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Novel Mechanism for Fluoroquinolone Resistance in *Acinetobacter baumannii*

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An EZ::TN<R6Kγori/KAN-2>Tnp transposon insertion in an open reading frame of unknown function (*ncr*) in *Acinetobacter baumannii* resulted in an 8-fold increase in ciprofloxacin resistance (Cip^r). Transposon insertions in an *ncr* mutant that reduced Cip^r back to wild type mapped to three genes encoding subunits of the RecCBD exonuclease. The *ncr* mutation increased transcription of the *recCBD* genes, and overexpression of the *recCBD* genes in a wild-type background resulted in a 4-fold increase in Cip^r.

Acinetobacter baumannii is a Gram-negative bacterium that is capable of causing a wide variety of human infections (2, 3, 7, 10, 16). The ability to treat these infections has been complicated by the rapid increase in antibiotic resistance in this bacterium (8, 12, 13, 15, 17, 18). To better define the mechanisms that contribute to antibiotic resistance, a transposon insertion library, created in *A. baumannii* strain M2 with the EZ::TN<R6Kγori/KAN-2>Tnp transposon (Epicentre, Madison WI), was used to identify mutants with increased resistance to ciprofloxacin. Our original goal was to identify insertions that increased the expression of efflux systems resulting in multiple antibiotic resistance. One mutant, designated AB-4B, was obtained that exhibited an 8-fold increase in ciprofloxacin resistance (Cip^r): 2 μg/ml versus 0.25 μg/ml for the wild-type M2 parent (Table 1). The mutation in AB-4B also resulted in a 4-fold increase in the levels of resistance to ofloxacin and gatifloxacin (data not shown). However, the mutation in mutant AB-4B did not significantly alter the levels of resistance to chloramphenicol, gentamicin, tigecycline, rifampin, or ampicillin (data not shown). This suggested that the fluoroquinolone resistance was not due to increased expression of a multidrug efflux system. The EZ::TN<R6Kγori/KAN-2>Tnp insertion and flanking *A. baumanii* DNA were isolated by rescue cloning after digestion of chromosomal DNA with XbaI, followed by religation and transformation into *Escherichia coli* CC118 to identify plasmids containing chromosomal DNA along with the R6K plasmid origin present in the transposon. The site of insertion was mapped to an open reading frame (ORF) designated A1S_0815, based on the genome sequence of ATCC 17978 (19), and this gene is present in all *A. baumannii* isolates sequenced to date. In ATCC 17978, this ORF encodes a protein of 132 amino acids. However, in other *A. baumannii* isolates, this protein was 186 amino acids. Further analysis of the sequence surrounding the A1S_0815 gene indicated a frameshift error that likely accounted for the smaller size of the protein. In the AB-4B mutant, the EZ::TN<R6Kγori/KAN-2>Tnp transposon inserted at a position corresponding to amino acid 50 of the 186-amino-acid protein. The protein encoded by A1S_0815 had a conserved domain (DUF177 superfamily) that suggested a possible metal binding function. The protein encoded by A1S_0815 was similar to those present in other bacteria, including *Psychrobacter arcticus* (37% identity, 59% similarity; YP 263815.1), *Azotobacter vinelandii* (37% identity, 54% similarity; YP002798678.1), *Pseudomonas aeruginosa* (31% identity, 53% similarity), and *E. coli* YcdE (27% identity, 44% similarity). Given the role for this gene in Cip^r, it was renamed *ncr* (*n*ovel *c*iprofloxacin *r*esistance). In *A. baumannii* and the bacteria listed above, the A1S_0815-like gene was encoded immediately upstream of a putative *rpmF* (A1S_0816) gene encoding the 50S ribosomal protein L32. Reverse transcription-PCR (RT-PCR) analysis indicated that both genes formed an operon (data not shown).

