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aarC, an Essential Gene Involved in Density-Dependent Regulation of the 2′-*N*-Acetyltransferase in *Providencia stuartii*

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The 2′-*N*-acetyltransferase [AAC(2′)-Ia] in *Providencia stuartii* has a dual function where it is involved in the acetylation of peptidoglycan and certain aminoglycosides. A search for negative regulators of the *aac(2′)-Ia* gene has resulted in the identification of *aarC*. A missense allele (*aarC1*) resulted in an 8.9-fold increase in β-galactosidase accumulation from an *aac(2′)-lacZ* transcriptional fusion. Northern blot analysis demonstrated an increase in *aac(2′)-Ia* mRNA accumulation that was specific to cells at high density. In addition, the *aarC1* allele also resulted in a substantial increase in the expression of *aarP*, a transcriptional activator of the *aac(2′)-Ia* gene. The wild-type *aarC* gene was isolated by complementation and encodes a predicted protein of 365 amino acids with a molecular mass of 39,815 Da. The predicted AarC protein exhibited 88% amino acid homology to the previously identified GcpE protein of *Escherichia coli* and 86% homology to a gene product from *Haemophilus influenzae*. The *E. coli gcpE* gene was able to functionally complement the *aarC1* allele in *P. stuartii*. The *aarC1* allele was identified as a T to G transversion that resulted in a valine to glycine substitution at position 136 in the AarC protein. The *aarC* gene appears to be essential for cell viability as construction of a disrupted copy (*aarC*::*lacZ*) was possible only in cells that carried an episomal copy of *aarC* or *gcpE*.

Providencia stuartii is a gram-negative enteric bacterium responsible for a variety of human infections, especially those of the urinary tract (11, 16, 25). Among the family *Enterobacteriaceae, P. stuartii* and other members of the tribe *Proteeae* (*Proteus* and *Morganella* spp.) all contain a unique modification of peptidoglycan corresponding to O acetylation at the C-6 hydroxyl of muramic acid (4). This modification is proposed to control the activity of autolysins, either by serving as a substrate or by blocking their activity (4–6, 19). Recently, the chromosomally encoded aminoglycoside 2′-*N*-acetyltransferase has been shown to have a role in the O acetylation of peptidoglycan in *P. stuartii* (19). This enzyme, encoded by the *aac(2′)-Ia* gene, is universal to *P. stuartii* and was initially identified by its role in aminoglycoside resistance (3, 20, 26). Presumably, aminoglycosides are not the true substrate of AAC(2′)-Ia but are acetylated due to their structural similarity with peptidoglycan.

Regulation of *aac(2′)-Ia* expression is complex. Recessive mutations in at least four loci, *aarA*, *aarB*, *aarD*, and *aarG*, that increase *aac(2′)-Ia* mRNA accumulation have been identified (15, 20, 23). In addition, a transcriptional activator, *aarP*, has been identified (14). An additional level of regulation includes repression mediated by a diffusible extracellular factor, AR-factor, which acts by an unknown mechanism to decrease *aac(2′)-Ia* mRNA accumulation as cells approach high density (22).

This study reports the identification and characterization of the *aarC* gene in *P. stuartii*. The deduced AarC protein displayed extensive identity to GcpE of *Escherichia coli*, a gene product whose function is unknown but which has been reported to be essential (1, 7). In *P. stuartii*, the role of the *aarC* gene product in regulation of *aac(2′)-Ia* is specific to cells at high density; therefore, *aarC* may act in a pathway by which *P. stuartii* responds to a density-dependent signal.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. stuartii* PR50 is a wild-type strain that has been described previously (20) and was the parent strain for the isolation of all mutants (Table 1). *E. coli* XL1-Blue (Stratagene) and DH5α (Gibco/BRL) were used as hosts for the propagation of plasmids. In addition, to propagate R6K *pir* mutant derivatives, strains DH5α λ*pir* (14) and SM10 λ*pir* (18) were used. Plasmids pACYC184 (2), pBluescript SK(−) (Stratagene), pBCKS (−) (Stratagene), and pKNG101 (13) were used as cloning vectors. All bacteria were grown with Luria-Bertani (LB) medium supplemented with 15 g of agar/liter as needed. Antibiotics were used at the following concentrations for growth of *E. coli*: ampicillin, 150 μg/ml; chloramphenicol, 25 μg/ml; streptomycin, 25 μg/ml; tetracycline, 15 μg/ml; and kanamycin, 20 μg/ml. For growth of *P. stuartii*, antibiotics were used at the following concentrations: ampicillin, 150 μg/ml; chloramphenicol, 100 μg/ml; streptomycin 75, μg/ml; and tetracycline, 30 μg/ml. Selection for sucrose resistance in *P. stuartii* was done at 30°C on LB agar plates without NaCl and containing 5% sucrose.

