[Frontiers in Bioscience 4, d132-140, February 1, 1999]

# THE CHROMOSOMAL 2'-*N*-ACETYLTRANSFERASE OF *PROVIDENCIA STUARTII:* PHYSIOLOGICAL FUNCTIONS AND GENETIC REGULATION

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## 1. ABSTRACT

Instrisic chromosomal acetyltransferases involved in aminoglycoside resistance have been identified in a number of bacteria. In *Providencia stuartii,* a chromosomal acetyltransferase (AAC(2')-Ia) has been characterized in detail. In addition to the ability to acetylate aminoglycosides, the AAC(2')-Ia enzyme has at least one physiological function, which is the acetylation of peptidoglycan. This modification is likely to influence the autolytic system in *P. stuartii.* The regulation of *aac(2')-Ia* expression is extremely complex involving at least seven regulatory genes acting in at least two pathways. This complexity in regulation indicates that *aac(2')-Ia* expression must be tightly controlled in response to different environmental conditions. This presumably reflects the importance of maintaining correct levels of peptidoglycan acetylation. In this review, a summary of data will be presented involving both the physiological and genetic aspects of *aac(2')-Ia* in *P. stuartii* .

## 2. INTRODUCTION

### 2.1. Aminoglycoside resistance mechanisms

The aminoglycosides are a large family of antibiotics which have been used extensively in the clinic since the introduction of Streptomycin over 50 years ago. Aminoglycosides inhibit protein synthesis by binding to the ribosome and are bactericidal (1, 2, 3). Bacteria may acquire aminoglycoside resistance by decreased uptake/permeability, alterations of the ribosome, or through the acquisition of aminoglycoside modifying enzymes (4, 5, 6, 7). The first two mechanisms are rare and usually account for low-level resistance. High level resistance arising from the acquisition of aminoglycoside modifying enzymes is the most common and clinically important mechanism (6). Modification by aminoglycoside modifying enzymes results in inactivation of the aminoglycoside by decreasing it's affinity for ribosomes (8). Three types of aminoglycoside modifying enzymes have been described: the aminoglycoside phosphotransferases (APH) which phosphorylate hydroxyl groups; the aminoglycoside nucleotidyltransferases (ANT) which adenylylate hydroxyl groups; and the aminoglycoside acetyltransferases (AAC) which acetylate amino groups. The aminoglycoside modifying enzymes are further subdivided based on the position of the aminoglycoside that is modified. The AAC(2')-Ia protein reviewed here acetylates amino groups present at the 2' carbon of the deoxystreptamine core (9, 10, 11). For a detailed description of the nomenclature of the aminoglycoside modifying enzymes see Shaw *et al.,* 1993 (6).

### 2.2. Aminoglycoside resistance genes

The majority of genes encoding aminoglycoside modifying enzymes are carried on plasmids or other mobile genetic elements (6). This association has contributed to the rapid spread of aminoglycoside resistance throughout the bacterial kingdom. Recently, a number of chromosomally encoded *N*-acetyltransferases have been identified which are not associated with mobile genetic elements. These include AAC(2')-Ia from *Providencia stuartii* (10), AAC(2')-Ib from *Mycobacterium fortuitum* (12), AAC(2')-Ic from *Mycobacterium tuberculosis*, AAC(2')-Id from *Mycobacterium smegmatis* (13), AAC(6')-Ic from *Serratia marcescens* (14), AAC(6')-If from *Acinetobacter* sp. 13 (15), AAC(6')-Ig from *Acinetobacter haemolyticus* (16), AAC(6')-Ii from *Enterococcus faecium* (17), and AAC(6')-Ik from *Acinetobacter* sp. 6 (18). In each case, the resistance gene has been found to be universally present in the species from which they were identified, regardless of the aminoglycoside resistance phenotype, suggesting that they may be involved in a housekeeping function separate from acetylating aminoglycosides.

