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Identification and Characterization of *aarF*, a Locus Required for Production of Ubiquinone in *Providencia stuartii* and *Escherichia coli* and for Expression of 2′-*N*-Acetyltransferase in *P. stuartii*

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***Providencia stuartii* contains a chromosomal 2′-*N*-acetyltransferase [AAC(2′)-Ia] involved in the O acetylation of peptidoglycan. The AAC(2′)-Ia enzyme is also capable of acetylating and inactivating certain aminoglycosides and confers high-level resistance to these antibiotics when overexpressed. We report the identification of a locus in *P. stuartii*, designated *aarF*, that is required for the expression of AAC(2′)-Ia. Northern (RNA) analysis demonstrated that *aac(2′)-Ia* mRNA levels were dramatically decreased in a *P. stuartii* strain carrying an *aarF*::Cm disruption. The *aarF*::Cm disruption also resulted in a deficiency in the respiratory cofactor ubiquinone. The *aarF* locus encoded a protein that had a predicted molecular mass of 62,559 Da and that exhibited extensive amino acid similarity to the products of two adjacent open reading frames of unknown function (YigQ and YigR), located at 86 min on the *Escherichia coli* chromosome. An *E. coli yigR*::Kan mutant was also deficient in ubiquinone content. Complementation studies demonstrated that the *aarF* and the *E. coli yigQR* loci were functionally equivalent. The *aarF* or *yigQR* genes were unable to complement *ubiD* and *ubiE* mutations that are also present at 86 min on the *E. coli* chromosome. This result indicates that *aarF* (*yigQR*) represents a novel locus for ubiquinone production and reveals a previously unreported connection between ubiquinone biosynthesis and the regulation of gene expression.**

The gram-negative bacterium *Providencia stuartii* is a member of the *Proteeae*, which includes the genera *Proteus*, *Morganella*, and *Providencia*. Members of the *Proteeae* possess peptidoglycan that is O acetylated at the C-6 hydroxyl position of *N*-acetylmuramyl residues (8). This modification confers resistance to muramidases such as lysozyme and has been speculated to modulate the activity of endogenous peptidoglycan-specific hydrolases, termed autolysins (8, 9, 16). *P. stuartii* contains a chromosomal 2′-*N*-acetyltransferase, encoded by the *aac(2′)-Ia* locus, that has been implicated in this process (7, 29, 34, 38). This enzyme is also capable of acetylating and inactivating certain aminoglycoside antibiotics and was originally identified in clinical strains of *P. stuartii* overexpressing the enzyme (7, 38).

The *aac(2′)-Ia* gene is expressed at low levels in wild-type *P. stuartii* (34). The expression of *aac(2′)-Ia* is controlled in part by a small transcriptional activator, AarP, that is related to members of the XylS-AraC family of positive activators (18, 24). Recessive mutations that result in increased *aac(2′)-Ia* mRNA accumulation have also been identified in five loci (*aarA*, *aarB*, *aarC*, *aarD*, and *aarG*) (25, 32–35). The expression of *aarP* has been shown to be increased in the *aarB*, *aarC*, and *aarG* mutant backgrounds. These results suggest that *aarP* may play a central role in the activation of *aac(2′)-Ia* expression (32, 35).

In this study, we report the identification of the *aarF* gene of *P. stuartii* and demonstrate that *aarF* function is required for the expression of *aac(2′)-Ia*. We also present evidence suggesting that *aarF* is functionally equivalent to *Escherichia coli yigQR* and that both *aarF* and *yigQR* represent novel loci required for the production of ubiquinone.

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MATERIALS AND METHODS

Bacterial strains and plasmids. All bacteria, bacteriophages, and plasmids used in this study are described in Table 1.

Media and bacterial growth. Bacteria were routinely grown in Luria-Bertani (LB) broth at 37°C. To test for the aerobic utilization of nonfermentable carbon sources, M9 minimal agar plates (26) containing either 0.2% glucose or 0.5% succinate were used. For the growth of *E. coli* AN66 *ubiD* and AN70 *ubiE*, M9 plates were supplemented with L-leucine, L-threonine, and L-methionine each at a final concentration of 0.2 mM and with thiamine at a final concentration of 0.02 μM.

Gentamicin resistance determinations. MICs for gentamicin were determined by an agar dilution method with twofold increasing concentrations of gentamicin. The MIC was defined as the lowest concentration of gentamicin that prevented the formation of single colonies.

Plasmid constructions. A genomic library of *P. stuartii* DNA was constructed by ligation of partial *Sau*3AI fragments into *Bam*HI-digested and dephosphorylated pACYC184 and was described previously (6, 24). Plasmid pAFM12 is a pACYC184 recombinant with a 3.6-kb *Sau*3AI fragment of *P. stuartii* DNA containing the *aarF* gene. Plasmid pSK.aarF was constructed by inserting a 1.9-kb *Sph*I fragment from pAFM12 into pBluescript SK(−) linearized with *Sma*I. A genomic library of *E. coli* partial *Sau*3AI fragments constructed in pET21a was kindly provided by P. deBoer, Case Western Reserve University. Plasmid pEF1 is a pET21a recombinant and contains a 3.5-kb insert. Plasmid pSK-2.6 contains a 2.6-kb *Sal*I fragment from pEF1 ligated into the *Sal*I site of pBluescript SK(−). Plasmid pSK.yigQR was constructed by digesting pSK-2.6 with *Eco*RV to release a 457-bp fragment and religating the linearized plasmid. Plasmid pSK.yigQRΔ*Dra*I was constructed by linearizing pSK.yigQR with *Hin*cII followed by partial digestion with *Dra*I to release a 467-bp fragment and religation. Plasmid pSK.yigR was constructed by digesting pSK-2.6 with *Cla*I and *Nar*I to release a 701-bp fragment and religating the linearized plasmid.