Isolation and subcloning of *aarC*. A *Sau*3AI recombinant library of *P. stuartii* chromosomal DNA in pACYC184 has been described previously (14). Plasmid pACYC184.aarC contains an approximately 1.7-kb *Sau*3A partial fragment in pACYC184. Digestion of this plasmid with *Sal*I released a 2.0-kb fragment containing most of the *P. stuartii* insert DNA along with 276 bp of pACYC184 DNA from the *Bam*HI cloning site to the *Sal*I site. This 2.0-kb *Sal*I fragment was cloned into the *Sal*I site of pBluescript II SK(−), resulting in pSK-2.0. Digestion of pSK-2.0 with *Bam*HI released a 600-bp fragment, and subsequent religation of the vector-containing fragment resulted in pSK.aarC1.4, which contained a 1.4-kb insert entirely composed of *P. stuartii* DNA. This 1.4-kb insert was then cloned into pBCKS (−) as a 1.4-kb *Bam*HI-*Sal*I fragment, resulting in pBC.aarC1.4. Plasmid pBC.aarC1.4 was further subcloned by digestion with *Ssp*I and *Bam*HI, which released an approximately 1.0-kb fragment that was subcloned into *Eco*RV- and *Bam*HI-digested pBCKS (−) and pBluescript II SK(−). This resulted in pBC.aarC1.0 and pSK.aarC1.0, respectively. To isolate a DNA fragment containing a full-length version of the *aarC* gene, chromosomal DNA from PR50 was digested with *Sal*I and fragments of 4 to 6 kb were ligated to pBluescript II SK(−). Previously, Southern blot analysis demonstrated that *aarC* was within an approximately 5-kb *Sal*I fragment. Colony hybridization with a 309-bp *Eco*RV fragment from pBC.aarC1.4 was used to identify recombinant plasmids containing the 5-kb *Sal*I fragment with the full-length *aarC* gene. This plasmid was designated pSK.aarC5.0. Plasmid pPR25 contains an approximately 0.7-kb *Nru*I fragment from pSK.aarC5.0 cloned into the *Eco*RV site of pBCKS (−). Plasmid pPR26 contains a 3.9-kb *Nru*I-*Sal*I fragment from pSK.aarC5.0 cloned into the *Eco*RV and *Sal*I sites of pBCKS (−).

To isolate the *aarC1* mutation, chromosomal DNA from PR60 was digested

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and relevant markers	Source
Strains		
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*lac-pro* [F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*]	Stratagene
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*lacZYA-argF U169* ϕ80d*lacZ*ΔM15	Gibco/BRL
DH5α λ*pir*	DH5α lysogenized with λ*pir*	Macinga et al. (14)
SM10 λ*pir*	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu Km^r λ*pir*	Miller and Mekalanos (18)
P. stuartii		
PR50	Wild type	Rather et al. (20)
PR500	PR50 containing pR401 and pACYC184.aarG	This study
PR60	PR50 *aarC1*	This study
Plasmids		
pACYC184	Medium-copy-number vector; Cm^r Tc^r	Chang and Cohen (2)
pKNG101	R6K-derived suicide plasmid containing Str^r and *sacB*	Kaniga et al. (13)
pBluescript SK(−)	High-copy-number vector; Ap^r	Stratagene
pBCKS (−)	High-copy-number vector; Cm^r	Stratagene
pR401	*aac*(*2′*)-*lacZ* transcriptional fusion	Rather and Orosz (21)
pACYC184.aarC	pACYC184 containing 1.7-kb *P. stuartii* chromosomal fragment	This study
pSK-2.0	pBluescript SK(−)::2.0-kb *Sal*I fragment from pACYC184.aarC	This study
pSK.aarC1.4	pBluescript SK(−)::1.4-kb *Sal*I-*Bam*HI fragment from pSK-2.0	This study
pBC.aarC1.4	pBCKS (−)::1.4-kb *Sal*I-*Bam*HI fragment from pSK-2.0	This study
pSK.aarC1.0	pBluescript SK(−)::1.0-kb *Ssp*I-*Bam*HI fragment from pSK.aarC1.4	This study
pBC.aarC1.0	pBCKS (−)::1.0-kb *Ssp*I-*Bam*HI fragment from pSK.aarC1.4	This study
pBC.aarC::lacZ	pBC.aarC1.0::lacZ inserted at unique *Nru*I site	This study
pKNG101.aarC::lacZ	pKNG101::4.5-kb *Sal*I-*Bam*HI fragment from pBC.aarC::lacZ	This study
pSK.aarC5.0	pBluescript SK(−)::5.0-kb *Sal*I chromosomal fragment from PR50	This study
pPR25	pBluescript SK(−)::0.7-kb *Nru*I fragment from pSK.aarC5.0	This study
pPR26	pBluescript SK(−)::3.9-kb *Nru*I-*Sal*I fragment from pSK.aarC5.0	This study
pSK.aarC1.4mut	pBluescript SK(−)::1.4-kb *Sal*I-*Bam*HI fragment containing *aarC1* allele	This study
pSK.aarC1.0mut	pSK.aarC1.0 with substituted 320-bp *Nru*I-*Bcl*I fragment from pSK.aarC1.4mut	This study

with *Bam*HI and *Sal*I, and fragments in the range of 1 to 2 kb were ligated to pBluescript II SK(−) digested with *Bam*HI and *Sal*I. Colony hybridization as described above was then used to identify plasmids with a 1.4-kb fragment containing the mutant *aarC* gene. This plasmid was designated pSK.aarCmut.1.4.