## 3. AAC(2')-IA IN *PROVIDENCIA STUARTII*

### 3.1. Identification of the AAC(2')-Ia enzyme

Early studies by both Chevereau et. al., and Yamaguchi et. al. led to the identification of the AAC(2') enzyme in *Providencia* (9, 11), referred to hereafter as AAC(2')-Ia. Studies using the purified enzyme from *Providencia* strain GN1544 demonstrated that AAC(2')-Ia exhibited a pH optimum of 6.5 for lividomycin-A inactivation, and that the enzyme lost activity upon exposure to 65$^{o}$C for 5 minutes (11). Interestingly, the pH optimum was dependent on the substrate used in the assays. The failure to transfer the AAC(2') resistance phenotype by conjugation, together with the inability to eliminate the resistance phenotype from *Providencia* strains by curing with acridine orange or ethidium bromide, led to the first suggestion that the *aac(2')-Ia* gene was chromosomally encoded (11). In a subsequent study it was shown that all aminoglycoside sensitive *P. stuartii* strains possessed low-level 2'-*N*-acetylating activity by the phosphocellulose binding assay (19). This data suggested that these *P. stuartii* strains all contained the *aac(2')-Ia* gene and expressed it at low-levels. Furthermore, McHale *et al*. demonstrated no direct correlation between AAC(2') resistance profiles and the presence of plasmid DNA in clinical isolates of *P. stuartii* (20).

Studies by Swiatlo and Kocka suggested that *aac(2')-Ia* expression in *P. stuartii* was inducible by exposure to aminoglycosides (21). In this study, two sensitive *P. stuartii* isolates were serially passed in media containing increasing amounts of gentamicin. By the fifth transfer, cells had gentamicin MICs that were at least 20-fold higher than the starting isolate and AAC(2') enzyme activity showed a 10-fold increase. Passage of these highly resistant isolates for five transfers, in the absence of aminoglycoside, resulted in both the gentamicin MIC and enzyme activities returning to the normal levels seen in the starting isolates. This led the authors to conclude that the AAC(2')-Ia expression is inducible by the presence of aminoglycosides. However, it was never shown if exposure to subinhibitory amounts of aminoglycoside increased AAC(2')-Ia expression. This would have determined if a true induction event took place. Data from our lab does not support these results. We have demonstrated that AAC(2')-Ia expression is not inducible by aminoglycosides as previously suggested (21). The conversion of sensitive *P. stuartii* isolates to those with high-level *aac(2')-Ia* expression, primarily involves mutations in a number of regulatory genes which are discussed in detail below.

### 3.2. Isolation of the *aac(2')-Ia* gene

The ability of the AAC(2')-Ia enzyme to acetylate genatmicin was exploited to clone the *aac(2)-Ia* structural gene in the gentamicin sensitive *E. coli* strain DH5α (10). A library of partially digested *Sau*3AI fragments was prepared in pACYC184 from a clinical *P. stuartii* isolate (SCH75082831A), referred to hereafter as PR50 (10). Recombinants containing the *aac(2')-Ia* gene were identified on plates containing 5 μg/ml gentamicin. *E. coli* recombinants containing the *aac(2')-Ia* gene expressed a low level resistance to gentamicin, tobramycin, netilmicin and 6'-*N*-netilmicin, but not to 2'-*N*-netilmicin. This resistance profile was consistent with 2'-*N*-acetylating activity and phosphocellulose binding assays confirmed acetylation of 6-*N*-netilmicin, whereas 2'-*N*-netilmicin was not acetylated (10). DNA sequence analysis of the cloned insert and mutagenesis experiments allowed for identification of the *aac(2')-Ia* open reading frame which is predicted to encode a protein of 20.1 kDa. The deduced AAC(2')-Ia protein displays extensive similarity to a family of 2'-*N*-acetyltransferases identified in a number of *Mycobacterial* species and limited similarity to the AAC(6')-Ic protein of *Serratia marcescens* .

### 3.3. Distribution of *aac(2')-Ia* in *Providencia*

The prevelance of *aac(2')-Ia* or related genes in other *Providencia* species has been investigated using Southern hybridization at low stringency. As expected, the *aac(2')-Ia* gene was present in all isolates of *P. stuartii* tested (22). Sequences with homology to *aac(2')-Ia* were also observed in *Proteus penneri* and *Providencia rettgeri* (22).