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*	24
SM10 λ*pir*	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu Km^r λ*pir*	28
BL21(DE3)	(B strain; r_B^- m_B^-) F$^-$ *ompT hsdS*$_B$; λ prophage carries T7 RNA polymerase gene	Novagen
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150*(Str^r) *relA1 flbB5301 deoC1 ptsF25 rbsR*	36
DM111	MC4100 *lamB*	This study
DM113	MC4100 *lamB* Rif^r	This study
DM115	MC4100 *lamB* Rif^r *yigR*::Kan	This study
RM1734	(MG1655) λ$^-$ F$^-$ *rph-1*	R. Maurer (20)
DM123	RM1734 *yigR*::Kan [P1 (DM115) × RM1734]	This study
AN66	*thr-1 leuB6 lacZ4 glnV44 rpsL8*(Str^r) *ubiD410*	12
AN70	*metB ubiE401*	39
P. stuartii		
PR50	Wild type	34
PR50.AFM12	*aarF1*	This study
PR54	*aarF*::Cm	This study
Plasmids		
pUC4.KIXX	Cloning vector, Ap, Kan	Pharmacia
pACYC184	Medium copy vector, Cm, Tc	6
pACYC184.lacI^q	pACYC184::1.3-kb *Xba*I-*Cla*I fragment containing *lacI*^{q}	This study
pAFM12	pACYC184::3.6-kb *Sau*3AI containing *aarF*	This study
pMJR1560	Cloning vector, *lacI*^{q}	Amersham
pBluescript SK(−)	High-copy-number vector, Ap	Stratagene
pSK.aarF	pBluescript SK(−)::1.9-kb *Sph*I fragment from pAFM12	This study
pBluescript KS(−)	High-copy-number vector, Ap	Stratagene
pKS.aarF	pBluescript KS(−)::1.9-kb *Sph*I fragment from pAFM12	This study
pKS.*Nhe*I	Derivative of pSK.aarF with a frameshift mutation inserted at a unique *Nhe*I site internal to the *aarF* coding region	This study
pSK.aarF::Cm	pSK.aarF containing a 3.6-kb chloramphenicol resistance cassette inserted within a unique *Nru*I site in *aarF*	This study
pKNG101	R6K-derived suicide plasmid containing Str and *sacB*	22
pKNG101.aarF::Cm	pKNG101::6-kb *Bam*HI-*Apa*I fragment from pSK.aarF::Cm	This study
pET21a	T7 expression plasmid, Ap	Novagen
pEF1	pET21a::3.5-kb *Sau*3AI fragment containing *E. coli yigOPQR* genes	This study
pSK-2.6	pBluescript SK(−)::2.6-kb *Sal*I fragment from pEF1 (*yigPQR*)	This study
pSK.yigQR	Derivative of pSK-2.6 with *yigP* deleted	This study
pSK.yigR	Derivative of pSK-2.6 with *yigP* and initiating codon of *yigQ* deleted	This study
pSK.yigQRΔ*Dra*I	Derivative of pSK.yigQR with C-terminal *yigR* deletion	This study
pSK.yigR::Kan	pSK-2.6 containing a 1.3-kb kanamycin resistance cassette inserted within a unique *Bsm*I site in *yigR*	This study
pKNG101.yigR::Kan	pKNG101::3.8-kb *Sal*I fragment from pSK.yigR::Kan	This study

Identification of AarF. To identify the *aarF* gene product, a *Xho*I-*Xba*I fragment containing the *aarF* gene was excised from pSK.aarF and ligated into pBluescript KS(−) to create pKS.aarF. In pKS.aarF, the *aarF* gene is downstream from and in the same orientation as the T7 promoter. To create a negative control plasmid, pKS.aarF was linearized with *Nhe*I, which cuts internal to the *aarF* coding region, end filled with the Klenow fragment and deoxynucleoside triphosphates (dNTPs), and religated. The resulting plasmid, pKS.*Nhe*I, carries a frameshift mutation that truncates the predicted AarF protein after amino acid 99. To ensure that the *aarF* gene would not be expressed in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG), the *lacI*^{q} gene was introduced into plasmid pACYC184 (6) as follows. A 1.3-kb fragment containing the *lacI*^{q} gene was released from plasmid pMJR1560 (Amersham) by digestion with *Eco*RI and *Hin*dIII and was cloned into pBluescript SK(−) that had been digested with the same enzymes to create pSK.lacI^q. The *lacI*^{q} gene was then excised from pSK.lacI^q as a 1.3-kb *Xba*I-*Cla*I fragment and was cloned into pACYC184 that had been digested with the same enzymes to create pACYC184.lacI^q. Plasmid pACYC184.lacI^q and each of the *aarF* derivative plasmids were cointroduced into *E. coli* BL21(DE3) (Novagen). Cultures were shaken in LB broth at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 and induced with 1 mM IPTG. After 30 min, rifampin was added to a final concentration of 100 μg/ml, and cultures were shaken for an additional 2.5 h. Cells were harvested, and 15-μl aliquots were dissolved in sodium dodecyl sulfate (SDS) loading dye, boiled, and run on SDS–10% polyacrylamide gels. Total cellular protein was visualized after Coomassie blue staining.

Construction of chromosomal *aarF* and *yigR* disruptions. To construct an *aarF* null allele in *P. stuartii*, plasmid pSK.aarF was linearized at a unique *Nru*I site present midway in the *aarF* coding region at position 956. A chloramphenicol resistance cassette from pUT::mini-Tn*5*Cm (15), present as a 3.6-kb *Hin*dIII fragment, was end filled with the Klenow fragment of DNA polymerase I and dNTPs and ligated into *Nru*I-linearized pSK.aarF to produce pSK.aarF::Cm. To recombine the *aarF*::Cm disruption into the *P. stuartii* chromosome, a 6-kb *Bam*HI-*Apa*I fragment was excised from pSK.aarF::Cm and ligated into suicide vector pKNG101 (22) that had been digested with the same enzymes. The resulting plasmid, designated pKNG101.aarF::Cm, was integrated into the chromosome of strain PR50 by conjugal mating as described previously (24). The merodiploid was resolved by selection on 5% sucrose, and strains containing the disrupted *aarF* locus were identified on the basis of chloramphenicol resistance. Southern analysis confirmed that the chromosomal *aarF* locus had been disrupted by the chloramphenicol resistance cassette.

