Identification of the enzyme responsible for \(N\)-acetylation of norfloxacin by *Microbacterium* sp. strain 4N2-2

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ABSTRACT

*Microbacterium* sp. 4N2-2, isolated from a wastewater treatment plant, converts the antibacterial fluoroquinolone, norfloxacin, to *N*-acetylnorfloxacin and three other metabolites. Because *N*-acetylation results in loss of antibacterial activity, identification of the enzyme responsible is important for understanding fluoroquinolone resistance. The enzyme was identified as glutamine synthetase (GS); *N*-acetylnorfloxacin was produced only under conditions for GS expression. The GS gene (*glnA*) was cloned and the protein (53 kDa) was heterologously expressed and isolated. Optimal conditions and biochemical properties (*K_m* and *V_max*) of purified GS were characterized; the purified enzyme was inhibited by Mn^{2+}, Mg^{2+}, ATP and ADP. The contribution of GS to norfloxacin resistance was shown by using a norfloxacin-sensitive *Escherichia coli* strain carrying *glnA* derived from *Microbacterium* sp. 4N2-2. The GS of *Microbacterium* sp. 4N2-2 was shown to act as an *N*-acetyltransferase for norfloxacin, which produced low-level norfloxacin resistance. Structural and docking analysis identified potential binding sites for norfloxacin at the ADP-binding site and for acetyl-CoA at a cleft in GS. The results suggest that environmental bacteria whose enzymes modify fluoroquinolones may be able to survive in the presence of low fluoroquinolone concentrations.

INTRODUCTION

Fluoroquinolones are widely used as human and veterinary antimicrobial agents (19). Because their persistence in the environment (18) may act as selective pressure for naïve strains to acquire resistance (44), an understanding of the fate of these drugs should help to prevent drug resistance. The principal resistance mechanisms for fluoroquinolones include: (i) mutation of
genes for the drug targets (GyrA, GyrB and ParC) (20, 21, 41), (ii) mimicking the drug targets (QnrA, QnrB, and QnrS) (16, 22, 28), (iii) reduction of drug accumulation in the cells (AcrAB-TolC and QepA) (6, 20, 48), and (iv) enzymatic modification of the drug [N-acetylation by AAC(6')-Ib-cr] (23, 34, 39).

In some strains of *Escherichia coli*, a mutated aminoglycoside acetyltransferase, AAC(6')-Ib-cr, acetylates fluoroquinolones at the N-terminal of the piperazine ring (23, 34, 39) and enhances bacterial resistance to these drugs (39). Production of N-acetylnorfloxacin and N-nitrosonorfloxacin by environmental *Mycobacterium* sp. strains has also been found, but the enzymes responsible for modifications are unknown (1) and the aac(6')-Ib-cr variant gene has not been detected in these strains (23).

A norfloxacin-modifying bacterium, *Microbacterium* sp. strain 4N2-2, was isolated from wastewater (25). Some strains of this genus have been isolated from human clinical specimens; and the majority of them have shown fluoroquinolone resistance (12). *Microbacterium* sp. 4N2-2 transforms norfloxacin into four different metabolites, including N-acetylnorfloxacin, and cell extracts of this strain catalyze the N-acetylation of norfloxacin (25). N-Acetylation is enhanced by Casamino acids and inhibited by ammonium and nitrate (25), suggesting that the enzyme responsible for norfloxacin N-acetylation might also be involved in nitrogen metabolism.

In the present study, an enzyme with N-acetyltransferase activity was isolated from *Microbacterium* sp. 4N2-2 and identified. The coding gene was cloned and heterologously expressed in *E. coli* for biochemical characterization of the enzyme and its contribution to norfloxacin resistance. Binding models of the enzyme with norfloxacin and cofactor were proposed by *in silico* structure modeling and ligand-binding (docking) analysis. The results
provide insight into the potential for enzymes of environmental bacteria to contribute to drug resistance.

**MATERIALS AND METHODS**

**Strains, chemicals and media.** *Microbacterium* sp. strain 4N2-2 was isolated from a wastewater treatment plant in Little Rock, Arkansas (25). *E. coli* BL21 (DE3) pLysS, for heterologous overexpression of the target protein, was purchased from Novagen (EMD Millipore, Billerica, MA). Both strains were stored in 20% glycerol at −80°C.

