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# Insight into Norfloxacin Resistance of *Acinetobacter oleivorans* DR1: Target Gene Mutation, Persister, and RNA-Seq Analyses S

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Antibiotic resistance of soilborne *Acinetobacter* species has been poorly explored. In this study, norfloxacin resistance of a soil bacterium, *Acinetobacter oleivorans* DR1, was investigated. The frequencies of mutant appearance of all tested non-pathogenic *Acinetobacter* strains were lower than those of pathogenic strains under minimum inhibitory concentration (MIC). When the quinolone-resistance-determining region of the *gyrA* gene was examined, only one mutant (His78Asn) out of 10 resistant variants had a mutation. Whole transcriptome analysis using a RNA-Seq demonstrated that genes involved in SOS response and DNA repair were significantly up-regulated by norfloxacin. Determining the MICs of survival cells after norfloxacin treatment confirmed some of those cells were indeed persister cells. Ten colonies, randomly selected from among those that survived in the presence of norfloxacin, did not exhibit increased MIC. Thus, both the low mutation frequency of the target gene and SOS response under norfloxacin suggested that persister formation might contribute to the resistance of DR1 against norfloxacin. The persister frequency increased without a change in MIC when stationary phase cells, low growth rates conditions, and growth-deficient *dnaJ* mutant were used. Taken together, our comprehensive approach, which included mutational analysis of the target gene, persister formation assays, and RNA sequencing, indicated that DR1 survival when exposed to norfloxacin is related not only to target gene mutation but also to persister formation, possibly through up-regulation of the SOS response and DNA repair genes.

**Keywords**: Bacteria, mutation, antibiotics, norfloxacin, persister, RNA-Seq

## Introduction

*Acinetobacter* species are ubiquitous in the environment and some have emerged as pathogens, such as *Acinetobacter baumannii* [4]. Resistance to many environmental chemicals, including antibiotics, is a key factor for enabling the survival of these organisms [4]. Fluoroquinolone is one of the most potent antibiotics against bacteria [1]. A feature of fluoroquinolones (*e.g.*, norfloxacin) is the rapid emergence of bacterial resistance. Fluoroquinolones bind to bacterial type II topoisomerases (DNA gyrase and topoisomerase IV) and inhibit bacterial DNA replication, resulting in double-strand breaks [30]. Repair processing of double-strand breaks cause the induction of the SOS response in *E. coli* [9]. Resistance to fluoroquinolones is known to involve mutations in their target gene, *gyrA*. In *Escherichia coli*, resistance to fluoroquinolones appears to be caused by mutations in the quinolone-resistance-determining region (QRDR) of the *gyrA* gene [42, 43]. Antibiotic-targeted gene mutation has been obsereved in *Acinetobacter* species for which a correlation between target gene mutations and alterations of physiological characteristics has been noted in our previous study [22].

Although target gene mutation could give rise to antibiotic resistance, some cells in the population are able to survive in the presence of antibiotics by persister formation. Persisters can be tolerant to antibiotics through metabolic inactivation, without the involvement of genetic mutations. Such dormant cells do not grow, or grow slowly, in the presence of antibiotics. However, these cells may

become active when conditions have enhanced and may re-initiate growth [6]. Although some global regulators such as DksA and HupAB of *E. coli* are reported to be involved in persister formation because knockout strains have decreased persister formation [27], little is known about the mechanisms and physiology of persister formation in *Acinetobacter* species.

With the recent introduction of next-generation sequencing, RNA sequencing has become more preferable for transcriptome analysis, where it has been overtaking microarray-based methods [37]. There are many advantages of transcriptome analysis through RNA sequencing, or RNA-Seq, such as the ability to detect virtually all transcripts, identification of sequence variations (*e.g.*, SNPs) in the transcribed regions, detection of a large dynamic range of expression levels, and a high degree of reproducibility [44]. Thus far, in *Acinetobacter* species, there is only one published report on RNA-Seq of the *A. baumannii* transcriptome, under ethanol treatment [7]. Most antibiotic resistance research on *Acinetobacter* species has focused especially on pathogenic *Acinetobacter* strains (*e.g.*, *Acinetobacter baumannii*), but the natural environment (*e.g.*, soil) may be a source of acquisition of antibiotic resistance [12]. Thus, the mechanisms of antibiotic resistance in soilborne *Acinetobacter* species, which have been only rarely explored, are a valuable line of study. In this study, we examined mutant frequency caused by norfloxacin in *Acinetobacter* species, which exist abundantly in both clinical and environmental sources. To understand the antibiotic resistance mechanisms of soilborne *Acinetobacter* species, *A. oleivorans* DR1 was chosen for our study in the presence of norfloxacin. Here, we present the data showing that not only target gene mutation but also persister formation are important for norfloxacin resistance in *A. oleivorans* DR1.

## Materials and Methods

### Antibiotics, Culture Media, Bacterial Strains, and Growth Conditions

The novel diesel-degrading *Acinetobacter oleivorans* DR1 was isolated from the soil of a Korea University paddy field (Deokso, Gyeonggi-Do, Korea) and its genome was completely sequenced in a previous study [18, 21]. Nutrient broth (NB) and nutrient agar (NA) plates were used to culture *Acinetobacter* species at 30°C. To modulate the growth rate of DR1 cells, Minimal Salts Basal media (MSB) [39], containing 10 mM succinate or 5 mM salicylate and diluted NB were used. Commercial antibiotics were purchased from Sigma (norfloxacin) and Bioshop (kanamycin). Growth was monitored by measuring the $OD_{600}$ of the liquid cultures using a Biophotometer (Eppendorf, Germany).