To construct a *yigR* null allele in *E. coli*, plasmid pSK-2.6 was linearized at a

unique *Bsm*I site internal to the *yigR* open reading frame and treated with T4 DNA polymerase and dNTPs to produce blunt ends. A 1.3-kb *Sma*I fragment containing a kanamycin resistance cassette was excised from pUC4::KIXX (Pharmacia) and ligated into linearized pSK-2.6 to produce pSK.yigR::Kan. The 3.8-kb *yigR*::Kan disruption was then excised from pSK.yigR::Kan with *Sal*I and ligated into the unique *Sal*I site of suicide vector pKNG101. The resulting plasmid, designated pKNG101.yigR::Kan, was introduced into the chromosome of strain DM113 by conjugal mating essentially as described previously (24), with the exception that rifampin was used at 100 μg/ml to counterselect against the donor strain. The merodiploid was resolved by selection on 5% sucrose, and strains containing the disrupted *yigR* locus were identified on the basis of kanamycin resistance. Southern analysis confirmed that the chromosomal *yigR* locus had been disrupted by the kanamycin resistance cassette. The chromosomal *yigR*::Kan disruption was then introduced into wild-type *E. coli* RM1734 via a P1 lysate derived from DM115. Transductants were obtained on LB agar plates containing 50 μg of kanamycin per ml, and the *yigR*::Kan disruption was confirmed by Southern analysis. A representative strain was designated DM123.

β-Galactosidase assays. Plasmid pR401 containing an *aac(2′)-lacZ* transcriptional fusion was described previously (33). β-Galactosidase assays were performed in triplicate with cell samples harvested at the early log phase, and activity was expressed in Miller units (26). Reported values represent the average for triplicate samples.

RNA analysis. To examine *aac(2′)-Ia* mRNA levels in *P. stuartii* strains, cultures were grown in LB broth at 37°C to an A_{600} of 0.2, and RNA was prepared with TRIazol reagent (Gibco/BRL). RNA was loaded in duplicate, fractionated on a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a nylon membrane by capillary transfer. Filters were probed with a digoxigenin-labeled 602-bp *Taq*I-*Ssp*I fragment containing the *aac(2′)-Ia* coding sequence. As an internal control for loading, probes were "spiked" with a labeled fragment internal to the *E. coli* 23S rRNA coding sequence. Filters were developed with Lumi-Phos 530 (Boehringer Mannheim Biochemicals) and exposed to autoradiography film.

Ubiquinone analysis. Cells were first grown in LB medium supplemented with 0.5% glucose in 2-liter flasks. The cultures were shaken overnight as starter cultures of 50 ml in 250-ml flasks. Cells were then inoculated into 500 ml of the same medium to an OD_{600} of 0.05 and shaken at 37°C. Cells were harvested at an OD_{600} of 2.0. Typically, 3 liters of culture was used for analysis. Cells were harvested, and pellets were washed twice in 50 mM potassium phosphate buffer and stored at −20°C. Quinone extraction was performed as described by Collins (10). Thawed cells, 5 g (wet weight), in 10 ml of phosphate buffer were broken by sonication at 1-min intervals, with 1 min of cooling in between the intervals, for 5 min. Lysis was confirmed by microscopic examination. Lysed cells were resuspended in 100 ml of acetone and left to digest for 12 h at 4°C with stirring. Cell debris was removed by filtration through Whatman no. 1 filter paper. The filtrate was then evaporated to 1 ml in a rotary evaporator at 40°C. The sample was freeze-dried, and the residue was dissolved in 2 ml of acetone. Samples (100 μl) were applied to a Silica Gel F_{254} plastic-backed thin-layer chromatography plate (Merck item 5735), which was developed in hexane-diethyl ether (85:15, wt/vol). Coenzyme Q8 was used as a standard. The coenzyme Q8 spots were visualized by UV illumination. The spots were cut out, and quinones were eluted with 100% ethanol. The silica gel powder was removed by centrifugation, and the spectra of the clear supernatants were recorded with a Variant DMS-90 spectrophotometer.

Nucleotide sequence accession number. The nucleotide sequence of *aarF* has been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Library under accession no. AF002165.

RESULTS

Identification of the *aarF* locus. PR50.AFM12 is a spontaneous gentamicin-resistant derivative of wild-type *P. stuartii* PR50. Gentamicin resistance in PR50.AFM12 was increased 256-fold (1,024 μg/ml) over that observed for wild-type PR50 (4 μg/ml). To determine whether *aac(2′)-Ia* expression was increased in PR50.AFM12, plasmid pR401, containing an *aac(2′)-lacZ* transcriptional fusion, was introduced. PR50.AFM12/pR401 formed dark blue colonies when grown on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. In contrast, isogenic strain PR50/pR401 formed white colonies when grown on the same plates. The mutant allele in PR50.AFM12 was therefore designated *aarF1*. The regulatory effects of *aarF1* on *aac(2′)-Ia* expression were examined in further detail (see below).

PR50.AFM12 demonstrated a reduced growth rate compared to wild-type PR50 and formed significantly smaller colonies on LB agar plates. Because PR50.AFM12 was selected spontaneously, it seemed likely that a single mutation was responsible for both the increased gentamicin resistance and the reduced growth rate observed in this strain. Therefore, to complement the *aarF1* mutation, a library of PR50 genomic DNA was constructed in pACYC184 and introduced into PR50.AFM12 (6, 24). Transformants that exhibited a wild-type growth rate were easily visible in the background of microcolonies. Plasmid DNA was purified from several large colonies and retransformed into PR50.AFM12, resulting in 100% of the transformants exhibiting a wild-type growth rate. Transformants forming large colonies also exhibited gentamicin resistance that was indistinguishable from that of wild-type *P. stuartii* (data not shown). Analysis of a complementing plasmid, pAFM12, indicated the presence of a 3.6-kb insert.

DNA sequence analysis. A 1.9-kb *Sph*I fragment from pAFM12 was subcloned into pBluescript SK(−), resulting in plasmid pSK.aarF. The introduction of pSK.aarF into pAFM12 also resulted in transformants exhibiting a wild-type growth rate (Fig. 1B). The nucleotide sequence of the 1,877-bp fragment in pSK.aarF was determined on both strands. A single open reading frame of 1,632 bp, predicted to encode a 544-amino-acid polypeptide (Fig. 1A), was identified. To determine whether this open reading frame encoded *aarF*, a chloramphenicol resistance cassette from pUT::mini-Tn*5*Cm (15) was inserted into a unique *Nru*I site within this open reading frame. The resulting plasmid, pSK.aarF::Cm, was unable to restore a wild-type growth rate when introduced into PR50.AFM12 (Fig. 1B). Based on this result and the data presented below, this open reading frame has been designated *aarF*.