Norfloxacin (Sigma-Aldrich, St. Louis, MO) was prepared as a 10 mg ml⁻¹ stock solution in 40 mM KOH for cultures and enzyme assays.

OM medium (25) was used as the basal medium for cultures of *Microbacterium* sp. 4N2-2. For extraction of proteins, cultures were grown in OM medium with 2.0 g l⁻¹ Casamino acids instead of ammonium and nitrate (25). When testing the effects of nitrogen sources on N-acetylation, 1.0 g l⁻¹ of glutamate, glutamine or ammonium was substituted. Cultures of *Microbacterium* sp. 4N2-2 were incubated at 30°C with shaking at 200 r.p.m., for 9 days for protein extraction or for 10-17 days for N-acetylation analysis.

LB broth with 10 g l⁻¹ NaCl (BD Biosciences, Franklin Lakes, NJ) was used for cultures of *E. coli* and LB agar (20 g l⁻¹ agar) was used for the norfloxacin disk assay. Cultures of *E. coli* were incubated aerobically at 37°C with shaking at 200 r.p.m. for 18 h.

**High-performance liquid chromatography.** After cultures were centrifuged (13,000 × g) and filtered (0.45 μm), 10 μl of the spent medium was directly injected for HPLC analysis of norfloxacin N-acetylation at 280 nm as previously described (25).
**N-Acetylation by cell extracts.** After incubation in OM medium with Casamino acids for 10-17 days, cells of *Microbacterium* sp. 4N2-2 were washed twice and suspended in 50 mM HEPES buffer (pH 7.5) containing 10% glycerol. Four ml of cell suspension was disrupted by passing five times through a French Pressure Cell at 137,000 kPa. Cell debris was removed by centrifugation (13,000 × g) and filtration (0.22 μm). Various pH values, temperatures, and amounts of enzyme, substrate, and cofactors were used to evaluate the optimum conditions for the reaction. Cell extracts were incubated with 0.06 mM norfloxacin, 0.2 mM acetyl-CoA (39) and 5.0 mM CaCl₂ in 50 mM Tris-HCl buffer (pH 8.0) at 45°C. Production of N-acetylnorfloxacin was monitored by direct HPLC analysis of incubated reaction mixtures and controls.

**Purification of the enzyme responsible for N-acetylation.** Ammonium sulfate was added to cell extracts to a final concentration of 1.0 M; the mixture was centrifuged and filtered (0.45 μm). The filtrate was applied to a HiPrep phenyl FF 16/10 column (GE Healthcare, Piscataway, NJ). The proteins were eluted as follows: 60 ml of 1 M (NH₄)₂SO₄, 60 ml of 0.5 M (NH₄)₂SO₄, 60 ml of 0.2 M (NH₄)₂SO₄, and 60 ml of water. The active fractions [eluted by 0.2 M (NH₄)₂SO₄], as shown by HPLC, were collected, concentrated and diluted in 25 mM piperazine-HCl buffer (pH 5.0). The fractions were applied to a Q XL column (GE Healthcare). The proteins then were eluted as follows: 60 ml of 25 mM phosphate buffer, 60 ml of a linear gradient to 0.2 M NaCl, 60 ml of 0.5 M NaCl, and 60 ml of 1 M NaCl. The fractions with N-acetylation activity eluted by 0.5 M NaCl were collected and applied to a Mono Q HR 5/5 column (GE Healthcare). The proteins were eluted with a linear gradient of 0 to 1 M NaCl.

**Protein identification by mass spectrometry.** Proteins from the Mono Q column were separated on an SDS-PAGE gel (40). The bands were excised, destained with 25 mM
NH₄HCO₃/50% acetonitrile, cut into smaller pieces and dried in vacuo. Proteins were then digested with trypsin (Promega, Madison, WI) at 37°C for 16 h in 25 mM NH₄HCO₃ (pH 8.3). The resulting peptides were extracted with aqueous 70% acetonitrile/5% formic acid under sonication and lyophilized.

