates (data not shown). Additional Southern blots demonstrated that mini-Tn*5*Cm had inserted in separate locations within this common *Cla*I fragment (data not shown). The insertional mutations in strains PR50.A6 and PR50.B3 that increased *aac(2′)-Ia* expression were designated *aarA1* and *aarA2* respectively, and these strains were used for further study. The third mutant, PR50.A1, contained two mini-Tn*5*Cm insertions, identified by two hybridizing fragments in excess of 12 kb, and the mutant allele which increased *aac(2′)-la* expression was designated *aarC1*, on the basis of the inability of *aarA* to complement this mutation (see below).

Cloning and DNA sequence analysis of *aarA*. The wild-type *aarA* gene was cloned in a two-step process. First, a chromosomal fragment containing the *aarA*::mini-Tn*5*Cm insertion and flanking DNA was isolated from PR50.B3 by digesting chromosomal DNA with *Cla*I followed by ligation to *Cla*I-digested pBluescript SK(−) (Stratagene). This ligation was used to transform *E. coli* XL1, and cells were plated on LB agar containing chloramphenicol (20 μg/ml) to select for recombinants which had acquired the mini-Tn*5*Cm insertion along with flanking *P. stuartii* chromosomal DNA. As expected, chloramphenicol-resistant transformants contained a 6.6-kb *Cla*I fragment. Restriction mapping of this cloned fragment showed that the insert contained 2.2 and 0.8 kb of *P. stuartii* DNA flanking the Tn*5*Cm element. The 0.8-kb fragment was then used as a probe in colony hybridizations to isolate the wild-type version of *aarA* from PR50 by ligation of 2- to 4-kb *Cla*I fragments into pBluescript SK(−). Plasmid pSK.aarA, containing a 3.0-kb *Cla*I fragment encoding *aarA* was isolated in this manner.

To confirm that pSK.aarA contained a functional *aarA* gene, this plasmid was introduced by electroporation (25) into PR50.A6 and PR50.B3, both containing mini-Tn*5*Cm insertions within the *aarA* locus. The level of gentamicin resistance was decreased from 32 to 2 μg/ml in cells containing pSK.aarA, relative to the 32 μg/ml seen in cells transformed with the control vector pBluescript SK(−). This complementation resulted in gentamicin resistance levels that were slightly below the wild-type level of 4 μg/ml, which could be the result of a gene dosage effect. This verified that pSK.aarA contained a functional *aarA* gene and that both PR50.A6 and PR50.B3 contained mini-Tn*5*Cm insertions which affected *aarA* function. Plasmid pSK.aarA was unable to complement PR50.A1 (*aarC1*::mini-Tn*5*Cm) (data not shown).

Nucleotide sequence of *aarA*. The nucleotide sequence of the 3.0-kb *Cla*I fragment within pSK.aarA was determined by using previously described procedures (10, 25). The insert in pSK.aarA consisted of 3,016 nucleotides and contained three large open reading frames. To localize the potential *aarA* coding region, we used Southern blot analysis to determine the approximate site of the mini-Tn*5*Cm insertions in PR50.A6 and PR50.B3, and the insertions were localized to positions 700 and 800, respectively, as indicated in Fig. 1. Both insertions were located within an open reading frame of 846 nucleotides. To test whether this 846-bp open reading frame encoded *aarA*, subclones of pSK.aarA were constructed and then tested for their ability to complement the *aarA*::mini-Tn*5*Cm mutation in PR50.B3. Introduction of pSK.aarA ΔH/X into PR50.B3 resulted in a reduction of gentamicin resistance levels from 32 to 2 μg/ml (Fig. 1), indicating complementation of the *aarA*::mini-Tn*5*Cm mutation. Introduction of the control plasmid pBluescript SK(−) did not change the gentamicin resistance levels of PR50.B3, which remained at 32 μg/ml. Introduction of pSK.aarA ΔE/N, containing an in-frame 585-bp deletion between the *Eco*47III and *Nar*I sites within the 846-bp open reading frame (Fig. 1 and 2), did not complement the *aarA*