Identification of the *aarF* gene product. To determine whether the *aarF* locus encoded a polypeptide of the predicted size, the *aarF* gene was excised from pSK.aarF and subcloned into pBluescript KS(−) to enable transcription to be driven from the T7 promoter. The resulting plasmid, pKS.aarF, was transformed into the expression strain *E. coli* BL21(DE3)/pACYC184.lacI^q. A 67-kDa polypeptide observed upon induction with IPTG was not observed in an uninduced control culture (Fig. 2). No induction was observed in a control strain that was transformed with pBluescript KS(−). The size of the observed polypeptide correlated with the predicted size of 62.5 kDa. To confirm that the observed polypeptide was encoded by the *aarF* gene, a small insertion was introduced at a unique *Nhe*I site (Fig. 1B). This insertion resulted in a frameshift leading to a severe truncation of the putative AarF protein. When the resulting plasmid, designated pKS.*Nhe*I, was introduced into the *E. coli* expression strain, no induced polypeptide was observed upon the addition of IPTG (Fig. 2). The introduction of plasmid pKS.*Nhe*I into PR50.AFM12 also failed to restore a wild-type growth rate (Fig. 1B).

Cloning and analysis of the *E. coli yigQR* locus. The predicted amino acid sequence of the *aarF* open reading frame exhibited a high degree of homology to two putative adjacent open reading frames of unknown function, present at 86 min on the *E. coli* chromosome, and designated *yigQ* (75% identity) and *yigR* (77% identity) (14). The amino acid alignment of AarF with YigQ and YigR is illustrated in Fig. 3. Because of the high degree of homology between AarF and the putative YigQ and YigR proteins, it was hypothesized that the *yigQR* locus may be able to functionally substitute for *aarF* in *P. stuartii*. To test this hypothesis, a partial *Sau*3AI library prepared from a wild-type *E. coli* strain was introduced into PR50.AFM12, and transformants forming wild-type-size colonies were selected. Plasmids from 11 individual large colonies were analyzed by restriction mapping, and all were shown to contain inserts with a common region of DNA (data not shown). One plasmid, designated pEF1, contained a 3.5-kb insert and was chosen for further study. A 2.6-kb *Sal*I fragment

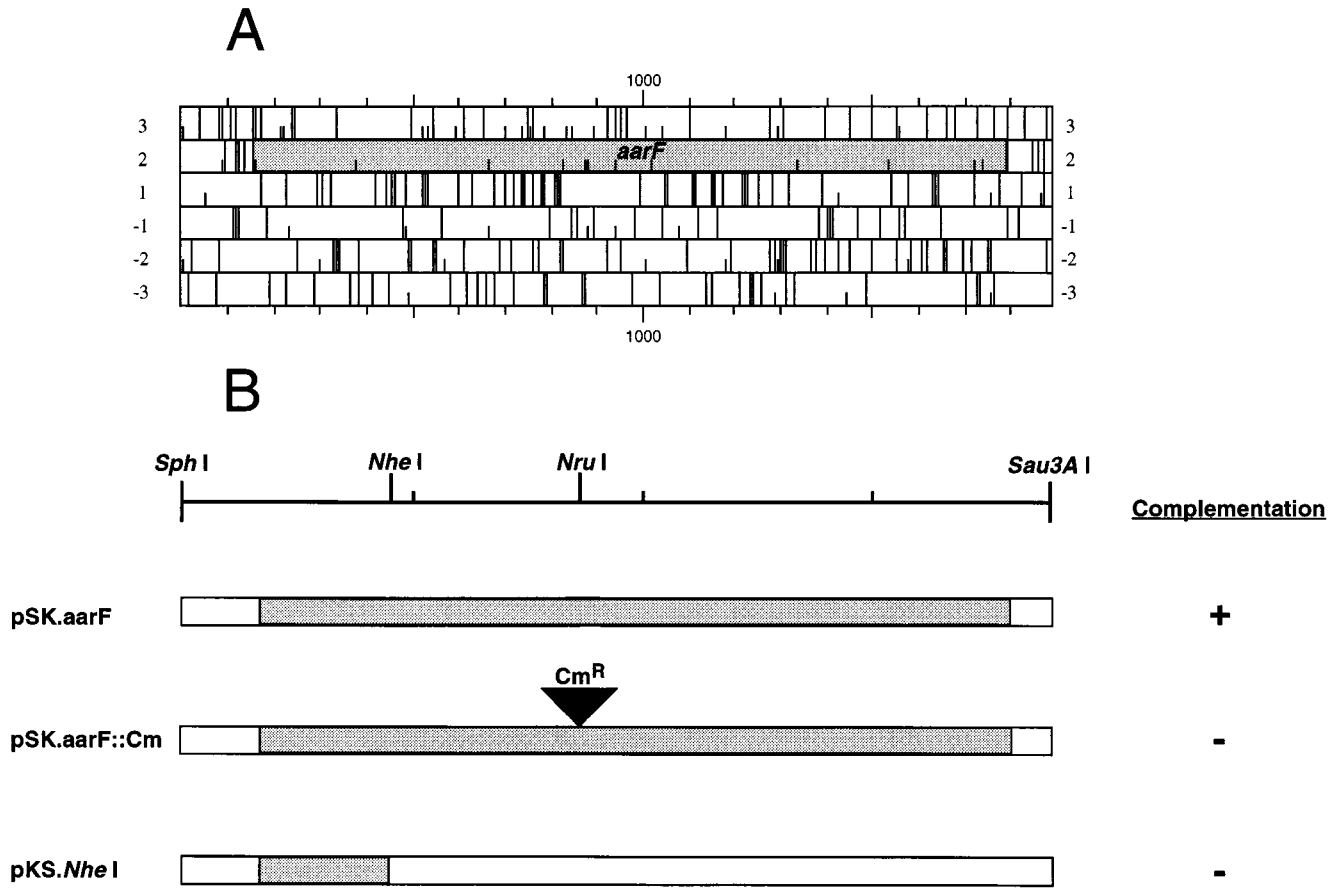

FIG. 1. Identification of the *aarF* coding region. (A) Open reading frame map of the 1,883-bp fragment of *P. stuartii* DNA in pSK.aarF among all six possible reading frames. Lines extending halfway through the reading frame represent potential start codons, and lines extending completely through the reading frame represent stop codons. (B) Complementation of the slow growth phenotype of PR50.AFM12 by various constructs derived from pAFM12. Shaded regions represent the extent of the *aarF* coding region in each construct. +, restoration of wild-type growth rate; −, failure to restore wild-type growth rate.