### Determination of MICs of Norfloxacin for *Acinetobacter* Species

The MICs were determined using the agar plating method [17]. Norfloxacin was used against *Acinetobacter* species. Eighteen *Acinetobacter* species colonies were suspended in 1 ml of phosphate-buffered saline (PBS, pH 7.4) and spread onto an NA plate containing each antibiotic at a different concentration. Norfloxacin MICs were tested in the range of 0 to 256 µg/ml. MICs were determined by spreading approximately $10^7$ CFU/ml of the PBS suspension. After spreading, the plates were incubated for 24 h at 30°C and examined for 99.9% killing. In the case of *Acinetobacter oleivorans* DR1 and the *dnaJ* mutant, MICs were determined by liquid nutrient medium using 96-well polystyrene microtiter plates (Costar, USA). Bacterial cells were inoculated into NB and incubated for 24 h at 30°C. Then, cells were harvested and washed three times with PBS. Each strain was inoculated at a density of $10^5$ CFU/ml in NB containing 0 to 256 µg/ml of norfloxacin and grown in 96-well plates for 24 h at 30°C. The MICs were estimated as the lowest concentration of norfloxacin at which the $OD_{600}$ was less than 0.01. Mutant frequency was calculated from the CFU/ml ratio (number of colonies in NA plate with the antibiotic corresponding to the MIC/number of colonies in NA plate without the antibiotic).

### Amplification and Sequencing of the *gyrA* Genes

In order to compare the sequences of the *gyrA* genes in norfloxacin-resistant variants of DR1 cells, the N-terminal of the quinolone-resistance-determining region were amplified *via* PCR using the gyrA17-F (5′-TCMGACCGATTGCCATTG-3′), and gyrA447-R (5′-DCCGTCGTAGTTRYCTTCCCARTC-3′) primers. This degenerate primer pair was designed by alignment of the *gyrA* sequences of *A. oleivorans* DR1 (GenBank Accession No. YP_003731109), *A. baumannii* (GenBank Accession Nos. NC_010400, NC_011586, NC_011595, NC_010611, and NC_010410), and *A. haemolyticus* (GenBank Accession No. ADMT01000199). Amplified PCR products (431 bp) were cloned into the pGEM-T easy vector *via* TA cloning and sequenced (Macrogen, Korea) for identification of mutation positions.

### RNA Extraction, Library Construction, and Sequencing

The cells were grown to exponential phase ($OD_{600}$ ~ 0.4) and treated with norfloxacin (4 µg/ml) over a period of 15 min. Total RNA was isolated from 5 ml of stationary cells, using the RNeasy Mini kit (Qiagen, USA), according to the manufacturer's instructions. All procedures for RNA sequencing and alignment of the transcriptome were conducted by Chunlab (Seoul, South Korea). The RNA was subjected to a subtractive Hyb-based rRNA removal process using the MICROBExpress Bacterial mRNA Enrichment Kit (Ambion). The following process, including library construction, was carried out as described previously [46]. RNA sequencing was performed using 2 runs of the Illumina Genome Analyzer IIx to generate single-ended 36 bp reads. The genome sequence and annotation information of *A. oleivorans* DR1 was obtained from the NCBI database (Accession No. NC_014259.1).

Quality-filtered reads were aligned to the reference genome sequence using the CLC Genomics Workbench 4.0 (CLC Bio). Mapping was based on a minimal length of 32 bp with an allowance of up to two mismatches. The relative transcript abundance was measured in reads per kilobase of transcript per million mapped sequence reads (RPKM) [32]. The genes that showed a fold change (RPKM of DR1-nor/RPKM of DR1-exp) larger than 2.0 and lower than 0.5 were regarded as up- and down-regulated genes, respectively. Mapped reads were visualized using the BamView and Artemis programs [8]. The RNA-Seq data were deposited in the National Center for Biotechnology Information (NCBI) GEO site under Accession No. GSE38340.

### Construction of the *dnaJ* Mutant in the DR1 Strain

The DR1 genome has been completely sequenced [18]. The fragment encompassing the partial *dnaJ* gene of *A. oleivorans* DR1 was amplified using primer pairs (dnaJ-f (5′-TGTCGTGGCCAA GGTAAAA-3′) and dnaJ-r (5′-TTGCCACGTAAACGGAATAAT-3′)). The PCR product for *dnaJ* was digested with the *Eco*RI restriction enzyme. Each fragment was subsequently inserted into the pVIK112 vector *via* ligation. The constructed plasmids were then transformed into *E. coli* S17-1λpir. Conjugation was conducted *via* biparental filter mating with the DR1 strain, thus creating a *dnaJ* mutant of the DR1 strain.

### Persistence Assay of DR1 Wild Type and the *dnaJ* Mutant with Norfloxacin

Wild-type and mutant cells were grown overnight in NB and subsequently diluted 100-fold. Then, the cells from exponential phase ($OD_{600}$ ~ 0.4, in the case of nutrient media culture) were exposed to norfloxacin (50 μg/ml, 12.5-fold MIC). Cells were collected at the indicated times (6 h in general cell survival test) and diluted in PBS, and spread onto NA plates. Colonies were counted on the following day, after overnight incubation at 30°C. Cell survival was determined from the relative percentage of CFU/ml ((CFU at the indicated times/CFU for the samples incubated for 0 h after adding antibiotics) × 100).