Construction of an *aarC*::*lacZ* disruption. Plasmid pBC.aarC1.0 containing a 1.0-kb portion of the *aarC* coding region was linearized by digestion with *Nru*I, which cuts at position 454 within the *aarC* coding region. A promoterless *lacZ* cassette was obtained from pQF50 (8) by digestion with *Sma*I and *Sca*I and ligated in the correct orientation to pBC.aarC, creating an *aarC*::*lacZ* transcriptional fusion. A DNA fragment containing this fusion was excised by digestion with *Bam*HI and *Sal*I and cloned into the suicide vector pKNG101 (13), creating pKNG101.aarC::lacZ. This plasmid contains a *pir*-dependent origin of replication, a counterselectable *sacB* gene, and a streptomycin resistance gene. To introduce pKNG101.aarC::lacZ into the *P. stuartii* chromosome, *E. coli* SM10 λ*pir* (18) containing pKNG101.aarC::lacZ was mated to *P. stuartii* strains as described previously (21). Mating mixtures were then plated on LB plates containing streptomycin (75 μg/ml), tetracycline (30 μg/ml), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). In the case of *P. stuartii* strains containing plasmid DNA, ampicillin was also added at a concentration of 300 μg/ml. Selection for loss of the integrated pKNG101.aarC::lacZ was achieved by growth at 30°C on LB plates without NaCl and containing 5% sucrose. Southern blot analysis was performed on strains by digestion of chromosomal DNA with *Sal*I followed by transfer to a nylon membrane. The *aarC* probe contained a 1.4-kb *Bam*HI-*Sal*I fragment. The probe specific for *lacZ* was a 3.2-kb *Pst*I fragment from pMC1871 (Pharmacia).

Northern blot analysis. RNA was prepared for Northern blot analysis by using the TRIzol reagent (Gibco/BRL) and was fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane by capillary transfer. To ensure both equal loading and transfer of RNA to the nylon membrane, RNAs were directly photographed on the nylon filter by UV illumination. Filters were then probed with a digoxigenin-labeled 602-bp *Taq*I-*Ssp*I fragment containing the *aac*(*2′*)-*Ia* coding sequence. Filters were developed with Lumi-Phos 530 (Boehringer Mannheim) and exposed to X-ray film.

DNA sequencing. Fluorescein-labeled universal and reverse primers were used to sequence double-stranded DNA by using the AutoRead sequencing kit (Pharmacia). Internal primers were used as needed to fill gaps in the contiguous sequence. Label was incorporated into these sequencing reactions by using fluorescein-labeled dATP. All sequencing reactions were analyzed on an A.L.F. automated sequencer (Pharmacia). The entire nucleotide sequence of both strands of the *aarC* gene and flanking DNA was determined in this manner. Specific templates for sequencing were generated as follows. Digestion of pBC.aarC1.4 with *Eco*RV released fragments of 335, 420, 267, 309, and 80 bp, each of which was cloned into the *Eco*RV site of pBluescript II SK(−) and used as a sequencing template. DNA sequence analysis of the insert within pBC.aarC1.4 indicated the presence of a partial open reading frame of 738 bp capable of encoding a protein of 246 amino acids with a truncated carboxy terminus. To sequence the remainder of the *aarC* coding region, we cloned a 0.5-kb fragment from pACYC184.aarC containing additional 3′ coding region. This resulted in an additional 266 nucleotides downstream of the previous sequence; however, a stop codon for this open reading frame had not yet been reached. To complete the sequence, plasmid pSK.aarC5.0, containing the entire *aarC* gene on a 5.0-kb *Sal*I fragment, was isolated. Custom primers were then used to finish the DNA sequence analysis. Protein homology searches were performed by using the blastp program at the National Center for Biotechnology Information.

β-Galactosidase assays. Plasmid pR401 containing an *aac*(*2′*)-*lacZ* transcriptional fusion has been described previously (21). β-Galactosidase activity is reported as Miller units (17). Reported values represent the averages of triplicate samples from three independent experiments.

Nucleotide sequence accession number. The sequence of *aarC* has been submitted to GenBank and assigned accession number U67933.

RESULTS

Isolation of the *aarC1* allele. The chromosomal 2′-*N*-acetyltransferase [AAC(2′)-Ia] has a role in the O acetylation of peptidoglycan in *P. stuartii* (19) and also acetylates some aminoglycosides (3, 20, 26). As a result, *P. stuartii* cells with increased *aac*(*2′*)-*Ia* expression become resistant to higher levels of aminoglycosides (14, 15, 20, 21). To identify genes involved in regulating *aac*(*2′*)-*Ia* expression, our strategy was to generate mutations that simultaneously increased expression of the chromosomal copy of *aac*(*2′*)-*Ia* and of an *aac*(*2′*)-*lacZ* fusion present on plasmid pR401 (21). In addition, we wanted to prevent the reisolation of *aarG* mutations which alter the regulation of *aac*(*2′*)-*Ia* (23). Therefore, we used strain PR500, which is a PR50 derivative containing pR401 [*aac*(*2′*)-*lacZ*] and pACYC184.aarG, a compatible pACYC184 derivative containing the *aarG* gene. PR500 forms white colonies on