from pEF1 was subcloned into pBluescript SK(−), creating pSK-2.6. The introduction of pSK-2.6 into PR50.AFM12 also restored a wild-type growth rate. To determine the identity of the cloned *E. coli* DNA in pSK-2.6, sequence data were obtained from both ends and was compared to the GenBank sequence databases. The cloned *E. coli* fragment in pSK-2.6 extends from the *Sal*I site at nucleotide 72625 to the *Sau3A*I site at nucleotide 75101, as reported by Daniels et al. (14). This fragment contains the *yigQ* and *yigR* open reading frames as well as an upstream open reading frame, designated *yigP* (14). To determine which open reading frame(s) was required for the complementation of PR50.AFM12, a series of derivatives of pSK-2.6 were created as described in Materials and Methods. These derivatives were introduced into PR50.AFM12 and scored for complementation by the ability of the insert to restore a wild-type growth rate (Fig. 4). Plasmid pSK.yigQR, which has a deletion of *yigP*, retained the ability to complement the *aarF1* mutation. In contrast, a deletion removing both the *yigP* open reading frame and the initiating ATG codon of the *yigQ* open reading frame (plasmid pSK.yigR) resulted in partial complementation of the growth defect in PR50.AFM12. Similarly, a deletion removing the C-terminal 96 amino acids of the *yigR* open reading frame (plasmid pSK.yigQRΔ*Dra*I) also resulted in partial complementation of PR50.AFM12. Finally, insertion of a kanamycin resistance cassette into a unique *Bsm*I site internal to the *yigR* open reading frame

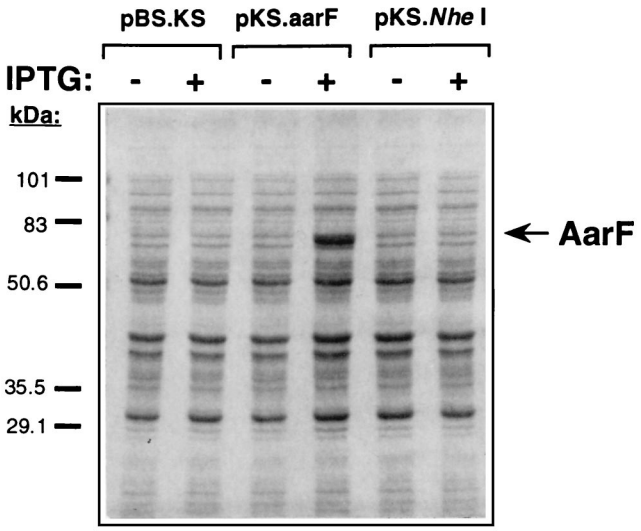

FIG. 2. Identification of the *aarF* gene product. The expression strain BL21(DE3)/pACYC184.lacI^q contained one of the following plasmids: pBS.KS (control vector), pKS.aarF (intact *aarF* gene transcribed by the T7 promoter), or pKS. *Nhe*I (frameshift in *aarF* coding sequence). Strains were grown in LB broth and induced with IPTG as indicated. Total cellular protein was visualized by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining. The size of the AarF polypeptide (67 kDa) was estimated by the relative mobility with respect to prestained low molecular-mass markers (Bio-Rad).

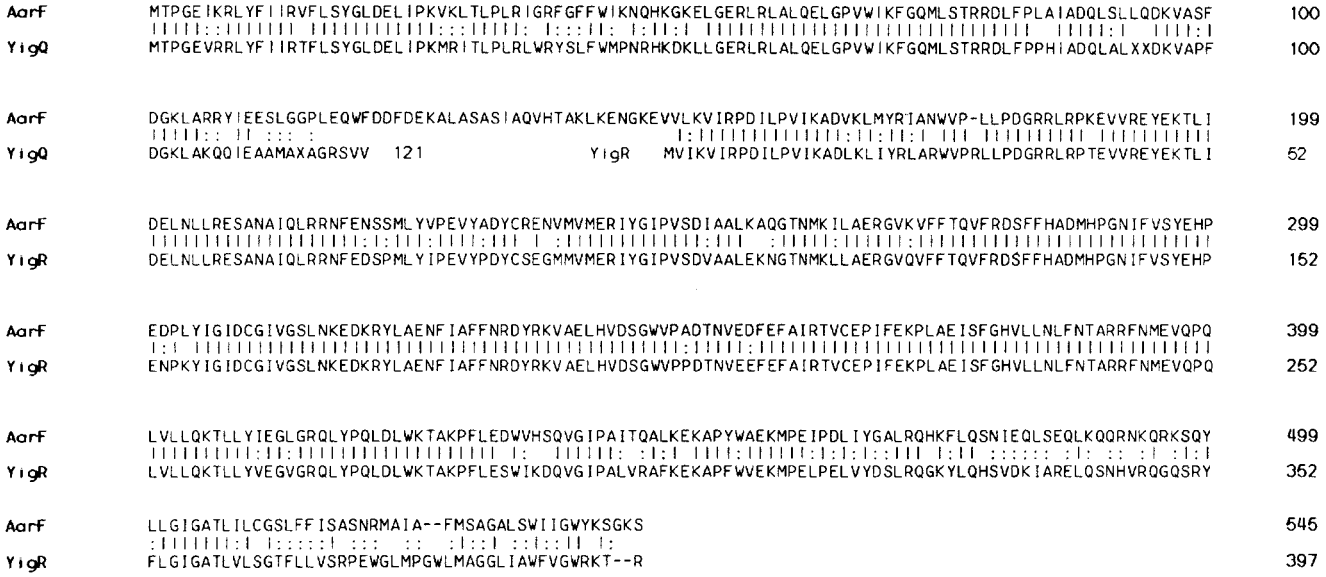

FIG. 3. Homology between AarF and the YigQ and YigR proteins. Proteins were aligned with the Clustal V program (21). Identical amino acids are indicated by vertical bars; similar amino acids are indicated by colons. X, ambiguity in the reported amino acid sequence of YigQ (14).

