Altered denA and anr gene expression in aminoglycoside adaptive resistance in Pseudomonas aeruginosa

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Adaptive resistance to aminoglycoside killing and cytoplasmic accumulation occurs in cultures of originally susceptible \textit{Pseudomonas aeruginosa} following an initial incubation with aminoglycoside. Anaerobiosis has also been reported to reduce bacterial killing and limit cytoplasmic aminoglycoside accumulation. We hypothesized that a common mechanism may facilitate reduced bacterial killing and aminoglycoside accumulation in both cases. Northern blot analysis of \textit{P. aeruginosa} adaptively resistant to gentamicin demonstrated increased mRNA levels of both denA (nitrite reductase), which facilitates terminal electron acceptance in the anaerobic respiratory pathway, and its regulatory protein, ANR, in the absence of promoter DNA sequence changes, when compared with controls. These observations suggested that \textit{P. aeruginosa} may regulate the expression of genes in its anaerobic respiratory pathway in response to aminoglycosides and may explain, at least partially, \textit{P. aeruginosa} adaptive resistance to aminoglycosides.

\textbf{Introduction}

\textit{Pseudomonas aeruginosa} is one of the most common bacteria causing difficult-to-treat nosocomial infections.\textsuperscript{1} Aminoglycosides, in combination with \(\beta\)-lactams such as ceftazidime, provide effective therapy for the treatment of these infections.\textsuperscript{2} Occasionally, however, therapeutic failures occur, some of which have been attributed to transient aminoglycoside resistance.\textsuperscript{3,4,5} The reproducible pattern by which initially susceptible \textit{P. aeruginosa} demonstrate transient resistance to aminoglycosides (reduced bacterial killing) following initial incubation with aminoglycosides, that is reversed with growth in aminoglycoside-free media, has been termed adaptive resistance.\textsuperscript{6}

A adaptive resistance correlates with a marked reduction in intracellular accumulation of aminoglycoside.\textsuperscript{4,5,7} However, the specific mechanism of, and the cellular structure(s) responsible for aminoglycoside adaptive resistance in \textit{P. aeruginosa} remain enigmatic.

A anaerobiosis has also been demonstrated to limit cytoplasmic accumulation of aminoglycoside and to reduce bacterial killing.\textsuperscript{8-10} These reports studied the facultative bacteria \textit{Escherichia coli} and \textit{Staphylococcus aureus}\textsuperscript{9,10} and the strict anaerobes \textit{Clostridium perfringens} and \textit{Bacteroides fragilis}.\textsuperscript{8} It has also been reported that streptomycin and gentamicin were transported less effectively when nitrate was substituted for oxygen as a terminal electron acceptor in \textit{P. aeruginosa}.\textsuperscript{8} Further anaerobic work with \textit{P. aeruginosa} may not have been performed because this species is commonly, although somewhat misleadingly, classified as a strict aerobe.

Despite its preference for aerobic environments, \textit{P. aeruginosa} is well adapted to conditions of oxygen limitation given the presence of a suitable terminal electron acceptor molecule (e.g. nitrite) or one of its derivatives (e.g. nitrate).\textsuperscript{11} \textit{P. aeruginosa} has an aerobic and an anaerobic respiratory pathway, both of which generate ATP exclusively via oxidative phosphorylation.\textsuperscript{11} The aerobic pathway terminates at a cytochrome c oxidase and is cyanide-sensitive, with a KCN \(50\%\) inhibitory concentration (IC\textsubscript{50}) of 5 \(\mu\)M KCN.\textsuperscript{11} The anaerobic pathway terminates at nitrite reductase (DENA) and is considered cyanide-resistant, with a KCN IC\textsubscript{50} of 30 mM.\textsuperscript{11} Nitrite reductase is also expressed under aerobic conditions in the absence of nitrite or nitrate supplementation\textsuperscript{12} and contributes to \(O_2\) reduction to \(H_2O\), albeit at a reduced rate when compared with cytochrome c oxidase.\textsuperscript{13,14} Transcription of the nitrite reductase gene (denA) is positively
regulated by ANR, a regulatory protein that is essential for nitrate respiration, anaerobic arginine degradation and KCN production in *P. aeruginosa*.\(^\text{15,16}\)

The objective of this study was to determine whether the reduced intracellular accumulation of aminoglycoside and bacterial killing demonstrated by adaptively resistant *P. aeruginosa*,\(^\text{4,6,7}\) and that previously reported for anaerobically grown *P. aeruginosa*,\(^\text{5}\) are related. Northern blots were performed to assess denA and anr mRNA levels in wild-type, adaptively resistant, post-adaptively resistant and anaerobically grown cultures to determine whether *P. aeruginosa* adaptively resistant to gentamicin increase their reliance upon the anaerobic respiratory pathway.