## Results

### Determination of MICs and Mutant Frequency of *Acinetobacter* Species

The *Acinetobacter* species tested in this study have different MICs in the presence of norfloxacin (Table 1). The range of MIC was 0.25 to 16 μg/ml in *Acinetobacter* species. *A. baumannii*, which is well known as a multidrug-resistant pathogen, showed the highest MIC values. A comparison of the sizes of the genomes available from NCBI did not show a relationship between the genome size and high MIC values of these *Acinetobacter* species. However, analysis of the mutant frequency showed that non-pathogenic and soilborne strains (*A. oleivorans* DR1, *A. calcoaceticus*, and *A. baylyi*) had a 10-fold lower frequency in the presence of norfloxacin compared with other *Acinetobacter* species. Interestingly, *Acinetobacter* species PY6, PR8, GH02, and B113 [25], which were recently isolated, have higher MICs, but lower mutant frequencies. It has been reported that other pathogenic bacteria such as *E. coli* and *Salmonella*

**Table 1.** Norfloxacin MICs and mutant frequency of *Acinetobacter* species.

| *Acinetobacter* species. | Genome size (Mb) | Source | MIC (μg/ml) | Mutant frequency |
|---|---|---|---|---|
| *Acinetobacter oleivorans* DR1 | 4.15 | Soil | 4 | $(2.89 \pm 0.55)10^{-8}$ |
| *Acinetobacter calcoaceticus* KACC 11541 | 3.86 | Soil | 0.25 | $(1.44 \pm 0.15)10^{-8}$ |
| *Acinetobacter* sp. GH02 | NA | Soil | 4 | $(3.04 \pm 0.31)10^{-8}$ |
| *Acinetobacter* sp. B113 | NA | Soil | 0.5 | $(3.03 \pm 1.12)10^{-9}$ |
| *Acinetobacter baylyi* KACC12224 | 3.60 | Activated sludge | 1 | $(1.48 \pm 0.06)10^{-8}$ |
| *Acinetobacter gerneri* KACC12227 | NA | Activated sludge | 0.5 | $(2.65 \pm 0.49)10^{-7}$ |
| *Acinetobacter* sp. PY6 | NA | Plant | 8 | $(6.61 \pm 1.63)10^{-10}$ |
| *Acineotbacter* sp. PR8 | NA | Plant | 8 | $(2.36 \pm 1.14)10^{-9}$ |
| *Acinetobacter baumannii* KCTC2508 | 3.97 | Human | 16 | $(2.61 \pm 1.11)10^{-7}$ |
| *Acinetobacter haemolyticus* KACC 12049 | 3.37 | Human | 4 | $(5.49 \pm 1.87)10^{-7}$ |
| *Acinetobacter johnsonii* KACC 12053 | 3.69 | Human | 2 | $(2.99 \pm 0.59)10^{-7}$ |
| *Acinetobacter junii* KACC12456 | 3.46 | Human | 4 | $(1.14 \pm 0.38)10^{-7}$ |
| *Acinetobacter ursingii* KACC12453 | NA | Human | 2 | $(3.06 \pm 1.33)10^{-7}$ |

The strains used in this study were *A. oleivorans* DR1 (KCTC 23045 = JCM16667), *A. calcoaceticus* KACC 11541$^{T}$ (= DSMZ 30006$^{T}$), *Acinetobacter* sp. GH02 (GenBank Accession No. GU593001.1), *Acinetobacter* sp. B113 (GenBank Accession No. EU883929.1), *A. baylyi* KACC 12224$^{T}$ (= DSMZ 14961$^{T}$), *A. gerneri* KACC 12227$^{T}$ (= DSMZ 14967$^{T}$), *Acinetobacter* sp. PY6, *Acinetobacter* sp. PR8, *A. baumannii* KCTC2508$^{T}$ (= DSMZ 30007$^{T}$), *A. haemolyticus* KACC 12049$^{T}$ (= DSMZ 6962$^{T}$), *A. johnsonii* KACC 12053$^{T}$ (= DSMZ 6963$^{T}$), *A. junii* KACC 12456$^{T}$ (= DSMZ 6964$^{T}$), and *A. ursingii* KACC 12453$^{T}$ (= DSMZ 16037$^{T}$). NA, not available.

showed hypermutability (over 1%) [26]. Thus, we speculated that some strains, which showed both low mutant frequency and higher MIC, had specific mechanisms of antibiotic resistance. *Acinetobacter oleivorans* DR1 was chosen for further study because it is non-pathogenic, had a lower mutant frequency but a relatively high MIC of norfloxacin among environmental strains, and its complete genome sequence is available.

**Target Gene Mutation in Norfloxacin-Resistant Variants**

We examined target gene mutations caused by norfloxacin in *Acinetobacter oleivorans* DR1. Ten resistant variants of DR1 were obtained by plating cells on NA plates containing norfloxacin (4 μg/ml, corresponding to norfloxacin MIC). In order to compare the sequences of the *gyrA* genes in norfloxacin-resistant variants of DR1 cells, the sequence of QRDR in norfloxacin-resistant variants was amplified and then sequenced. We did not find mutations in *gyrA*, except in one norfloxacin-resistant variant (His78Asn). Our data showed that mutation in the QRDR was very rare in DR1 cells.