### 3.4. Physiological functions for AAC(2')-Ia in *P. stuartii*

The universal presence of the *aac(2')-Ia* gene in *P. stuartii* suggested a physiological role in metabolism. The similarity of aminoglycosides to cellular substrates containing amino sugars, such as *N*-acetylglucosamine and *N*-acetylmuramic acid led to the proposal that AAC(2')-Ia may be involved in processes related to peptidoglycan or lipopolysaccharide (LPS) metabolism (10, 22, 23, 24). Reactions involving the acetylation of peptidoglycan have been demonstrated in *P. stuartii* and a relationship between AAC(2')-Ia and peptidoglycan acetylation has been established by the work of K. Payie and A. Clarke (23, 24). Analysis of *P. stuartii* mutants with increased *aac(2')-Ia* expression has revealed a corresponding increase in the levels of

**Table 1.** Regulatory genes of aac(2')-Ia

| GENE | COMMENTS |
|---|---|
| *aarA* | Negative regulator. Probable integral membrane protein. Involved in response to AR-factor |
| *aarB* | Negative regulator. Identity is unknown. |
| *aarC* | Negative regulator at high cell density. Highly conserved in bacteria. Essential gene. |
| *aarD* | Negative regulator. CydD homolog |
| *aarE* | Positive effector. UbiA homolog required for ubiquinone biosynthesis. |
| *aarF* | Positive effector. Novel locus required for ubiquinone biosynthesis. |
| *aarG* | Negative regulator. Sensor kinase. |
| *aarP* | Transcriptional activator. Related to the MarA/SoxS family. |

*O*-acetylation. Interestingly, biochemical analysis of the AAC(2')-Ia enzyme has confirmed that it is also capable of acetylating aminoglycosides using acetate obtained from either acetyl CoA, soluble peptidoglycan fragments or *N*-acetylglucosamine (24). An additional phenotype associated with *aac(2')-Ia* overexpression is altered cell morphology with cells appearing as shortened coccobaccili or as chains of cells (23).

To address the physiological function of AAC(2')-Ia, a *P. stuartii* mutant (PR100) has been constructed which contains a frameshift mutation in the *aac(2')-Ia* gene. This frameshift is predicted to result in a loss of function as it would result in a truncated polypeptide missing almost the entire COOH terminal portion of AAC(2')-Ia. The *aac(2')-Ia* frameshift results in a reduction in the intrinsic levels of aminoglycoside resistance from 8 μg/ml to 0.5 μg/ml and phosphocellulose binding assays have revealed a complete loss of gentamcin acetylation in extracts prepared from PR100 (24). Thus AAC(2')-Ia is the sole acetyltransferase in the cell for aminoglycoside acetylation. The loss of the AAC(2')-Ia enzyme resulted in a significant decrease in the levels of peptidoglycan *O*-acetylation (42%), relative to the 54% seen in the isogenic wild-type parent (23). The residual levels of *O*-acetylation are likely to result from a second *O*-acetyltransferase, which appears to be responsible for the majority of peptidoglycan *O*-acetylation. Furthermore, the loss of AAC(2')-Ia results in significant changes in cell morphology with the formation of distorted rod-shaped cells which failed to constrict during cell division. These cells also displayed altered staining properties with uranyl acetate (23). It is known that *O*-acetylation blocks the activity of autolytic enzymes (muramidases) involved in peptidoglycan turnover. Moreover, *O*-acetylation also appears to be required for the activity of other autolysins (25, 26). These phentoypes observed in the *aac(2')-Ia* frameshift mutant are consistent with altered levels of *O*-acetylation leading to significant changes in the autolytic system involved in cell wall turnover.