TABLE 1 Ciprofloxacin resistance levels of *A. baumannii* strains in this study

Strain	Ciprofloxacin MIC (μg/ml)
M2	0.25
AB-4B *ncr*::EZ::TN<R6Kγori/KAN-2>Tnp	2
AB-0815 *ncr*::Sm^r	2
AB-4B/pWH1266	2
AB-4B/pWH1266-*ncr*	1
AB-4B::pKNG101-*ncr*	0.25

To verify that the *ncr*::EZ::TN<R6Kγori/KAN-2>Tnp insertion was responsible for the increased Cip^r in strain AB-4B, a null allele in the *ncr* gene of the wild-type M2 strain was recreated by the insertion of a suicide plasmid containing an internal region of the *ncr* gene generated by PCR using the primers 5′-GTGATCTA GATGCTCGTATTGCTCGTGAAG-3′and 5′-ACATGTCGACT GATGTTTATGTTCACAAGC-3′. These primers contained restriction sites for XbaI and SalI that were used to clone the fragment into pKNG101 (11). The plasmid was moved into *A. baumannii* by conjugation with *E. coli* SM10 λ*pir*. Exconjugants were verified to contain the *ncr*::pKNG101 (Sm^r) disruption by Southern blot analysis, and one strain, designated AB-0815, was

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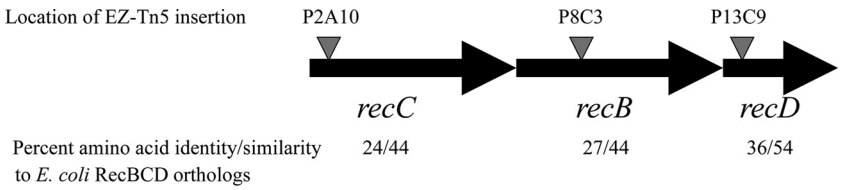

FIG 1 Transposon insertions that reverse ciprofloxacin resistance in AB-4B. The location of EZ::TN transposon insertions that reverse the high-level ciprofloxacin resistance is shown. The organization of the *recC*, *recB*, and *recD* genes is shown, and the percentages of amino acid identity and similarity to the corresponding *E. coli* gene products are shown below each gene.

used for further analysis. AB-0815 *ncr*::*Sm*^{r} exhibited a similar phenotype to strain AB-4B, with an 8-fold increase in Cip^r (Table 1). Both the *ncr*::*Sm*^{r} mutant and the original *ncr*::EZ::TN<R6Kγori/KAN-2>Tnp mutant exhibited a slow-growth phenotype and formed smaller colonies on agar plates. To further verify the role of the *ncr* mutation in resistance, we amplified the wild-type *ncr* gene by PCR using the primers 5′-TGCACCACGTCAGGTAAAAGAG-3′ and 5′-GCGCGGATCCAAATGGATCAGAACAAATTC-3′ and cloned this insert as a BamHI fragment into the *A. baumannii*/*E. coli* shuttle plasmid pWH1266 (9). In this plasmid (pWH1266-*ncr*), transcription of the *ncr* gene is driven by its native promoter. When pWH1266-*ncr* was introduced into the AB-4B *ncr*::EZ::TN<R6Kγori/KAN-2>Tnp mutant, the levels of Cip^r were decreased 2-fold to 1 μg/ml (Table 1), a level intermediate between those of the wild type and the *ncr* mutant. The basis for the partial complementation is unclear. However, when the same *ncr*-containing fragment was cloned into the suicide vector pKNG101 and integrated in single copy in AB-4B, the levels of Cip^r were reduced to 0.25 μg/ml.

As mentioned above, the genomic organizations of the *A. baumannii ncr* and *rpmF* genes were identical to that seen in *E. coli*, where the *ncr* homolog is designated *ycdE*. Null alleles of the *E. coli ycdE* and *rpmF* genes were obtained from the Keio collection (1) to determine if mutations in either of these genes resulted in a ciprofloxacin-resistant phenotype. Neither mutation had a significant effect on Cip^r, with MICs for the parent strain BW25113 of 0.5 μg/ml (data not shown).