**Transcriptional Profiling of *A. oleivorans* DR1 by RNA Sequencing**

To identify genes important for the norfloxacin resistance in *A. oleivorans* DR1, we conducted RNA sequencing. To determine the appropriate concentration of norfloxacin for differential gene expression, we measured the MIC in nutrient liquid media (4 μg/ml). The number of reads for two samples, which were designated as DR1-exp for exponential phase cells and DR1-nor for norfloxacin-treated cells, were 6,583,302 and 19,081,119, respectively (Supplementary Table S1). More than 98% of the reads were mapped to the reference genome sequence, demonstrating considerable sequencing quality. However, as shown in Supplementary Table S1, we detected 53 genes and 74 genes with a zero RPKM value in DR1-exp and DR1-nor samples, suggesting that some genes are expressed at a low level or that these are not easily detected because of experimental bias. Our analysis demonstrated that a total of 418 genes were expressed differentially by more than 2-fold (121 genes up-regulated and 297 genes down-regulated) in the DR1-nor cells, as compared with the DR1-exp cells (Supplementary Tables S2 and S3). Among both up- and down-regulated genes, approximately 44% (in up-regulated genes) and 46% (in down-regulated genes) were hypothetical proteins whose functions remained to be characterized. Interestingly, a large portion of these norfloxacin-induced hypothetical proteins exist in phage-related genomic regions, from 1,605,948 to 1,630,518 bp and from 1,659,243 to 1,709,595 bp (Fig. 1 and Supplementary Fig. S2). There are two putative-phage regions within the

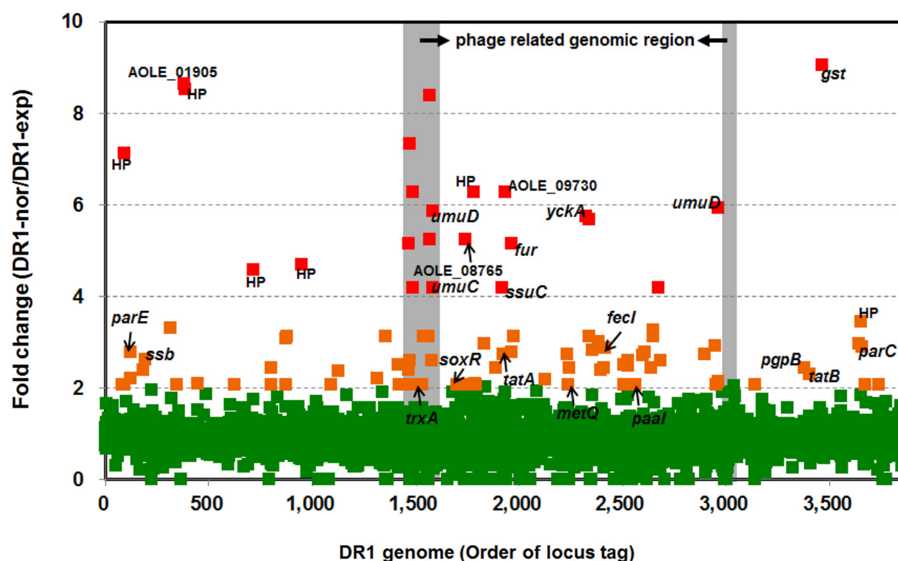


**Fig. 1.** Patterns of gene expression induced by norfloxacin in DR1.

Fold change (RPKM of DR1-nor/RPKM of DR1-exp) values were graphed according to the DR1 genome locus tag. Genes that had a fold change >4 are displayed in red, whereas those with a fold change ≤4, but ≥2, are displayed in orange. Green spots indicate genes that showed no significant change of expression, or had reduced expression. Phage-related genomic regions are displayed in gray. Hypothetical proteins are designated as HP.

DR1 genome [19] and one of the two regions, which was influenced by norfloxacin in this study, is a DR1-specific genomic region among the *Acinetobacter* species. Moreover, the expression of AOLE_07390, encoding a phage integrase, was increased 2.1-fold by norfloxacin. On the other hand, under ethanol stress conditions, a phage-related gene (AS1_0661, phage intergrase family protein, P-4 like integase) was down-regulated in *A. baumannii* [7]. Another study found that 22 phage-related genes were up-regulated by low concentrations of norfloxacin (200 ng/ml) in *E. coli* O157:H7 [15]. To explore the plaque or phage particles formation by norfloxacin treatment, we tested phage induction assays as described in previous studies [3, 40, 41]. DR1 cells were treated with 4 µg/ml of norfloxacin, 0.25 mM of hydrogen peroxide, and UV irradiation for 60 sec to induce any bateriophage. However, we could not observe any plaque and phage particles. We speculated that antibiotic stress induces phage-related genes and that might contribute to the genetic rearrangement of the bacterial strain. The genes involved in replication, recombination, and repair and in inorganic ion transport and metabolism showed an increased expression level in the DR1-nor cells (Table 2 and Supplementary Fig. S1).

Norfloxacin inhibits type II topoisomerases, which include DNA gyrase and topoisomerase IV [16]. In RNA-Seq analysis, DNA topoisomerase IV was up-regulated (AOLE_18380, DNA topoisomerase IV subunit A, 2.92-fold change; AOLE_00595, DNA topoisomerase IV subunit B, 2.80-fold change), but there was no difference in the expression of DNA gyrase (1.24-fold change in AOLE_00020, DNA gyrase B subunit; 0.99-fold change in AOLE_04195, DNA gyrase A subunit). We speculated that the 5.17-fold increase in the expression of *recA* (AOLE_07375), which functions in the repair of DNA [2], was a result of increased recombinational repair of double-strand DNA damage induced by the inhibition of type II topoisomerases in norfloxacin-treated cells. AOLE_09730 (peptide ABC transporter membrane protein), AOLE_11685 (*yckA*, ABC-type amino acid transport system, permease component), AOLE_09655 (*ssuC*, ABC transporter permeaseABC-type metal ion transport system), and AOLE_11250 (*metQ*, ABC-type metal ion transport system) were induced by norfloxacin (Fig. 1), and the products of these genes participate in transport mechanisms and are membrane-related. The up-regulation of membrane-related genes involved in transport could contribute to antibiotic resistance. A greater number of genes were down-regulated, than up-regulated, by norfloxacin treatment (Supplementary Table S3). In particular, the transcriptomes of *A. oleivorans* DR1 showed that genes involved in energy production and amino acid metabolism were down-regulated by norfloxacin (Supplementary Table S3).