## 4. *AAC(2')-IA* REGULATION

### 4.1. Strategies for isolation of regulatory genes

Studies on *aac(2')-Ia* regulation have been conducted primarily at the transcriptional level. Primer extension analysis has been used to identify a promoter for *aac(2')-Ia* which contains a -10 consensus sequence (TATAAT) for the □70 form of RNA polymerase and the sequence CTTTTT at the -35 region (10). This -35 sequence does not conform to known consensus sequences and predicts that *aac(2')-Ia* transcription may require ancillary factors (see *aarP* below). The regulation of *aac(2')-Ia* has been studied using transcriptional *lacZ* fusions to the *aac(2')-Ia* promoter region. In a wild-type *P. stuartii* strain, such as PR50, the expression of an *aac(2')-lacZ* fusion is low, resulting in a pale blue colony phenotype on X-gal plates. Using this fusion, we have determined that transcription of *aac(2')-Ia* is not inducible by aminoglycosides (10). However, aminoglycoside resistant mutants arise a high frequency ($10^{-6}$-$10^{-7}$) when selected at 4X the MIC for gentamicin. In addition, the majority of these mutants display a dark blue phenotype on X-gal plates indicating increased transcription of the *aac(2')-Ia* gene. This demonstrates that the mutations leading to aminoglycoside resistance are *trans*-acting since they simultaneously activated the chromosomal copy of *aac(2')-Ia* and the *aac(2')-lacZ* fusion present on a low copy plasmid. Selection of spontaneous and mini-Tn*5Cm* induced mutations which activate both copies of the *aac(2')-Ia* promoter has been our strategy to identify negative regulators of *aac(2')-Ia.* This approach has led to the identification of a complex regulatory network that involves genes designated *aar* (*a*minoglycoside *a*cetyltransferase *r*egulator) which are summarized in table 1.

### 4.2. Regulatory genes

#### 4.2.1. *aarA*

The *aarA* gene was identified as a mini-Tn*5Cm* insertion that increased expression of an *aac(2')-lacZ* fusion 3-4 fold in liquid growth conditions (27). Loss of function mutations in *aarA* also resulted in a gentamicin resistance level that was increased 8-fold above wild-type. Null mutations in *aarA* are highly pleiotrophic and additional phenotypes include; loss of production of a diffusible yellow pigment and altered morphology with abberant cell separation after division. This results in a very distinctive cell chaining phenotype that is most prominent in cells at mid-log phase. Furthermore, the effects of the *aarA* mutation are much stronger when cells are grown on agar plates, where the expression of an *aac(2')-lacZ* fusion is increased 8-10 fold. The AarA polypeptide is 31.1 kDa in size and very hydrophobic with at least two possible transmembrane domains. Homology searches of the databases with AarA resulted in no significant matches to other proteins. Genetic evidence implicates AarA in a pathway required for response to an extracellular pheromone signal, AR-factor, that acts to reduce *aac(2')-Ia* expression (see below). Furthermore, an additional *P. stuartii* gene (*cma37*) which is regulated by quorum sensing is strongly dependent on a functional *aarA* gene for expression. Studies with this fusion again indicate that AarA is required for cells to sense the extracellular signal which activates the *cma37* fusion. The stronger phenotypes associated with *aarA* deletions seen on solid media, relative to liquid growth are consistent with a role in response to an extracellular signal due to the increased

accumulation of AR-factor in the surrounding agar vs. diffusion in liquid. Therefore, the pleiotrophic phenotypes, such as loss of pigmentation and defective cell division seen in cells with the *aarA* gene deleted, may result from a defect in sensing the quorum signal AR-factor.

#### 4.2.2. *aarB*

The *aarB3* mutation originally designated *aar3* (10) results in a 10 to 12-fold increase in *aac(2')-Ia* transcription. In the *aarB3* background, the levels of aminoglycoside resistance are increased 128-fold above wild-type, suggesting that this mutation further increases aminoglycoside resistance in a manner independent of *aac(2')-Ia* expression. The *aarB3* mutation results in a small colony phenotype and cells which are dramatically shortened. The identity of the *aarB* gene remains to be determined. However, the use of a plasmid library to complement the *aarB* mutation has resulted in the identification of a high copy suppressor which contains the *P. stuartii hemB* homolog. This raises the possibility that the *aarB* mutation is in a component of the electron transport chain. This would be consistent with the levels of aminoglycoside resistance increased in a manner that is not proportional to *aac(2')-Ia* expression.