**Materials and methods**

**Bacterial strains**

One reference strain (ATCC 27853) and two blood culture isolates (F991, E1481) of *P. aeruginosa* were tested.\(^\text{7}\) A righthand MICS of gentamicin (Schering Canada, Pointe-Claire, Canada) had been previously determined as 3.6, 3.5 and 3.2 mg/L for ATCC 27853, F991 and E1481, respectively.\(^\text{7}\)

**Aminoglycoside adaptive resistance**

A daptive resistance experiments were performed at 37°C in Mueller–Hinton broth (Becton Dickinson, Cockeysville, MD, USA). Twenty millilitre logarithmic phase cultures of *P. aeruginosa* containing 10\(^7\) cfu/mL were exposed to gentamicin at a concentration of 8 mg/L. Following a 24 h incubation, 100 \(\mu\)L of this culture was transferred to fresh Mueller–Hinton broth supplemented with 8 mg/L gentamicin and allowed to grow to a density of approximately 10\(^8\) cfu/mL.\(^\text{7}\) Following a second identical subculture, cultures were confirmed to be adaptively resistant using a previously described method.\(^\text{6}\) Post-adaptively resistant cultures were grown for 72 h in antimicrobial-free Mueller–Hinton broth and confirmed to have wild-type gentamicin susceptibility as previously described.\(^\text{7}\)

**Anaerobic growth conditions**

A n aerobic growth conditions were maintained using a Coy Laboratories (Ann Arbor, MI, USA) anaerobic chamber. The atmosphere inside the anaerobic chamber consisted of a mixture of 80% N\(_2\), 10% H\(_2\) and 10% CO\(_2\). Mueller–Hinton broth was allowed to degas for 3 days in the anaerobic chamber before use. Broth treated in this manner did not support the growth of *P. aeruginosa* ATCC 27853 (negative control), but did support the growth of *E. coli* ATCC 25922 (positive control). A aerobic Mueller–Hinton broth supplemented with 50 mM KNO\(_3\) supported anaerobic growth of all *P. aeruginosa* isolates tested.

**Northern transfer**

A previously described method was used to isolate total RNA from *P. aeruginosa*.\(^\text{17}\) The purity and quantity of RNA were determined spectrophotometrically. Twenty micrograms of total RNA was loaded per lane on to a 1% agarose gel containing 0.22 M formaldehyde and electrophoresed.\(^\text{17}\) Duplicate samples were loaded on the same gel and visualized by ethidium bromide staining following electrophoresis. 16S and 23S ribosomal RNA standards (Sigma, Mississauga, Canada) and a 100 bp DNA ladder (Boehringer Mannheim, Laval, Canada) were also run to estimate the size of the probed mRNA. Samples on the other side of the gel were transferred to a piece of positively charged nylon membrane (Boehringer Mannheim).\(^\text{17}\)

**Probe selection and preparation**

The complete DNA sequences of denA, anr and their respective flanking regions have been published.\(^\text{18,19}\) A 699 bp internal denA region was amplified by PCR and then labelled with digoxigenin using primers selected using PCGene computer software (University of Geneva, Geneva, Switzerland). Sequences of the denA sense and antisense primers were 5'-ATGCCTGGAGCTCAATG-3' and 5'-GACGGTGCTGTGATCGATCA-3', respectively (annealing temperature 52°C). Similarly, a 615 bp internal anr region was amplified by PCR and labelled using the sense and antisense primers 5'-CCCCAAGCACTGCAAGGATTGC-3' and 5'-TTCTGCTGAGACGGCGGTGTAAGAGCC-3', respectively (annealing temperature 62°C). Outer membrane protein F (ompF) mRNA was also probed to serve as an RNA loading control.\(^\text{20}\) Sequences of the ompF sense and antisense primers were 5'-AGGGCCCAAGACTCCTGTTGACTAG-3' and 5'-AATCTCGGCATCTTGCACACCGCGAGCG-3', respectively (annealing temperature 60°C) and gave an amplified product of 955 bp.