**Persister Formation of *A. oleivorans* DR1 Under Norfloxacin Treatment**

SOS response induces persistence to fluoroquinolone antibiotics in *E. coli* [5, 11]. Our transcriptomic analyses showed that the expression of many genes (*recA*, *umuCD*, *gst*, and *trxA*; see Table 2) involved in SOS response was changed under norfloxacin treatment. In addition, the result that DR1 showed a lower mutant frequency but a higher MIC of norfloxacin led us to examine other specific mechanisms of antibiotic resistance. Thus, we focused on norfloxacin resistance cells for further analysis of persister formation. Persisters are specialized cells that are highly

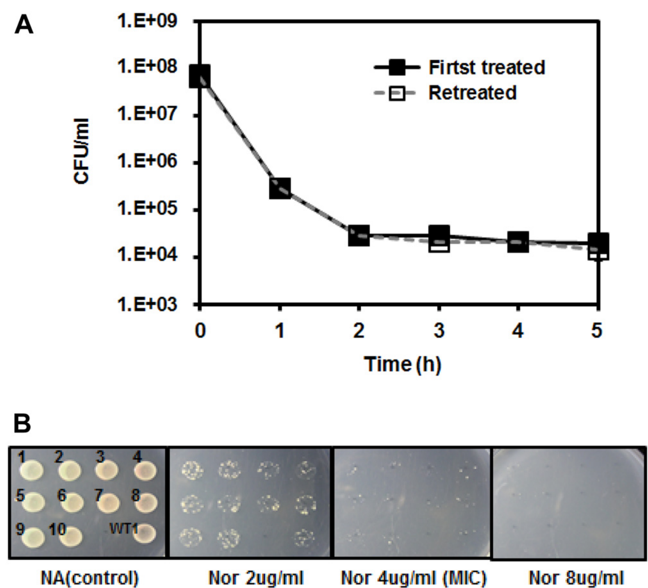


**Fig. 2.** Persister formation of DR1 in the presence of norfloxacin.

(**A**) Time-dependent killing of DR1 by norfloxacin. Exponentially grown DR1 cells were exposed to norfloxacin (50 µg/ml). Each time after addition of antibiotics, the cells were harvested and spread onto antibiotic-free plates. Cell survival was determined from the relative percentage of CFU/ml ((CFU after the each time incubation with antibiotics/CFU 0 h after addition of antibiotics) × 100). All data show the average of three replicates, and error bars indicate the standard deviation. (**B**) Confirmation for alterations of MIC in survived cells. Ten colonies randomly selected from those that survived on antibiotic-containing plates (approximately $10^5$ CFU/ml) were spotted onto NA, with or without antibiotic. All experiments were performed three times, and one representative image is shown. NA, no antibiotic; Nor, norfloxacin.

**Table 2.** Up-regulated genes under norfloxacin treatment condition in *A. oleivorans* DR1 compared with no treatment during the exponential phase.

| Locus tag | Product | Fold change |
|---|---|---|
| | **Amino acid metabolism and transport** | |
| AOLE_09730 | Peptide ABC transporter membrane protein | 6.29 |
| AOLE_11685 (*yckA*)[a] | ABC-type amino acid transport system, permease component | 5.77 |
| AOLE_08765 | Shikimate dehydrogenase | 5.24 |
| AOLE_11990 | 2-Aminoethylphosphonate:pyruvate transaminasee | 3.02 |
| | **Lipid metabolism** | |
| AOLE_11230 | Acyl-CoA dehydrogenase | 2.75 |
| AOLE_16945 (*pgpB*)[a] | Membrane-associated phospholipid phosphatase | 2.45 |
| AOLE_08710 | 3-Oxoacyl-(acyl-carrier-protein) reductase | 2.10 |
| | **Transcription** | |
| AOLE_12045 | Transcriptional regulator, TetR family protein | 6.66 |
| AOLE_14875 (*umuD*)[a] | DNA polymerase V component | 5.94 |
| AOLE_07970 (*umuD*)[a] | DNA polymerase V component | 5.87 |
| AOLE_11745 | SOS-response transcriptional repressor | 3.15 |
| AOLE_14800 (*fecI*) | RNA polymerase sigma factor FecI | 2.94 |
| AOLE_12135 (*soxR*) | Redox-sensitive transcriptional activator SoxR | 2.88 |
| AOLE_08565 | AraC-type DNA-binding domain-containing protein | 2.10 |
| AOLE_12595 | TetR family transcriptional regulator | 2.10 |
| | **Replication, recombination, and repair** | |
| AOLE_07375 (*recA*) | Recombinase A | 5.17 |
| AOLE_07965 (*umuC*)[a] | DNA-directed DNA polymerase UmuC | 4.19 |
| AOLE_18380 (*parC*) | DNA topoisomerase IV subunit A | 2.92 |
| AOLE_00595 (*parE*) | DNA topoisomerase IV subunit B | 2.80 |
| AOLE_00960 (*ssb*)[a] | ssDNA-binding protein controls activity of RecBCD nuclease | 2.64 |
| AOLE_00910 (*uvrA*)[a] | Excinuclease ABC subunit A | 2.40 |
| AOLE_14880 | DNA-directed DNA polymerase UmuC | 2.13 |
| AOLE_07390 | Phage integrase | 2.10 |
| AOLE_04385 (*ruvB*) | Holliday junction DNA helicase RuvB | 2.09 |
| | **Posttranslational modification, protein turnover, chaperones** | |
| AOLE_17410 (*gst*) | Glutathione *S*-transferase | 9.06 |
| AOLE_07635 (*trxA*) | Thioredoxin | 2.10 |
| | **Inorganic ion transport and metabolism** | |
| AOLE_09875 (*fur*)[a] | Ferric uptake regulation protein (ferric uptake regulator) | 14.34 |
| AOLE_09655 (*ssuC*)[a] | ABC transporter permease | 4.19 |
| AOLE_01565 | Putative regulatory or redox component complexing with Bfr | 3.32 |
| AOLE_09215 | Iron ABC transporter ATP-binding protein | 2.97 |
| AOLE_12705 | Substrate-binding region of ABC-type glycine betaine transport system | 2.49 |
| AOLE_11250 (*metQ*)[a] | ABC-type metal ion transport system | 2.10 |
| | **Secondary metabolites biosynthesis, transport, and catabolism** | |
| AOLE_12875 (*paaI*)[a] | Phenylacetic acid degradation protein | 2.10 |
| | **Intracellular trafficking, secretion, and vesicular transport** | |
| AOLE_09665 (*tatA*)[a] | Sec-independent protein secretion pathway, translocase protein | 2.75 |
| AOLE_17090 (*tatB*)[a] | Sec-independent protein translocase protein | 2.31 |