#### 4.2.3. *aarC*

The *aarC* gene was identified by a mutation (*aarC1*) which simultaneously activated the chromosomal *aac(2')-Ia* gene and a plasmid encoded *aac(2')-lacZ* transcriptional fusion (28). The deduced AarC protein is 40 kDa and is highly conserved to a family of proteins that is widespread in bacteria. The *E. coli* homolog is GcpE and studies in our lab have demonstrated that *gcpE* is essential for *E. coli* viability and have also shown that *aarC* is an essential gene in *P. stuartii* (28). Furthermore, complementation experiments have shown that *aarC* and *gcpE* are functionally equivalent. The missense allele, *aarC1*, results in a number of pleiotrophic phenotypes including; slow growth, altered cell morphology, and increased *aac(2')-Ia* expression at high cell density. The biochemical function of AarC remains to be determined.

#### 4.2.4. *AarD*

The *aarD* gene is a *trans*-acting negative regulator of *aac(2')-Ia* which was identified as a mini-Tn*5*Cm insertion resulting in the activation of an *aac(2')-IacZ* transcriptional fusion (29). The mini-Tn*5*Cm insertion (designated *aarD1*) results in a 5-fold activation of the *aac(2')-lacZ* fusion, a 3-fold increase in the levels of *aac(2')-Ia* mRNA accumulation, and a 32-fold increase in aminoglycoside resistance over that of wild-type *P. stuartii*.

The *aarD* locus has been cloned by complementation and encodes two polypeptides, AarD and OrfX, which exhibit extensive homology to the *Escherichia coli* CydD and CydC proteins respectively (29, 31, 32). The CydD and CydC proteins comprise a heterodimeric ABC transporter complex which is involved in formation of a functional cytochrome *d* oxidase complex (32, 33, 34). Mutations in *cydD* and *cydC* result in the loss of the cytochrome *d* oxidase both spectroscopically and functionally (32, 34, 35). *P. stuartii aarD* mutants exhibit phenotypic characteristics consistent with a defect in the cytochrome *d* oxidase including hyper-susceptibility to the respiratory inhibitors $Zn^{2+}$ and toluidine blue (29, 30, 34). Introduction of the *E. coli cydDC* genes into the *aarD1* background leads to complementation of all mutant phenotypes suggesting that the two loci are functional homologues (29).

The increased *aac(2')-Ia* expression observed in the *aarD1* background contributes minimally to the overall increase in gentamicin resistance since introduction of the *aarD1* mutation into an *aac(2')-Ia* mutant strain also results in a 32-fold increase in gentamicin resistance. Since previous studies have demonstrated that uptake of aminoglycosides is dependent on the presence of a functional electron transport system (7, 36, 37), and since electron transport is defective in the *aarD1* background (29), it is probable that a decrease in aminoglycoside uptake accounts for the high level of resistance observed in *aarD* mutants. It seems unlikely that *aarD* plays a direct role in the regulation of *aac(2')-Ia* since ABC transporters are not known to function as transcriptional regulators (38). It has recently been proposed that *aac(2')-Ia* expression is influenced by an uncharacterized regulatory pathway that responds to changes in the redox state of the membrane (see below) (39). Mutations in *aarD* are predicted to alter the redox state of the membrane and thus indirectly affect *aac(2')-Ia* expression.

An interesting phenotype of the *aarD1* mutation is sensitivity to a self-produced extracellular factor (29). This phenotype is not unique to *P. stuartii,* as *E. coli* mutants (*cydD*, *cydAB*) lacking cytochrome *d* oxidase are also sensitive to a self-produced extracellular factor. The identity of this factor has not been established.