plates containing X-Gal and has aminoglycoside resistance to 4 μg of gentamicin/ml. Selection of mutants was achieved by plating approximately 10^8 cells of PR500 on LB plates containing 15 μg of gentamicin/ml, X-Gal (to select and identify regulatory mutants), ampicillin (150 μg/ml; to maintain pR401), and chloramphenicol (100 μg/ml; to maintain pACYC184.aarG). As observed previously, colonies arose at a frequency of 10^{-6} to 10^{-7} (20). Colonies which grew under these conditions and exhibited blue color were analyzed further. These colonies presumably resulted from mutations in a *trans*-acting regulator of *aac(2′)-Ia* since expression of both the chromosomal copy of *aac(2′)-Ia* and the plasmid-encoded *aac(2′)-lacZ* fusion was increased. One class of mutants which formed small blue colonies was identified, and a representative isolate, PR60/pR401/pACYC184.aarG, was characterized. This strain was then cured of pACYC184.aarG, resulting in PR60/pR401, which displayed a phenotype identical to PR500, indicating that the presence of *aarG*, in multicopy did not alter the effects of the *aarC1* mutation. Introduction of the previously cloned *aarA*, *aarB*, and *aarD* genes (15, 20, 21) into PR60/pR401 did not result in complementation (data not shown), indicating that PR60 contained a new regulatory mutation, which was designated *aarC1.*

***aarC1* increases *aac(2′)-Ia* at high cell density.** The accumulation of β-galactosidase in wild-type PR50/pR401 and PR60 *aarC1*/pR401 was measured in cells at mid-log phase, and an 8.9-fold increase in β-galactosidase accumulation was observed in PR60 (32.1 ± 2.4 Miller units) relative to PR50 (3.6 ± 0.2 Miller units). This experiment was repeated two times and gave increases of 8.8- and 9.2-fold, respectively, in PR60. Northern blot analysis was then used to directly examine the effects of the *aarC1* allele on *aac(2′)-Ia* mRNA accumulation. RNA was prepared from wild-type PR50 and from PR60 (*aarC1*) cured of plasmid pR401 at log phase (optical density at 600 nm [OD_{600}] = 0.2) and examined by Northern blot analysis. Unexpectedly, the levels of *aac(2′)-Ia* mRNA accumulation in PR60 (*aarC1*) were not significantly different from wild type and in some instances appeared to decrease. Based on this result, the effects of the *aarC1* allele on *aac(2′)-Ia* expression were examined at various stages of growth. RNA was prepared from cells at log phase (OD_{600} = 0.2) and stationary phase (OD_{600} = 1.2), and *aac(2′)-Ia* mRNA accumulation was examined by Northern blot analysis (Fig. 1A). The levels of *aac(2′)-Ia* mRNA in cells of PR50 (wild type) and PR60 (*aarC1*) were approximately equal at log phase (Fig. 1). However, at late log/early stationary phase, PR60 demonstrated a considerable increase in the accumulation of *aac(2′)-Ia* mRNA relative to wild-type PR50.

Increased expression of the transcriptional activator *aarP* in an *aarC1* background. Previously, we had identified a transcriptional activator, AarP, which activates the *aac(2′)-Ia* gene (14). The increased *aac(2′)-Ia* expression that was observed in the *aarC1* background may have resulted from increased *aarP* expression. The accumulation of *aarP* mRNA was examined by Northern blot analysis as shown in Fig. 1B. At low cell density, the *aarC1* allele did not significantly affect the accumulation of *aarP* mRNA in PR60 relative to wild-type PR50. However, in cells at high density, the accumulation of *aarP* mRNA was significantly increased in the *aarC1* background.

Cloning and analysis of *aarC*. PR60 *aarC1* formed colonies that were considerably smaller than wild-type PR50 after 24 h of growth. Since the *aarC1* mutation was selected spontaneously, we reasoned that a single mutation may be responsible for both the slow growth phenotype and the increased *aac(2′)-Ia* expression. Therefore, to isolate the wild-type *aarC* gene, a recombinant library of *P. stuartii* DNA in pACYC184 (2, 14) was introduced by electroporation into PR60/pR401 [*aac(2′)-lacZ*] and colonies with a normal growth phenotype were isolated. This resulted in the isolation of plasmids with different inserts, each containing a common region of DNA based on restriction mapping. Retransformation of a representative plasmid, pACYC184.aarC, demonstrated that the insert *P. stuartii* DNA was responsible for correcting the slow growth phenotype. In addition, the resulting transformants now formed white colonies on X-Gal plates. Since both the slow growth phenotype and increased *aac(2′)-Ia* expression were corrected to wild type by introduction of the plasmids, it seemed reasonable that the recombinant plasmids contained the wild-type *aarC* gene.