[a]Gene symbols from *Acinetobacter calcoaceticus* PHEA-2. Similarities of these genes between *A. oleivorans* and *A. calcoaceticus* PHEA-2 are more than 70%.

tolerant to antibiotics without mutation. It has been reported that persister formation can be initiated after DNA damage and the induction of the SOS response, or by signaling molecules produced by neighboring cells [27]. Although persister formation has been widely studied in *E. coli*, the mechanism and pattern of persister formation in other species has received little attention. To test the ability of *A. oleivorans* DR1 to form persister cells, exponentially grown $10^8$ CFU/ml cells were exposed to norfloxacin (50 μg/ml). At the indicated time, cells were harvested and spread onto antibiotic-free plates. Approximately $10^4$ CFU/ml of cells survived after 5 h of treatment with norfloxacin (Fig. 2A). We determined the MICs of surviving cells after antibiotic treatment to confirm whether these cells were indeed persister cells. Ten colonies, randomly selected from among those that survived in the presence of norfloxacin, did not exhibit increased MICs. The surviving cells were re-treated with norfloxacin and spread onto an NA plate again. The time-dependent survival curve showed the same pattern as that of the initial cells (Fig. 2A).

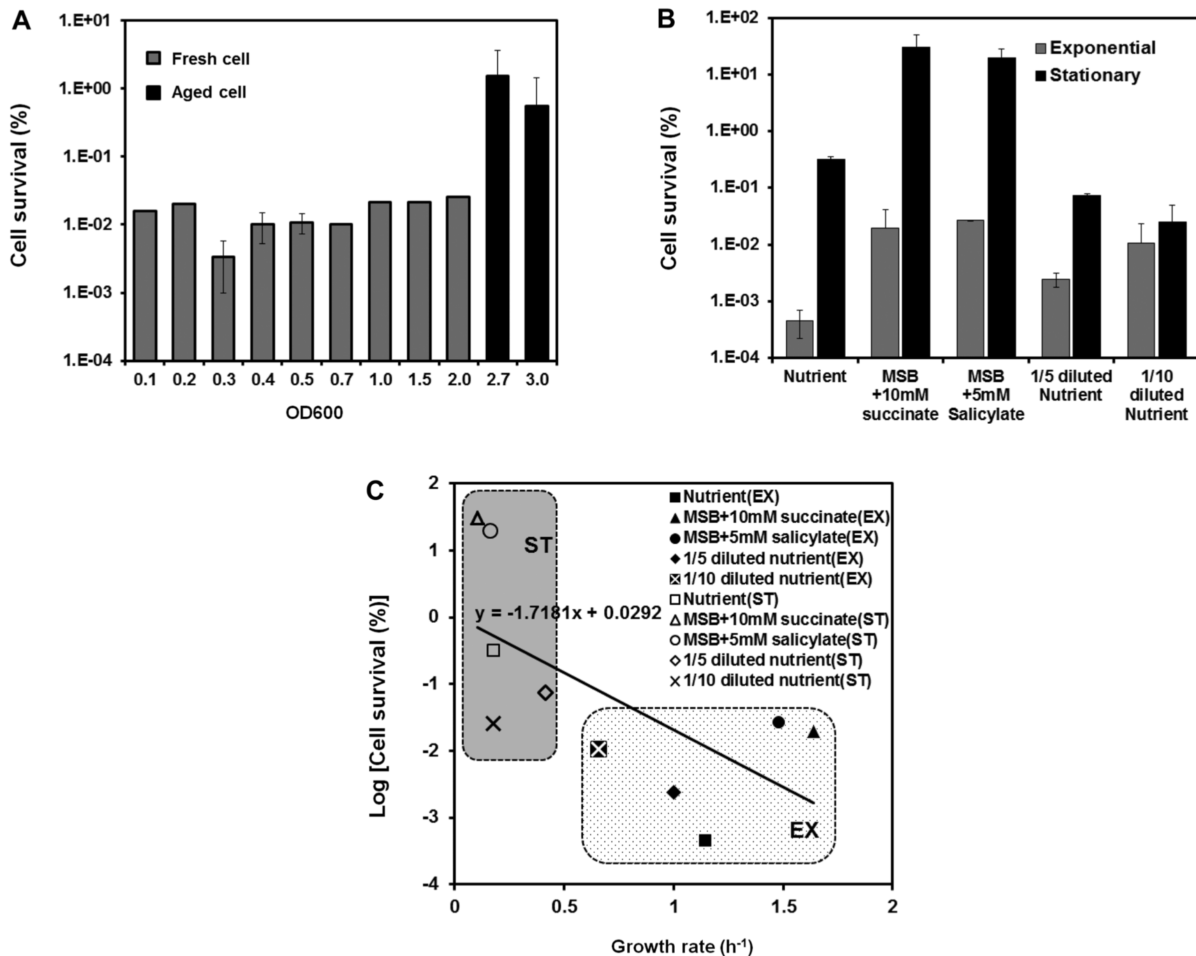


**Fig. 3.** The growth rate strongly influences persister cell formation.

(**A**) The cultures were diluted 100-fold in fresh media and incubated until cells reached each $OD_{600}$ (these cells were designated as fresh cells; gray bar). At this time point, cells were treated with norfloxacin (50 μg/ml) and incubated for 6 h. Wild-type cells were grown for 48 and 72 h (these cells were designated as aged cells; black bar). (**B**) The cell survival test was performed on cells at the exponential (EX; gray bar) and stationary (ST; black bar) growth phases in five different media. Cell survival was determined as the relative percentage of CFU/ml ((CFU after the 6-h incubation with antibiotics/CFU 0 h of incubation after adding antibiotics) × 100). All data show the average of three replicates, and error bars indicate the standard deviation. (**C**) The scatter diagram between growth rate and persister formation. A statistically significant difference ($p$ = 0.02) was observed between the two groups (EX, dotted pattern box; ST, gray box) by using Student's $t$-test. Regression analysis yielded a β coefficient of -1.7181 ($p$ = 0.04), indicating a statistically significant negative correlation between the growth rate and persister formation. All statistical analyses were performed using SPSS, ver. 12.0.

Thus, we confirmed that all remaining cells were persisters that did not have any heritable changes.