#### 4.2.5. *AarE*

The *aarE* gene was identified by selecting for *P. stuartii* mutants resistant to gentamicin (40). The *aarE1* allele resulted in a level of gentamicin resistance that is increased to 256 μg/ml, relative to the 4 μg/ml observed in the isogenic parent. Surprisingly, the accumulation of *aac(2')-Ia* mRNA was significantly reduced in the *aarE1* background. Analysis of the *aarE* gene has shown it to be the *ubiA* homolog, which encodes an octaprenyltransferase required for the second step of ubiquinone biosynthesis. The loss of ubiquinone function is predicted to decrease the uptake of aminoglycosides, which is likely to explain the high-level aminoglycoside resistance. The decreased *aac(2')-Ia* mRNA accumation may reflect a requirement for uniquinone, either directly or indirectly in a regulatory process involved in *aac(2')-Ia* mRNA stability.

#### 4.2.6. *AarF*

The *aarF* locus of *P. stuartii* is a positive regulator of *aac(2')-Ia* expression with the level of *aac(2')-Ia* mRNA being dramatically decreased in an *aarF* null mutant (39). Despite the lack of *aac(2')-Ia* expression, *aarF* null mutants exhibit a 256-fold increase in gentamicin resistance over the wild-type strain. *P. stuartii aarF* null mutants also exhibit severe growth defects under aerobic

growth conditions and have been found to lack detectable quantities of the respiratory cofactor ubiquinone.

The wild-type *aarF* gene has been cloned and encodes a 62.5 kDa polypeptide which exhibits extensive amino acid identity to two putative adjacent open reading frames from *Escherichia coli* designated *yigQ* and *yigR* (39, 41). Disruption of the *yigR* gene has confirmed that this locus is required for ubiquinone production in *E. coli* (39). Heterologous complementation studies demonstrate that *aarF* and the *E. coli yigQR* loci are functionally equivalent. Three ubiquinone biosynthesis genes, *ubiB*, *ubiD*, and *ubiE*, have been mapped near *yigQR* at minute 86 on the *E. coli* chromosome (41, 42, 43, 44). Complementation experiments with known *ubi* mutants have demonstrated that the *yigQR* locus is genetically distinct from *ubiB*, *ubiD* and *ubiE* suggesting that *yigQR* (*aarF*) represents a novel locus required for ubiquinone production.

Previous studies have shown that ubiquinone deficient *E. coli* mutants accumulate gentamicin poorly and as a result exhibit increased gentamicin resistance (36, 37). Therefore, the high-level gentamicin resistance observed in the *aarF* and *yigR* mutants is likely associated with decreased accumulation of the drug resulting from the absence of aerobic electron transport. It seems unlikely that *aarF* is directly involved in the regulation of *aac(2')-Ia*. It has been proposed that a reduced form of ubiquinone acts as an effector molecule in an uncharacterized regulatory pathway that activates the expression of *aac(2')-Ia* (39). In ubiquinone deficient *aarF* mutant strains, this regulatory cascade would be disrupted resulting in decreased *aac(2')-Ia* expression (see below).

#### 4.2.7. *AarG*

The *aarG* gene was identified in a genetic screen for negative regulators *of aac(2')-Ia.* A recessive mutation (*aarG1*) results in an 18-fold increase in the expression of β-galactosidase from an *aac(2')-lacZ* fusion (45). Direct measurments of RNA from the chromosomal copy of *aac(2')-Ia* have confirmed this increase at the level of RNA accumulation. Taken together, these results demonstrate that loss of *aarG* results in increased *aac(2')-Ia* transcription. The *aarG1* allele also results in enhanced expression of *aarP,* encoding a transcriptional activator of *aac(2')-Ia* (45). Genetic experiments have shown that in an *aarG1, aarP* double mutant, the expression of *aac(2')-Ia* is significantly reduced over that seen in the *aarG1* background. However, the levels of *aac(2')-Ia* in this double mutant are still significantly higher than in a strain with only an *aarP* mutation. Therefore, the *aarG1* mutation increases *aac(2')-Ia* expression by both *aarP* dependent and independent mechanisms.