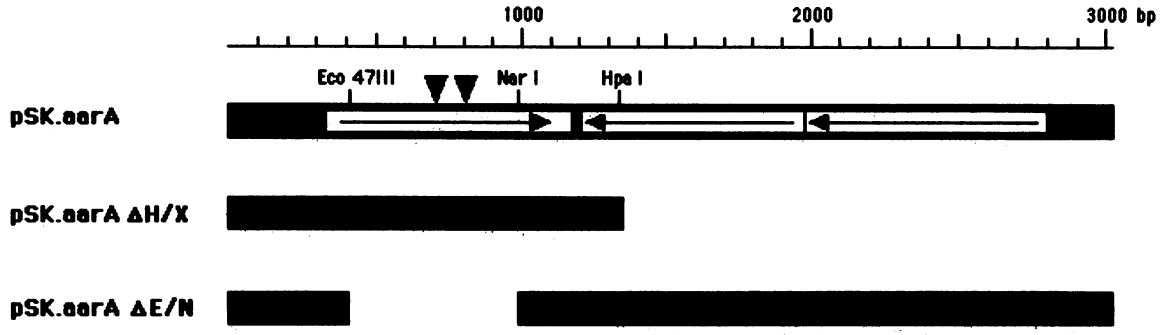

FIG. 1. Identification of *aarA* coding region. Plasmid pSK.aarA and several deletion derivatives are shown, and the closed areas represent DNA present in each of the plasmids. The location and orientation of three potential open reading frames are shown as open areas within pSK.aarA. Arrowheads denote the sites of the mini-Tn*5*Cm insertion in the *aarA* mutants.

mutation in PR50.B3, which retained a gentamicin resistance level of 32 μg/ml. These results strongly suggested that the 846-bp open reading frame encoded *aarA*. Figure 2 displays the nucleotide sequence of the 1,340-bp insert within pSK.aarA ΔH/X containing the 846-bp *aarA* open reading frame.

Properties of the AarA protein. The deduced AarA protein was 31,086 kDa and a search of both the National Biomedical Research Foundation-Protein Identification Resource and Swiss-Prot databases identified a region of AarA with 27% identity over a stretch of 78 amino acids to the GlpG protein of *E. coli*. The function of GlpG has been reported as unknown (31). No other significant homologies to AarA were identified. The Kyte-Doolittle hydropathy profile of the AarA protein demonstrated that it was extremely hydrophobic with potential transmembrane domains present between amino acids 15 to 40 and 135 to 170 (Fig. 2).

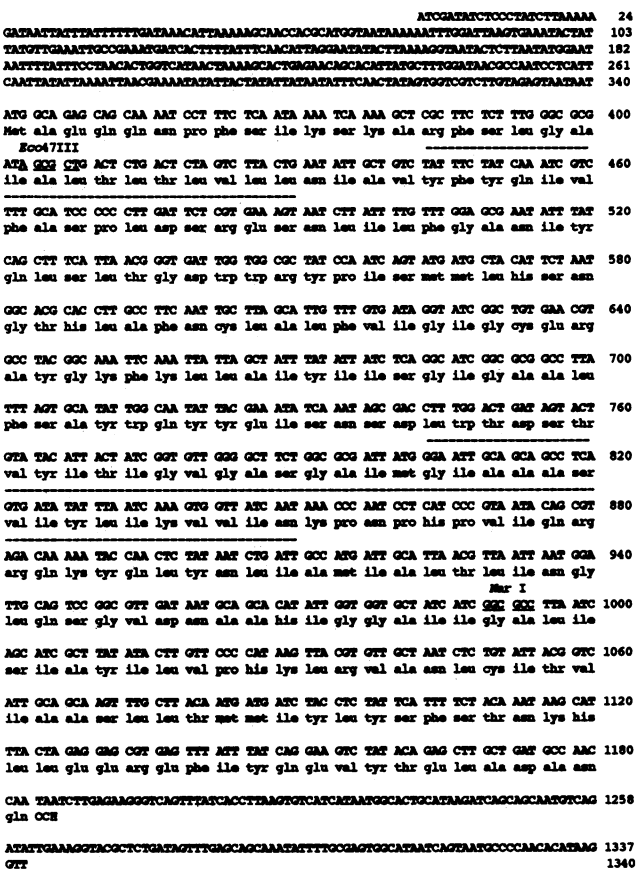

FIG. 2. Nucleotide sequence of *aarA*. The nucleotide sequence of a 1,340-bp fragment containing *aarA* is shown. In addition, relevant restriction sites are indicated above the corresponding nucleotide sequence. The deduced amino acid sequence of the AarA protein is shown below its corresponding nucleotide sequence, and potential membrane spanning regions are indicated by a dashed line below the corresponding amino acids.