**Influence of Growth Rate on Persister Formation**

A previous report showed that persisters are strongly influenced by the age of the inoculum and the media conditions [29]. We speculated that the growth rate might affect the production of dormant persister cells. To test this hypothesis directly, persister formation was compared at each growth phase with the same amounts of cells. DR1 cells were inoculated into NB and incubated for 24 h at 30°C. The cultures were 100-fold diluted in fresh nutrient and incubated until cells reached specific values of $OD_{600}$ (0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 1.0, 1.5, and 2.0) (These cells were designated as fresh cells.). At each time point, cells were treated with norfloxacin (50 μg/ml) and incubated for 6 h. In addition, persister formation was observed using cells grown for 48 and 72 h; these were designated as aged cells. Wild-type cells were grown for 48 and 72 h, and then norfloxacin was added to these aged cells. Six hours after the addition of antibiotics, cells were harvested and spread onto NA plates. Cell survival was calculated as the percentage of the initial cells that remained after treatment with antibiotics. Interestingly, the aged cells had an increased survival, more than 100-fold and 36-fold under norfloxacin stress, compared with the average percent survival of fresh cells (Fig. 3A).

To further delineate the relationship between growth rate and persister formation, we attempted to artificially change the growth rate using different growth media, including two carbon sources and diluted NB. We performed the cell survival test on cells at the exponential (EX) and stationary (ST) growth phases in each of the five growth media. In all of the tested media, stationary cells formed more persisters than exponential phase cells; 705-fold in nutrient media, 1,585-fold in MSB containing 10 mM succinate, 511-fold in MSB containing 5 mM salicylate, 27-fold in 1/5 diluted NB, and 25-fold in 1/10 diluted NB (Fig. 3B). We plotted the percentage of cell survival against the growth rate at each cellular growth phase (Fig. 3C). We found a statistically significant difference ($p = 0.02$) between the two groups (EX and ST) by using Student's *t*-test. By performing regression analysis, we found a negative correlation between growth rate and persister formation (the β coefficient was -1.7181, $p = 0.04$). Disruption of DnaJ decreased growth of DR1 in nutrient media (Fig. 4A). The *dnaJ* mutant had a longer lag phase (~7 h) compared with that of the wild-type parent (~2 h), and the growth rates ($h^{-1}$) of wild-type and *dnaJ* mutant DR1 were 2.01 ± 0.01 and 0.78 ± 0.02, respectively. We compared the persister cell formation of wild-type DR1 and the *dnaJ* mutant with 50 μg/ml norfloxacin. The *dnaJ* mutant formed more number of persister cells (more than 10-fold) than the wild-type strain did (Fig. 4B). We identified that the slow growth of the *dnaJ* mutant had a great influence on persister formation.