The Boehringer Mannheim PCR digoxigenin probe synthesis kit, which incorporates digoxigenin-11-dUTP into PCR products, was used according to the manufacturer’s instructions to label probes. A 50 \(\mu\)L reaction volume was placed in a GeneAmp PCR 9600 series thermocycler (Perkin–Elmer, Norwalk, CT, USA), heated at 94°C for 3 min and then cycled through the following protocol: denaturation at 94°C for 1 min, annealing at 52–62°C (primer pair dependent) for 1 min and extension at 72°C for 2 min. After completion of 35 cycles a final 4 min extension step was performed at 72°C. Electrophoresis in 1% agarose gels was used to remove primers from PCR-amplified DNA. Gel portions containing the PCR products were cut from the gels and the DNA fragments harvested using the Prep-A-Gene matrix system, protocol 2.1 (Bio-Rad, Mississauga, Canada). The yield of digoxigenin-labelled probe was then quantified.
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Northern hybridization protocol

The blot was placed into a hybridization bag containing 20 mL prehybridization solution (5 x SSC, 1.0% salmon sperm DNA, 0.1% N-lauroylsarcosine, 0.2% SDS), then the bag was sealed and incubated at the anticipated hybridization temperature for 2 h. The double-stranded DNA probes used were boiled for 10 min to denature the DNA and immediately placed on ice. Digoxigenin-11-dUTP-labelled probes were diluted in 20 mL of hybridization solution (identical to prehybridization solution) to a final probe concentration of 20 μg/L. The prehybridization solution was poured from the bag, the hybridization solution containing digoxigenin-11-dUTP-labelled probe added, and the blot incubated overnight at the hybridization temperature. The next morning, following stringency washes, the blots were subjected to immunological detection using anti-digoxigenin antibody conjugated to alkaline phosphatase and disodium 3-(4-methoxyspiro{|L,2-dioxetane-3,2’-(5’-chloro)tricyclo}[3.3.1.3’3,7]|decan}-4-yl) phenylphosphate (CSPD; Boehringer Mannheim).

DNA sequencing

The entire promoter region of denA, including the putative binding sequence of the ANR regulatory protein, was sequenced (215 bp).18 Primers were selected using PC-Gene computer software. Sequences of the denA sense and antisense primers were 5’-CTTGAGCAATACCGGCAGGC-3’ and 5’-GCTTTCATGTCGTCCTTGGCGTGA-3’, respectively (annealing temperature 58°C). Two hundred and twenty-nine nucleotides were sequenced upstream of the anr start codon.19 Sequences of the sense and antisense primers were 5’-AGGGTCAACATTCCAGTCACTCCG-3’ and 5’-GCAATCCTTGCAGTGCTTGG-3’, respectively (annealing temperature 60°C). PCR was performed as described above. The aforementioned primers doubled as sequencing primers.

The PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used to amplify DNA for sequence analysis on the A Pplied Biosystems model 373A automated DNA sequencing system. The manufacturer’s protocol was followed exactly. Centri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA) were used to remove excess DyeDeoxy terminators from completed sequencing reactions and reaction products were run on a 6% (acrylamide:bis-acrylamide 19:1) polyacrylamide gel.

Results

Northern analysis

The levels of denA and anr mRNA were discernably higher in P. aeruginosa cultures adaptively resistant to gentamicin than in wild-type and post-adaptively-resistant cultures (Figures 1 and 2). Similar results were demonstrated with the reference strain A TCC 27853 and the two blood culture isolates (F991, E1481) of P. aeruginosa tested. These experiments were conducted in the absence of nitrate or nitrite supplementation of Mueller–Hinton broth. In comparison with cultures adaptively resistant to gentamicin (lanes 1A, 2A and 3A in Figures 1 and 2), anaerobically grown P. aeruginosa ATCC 27853 (lane 4 in Figures 1 and 2) produced much higher levels of denA and anr mRNA. A aerobic cultures were supplemented with 50 mM KNO₃. The levels of ompF mRNA were determined by stripping and reprobing Northern blots according to the aforementioned Boehringer Mannheim

Figure 1. Northern analysis of denA. Lane 1, wild-type A TCC 27853; lane 1A, adaptively resistant A TCC 27853; lane 1B, post-adaptively resistant A TCC 27853. Similarly, lanes 2, 2A, 2B contain F991 and lanes 3, 3A, 3B contain E1481. Lane 4 shows denA expression in anaerobically grown P. aeruginosa A TCC 27853.
protocol. Each lane in Figures 1 and 2 contained a similar quantity of ompF mRNA (data not shown).