Effects of *aarA* deletion on *aac(2′)-Ia* expression. An unmarked, in-frame 585-bp deletion of the chromosomal *aarA* gene was constructed by allelic replacement by using plasmid pADV1, a pKNG101 (12) derivative containing *P. stuartii* DNA corresponding to the insert in pSK.aarA ΔE/N (Fig. 1). In addition, pADV1 contains a mobilization region of RK2, the *sacB* gene which confers sucrose sensitivity (8, 27), and an R6K origin of replication that will allow replication only in strains providing the *pir* gene product (π protein) in *trans* (14). Integration of pADV1 into the *P. stuartii* chromosome was achieved by a filter mating between *P. stuartii* PR50 containing pR400 [*aac(2′)-lacZ*] (25) and *E. coli* SM10 λ*pir* (20) containing pADV1. Since pADV1 cannot replicate in *P. stuartii*, selection for integration at the *aarA* locus was achieved by plating the mating mixture on LB media containing streptomycin (75 μg/ml), along with ampicillin (400 μg/ml) to maintain pR400 and tetracycline (15 μg/ml) to counterselect the *E. coli* donor. Integration of pADV1 at the *aarA* locus results in a duplication of *aarA*, one copy of the wild type and one copy containing the desired deletion. Resolution of the *aarA* duplication by a second crossover event was achieved by plating on LB agar plates without NaCl but containing ampicillin and 5% sucrose. Sucrose-resistant survivors were obtained at a frequency of 7.1×10^{-2}. Sucrose-resistant colonies containing the *aarA* deletion were identified at a frequency of 10.7% (14 of 131) as blue colonies on media containing X-Gal, which indicated activation of the *aac(2′)-lacZ* fusion by loss of *aarA*. Several colonies with this phenotype were cured of pR400 by growing in the absence of ampicillin, and Southern blot analysis was used to verify the correct deletion in strain PR51 (data not shown).

The *aarA* deletion in PR51 resulted in an eightfold increase in gentamicin resistance levels (32 μg/ml), relative to levels in wild-type PR50 (4 μg/ml). Introduction of pSK.aarA ΔH/K, containing only the *aarA* gene, into PR51 resulted in complementation, with a reduction of gentamicin resistance to 2 μg/ml. Therefore, the increased *aac(2′)-Ia* expression resulted

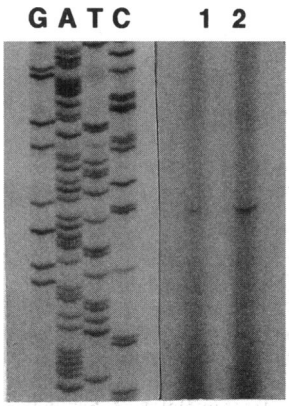

FIG. 3. Effect of *aarA* deletion on *aac(2')-Ia* mRNA accumulation. Total RNA (10 μg) prepared from PR50 (lane 1) and PR51 (lane 2) was annealed to 0.6 pmol of the oligonucleotide 5'-GCGAAATCGT CATGCGAAAAATCG-3' and extended with avian myeloblastosis virus reverse transcriptase as described previously (25), with the exception that extensions were carried out with unlabelled primer in the presence of 20 μCi of α-^{35}S-dATP at 48°C for 30 min. Lanes G, A, T, and C represents a dideoxy sequence ladder prepared by using the same primer and pSCH4500 (25) containing the cloned *aac(2')-Ia* gene. Reaction products were run on 6% acrylamide gels containing 8 M urea.

from loss of *aarA* function and was not due to polarity. To determine if transcriptional changes in *aac(2')-Ia* accounted for the increased gentamicin resistance levels observed in the *aarA* deletion mutant, we examined the levels of *aac(2')-Ia* mRNA by primer extension analysis. The results shown in Fig. 3 demonstrated increased accumulation of *aac(2')-Ia* mRNA in PR51 (Δ*aarA*), relative to that in PR50 (wild type).

To further quantitate this increase in *aac(2')-la* expression, we measured the accumulation of β-galactosidase from an *aac(2')-lacZ* fusion, present on plasmid pR401, a pQF50 (7) derivative containing an 800-bp fragment of the *aac(2')-la* promoter region fused to *lacZ*. In wild-type PR50 containing pR401, β-galactosidase activity was measured at 9.4 ± 0.38 U, as defined by Miller (19). In PR51 (pR401), this activity was measured at 32.7 ± 1.3 U, representing a 3.5-fold increase relative to that of the wild type.

Pleiotropic effects associated with loss of *aarA*. PR51 containing the *aarA* deletion displayed several prominent phenotypes. First, a secreted yellow pigment seen in wild-type cells was absent or greatly reduced in the *aarA* mutant. Second, *aarA* mutants displayed a change in cell morphology relative to that of the wild type. In Fig. 4, the phenotype of PR51 is compared with that of wild-type PR50. The *aarA* deletion resulted in cells which appear to be a defective in the ability to separate during division, resulting in paired cells along with chains of cells. Introduction of pSK.aarA ΔX/H, containing only the *aarA* coding region, restored both pigment production and normal cell morphology to PR51, confirming that these phenotypes resulted from loss of *aarA* and were not due to polar effects.