## Discussion

In this study, we determined the MICs and mutant frequencies of 13 *Acinetobacter* species in the presence of

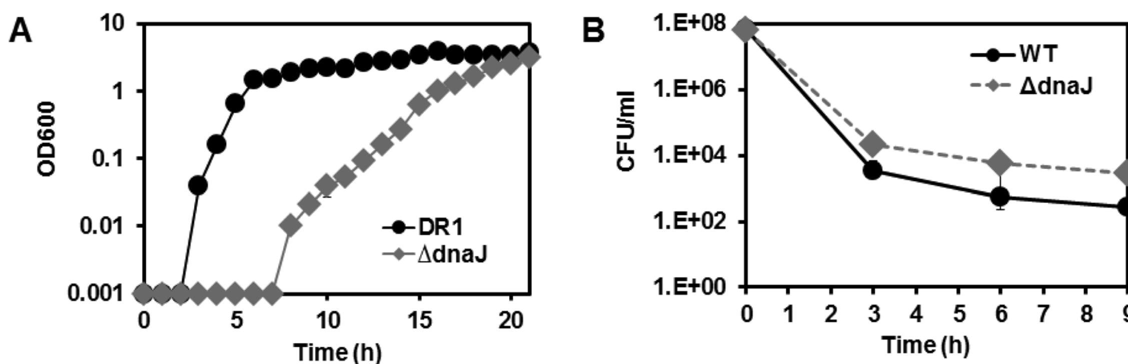


**Fig. 4.** Pattern of persister formation of wild-type DR1 and the *dnaJ* mutant.

(**A**) Growth curve of wild-type DR1 (black circle) and the *dnaJ* (gray diamond) mutant. Cells were grown at 30°C and the growth of each strain was monitored by measuring the $OD_{600}$ of cultures. (**B**) Persister formation of wild-type DR1 (black circle) and the *dnaJ* mutant (gray diamond). Wild-type and mutant cells were grown overnight in NB and subsequently diluted 100-fold. Then, when the diluted cells reached the exponential phase, they were exposed to norfloxacin (50 μg/ml). Aliquots of cells were withdrawn at the indicated times and diluted in PBS, and spread onto NA plates. Colonies were counted on the following day, after overnight incubation at 30°C. All data show the average of three replicates, and error bars indicate the standard deviation.

norfloxacin. The fluoroquinolones such as norfloxacin are widely used antibiotics that inhibit DNA gyrase (encoded by *gyrA*) or topoisomerase IV (encoded by *parC*) [20]. The level of resistance to fluoroquinolone and the mutation frequency of *gyrA* and *parC* are different among bacterial strains [20, 31]. In this study, we only identified missense mutations in *gyrA*. Thus, further studies will be needed to test whether mutations of *parC* or other genes also occur in antibiotic-resistant strains of DR1, to more deeply understand their nofloxacin resistance mechanisms. The low frequency of target gene mutation induced by norfloxacin led us to investigate persister formation, which is one of the mechanisms of acquisition of antibiotic resistance.

Fluoroquinolones can induce the SOS response [34], which is induced by a variety of environmental factors such as UV radiation, chemicals, and antibiotics. The *recA* gene (AOLE_07375), which plays key roles in the SOS response, was highly induced by norfloxacin in DR1. RecA is vital to the defense of DNA damage induced upon UV irradiation, antimicrobial compounds, and different stressors in *Acinetobacter* species [2, 14, 35]. Atypical SOS responses of *Acinetobacter* is worthy of further investigation because LexA-like tanscriptional repressor of the SOS gene network has not yet been identified in any *Acinetobacter* species [33, 36]. In a previous study from our group, this pattern was also observed in *Pseudomonas putida* and *Pseudomonas aeruginosa* when bacteria were exposed to ampicillin [45]. Many replication and DNA repair genes are strongly up-regulated, in both RNA-Seq analysis from this study and in microarray-based analysis [45], despite the difference in bacterial strains and types of antibiotics. Thus, the response of antibiotic-induced DNA damage can be a common mechanism for acquiring resistance. Iron availability affects antibiotic-mediated cell death in *Pseudomonas* [45]. In our transcriptome analysis, genes involved in iron uptake (*fur*, AOLE_09875, ferric uptake regulation protein; AOLE_09215, iron ABC transporter ATP-binding protein; AOLE_11250, ABC-type metal ion transport system) were up-regulated by norfloxacin. These results suggested that iron homeostasis might play an important role in norfloxacin-treated DR1 cells. In our RNA-Seq analysis, genes involved in drug transport were up-regulated by norfloxacin. Similarly, in *Acinetobacter baumannii*, which is a major pathogen, major fusion protein (MFP), resistance-nodulation-cell division (RND) efflux protein, and outer membrane factor (OMF) were shown to have important roles in norfloxacin resistance [10].

Here, we showed that persister formation of DR1 cells was strongly influenced by the growth rate in the presence of norfloxacin, which is consistent with other observations with *P. aeruginosa*, *E. coli*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* in the presence of fluoroquinolone antibiotics [23, 24, 38]. It has been also reported that growth-deficient mutants of *E. coil* were more tolerant to antibiotics owing to slower growth rate [13, 28]. These findings were supported by the fact that persister cells repressed genes involved in oxidative phosphorylation and consequentially induced a shutdown of metabolism [28]. Through analysis of the pattern of differentially expressed genes identified by RNA-Seq, we demonstrated that cells were damaged when exposed to norfloxacin, at a concentration corresponding to the MIC (4 μg/ml), and arrested transcription of genes involved in various metabolic and energy-producing processes. Here, our data suggested that acquisition of norfloxacin resistance in soilborne *A. oleivorans* DR1 is more complex than we thought: target gene mutation, persister formation, and up-regulation of SOS response and DNA repair genes.

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