Mutations in aarE, the ubiA Homolog of Providencia stuartii, Result in High-Level Aminoglycoside Resistance and Reduced Expression of the Chromosomal Aminoglycoside 2′-N-Acetyltransferase

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The aarE1 allele was identified on the basis of the resulting phenotype of increased aminoglycoside resistance. The aarE1 mutation also resulted in a small-colony phenotype and decreased levels of aac(2′)-Ia mRNA. The deduced AarE gene product displayed 61% amino acid identity to the Escherichia coli UbiA protein, an octaprenyltransferase required for the second step of ubiquinone biosynthesis. Complementation experiments in both Providencia stuartii and E. coli demonstrated that aarE and ubiA are functionally equivalent.

The regulation of a 2′-N-acetyltransferase [encoded by the aac(2′)-Ia gene] involved in peptidoglycan acetylation in the gram-negative enteric bacterium Providencia stuartii (8, 9, 29) has been extensively studied in our laboratory. This housekeeping gene was originally identified as an aminoglycoside resistance gene because of the acetylation of certain aminoglycosides by the AAC(2′)-Ia protein (7, 34, 39). A number of regulatory genes that influence expression of aac(2′)-Ia have been identified, including aarA, aarB, aarC, aarD, and aarp (21, 22, 33–35). The aarD gene in P. stuartii encodes CydD (22), a heterodimeric component of an ABC transporter required for the formation of a functional cytochrome bd oxidase (30, 31). This oxidase is required for the terminal step in electron transport under aerobic conditions. A null allele of aarD increased aac(2′)-Ia transcription (22), suggesting that the regulation of aac(2′)-Ia expression is coupled to some aspect of electron transport.

The process of electron transport in bacteria requires the molecule ubiquinone, which is capable of accepting electrons from a variety of dehydrogenases and transferring them to a terminal oxidase. At least nine genes (ubiH and ubiX) that are required for ubiquinone biosynthesis have been identified (11, 12, 18, 19, 24, 28, 36–38, 40, 41). The primary role of ubiquinone is in electron transport; however, it has been reported that ubiquinone mutants have reduced numbers of flagella and are nonmotile (15). This phenotype may be related to defects in electron transport. In this study, we found that a recessive mutation in the aarE gene, a homolog of ubiA, severely decreases aac(2′)-Ia mRNA accumulation. The product of ubiA is parahydroxybenzoate octaprenyltransferase, an enzyme involved in the second step of ubiquinone biosynthesis (38, 41). Based on the data obtained in this study, we propose a model in which ubiquinone or a functional electron transport chain is required for a process that influences aac(2′)-Ia mRNA accumulation. Our data suggest that this process does not involve the previously identified transcriptional activator AarP (21).

Bacterial strains and plasmids. Escherichia coli XL1 Blue (Stratagene) and DH5α (Gibco/BRL) were used as hosts for transformations. E. coli DH5α λ pir (21) and SM10 λ pir (26) were used as hosts for pKNG101 (16) derivatives. P. stuartii PR50 is a wild-type strain and has been described previously (34). Plasmids pACYC184 (6) and pBluescript II SK(--) (Stratagene) were used as cloning vectors. Electroporations were done as described previously (34).

Northern blot analysis. Total RNA was prepared by using the TRizol reagent (Gibco/BRL), and equal amounts were loaded, as determined on the basis of the intensities of the 23S and 16S rRNAs. The RNA was fractionated on a 2.2 M formaldehyde and transferred to nylon membranes by capillary transfer. To ensure that equal amounts of RNA were transferred to the nylon membranes, the nylon filters were photographed while under UV illumination. The filters were then probed with a digoxigenin-labeled 602-bp TaqI-SspI fragment containing the aac(2′)-Ia coding sequence. The filters were developed with the LumiPhos substrate.

DNA sequencing. Double-stranded templates were sequenced by using an AutoRead sequencing kit (Pharmacia) with fluorescein-labelled universal and reverse primers. Sequencing reactions were run on an A.L.F. automated sequencer (Pharmacia).