Concluding remarks. In a search for *trans*-acting negative regulators of *aac(2')-Ia*, we have identified the *aarA* gene. The increased accumulation of *aac(2')-la* mRNA in an *aarA* background and the increased β-galactosidase accumulation from an *aac(2')-lacZ* fusion suggest that *aarA* acts at the transcriptional level, although changes in mRNA stability have not been ruled out. The cloned *aarA* gene was unable to complement the previously isolated *aar3* mutation (25), suggesting that at

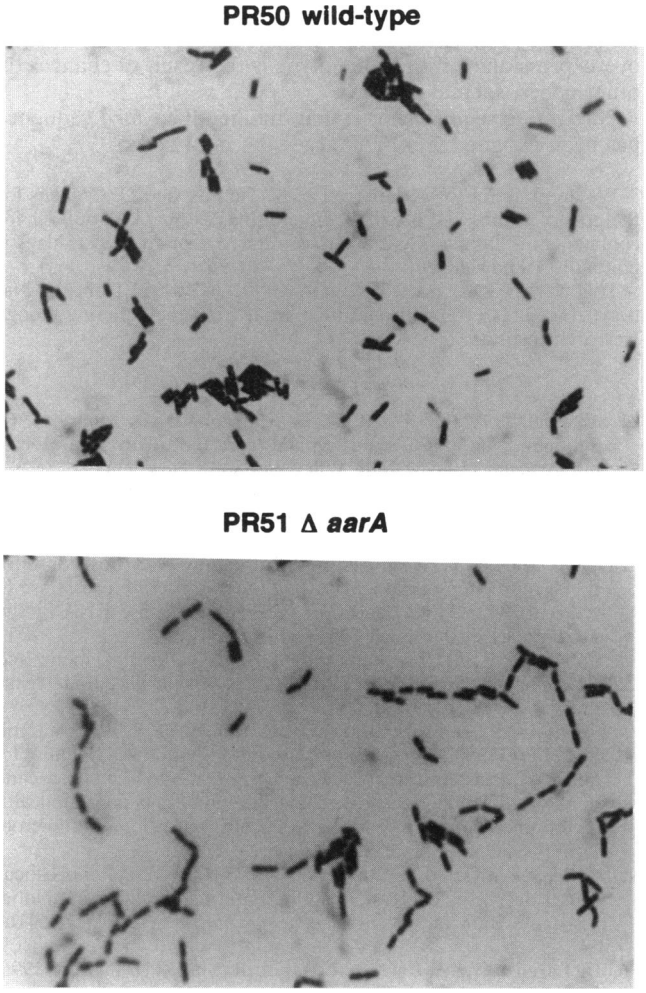

FIG. 4. Cell morphology of *P. stuartii* wild-type PR50 and (B) PR51 Δ*aarA* cells. In both cases, cells were taken from LB plates at an early stage of growth.

least two gene products negatively regulate *aac(2')-la* expression in *P. stuartii*.

Analysis of AarA demonstrated that it was very hydrophobic, with at least two potential transmembrane domains. This suggests the possibility that AarA is an integral membrane protein, which may act as a transcriptional regulator. Other examples of membrane-associated transcriptional regulators include the activators CadC (30), ToxR (21), and LuxR (13) and the transcriptional repressor PutA (23). However, it should be stressed that the putative membrane location of AarA and its ability to bind DNA have not been experimentally verified. Alternatively, regulation by AarA may be indirect, perhaps by AarA serving to transport a small ligand that interacts with a repressor.

Perhaps the most interesting phenotype of *aarA* mutants is their altered cell morphology. Relative to the rod-shaped cells of wild-type PR50, the *aarA* deletion resulted in cells that were connected together by remnants of septal material, suggesting a defect in the final stages of septation and subsequent cell separation. This chaining phenotype of *aarA* mutants is similar in appearance to *envA* mutants of *E. coli* (22). One explanation for this similarity is that *aac(2')-Ia* overexpression may antagonize the function of an EnvA homolog in *P. stuartii*. Experi-

ments are currently in progress to determine if the altered morphology of *aarA* mutants is the direct result of *aac(2')-Ia* overexpression or if this phenotype is the result of changes on other genes, such as *envA*.

Nucleotide sequence accession number. The *aarA* sequence has been assigned GenBank accession no. L28755.

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