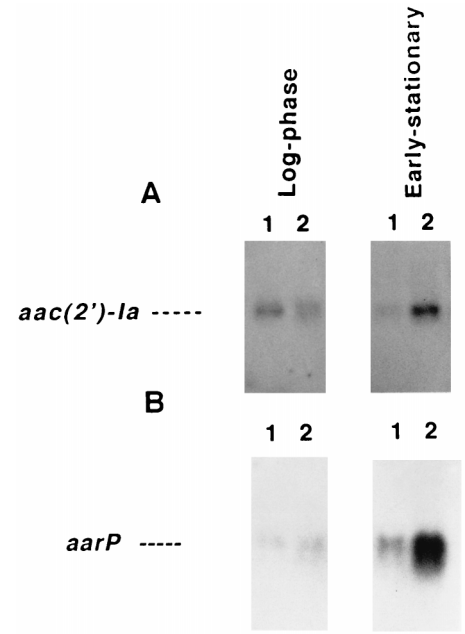

FIG. 1. *aarC* is a density-dependent regulator. Total RNA (20 μg) was prepared from PR50 (wild-type) or from PR60 (*aarC1*) cells at early log phase (OD_{600} = 0.2) and early stationary phase (OD_{600} = 1.2). In each experiment, lane 1 represents RNA from PR50 and lane 2 represents RNA from PR60 *aarC1*. (A) RNA was probed with a 602-bp *Taq*I-*Ssp*I fragment specific to *aac(2′)-Ia*. (B) RNA was probed with a digoxigenin-labeled 0.4-kb PCR-generated fragment specific to *aarP.*

The sequence of the *aarC* coding region was determined as described in Materials and Methods. The deduced AarC protein contained 365 amino acids and exhibited 88% identity over a stretch of 362 amino acids to the GcpE protein of *E. coli* (1) (Fig. 2). The function of GcpE is unknown; however, it has been proposed to be essential (1). Introduction of the *E. coli gcpE* gene on plasmid pSE401 (7) into PR60 *aarC1* restored wild-type growth levels, indicating that *gcpE* can functionally substitute for *aarC*. The deduced AarC protein also displayed 86% homology over 361 amino acids to an open reading frame identified in the *Haemophilus influenzae* genome (accession number H64063) (9) and 51% identity over 333 amino acids to YqfY in *Bacillus subtilis* (accession number D84432).

Identification of the *aarC1* allele. Plasmid pSK.5.0 containing the entire *aarC* gene and flanking DNA on a 5.0-kb *Sal*I fragment was capable of complementing the *aarC1* mutation. Subclones of this fragment were generated in pBluescript SK (−) that contained an approximately 700-bp *Nru*I fragment (pPR25) or a 3.9-kb *Nru*I-*Sal*I fragment (pPR26) (Fig. 3). Neither plasmid was capable of complementing the *aarC1* mu-

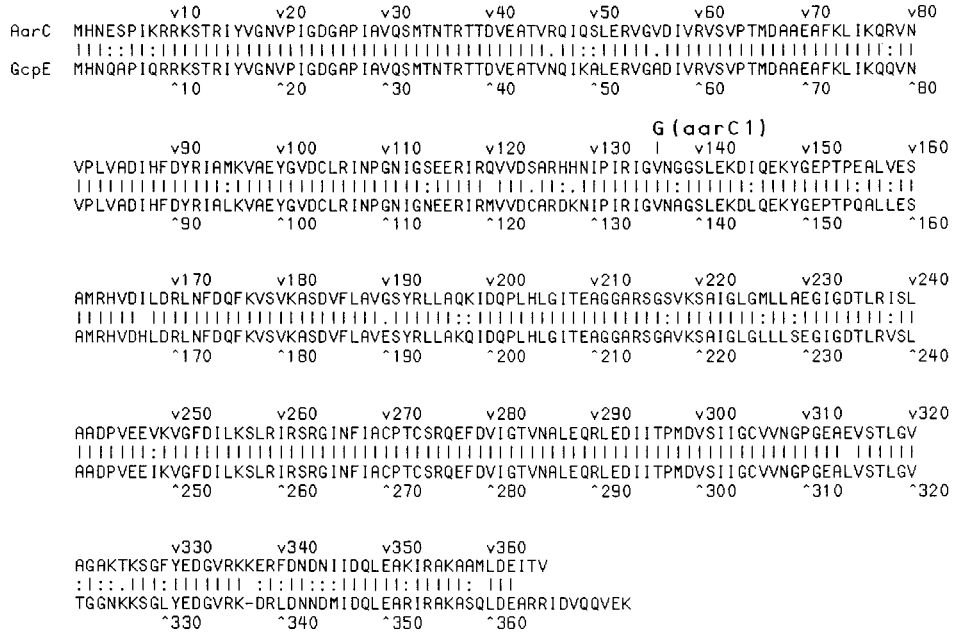

FIG. 2. Comparison between AarC and GcpE. The deduced amino acid sequences of AarC and GcpE are aligned. Vertical lines indicate identical amino acids, colons indicate conserved amino acids, and dots indicate amino acids with low similarity. The position of the valine to glycine substitution in the AarC mutant protein is indicated at position 136.

tation when introduced into PR60; however, PR60 cells containing pPR26 were very unstable and colonies rapidly became papillated with fast-growing sectors. In contrast, cells containing pPR25 remained small. The fast-growing cells could result from correction of the *aarC1* allele at high frequency by recombination of pPR26 into the chromosome. In this case, the *aarC1* mutation is predicted to be downstream of the *Nru*I site. Furthermore, plasmid pSK.aarC1.0 containing a 1.0-kb *Ssp*I-*Bam*HI fragment was shown to partially complement the *aarC1* allele. These results indicated that the *aarC1* mutation was likely to reside within the *Nru*I-to-*Bam*HI interval.