(plasmid pSK.yigR::Kan) abolished the complementation of PR50.AFM12. These data indicate that *yigP* is not required for the complementation of PR50.AFM12, whereas both the *yigQ* and *yigR* open reading frames are required for the complementation of PR50.AFM12.

Analysis of chromosomal *aarF* and *yigR* null mutants. A chromosomal *aarF*::Cm disruption in wild-type PR50 was constructed by allelic replacement as described in Materials and Methods, resulting in strain PR54. PR54 exhibited a slow growth phenotype similar to that of PR50.AFM12 (*aarF1*) and demonstrated resistance to gentamicin (1,024 μg/ml) that was equal to that of PR50.AFM12. The introduction of pSK.aarF into PR54 restored a wild-type growth rate, whereas the introduction of control plasmid pBluescript SK(−) did not affect either growth rate or gentamicin resistance (data not shown). Plasmid pSK-2.6, containing the wild-type *yigPQR* locus from *E. coli*, also restored a wild-type growth rate when introduced into PR54 (data not shown). The ability of the *E. coli yigQR*

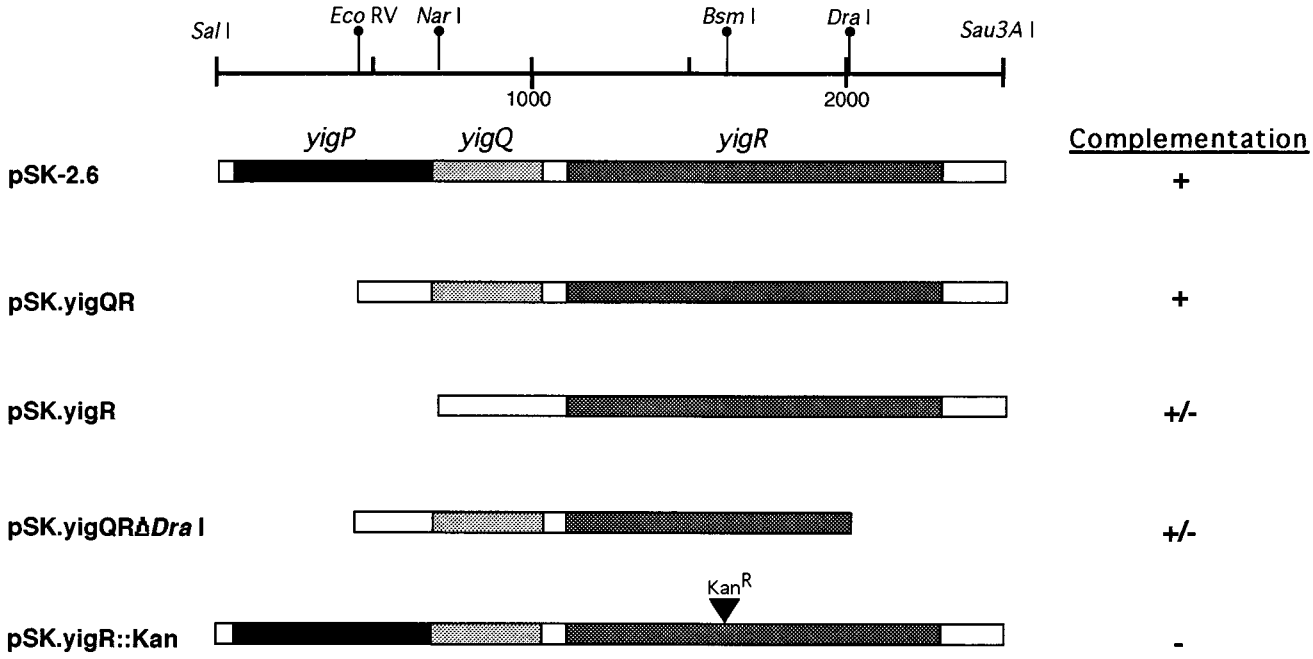

FIG. 4. Determination of sequences required for the complementation of PR50.AFM12. Complementation of the slow growth phenotype of PR50.AFM12 by various constructs derived from pSK-2.6 is shown. The positions of various restriction sites used to create the constructs are indicated. Shaded regions represent the extents of the *yigP*, *yigQ*, and *yigR* open reading frames present in each construct. +, restoration of wild-type growth rate; −, failure to restore wild-type growth rate; +/−, intermediate growth rate.

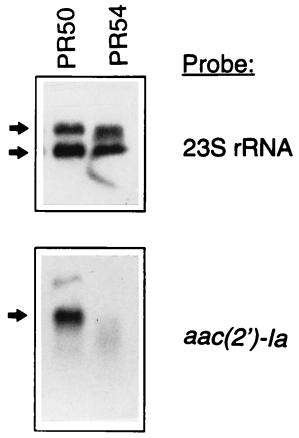

FIG. 5. Effects of *aarF*::Cm on *aac(2′)-Ia* expression. The accumulation of *aac(2′)-Ia* mRNA was determined by Northern analysis. Lanes: 1, 30 μg of RNA prepared from PR50 (wild type); 2, 30 μg of RNA prepared from PR54 (*aarF*::Cm). The filter was probed with the *aac(2′)-Ia* coding sequence (bottom panel). As an internal control for loading, the probe was spiked with a labeled fragment derived from the *E. coli* 23S rRNA gene (top panel). Arrows in the top panel denote the 23S rRNA. The arrow in the bottom panel denotes the *aac(2′)-Ia* message.

locus to substitute for *aarF* in PR54 suggested that the two loci are functionally equivalent. To examine the role of the *yigQR* locus in *E. coli*, the *yigR*::Kan disruption from pSK.yigR::Kan was introduced into the chromosome of wild-type *E. coli* RM1734 as described in Materials and Methods to produce DM123. DM123 exhibited a slow growth phenotype similar to that of the *P. stuartii aarF*::Cm mutant PR54. The introduction of either pSK-2.6 or pSK.aarF restored wild-type growth to DM123, whereas the introduction of the cloning vector pBluescript SK(−) did not affect the growth of DM123 (data not shown).