To understand the molecular basis for the increased ciprofloxacin resistance in the *ncr*::Sm^r mutant, we utilized EZ::TN<KAN-2>Tnp transposon mutagenesis to generate mutations in AB-0815 that reversed the high-level Cip^r. Three insertions with this phenotype were mapped to a contiguous set of open reading frames that encoded products highly similar to the *recCBD* genes encoding the gamma, beta, and alpha subunits of the exonuclease V complex (Fig. 1) (4). The *A. baumannii* proteins exhibited the following amino acid identities and similarities, respectively, compared to the *E. coli* proteins: RecC, 27% and 44%; RecB, 24% and 44%; and RecD, 36% and 54%. The insertion in strain P13C9 disrupted *recD*, encoding the alpha subunit. The insertion in mutant P8C3 disrupted *recB*, encoding the beta subunit, and the insertion in mutant P2A10 disrupted *recC*, encoding the gamma subunit (Fig. 1). Each of these insertions resulted in a reduction in Cip^r in the *ncr*::Sm^r background from 2 μg/ml down to 0.25 to 0.125 μg/ml (Table 2). To verify that the above insertions in the *recCBD* locus were responsible for the decreased Cip^r in each of the mutants, the wild-type *recCBD* genes were amplified by PCR using the primers 5′-CGTCGGATCCGTCAACGCATCCATTACAGG-3′ and 5′-TAGCGGATCCTATCTCGAATCCATGTAAGC-3′ and cloned into pWH1266. When the *ncr*::*Sm*^{r} mutant containing each of the EZ-Tn*5* insertions in the *recCBD* locus was transformed with pWH1266 plus *recCBD*, the levels of Cip^r were increased back to that seen in the original mutant (2 μg/ml), indicating that loss of RecCBD function was responsible for the ciprofloxacin sensitivity (Table 2).

The above data indicated that the *ncr* mutation required a functional *recCBD* locus to mediate increased Cip^r. This predicted that the Ncr gene product might be involved in negative regulation of the *recCBD* genes and that, in turn, their overexpression was responsible for ciprofloxacin resistance. To investigate this possibility, transcription of the *recCBD* genes was monitored by cloning a 510-bp fragment extending 458 bp upstream and 52 bp downstream of the ATG start codon for RecC into the transcriptional promoter probe plasmid pQF50 (5) to create a *recC-lacZ* transcriptional fusion. This plasmid is unable to replicate in *A. baumannii*, but it can integrate into the chromosome by homologous recombination at the *recCBD* region, creating a *lacZ* fusion in single copy. The expression of *recC-lacZ* was measured at 10.1 ± 0.5 Miller units in the wild-type M2 background and 25.0 ± 0.4 Miller units in the AB-4B background, indicating that transcription of the *recCBD* operon was increased 2.5-fold by the *ncr* mutation (Fig. 2A). To independently confirm the increase in *recCDB* transcription in the *ncr* mutant, total RNA was prepared from both the wild type and the *ncr* mutant and semiquantitative RT-PCR was used to examine *recCDB* transcript levels in both strains. Using this analysis, a similar 2-fold increase in *recCDB* transcript levels was observed in the *ncr* mutant (Fig. 2B).

To determine if overexpression of the *recCBD* genes was sufficient to confer increased Cip^r, the plasmid pWH-*recCBD* was introduced into the wild-type M2 strain. M2 cells containing only the pWH1266 vector exhibited a MIC of 0.25 μg/ml. However, the presence of pWH-*recCBD* increased ciprofloxacin resistance 4-fold, with a MIC of 1 μg/ml.

In this study, two separate null alleles in the *ncr* gene conferred the same phenotype—an 8-fold increase in Cip^r. This phenotype was exclusively due to loss of Ncr function, as the cloned *ncr* gene

TABLE 2 Effect of *recCBD* mutations on ciprofloxacin resistance

Strain	Genotype	Ciprofloxacin MIC (μg/ml)
AB-0815/pWH1266	*ncr*::Sm^r	2
P2A10/pWH1266	*ncr*::Sm^r *recB*::EZ::TN<KAN-2>Tnp	0.125
P8C3/pWH1266	*ncr*::Sm^r *recC*::EZ::TN<KAN-2>Tnp	0.125
P13C9/pWH1266	*ncr*::Sm^r *recD*::EZ::TN<KAN-2>Tnp	0.25
AB-0815/pWH-recCBD	*ncr*::Sm^r	2
P2A10/pWH-recCBD	*ncr*::Sm^r *recB*::EZ::TN<KAN-2>Tnp	2
P8C3/pWH-recCBD	*ncr*::Sm^r *recC*::EZ::TN<KAN-2>Tnp	2
P13C9/pWH-recCBD	*ncr*::Sm^r *recD*::EZ::TN<KAN-2>Tnp	2