The *aarG1* allele also confers a multiple antibiotic resistance phenotype (Mar) to *P. stuartii* resulting in increased resistance to tetracycline, chloramphenicol and fluoroquinolones. This Mar phenotype in the *aarG1* background is partially due to overexpression of *aarP,* which is know to confer a Mar phenotype in both *P. stuartii* and *E. coli* (see below). However, a mechanism independent of *aarP* overexpression also accounts for increased levels of intrinsic resistance in the *aarG1* background. This mechanism could involved increased expression of a second activator with a target specificity similar to that of AarP.

The *aarG* gene encodes a protein with similarity to sensor kinases of the two-component family with the strongest identity to PhoQ (57%). Immediately upstream of AarG is an open reading frame designated *aarR* which encoded a protein with 75% amino acid identity to PhoP, a response regulator (46). The regulatory phenotypes associated with the *aarG1* mutation may result from a failure to phosphorylate the putative response regulator AarR, which functions as a repressor of *aarP,* and possibly *aac(2')-Ia.*

#### 4.2.8. *aarP*

A central component in the activation of *aac(2')-Ia* expression is a small transcriptional regulator designated AarP. The *aarP* gene was originally isolated from a multicopy library of *P. stuartii* chromosomal DNA based on the ablility to activate *aac(2')-Ia* expression in *trans* (47). The presence of *aarP* in multiple copies led to an 8-fold increase in *aac(2')-Ia* mRNA accumulation. Studies utilizing an *aac(2')-lacZ* transcriptional fusion demonstrate that this increase results from an activation of *aac(2')-Ia* transcription. Chromosomal disruption of the *aarP* locus results in a fivefold reduction in *aac(2')-Ia* mRNA levels and eliminates the induction of *aac(2')-Ia* expression normally observed during logarithmic growth (48). These studies indicate that *aarP* is required for the normal expression pattern of *aac(2')-Ia* observed in wild-type *P. stuartii*. Furthermore, expression of *aarP* has been shown to be increased in the *aarB*, *aarC* and *aarG* mutants, demonstrating that *aarP* contributes to the overexpression of *aac(2')-Ia* in these mutant backgrounds (10, 28, 45).

The *aarP* gene encodes a 16 kDa protein which contains a putative DNA binding helix-turn-helix motif and belongs to the AraC/XylS family of transcriptional activators (47, 49). Several lines of evidence indicate that the AarP protein directly interacts with the *aac(2')-Ia* promoter region to activate transcription (48). *In vivo* transcriptional activation studies utilizing a series of 5' deletion derivatives of the *aac(2')-Ia* promoter demonstrate that sequences extending to -67 relative to the transcriptional start are required for activation by AarP. A 4-base insertion at -47 in the context of the full length promoter abolishes activation of the *aac(2')-Ia* promoter by AarP. Purified AarP protein binds to a wild-type *aac(2')-Ia* promoter fragment in electrophoretic mobility shift assays, but does not to bind a derivative containing the 4 base insertion at -47. Finally, DNaseI footprint analysis indicates that AarP protects a region of the *aac(2')-Ia* promoter extending from -29 to -46. This protected region partially overlaps the -35 region of the *aac(2')-Ia* promoter (10) suggesting that AarP functions as a Class II activator and mediates activation of *aac(2')-Ia* by interaction with the sigma subunit of RNA polymerase (50).

The AarP protein exhibits extensive homology with the *E. coli* MarA and SoxS proteins which are activators involved in the multiple-antibiotic-resistance phenotype and the oxidative stress response (47, 51- 55). Previous research has demonstrated that there is overlap in the *in vivo* targets for MarA and SoxS (52, 56- 60). This overlap is thought to be the result of the high degree of similarity in the helix-turn-helix domain of these proteins (54). AarP, which exhibits high homology to MarA and SoxS in the helix-turn-helix domain, was found to activate targets of both MarA and SoxS *in vivo* (47). The putative AarP binding site displays some similarities with the proposed binding sites for MarA and SoxS (48).