Effects of *aarF*::Cm on *aac(2′)-Ia* expression. Preliminary data suggested that the *aarF1* allele increased *aac(2′)-Ia* expression. To determine the phenotype of an *aarF*::Cm disruption, plasmid pR401 [*aac(2′)-lacZ*] was introduced into PR54. PR54/pR401 formed dark blue colonies when grown on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, whereas PR50/pR401 formed white colonies when grown on the same plates. In liquid assays, the accumulation of β-galactosidase in PR54/pR401 was measured at 19.9 ± 0.74 U and represented a 17-fold increase over the value for PR50/pR401 (1.14 ± 0.05 U). However, a control construct containing a lac_p-*lacZ* transcriptional fusion resulted in a 102-fold increase in β-galactosidase levels in PR54 relative to those observed in PR50 (data not shown). This result indicated that the *aarF*::Cm allele increased β-galactosidase expression or activity independently of the *aac(2′)-Ia* promoter. Preliminary data indicated that the copy number of pR401 is significantly higher in the *aarF*::Cm background (32). In addition, these data also suggested that *aarF*::Cm may actually decrease *aac(2′)-Ia* promoter activity. To confirm these results at the level of *aac(2′)-Ia* mRNA accumulation, RNA was prepared from PR54 and the parental strain, PR50, and was analyzed by Northern analysis with a probe specific to *aac(2′)-Ia*. The results shown in Fig. 5 demonstrated that *aac(2′)-Ia* mRNA levels were significantly lower in PR54 than in PR50.

***aarF* and *yigR* mutants are deficient in ubiquinone.** Because *aac(2′)-Ia* mRNA levels in the *aarF* mutant background are not increased above wild-type levels, an alternative mechanism must be responsible for the high-level gentamicin resistance.

TABLE 2. Phenotypes resulting from *aarF* and *yigR* disruptions

Strain	Growth[a] on:	
	Glucose	Succinate
P. stuartii		
PR50 (wild type)	+	+
PR54 (*aarF*::Cm)	+	−
PR54/pBluescript SK(−)	+	−
PR54/pSK.aarF	+	+
PR54/pSK-2.6	+	+
E. coli		
RM1734 (wild type)	+	+
DM123 (*yigR*::Kan)	+	−
DM123/pBluescript SK(−)	+	−
DM123/pSK.aarF	+	+
DM123/pSK-2.6	+	+
AN66 (*ubiD*)	+	±
AN66/pBluescript SK(−)	+	±
AN66/pSK.aarF	+	±
AN66/pSK-2.6	+	±
AN70 (*ubiE*)	+	−
AN70/pBluescript SK(−)	+	−
AN70/pSK.aarF	+	−
AN70/pSK-2.6	+	−
AN70/pEF1	+	+

[a] Strains were grown on M9 minimal agar plates containing either 0.2% glucose or 0.5% succinate. *E. coli* AN66 and AN70 were supplemented with L-leucine, L-threonine, and L-methionine each at a final concentration of 0.2 mM and with thiamine at a final concentration of 0.02 μM. +, wild-type growth rate; ±, poor growth; −, no growth.

Mutations that disrupt the aerobic respiratory electron transport chain have been reported to result in increased aminoglycoside resistance (2–5, 25, 27). One such class of mutants are those that are defective in ubiquinone biosynthesis (3, 4). Mutants that are defective in ubiquinone biosynthesis are unable to grow aerobically on nonfermentable carbon sources, such as malate or succinate (11). To determine whether PR54 was defective in ubiquinone production, the growth of PR50 and PR54 was compared aerobically on minimal media containing either glucose or succinate as the sole carbon source. Wild-type PR50 was able to utilize either glucose or succinate as a sole carbon source (Table 2). In contrast, PR54 was unable to utilize succinate as a sole carbon source aerobically. Similarly, *E. coli* RM1734 was able to utilize either glucose or succinate as a sole carbon source, whereas DM123 was unable to utilize succinate as a sole carbon source. The introduction of the complementating plasmids pSK.aarF and pSK-2.6 into PR54 and DM123, respectively, restored the ability to utilize succinate (Table 2).

Cell extracts were directly examined for ubiquinone content by thin-layer chromatography as described in Materials and Methods. Extracts from *P. stuartii* PR50 contained high levels of ubiquinone that comigrated with the coenzyme Q8 standard (R_f, 0.2). Extracts from PR54 contained no detectable ubiquinone but contained significant amounts of a precursor (R_f, 0.117). Analysis of the *E. coli yigR*::Kan mutant DM123 and the isogenic parental strain RM1734 yielded similar results. Thus, *aarF* in *P. stuartii* and *yigQR* in *E. coli* are required for the production of ubiquinone.

Three *E. coli* genes involved in ubiquinone biosynthesis (*ubiB*, *ubiD*, and *ubiE*) have been mapped to the same region on the chromosome as *yigQR* (min 86) (12, 14, 23, 39). The *ubiB* gene has been tentatively identified and lies approximately 4.7 kb downstream of *yigR* (14). Recently, the *ubiE* gene

was demonstrated to be equivalent to an open reading frame, designated *yigO*, that lies immediately upstream of the *yigP* open reading frame (14, 23). *E. coli* AN66 *ubiD* and AN70 *ubiE* were transformed with pSK.aarF and pSK-2.6 (*yigPQR*) (12, 39). Neither *E. coli* strain was complemented by pSK.aarF or pSK-2.6 (*yigPQR*), as scored by the restoration of growth on minimal succinate plates (Table 2). In contrast, transformation with plasmid pEF1 restored the ability of AN70 to utilize succinate as a sole carbon source. Subsequent sequence analysis demonstrated that plasmid pEF1 contained the intact *yigO* open reading frame in addition to *yigPQR* (data not shown). Therefore, the *aarF* gene of *P. stuartii* and the *yigQR* genes of *E. coli* are distinct from *ubiD* and *ubiE*.