Isolation of the aarE1 allele. Previous studies have shown that when P. stuartii is selected for gentamicin resistance at a level fourfold above the MIC, the resulting mutants usually display enhanced expression of the aac(2′)-Ia gene (22, 34, 35). These previous selections were done in cells that contained an aac(2′)-Ia fusion on the multicopy plasmid pR401 (33). To avoid the possibility that the presence of the aac(2′)-Ia promoter in multiple copies was altering the isolation of aac(2′)-Ia regulatory mutants, we resolated gentamicin-resistant mutants of PR50 lacking pR401. A mutant displaying high-level gentamicin resistance was isolated by plating PR50 (34) on Luria-Bertani plates containing 15 μg of gentamicin per ml. This mutant, PR11, was resistant to gentamicin at 256 μg/ml, relative to the isogenic parent, PR50, which was resistant to gentamicin at 4 μg/ml, and this mutant displayed a small-colony...
phenotype. The mutation responsible for this phenotype was designated aarE1, and the increased gentamicin resistance suggested that aarE1 may increase expression of the aac(2')-Ia gene. To examine this possibility, Northern blot analysis was used to determine the accumulation of aac(2')-Ia mRNA in both the mutant and parental strains at an optical density of 600 nm of 0.6. The results of this analysis are shown in Fig. 1; the levels of aac(2')-Ia mRNA were significantly lower in PR11 aarE1 than in the wild-type strain PR50. The decreased accumulation of aac(2')-Ia mRNA in PR11 aarE1 indicated that a mechanism independent of aac(2')-Ia must account for the high-level gentamicin resistance observed for this mutant.

Isolation and characterization of aarE. Strain PR11 exhibited a reduced growth rate and formed significantly smaller colonies than PR50 on Luria-Bertani agar plates. In addition, PR11 exhibited a reduced growth yield in liquid culture, with growth ceasing at an optical density at 600 nm of 0.4 to 0.5. Since PR11 was a spontaneous mutant, it seemed likely that a single mutation was responsible for both the reduced growth rate and the regulatory effects on aac(2')-Ia. Based on this assumption, a library of PR50 genomic DNA was constructed in pACYC184 (6) and introduced into PR11. Transformants with a normal growth rate were easily visible in the background of microcolonies. Plasmid DNA was purified from several large colonies and retransformed into PR11, with 100% of the transformants exhibiting a wild-type growth rate. Analysis of a complementing plasmid, pAFM11, indicated the presence of a 2.1-kb insert. The 2.1-kb Sall-EcoRV fragment was cloned into pBluescript II SK(−), resulting in plasmid pSK.aarE, which was also capable of complementing the aarE1 allele. The nucleotide sequence was determined from each end of the insert. At one end of the insert in pSK.aarE, a partial open reading frame encoding 79 amino acids with 76% identity to amino acids 710 to 789 of PIsB, which is the E. coli glycerol-3-phosphate O-acyltransferase, was identified (20). Nucleotide sequence analysis of the other end of the insert revealed two open reading frames. The first open reading frame, proximal to the end of the insert, encoded a truncated protein with 65% identity to the carboxy-terminal 48 amino acids of the E. coli UbiC protein (28). The second open reading frame, immediately adjacent to the first open reading frame, encoded a predicted product with 67% amino acid identity to amino acids 1 to 103 of the E. coli UbiA protein, which is p-hydroxybenzoate octaprenyltransferase (38, 41). Thus, the organization of this region of the P. stuartii chromosome is identical to that of E. coli, and it was predicted that the only functional gene contained within pSK.aarE would be the ubiA homolog. This indicated that the aarE1 mutation was within this ubiA homolog. On the basis of this information, the remainder of the ubiA sequence was determined, revealing a single complete open reading frame of 864 nucleotides capable of encoding a product of 288 amino acids. The product of this open reading frame, designated AarE, displayed 61% amino acid identity and 74% similarity to the E. coli UbiA protein, an octaprenyltransferase required for the second step of ubiquinone biosynthesis.

The aarE and ubiA genes are functionally equivalent. To determine whether PR11 was deficient in the production of ubiquinone, two tests were performed. First, growth on medium containing succinate as the sole carbon source requires ubiquinone under aerobic conditions (11). PR11 was unable to grow under these conditions, whereas the wild-type strain PR50 grew well. Next, cell extracts of PR11 (aarE1) and PR50 (wild type) were directly examined for ubiquinone by thin-layer chromatography as described previously (10). A ubiquinone standard (Q$_{8}$) on a control plate had an R$_{f}$ value of 0.19 and displayed a peak absorbance at 274 nm when eluted into ethanol. PR50 produced a strong signal which had an R$_{f}$ value of 0.20 in this system and a peak absorbance at 273 nm in ethanol. In contrast, extracts of PR11 aarE1 produced no detectable ubiquinone, and intermediates were not detected. These results are consistent with PR11 containing a mutation in the ubiA gene.