A chromosomal DNA fragment containing the *aarC1* allele was cloned from PR60 as a 1.4-kb *Sal*I-*Bam*HI fragment in pBluescript SK(−), resulting in plasmid pSK.aarC1.4mut. This plasmid failed to complement the *aarC1* allele when introduced into PR60. The presence of pSK.aarC1.4mut in either wild-type *P. stuartii* PR50 or *E. coli* XL1 substantially reduced the growth rate of cells, indicating that the mutant AarC gene product may have a dominant-negative effect. To further localize the *aarC1* mutation within the 1.4-kb fragment, restriction fragment exchanges were performed between pSK.aarC1.0 and pSK.aarC1.4mut. Substitution of the wild-type 320-bp *Nru*I-*Bcl*I fragment of pSK.aarC1.0 with the mutant fragment from pSK.aarC1.4mut resulted in *E. coli* transformants that were slow growing, and the resulting hybrid plasmid, pSK.aarC1.0mut, failed to complement PR60. This indicated that the *aarC1* mutation was within this 320-bp *Nru*I-*Bcl*I interval. The nucleotide sequence of this fragment revealed a single T to G transversion resulting in a valine to glycine substitution at position 136, a position conserved with the *E. coli* and *H. influenzae* homologs (Fig. 2).

***aarC* is an essential gene.** A previous report indicated that *gcpE* was an essential gene in *E. coli* (1). To assess the role of *aarC* in *P. stuartii*, we inserted a promoterless *lacZ* cassette into a unique *Nru*I site approximately one-third into the *aarC* gene. This *aarC*::*lacZ* insertion was transferred into the *sacB*-containing suicide plasmid pKNG101 (13), resulting in pKNG101. aarC::lacZ, which was then integrated into the chromosome of PR50 by a conjugal mating as described in Materials and Methods. This integration resulted in streptomycin-resistant (Sm^r) and sucrose-sensitive (Suc^s) colonies which were blue in the presence of X-Gal. Integration of pKNG101.aarC::lacZ could occur by either of two crossovers (Fig. 4) and would result in a strain diploid for *aarC*. Crossover 1 would yield a functional copy of *aarC*; however, crossover 2 would result in two non-

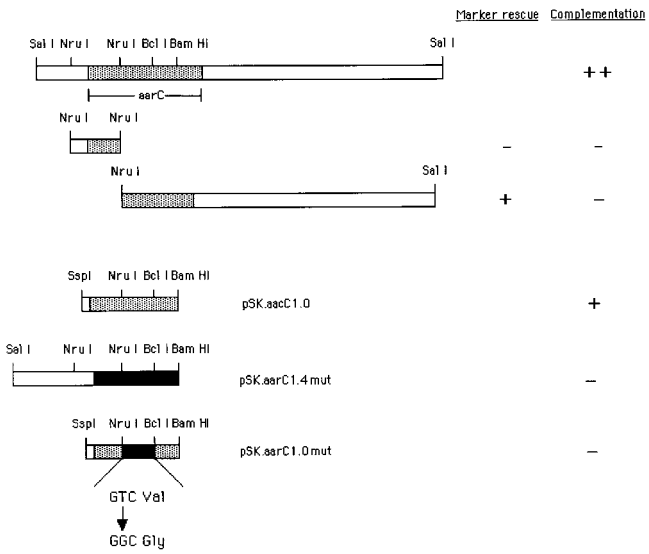

FIG. 3. Mapping of the *aarC1* allele. Recombinant plasmids containing the *aarC* region are shown. The shaded area represents the portion of the *aarC* gene within each plasmid. The ability of each plasmid to complement or marker rescue the *aarC1* mutation is shown. + (marker rescue), extensive formation of papillae in colonies; ++ (complementation), restoration of a wild-type growth rate to the *aarC1* mutant; + (complementation), partial complementation; −, no complementation.