DISCUSSION

In a search for regulators of the *aac(2′)-Ia* gene of *P. stuartii*, we identified *aarF*, a gene required for the production of the respiratory cofactor ubiquinone (coenzyme Q). The *aarF1* and *aarF*::Cm mutations resulted in a 256-fold increase in gentamicin resistance above wild-type levels and caused a severe defect in aerobic growth on rich media. Initial observations obtained through the use of an *aac(2′)-lacZ* transcriptional fusion suggested that *aac(2′)-Ia* expression was increased in the *aarF* mutant background. However, this expression appeared to be an artifact due to increased plasmid copy number in the *aarF* mutant background. Direct examination by Northern analysis revealed that *aac(2′)-Ia* mRNA levels in PR54 (*aarF*::Cm) were dramatically lower than those in wild-type PR50. To our knowledge, ubiquinone has never been implicated in gene regulation, so this finding is a novel one.

In light of the above data, it seems unlikely that ubiquinone is directly involved in the regulation of *aac(2′)-Ia*. We recently identified a locus in *P. stuartii*, designated *aarE*, that is also required for the expression of *aac(2′)-Ia*. The *aarE* gene was found to be the *P. stuartii* homolog of *ubiA* (32, 37). In contrast, the *aarD* locus (25), representing the *P. stuartii* homolog of *cydD* (30, 31), is required for the function of the cytochrome *d* terminal oxidase and is a negative regulator of *aac(2′)-Ia* expression. We propose a model for the regulation of *aac(2′)-Ia* expression by a regulatory cascade in which ubiquinone acts as an effector molecule (Fig. 6). In this model, the reduced form of ubiquinone (ubiquinol) serves as a signal to activate *aac(2′)-Ia* expression through an uncharacterized pathway. It is important to note that this pathway appears to be independent of the previously identified activator AarP (24). According to this model, in a cytochrome *d*-deficient strain, ubiquinol is predicted to accumulate and to result in the activation of *aac(2′)-Ia* expression. In ubiquinone-deficient *aarE* and *aarF* mutant strains, the regulatory cascade would be disrupted, resulting in decreased *aac(2′)-Ia* expression. Further studies with inhibitors of electron transport may provide evidence to support this model.

Because *aac(2′)-Ia* mRNA levels are decreased in PR50.AFM12 (*aarF1*) and PR54 (*aarF*::Cm), an alternative mechanism must be responsible for the large increase in gentamicin resistance observed in the *aarF* mutant background. Aminoglycoside uptake requires a sufficiently negative membrane potential as well as active electron transport (3, 13, 17). High-level, nonenzymatic resistance to the aminoglycosides usually arises from mutations in components of the aerobic respiratory chain (1, 3, 4, 13, 19). PR54 was unable to utilize succinate as a sole carbon source aerobically, and extracts from this strain were found to be devoid of ubiquinone. Ubiquinone-deficient *E. coli* mutants were previously shown to exhibit increased gentamicin resistance and were found to accumulate gentamicin poorly (3, 4). Therefore, the high-level gentamicin resistance observed in PR54 is likely associated with decreased accumulation of the drug resulting from the absence of aerobic electron transport.

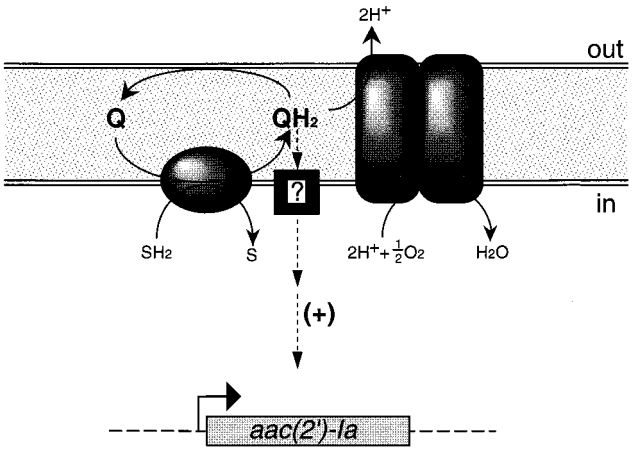

FIG. 6. Model for the regulation of *aac(2′)-Ia* expression. A schematic of the aerobic respiratory chain is depicted. A primary dehydrogenase couples the oxidation of a substrate (SH_2) and the reduction of ubiquinone (Q). A terminal oxidase complex oxidizes ubiquinol (QH_2) and reduces molecular oxygen to water, coupling the reaction to the generation of a proton gradient. The black box represents the putative sensor component of a regulatory cascade that responds to the levels of ubiquinol and increases *aac(2′)-Ia* expression.

The *aarF* locus was found to encode a single 544-amino-acid protein. The AarF polypeptide was identified with a T7 expression system and exhibited an apparent molecular mass of 67 kDa, in agreement with the predicted size of 62.5 kDa. The predicted AarF protein exhibited extensive amino acid identity with the products of two putative adjacent open reading frames, *yigQ* and *yigR*, present at 86.6 min on the *E. coli* chromosome (14). This region of the chromosome has been sequenced as part of the *E. coli* sequencing project, and frameshifts that could merge *yigQ* and *yigR* into one contiguous open reading frame are possible. It should be noted that we have no evidence suggesting that *yigQ* and *yigR* are contiguous.

An *E. coli yigR*::Kan mutant was found to be defective in ubiquinone biosynthesis. Three ubiquinone biosynthesis genes, *ubiB*, *ubiD*, and *ubiE*, map near *yigQ* and *yigR* at min 86 on the *E. coli* chromosome (12, 14, 23, 39). Complementation studies showed that the *yigQR* genes did not complement *ubiD* and *ubiE* mutations. In addition, the *ubiB* gene lies upstream of *yigQR* (14). Therefore, *aarF* (*yigQR*) represents a novel gene in the ubiquinone biosynthetic pathway. Extracts from both *P. stuartii aarF* and *E. coli yigR* mutants contained significant amounts of a ubiquinone precursor. Future studies to determine the identity of this precursor will be required to assign a function to the *aarF* (*yigQR*) locus.

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