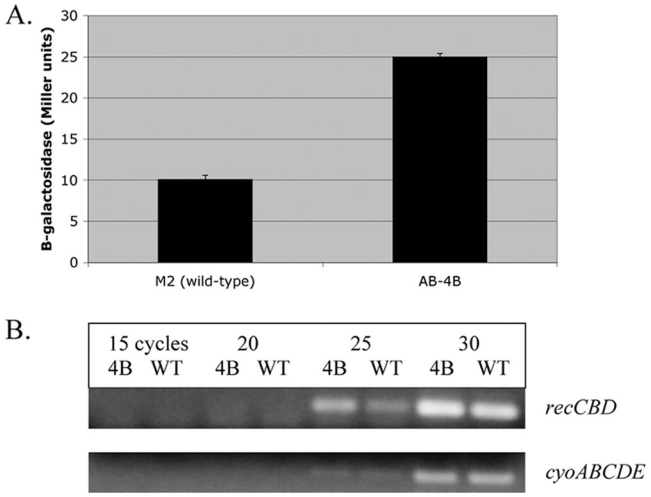

FIG 2 Effect of the *ncr* mutation on *recCBD* transcription. In panel A, the expression of a *recC-lacZ* fusion in both the M2 (wild-type) and AB-4B (*ncr*::EZ::TN<R6Kγori/KAN-2>Tnp) backgrounds is shown. In panel B, the expression of *recCBD* in wild-type M2 and AB-4B was monitored by semiquantitative RT-PCR. As an internal control, expression of the *cyoABCDE* operon was also examined from the same RNA samples. Samples were analyzed at 15, 20, 25, and 30 cycles. The absence of contaminating DNA from both samples was confirmed by the inability to generate PCR products in the absence of cDNA synthesis. The primers used for cDNA synthesis and subsequent RT-PCR for *recCBD* were 5′-ATTAAATGTAGCGTGTTCAG-3′, 5′-GAGCATCC TGAGCGCCAGAAG-3′, and 5′-CAATGTATTGCCCTAAACGGC-3′. For the *cyo* operon, the primers were 5′-ATGCGGATCCCAAGAGAAGATTTTC ACACC-3′, 5′-TGGTGATTCCTTCATTCATCATG-3′ and 5′-ACTAAATGC TCGATTTGGTGC-3′.

on a plasmid could complement these phenotypes, demonstrating that loss of *ncr* function and not polar effects on the downstream *rpmF* gene was responsible for Cip^r. Analysis of the Ncr protein did not reveal an obvious role in Cip^r, and although a number of Ncr orthologs are present in other bacteria, their function is unknown. The Ncr protein is annotated as having a metal binding domain (CO1399), and the lack of obvious signal sequences or transmembrane regions suggests it is localized to the cytoplasm. It was hypothesized that the loss of Ncr function altered the activity or expression of one or more gene products that then directly mediated Cip^r. A genetic analysis was then conducted to identify mutations that reversed the high-level Cip^r in the *ncr* mutant background. This revealed a key role for the *recCBD* genes in mediating the Cip^r. Moreover, the transcription of the *recCBD* genes was shown to be increased 2.5-fold in the *ncr* mutant. Taken together, this strongly suggested that the overexpression of the *recCBD* genes in the *ncr* background is responsible for the increased Cip^r. Consistent with this, overexpression of the *recCBD* genes on a multicopy plasmid was sufficient to increase the levels of Cip^r 4-fold. Although the Ncr protein acts as a negative regulator of the *recCBD* genes, the lack of clear homology to DNA binding proteins suggests this regulation may be indirect.

A relationship between the RecCBD system and Cip^r has been previously described by Gomez and Neyfakh, where loss of *recD* decreased Cip^r in *Acinetobacter baylyi* (6). Given that RecCBD functions in both DNA recombination and repair, the increased sensitivity to ciprofloxacin is likely due to the reduced ability to repair DNA damage mediated by the inhibition of DNA gyrase and topoisomerase. However, the ability of the *recCBD* genes to increase Cip^r when overexpressed appears to be a novel mechanism. Studies by Lopez et al. have demonstrated that ciprofloxacin stimulates recombination in a RecCBD-dependent manner in *E. coli* (14). Therefore, it is possible that increased expression of RecCBD in *A. baumannii* allows for a greater capacity to repair the DNA damage that results from the inhibition of DNA gyrase and topoisomerase.

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