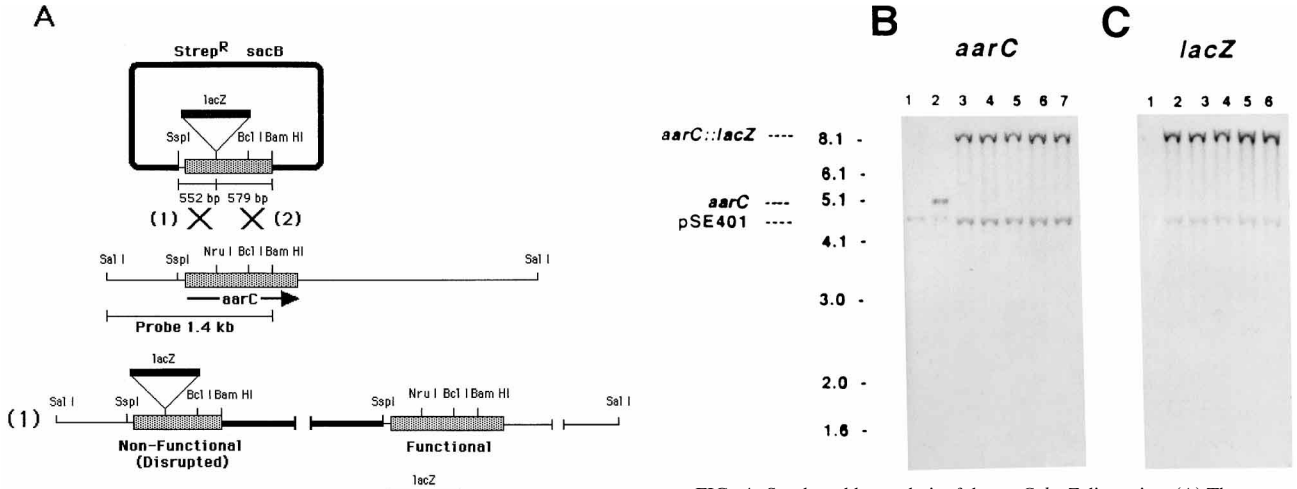

FIG. 4. Southern blot analysis of the *aarC*::*lacZ* disruption. (A) The structure of plasmid pKNG101.aarC::lacZ is depicted at the top, and the 5.0-kb *Sal*I chromosomal region of PR50 containing *aarC* is shown immediately below. Integration of pKNG101.aarC::lacZ into the chromosome can occur within the 552-bp interval 5′ to the inserted *lacZ* cassette as depicted in crossover 1 (part 1) or within the 579-bp interval 3′ to the inserted *lacZ* cassette as depicted in crossover 2 (part 2). The predicted structure of the chromosomal region in a strain containing an *aarC*::*lacZ* disruption is shown in part 3. The probe used for Southern blot analysis was the 1.4-kb *Sal*I-*Bam*HI fragment depicted below the PR50 chromosomal region. (B and C) Southern blot verification of the *aarC*::*lacZ* disruption. All DNAs were digested with *Sal*I. Panel B represents DNA probed with *aarC*. Lane 1, purified pSE401 plasmid DNA; lane 2, PR50/pSE401; lanes 3 to 7, putative PR50 *aarC*::*lacZ*/pSE401 mutants. Panel C represents DNA probed with the *lacZ* gene. All DNAs are *Sal*I digested. Lane 1, PR50/pSE401; lanes 2 to 6, putative PR50 *aarC*::*lacZ*/pSE401 mutants.

functional copies of *aarC*, one with a truncation and one containing the *lacZ* disruption. Southern blot analysis of 28 Sm^r Suc^s blue colonies demonstrated they all contained pKNG101.aarC::lacZ integrated by crossover 1 (data not shown) and thus retained a functional copy of *aarC*. This was unexpected, as there is more homology for crossover 2 (579 bp versus 552 bp for crossover 1). Excision of the integrated plasmid by selection on 5% sucrose failed to yield blue colonies corresponding to the *aarC*::*lacZ* disruption. These results were obtained in two independent experiments, suggesting that colonies containing the *aarC*::*lacZ* disruption were not viable.

To further test this possibility, we attempted to resolve the integrated copy of pKNG101.aarC-lacZ in PR50 transformed with plasmid pSE401 containing the *E. coli gcpE* gene (7). The *gcpE* gene was chosen because it was previously shown to complement the growth defect in PR60 *aarC1*, and the use of this gene should limit the potential problem of recombinational rescue of the *aarC*::*lacZ* disruption. Selection for sucrose resistance in this strain now resulted in approximately equal numbers of blue and white colonies. Southern blot analysis of five randomly chosen blue colonies confirmed the presence of the *aarC*::*lacZ* disruption, as demonstrated in Fig. 4B and C. When a probe specific to *aarC* was used, a *Sal*I fragment of 5 kb which corresponds to the *aarC* locus is present in PR50/pSE401 (Fig. 4B, lane 2). In addition, cross-hybridization is seen with a fragment from pSE401 (Fig. 4B, lanes 1 to 7). In the five putative *aarC*::*lacZ* mutants the 5.0-kb *Sal*I fragment is no longer present and is replaced with a fragment of approximately 8.5 kb in size (lanes 3 to 7). This is the expected size, as the *lacZ* cassette inserted into *aarC* is approximately 3.5 kb. To confirm this, a probe specific to *lacZ* was used and the five putative *aarC*::*lacZ* mutants demonstrated hybridization to the same 8.5-kb fragment (Fig. 4C, lanes 2 to 6). No hybridization was detected with DNA from PR50/pSE401 (Fig. 4C, lane 1).