Expression of *aarP* appears to be governed by a mechanism which differs from those controlling MarA and SoxS expression. Unlike the MarA and SoxS proteins, which are located in operons containing a gene which regulates their expression, the *aarP* message appears to be monocystronic. Expression of *aarP* was found to be slightly elevated in the presence of tetracycline but was not elevated in the presence of a potent inducer of MarA, salicylate (47). Recent studies of *aarP* expression have revealed that the AarP message accumulates as cell density increases (48). Furthermore, the addition of spent culture media leads to increased *aarP* message accumulation suggesting that *aarP* is subject to regulation by a density dependent cell signaling mechanism.

## 5. ROLE OF QUORUM SENSING IN *AAC(2')-IA* REGULATION

The regulation of *aac(2')-Ia* expression is also subject to control by cell to cell signaling or quorum sensing (61). The accumulation of *aac(2')-Ia* mRNA exhibits two levels of growth phase dependent expression. First, as cells approach mid-log phase, a significant increase is observed relative to cells at early-log phase. This increase at mid-log phase is the result of increased *aarP* expression. Second, as cells approach stationary phase, the levels of *aac(2')-Ia* mRNA are decreased to levels that are at least 20-fold lower than those at mid-log phase. This decrease at high density is mediated by the accumulation of an extracellular factor (AR-factor) (61). The growth of *P. stuartii* cells in spent (conditioned) media from stationary phase cultures resulted in the premature repression of *aac(2')-Ia* in cells at mid-log phase. The analysis of AR-factor has shown it to be between 500 and 1000Da in size, heat stable and sensitive to proteases. These characteristics are consistent with a small peptide, although the exact structure has not been determined.

## 6. PERSPECTIVE

The chromosomal *aac(2')-Ia* gene was originally identified as an aminoglycoside resistance gene. The universal presence of this gene in *P. stuartii* led to the identification of a role for AAC(2')-Ia in the O-acetylation of peptidoglycan. The possibility of additional roles for the AAC(2')-Ia enzyme in cellular metabolism, such as a role in LPS acetylation, have been suggested (10, 23, 24). However, the additional roles of AAC(2')-Ia in *P. stuartii,* if any, remain to be identified.

The complex regulatory networks controlling *aac(2')-Ia* expression presumably reflect the importance of maintaining correct levels of AAC(2')-Ia expression and subsequent O-acetylation of peptidoglycan. One pathway of *aac(2')-Ia* regulation involves a quorum sensing signal AR-factor. The response to AR-factor involves the putative integral membrane protein AarA, which may serve in a transport complex for AR-factor. In addition, the *aarC* gene may be involved in the AR-factor regulatory pathway, as *aarC* mutations only display a phenotype in cells at high density. A second pathway for *aac(2')-Ia* appears to involve an unidentified component of the electron transport chain, possibly a ubiquinone derivative. A model has been presented in which loss of the *aarD* gene may be predicted to lead to an accumulation of the reduced form of ubiquinone (ubiquinol) by preventing the formation of the cytochrome *bd* oxidase complex (39). Consistent with this model, *aarD* mutations lead to increased *aac(2')-Ia* expression and two mutations which block ubiquinone production (*aarE, aarF*) result in reduced *aac(2')-Ia* expresssion.

A third pathway of regulation involves the transcriptional activator *aarP*, which is a central activator of *aac(2')-Ia.* The *aarP* gene is subject to negative regulation by the *aarB, aarC* and *aarG* genes. The AarG sensor kinase acts to negatively regulate *aarP* and *aac(2')-Ia* by possibly phosphorylating the response regulator AarR. The signal which alters the kinase/phosphatase activity of AarG is unknown. However, the identification of this signal will be crucial for our understanding of regulation of *aac(2')-Ia* and of the multiple antibiotic resistance phenotype accompanied by AarP overexpression. In addition to the mutations described, we have identified additional regulatory mutations that remain to be characterized. The analysis of these genes may provide important clues regarding the physiological processes which are coupled to *aac(2')-Ia* regulation.

## 7. ACKNOWLEDGEMENTS

Work in my lab on the *aac(2')-Ia* gene has been supported by the National Institutes of Health, the National Science Foundation and the Department of Veterans Affairs.

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**Keywords:** *Providencia,* Aminoglycoside Resistance, Gene Regulation

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Received 8/26/98 Accepted 11/8/98