To determine if the aarE and ubiA genes are functionally equivalent, we conducted an unbiased search for E. coli genes that could complement the aarE1 allele in P. stuartii PR11. An E. coli genomic library of partial Sau3AI fragments constructed in pET21a (kindly provided by P. deBoer, Case Western Reserve University) was introduced into PR11 (aarE1), and complementing clones were identified as described previously for pAFM11. Analysis of all complementing plasmids revealed a common insert, and one plasmid with an insert of approximately 3 kb was used in further studies. The insert in this plasmid was shown to hybridize to Kohara phages IF8 and 12B4 (17), both of which are predicted to have the E. coli region containing pIsB and ubiCA. Therefore, based on the published sequence of ubiCA (28, 38), a 1,031-bp SmaI-BclI fragment predicted to contain only the ubiA gene was subcloned into pBC Ks(−), resulting in pBC.ubiA. Sequence analysis of the ends of pBC.ubiA verified that it contained only the ubiA gene. Introduction of pBC.ubiA into PR11 (aarE1) resulted in complementation of the aarE1 mutation, as indicated by the wild-type growth rate and the ability of the transformant to grow on succinate minimal medium (Table 1). As expected, pSK.aarE also restored the ability of PR11 to grow on succinate plates (Table 1). In addition, both pSK.aarE and pBC.ubiA were able to complement an E. coli ubiA mutant (HW273) (data not shown). Thus, aarE and ubiA are functionally equivalent.

Construction and characterization of an aarE::Km null allele. To confirm that the loss of aarE function was responsible for the observed phenotypes in PR11, an aarE::Km disruption
TABLE 1. Growth phenotypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth on M9 medium with:</th>
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<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>PR50 (wild type)</td>
<td>+</td>
</tr>
<tr>
<td>PR11 (aarE1)</td>
<td>+</td>
</tr>
<tr>
<td>PR11/pBluescript II SK(−)</td>
<td>+</td>
</tr>
<tr>
<td>PR11/pSK.aarE</td>
<td>+</td>
</tr>
<tr>
<td>PR11/pBC KS(−)</td>
<td>+</td>
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<tr>
<td>PR11/pBC.ubiA</td>
<td>+</td>
</tr>
<tr>
<td>PR11.D (aarE2::Km)</td>
<td>+</td>
</tr>
<tr>
<td>PR11.D/pBluescript II SK(−)</td>
<td>+</td>
</tr>
<tr>
<td>PR11.D/pSK.aarE</td>
<td>+</td>
</tr>
<tr>
<td>PR11.D/pBC KS(−)</td>
<td>+</td>
</tr>
<tr>
<td>PR11.D/pBC.ubiA</td>
<td>+</td>
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* Strains were grown on M9 minimal agar plates containing either 0.2% glucose or 0.5% succinate. +, wild-type growth rate; −, no growth.


discusssion from that of PR11-aarE and the kanamycin resistance cassette, indicated the presence of the aarE::Km disruption (data not shown).

Concluding remarks. Loss-of-function mutations in the aarE gene of P. stuartii resulted in high-level aminoglycoside resistance. The AarE gene product was 61% identical and functionally equivalent to the UbiA protein of E. coli. UbiA is an octaprenyltransferase which catalyzes the second step in ubiquinone biosynthesis, the addition of a prenyl group to 4-hydroxybenzoate (38, 41). Thus, in a ubiA mutant, ubiquinone biosynthesis is blocked, leading to a defect in electron transport and aerobic respiration. This results in the slow-growth phenotype observed in the aarE (ubiA) mutants and also explains the high-level aminoglycoside resistance that is independent of aarE. A number of previous studies have established that high-level aminoglycoside resistance can result from alterations in electron transport (1–5, 13, 14, 22, 25, 27, 36a), including a deficiency in ubiquinone (3, 4, 27).

A potentially important result from this study, in combination with our analysis of a new locus involved in ubiquinone biosynthesis (23), is the observation that mutants unable to produce ubiquinone have altered levels of aarE-specific mRNAs. The regulatory mechanism(s) which requires ubiquinone or a functional electron transport chain to maintain normal levels of aarE-specific mRNAs remains to be identified.

The decreased aarE-specific mRNA accumulation is probably not due to a general defect in electron transport, since cydD mutants display increased aarE-specific mRNA accumulation (22). In addition, the reduced aarE-specific mRNA levels in the aarE mutant background are probably not due to a reduced growth rate. Mutations in the aarE, aarC, and aarD genes all reduce the growth rate to a level similar to that of an aarE mutant, yet these mutations lead to 3- to 12-fold increases in aarE-specific mRNA accumulation relative to the wild type (22, 34, 35). Studies using an aarE lacZ fusion in a single copy indicate that the accumulation of β-galactosidase is similar in the wild type and aarE::Km mutants (32). Based on the above-described data, the decrease in aarE-specific mRNA accumulation in the aarE (ubiA) mutants appears to result from decreased mRNA stability. This could result from changes in the cellular levels of an RNase.

Nucleotide sequence accession number. The aarE nucleotide sequence has been deposited in GenBank under accession no. AF036609.

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REFERENCES


32. Rather, P. N. Unpublished results.