To determine if the viability of a strain with the *aarC*::*lacZ* disruption was now dependent on the presence of pSE401, we grew PR50 (*aarC*::*lacZ*)/pSE401 in the absence of ampicillin for approximately 12 generations and plated out for single colonies on LB plates. All resulting colonies (474 of 474 tested) remained ampicillin resistant, indicating they retained pSE401. In contrast, strain PR50/pKNG101.*aarC*::*lacZ*/pSE401, which is diploid for *aarC* (Fig. 4), was cured of pSE401 at frequencies above 50% under these same conditions. Thus, the presence of *gcpE* on pSE401 was essential for the construction of an *aarC*::*lacZ* disruption in the chromosome, and the resulting strain requires pSE401 for viability.

DISCUSSION

The *aarC* gene has been identified as a negative regulator of *aac(2′)-Ia* in *P. stuartii*. A missense allele (*aarC1*) resulted in the increased accumulation of *aac(2′)-Ia* mRNA when cells were at high density. The *aarC1* allele also increased the accumulation of mRNA specific to *aarP*, a gene encoding a transcriptional activator of *aac(2′)-Ia* (14). This increase in *aarP* expression was also specific to cells at high density (Fig. 1). A possible model consistent with these observations is that *aac(2′)-Ia* overexpression in the *aarC1* background is simply due to overexpression of *aarP*. To test this possibility, we have attempted to introduce an *aarP*::Cm disruption into the *aarC1* background; however, attempts to construct this double mutant have failed repeatedly.

The *aarC1* allele is recessive to wild type, suggesting that the regulatory consequences of *aarC1* are the result of loss of AarC function. Interestingly, the presence of the *aarC1* allele

on a high-copy-number plasmid was detrimental to the growth of both *P. stuartii* and *E. coli*. One interpretation of this result is that AarC functions as a multimer and overexpression of the mutant subunit interferes with the function of the wild-type subunits. Alternatively, AarC may interact in a complex with other proteins. Analysis of the AarC protein did not reveal any obvious functional motifs, and therefore, the mechanism by which it regulates mRNA accumulation remains to be determined. In addition, hydropathy analysis of AarC did not reveal any obvious signal sequence or hydrophobic regions, suggesting that AarC is cytoplasmic. AarC shared striking homology to GcpE of *E. coli* (1) and *H. influenzae* (9) and to the YqfY product of *B. subtilis*. The high degree of conservation between these three proteins suggests they are involved in an important housekeeping function. Attempts to disrupt *gcpE* in *E. coli* were possible only in cells that contained an episomal copy of *gcpE*, indicating that *gcpE* is essential (1). This study has demonstrated that *aarC* is also required for the viability of *P. stuartii*. In addition, AarC and GcpE are functionally equivalent, as introduction of pSE401 containing the *E. coli gcpE* gene into PR60 resulted in complementation of the *aarC1* allele and also allowed the construction of a chromosomal *aarC*::*lacZ* disruption. The presence of this gene in *E. coli* and *H. influenzae*, neither of which contains *aac*(*2′*)-*Ia*, suggests that additional functions are carried out by AarC. Consistent with this, the *aarC1* allele also resulted in a slow growth phenotype and altered cell morphology with the formation of very short rods, many of which were spherical.

The *aarC* gene can now be added to the list of genes, which includes *aarA*, *aarB*, *aarD*, and *aarG*, that serve as negative regulators of *aac*(*2′*)-*Ia* (15, 20, 21, 23). Among these genes only the *aarG* locus, which encodes a putative response regulator and a sensor kinase, exhibits clear similarity to transcriptional regulators (23). The *aarA* gene product appears to be an integral membrane protein, and the *aarD* gene encodes CydD, a component of an ABC transporter complex involved in the formation of a functional cytochrome *bd* oxidase (15). The product of *aarB* is presently unknown. The lack of similarity among these *aar* genes to transcriptional regulators suggests they act indirectly and may affect a common cellular process in which *aac*(*2′*)-*Ia* and/or *aarP* regulation is coupled. An obvious candidate for this process given the identification of *aarD* (*cydD*) is electron transport. However, this is purely speculative at this time.

We have shown that the accumulation of *aac*(*2′*)-*Ia* mRNA is decreased at high cell density due to the accumulation of an extracellular signaling molecule termed AR-factor (22). This type of regulation is termed quorum sensing and is mediated by the accumulation of diverse chemical signals, including amino acids, peptides, and acyl derivatives of homoserine lactone (10, 12, 24). The requirement for high cell density to observe the *aarC* mutant phenotype raises the possibility that AarC is involved in an extracellular signaling process. Several possibilities exist including a requirement for *aarC* in AR-factor production or in the ability to respond to AR-factor, and experiments are in progress to examine these possibilities. Alternatively, *aarC* may regulate gene expression in response to other density-dependent signals such as oxygen and/or nutrient depletion or growth rate. The physiological significance of this regulation may be related the role of *aac*(*2′*)-*Ia* in the O acetylation of peptidoglycan, a modification which affects degradation by muramidases and thus may control autolytic activity (4–6, 19). The decrease in *aac*(*2′*)-*Ia* at high density may occur in preparation for changes in cell morphology that occur during the transition to stationary phase. Although AarC acts at high density, the requirement for viability indicates that it probably has an essential function early in cell growth. The essential nature of *aarC* and the remarkable conservation of this gene product in three different gram-negative and one gram-positive bacteria make it an attractive target for the development of new antimicrobials.

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