DNA sequencing

No DNA sequence changes were identified in either the denA or anr promoter regions of adaptively resistant cultures when compared with wild-type and post-adaptively-resistant cultures (data not shown). Therefore it appeared that increased denA and anr mRNA levels were likely to have arisen as the result of one or more regulatory events.

Discussion

A daptive resistance to aminoglycosides correlates with a transient reduction in cytoplasmic accumulation of aminoglycoside and reduced bacterial killing. The transient and reproducible nature of adaptive resistance suggests that it is not the result of a mutational event, but rather a function of subpopulation selection, a regulatory event or a combination of both. Cross-resistance to other aminoglycosides, which is characteristic of adaptive resistance, also contrasts with enzymatic modification and ribosomal mutation, both of which tend to affect specific aminoglycosides. A daptive resistance persists with continuous aminoglycoside exposure, but is lost with subculture in aminoglycoside-free media. Stepwise increases in aminoglycoside concentration, beginning at the MIC, allow P. aeruginosa to grow in aminoglycoside concentrations (128 mg/L) that would normally eradicate it completely.

Adaptively resistant cultures with high MICs revert to parental susceptibility when grown in aminoglycoside-free media. The reduction in aminoglycoside accumulation in adaptively resistant P. aeruginosa suggests that the location of the impediment is either external to, or within the cytoplasmic membrane, as bacterial cell death requires aminoglycoside interaction with its 3OS ribosomal targets. Aminoglycoside adaptive resistance in P. aeruginosa appears to coincide with cytoplasmic membrane changes and is independent of changes in either lipopolysaccharide (LPS) or outer membrane protein (OMP).

This study demonstrated that P. aeruginosa cultures adaptively resistant to gentamicin have higher denA and anr mRNA levels than wild-type and post-adaptively resistant cultures. The increased mRNA levels are probably due to a cellular regulatory event rather than a mutational event, as DNA sequence changes could not be identified in the promoter region of either gene. Whether the elevated mRNA levels reflect increased mRNA stability, increased transcription or a combination of both remains to be determined. ANR has previously been demonstrated to regulate denA transcription positively; however, the factors regulating anr mRNA levels are presently unknown, as are the mechanism(s) by which adaptively resistant P. aeruginosa transmit the signal(s) for increased anr and denA mRNA levels.

A relationship between increased anaerobic respiratory pathway use and P. aeruginosa adaptive resistance to aminoglycosides may exist for several reasons. First, anaerobically grown P. aeruginosa accumulate aminoglycosides less effectively than aerobically grown P. aeruginosa. Second, cytoplasmic aminoglycoside accumulation has been shown to be dependent upon a functional aerobic respiratory pathway in addition to protonmotive force. P. aeruginosa cultures incubated with 1 mM KCN demonstrate significant reductions in aminoglycoside accumulation without appreciable change in cellular protonmotive force. Cyanochrome c oxidase is completely inhibited by 1 mM KCN, while nitrite reductase remains unaffected. Increased nitrite reductase expression implies less cellular reliance upon cytochrome c oxidase (aerobic respiratory pathway); however, this was not confirmed. Third, cyanide production in P. aeruginosa is also subject to ANR regulation. Increased anr mRNA levels during aminoglycoside adaptive resistance suggest that a coincident increase in cellular cyanide production may occur. This supposition again suggests that P. aeruginosa cultures adaptively resistant to aminoglycosides may possess reduced cytochrome c oxidase activity. A non-functional

Figure 2. Northern analysis of anr. Lane 1, wild-type ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853. Similarly, lanes 2, 2A, 2B contain F991 and lanes 3, 3A, 3B contain E1481. Lane 4 shows anr expression in anaerobically grown P. aeruginosa ATCC 27853.
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or suboptimally functioning cytochrome c oxidase implies minoglycoside resistance. Fourth, adaptively resistant P. aeruginosa commonly form small colonies and demonstrate reduced growth rates. Increased use of the anaerobic respiratory pathway, in the absence of preferred terminal electron acceptor molecules (e.g. nitrite, nitrate), would imply a lower culture growth rate, as the affinity of O₂ for nitrite reductase is significantly lower than for cytochrome c oxidase. Further, the observation that stationary phase cultures produce KCN (ANR-regulated) to a concentration of approximately 200–300 μM, suggesting decreased aerobic respiratory pathway function and increased cellular reliance upon the anaerobic respiratory pathway. In conclusion, these observations suggest that P. aeruginosa may regulate the expression of its two principal respiratory pathways in response to minoglycoside insult and this may explain, at least partially, P. aeruginosa adaptive resistance to minoglycosides.

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References


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