The tryptic peptides from each gel band were analyzed by reversed-phase nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) (10). Briefly, the peptides were redissolved in 0.1% formic acid and injected onto a 9 cm × 75 μm (i.d.) fused silica capillary electrospray ionization (ESI) C₁₈ column, which was coupled online to an Orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Electron, San Jose, CA). Mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) were delivered by a Dionex UltiMate 3000 Nano and Cap LC system (Dionex Softron GmbH, Germering, Germany). Peptides were separated using a step gradient of 2-42% solvent B for 40 min and 42-98% solvent B for 10 min at a flow rate of 250 nL/min. The mass spectrometer was operated in a data-dependent mode, in which the seven most abundant peptide molecular ions from an MS survey scan (acquired in the Orbitrap analyzer) were dynamically selected for collision-induced dissociation (CID) and analyzed in the linear ion trap using a normalized collision energy of 35%.

The raw data from tandem mass spectrometry were searched against the Microbacterium testaceum protein sequence database (31) from the National Center for Biotechnology Information (www.ncbi.nih.gov). The SEQUEST cluster, running BioWorks (Rev. 3.3.1 SP1, Thermo Electron), was used for the identification of peptides and proteins.

**Gene cloning.** Genomic DNA of Microbacterium sp. 4N2-2 was extracted with an UltraClean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer’s instructions. Degenerate primers (5’-GGM CAG CTB TTC GAY GGM TCV...
TCS ATC CG-3’ and 5’-GSA GCT CGT AGA GGT CCT TGT CGA YSG GYK C-3’) were used for PCR. After the sequence of the amplicon had been obtained, two new primers were prepared to amplify upstream and downstream sequences to give the full sequence of the target gene. A random nonamer primer (GE Healthcare) and a reverse primer for upstream (5’- GTC ACG TCG GGG ATG AGC TGC ATG TCG-3’) were used to amplify the start region. A random nonamer primer and a forward primer for downstream (5’- TCG AAC CCG AAG GCC AAG CGC ATC GAG-3’) were used to amplify the stop region. Sequence fragments were assembled in silico to give the full nucleotide sequence.

**Heterologous expression and purification of the protein.** The forward primer (5’-CCG TTC CTC CAG GAG TTG ACA TAT GTT CAC CAC C-3’) and reverse primer (5’-NNN NNN NGG ATC C TCA ASA CSC CGW AGT ACA GCT-3’) for the pET-11a plasmid (Novagen) were designed to amplify the full target gene (*glnA*) from the genomic DNA of *Microbacterium* sp. 4N2-2 (underlined letters are recognition sequences for *Nde*I and *Bam*HI, respectively). The sequence of the degenerate primer region was confirmed by comparing it with the full gene sequence obtained. The amplified target gene (*glnA*, 1,425 bp) was inserted at the *Nde*I and *Bam*HI sites of pET-11a and the expression construct was confirmed by restriction enzyme analysis and sequencing. The expression construct then was introduced into the *E. coli* BL21 (DE3) pLysS expression host to yield *E. coli* BL21 (DE3) pLysS / pET-11a-*glnA*. Expression of the target protein was controlled by the T7lac promoter of pET-11a by gradual addition of IPTG (final concentration 0.8 mM) to cultures in LB medium for 1 h at 28°C. After induction, the cultures were incubated for 4 h, harvested by centrifugation, washed twice with 10 mM phosphate buffer (pH 7.2), and centrifuged again. After a freeze-thaw step, they were resuspended in the same buffer. Cells from 1.5 L were suspended in 25 ml of
phosphate buffer and disrupted by sonication in an ethanol-ice bath. Cell debris was removed by centrifugation and filtration. Ammonium sulfate was gradually added, to a final concentration of 20-100%, to find the optimum concentration. The precipitate not containing the target protein, as shown by a norfloxacin N-acetylation assay and SDS-PAGE analysis, was pelleted and discarded. The target protein then was precipitated from the supernatant with more ammonium sulfate. The pellet was resuspended in 10 mM Tris-HCl (pH 7.8) and dialyzed with the same buffer up to 20 ml, using a Centricon centrifugal filter unit (EMD Millipore). A Hi Prep Q XL 16/10 column (GE Healthcare) was used for purification with a gradient of 0-2.0 M NaCl in 10 mM Tris-HCl (pH 7.8) at a flow rate of 1 ml min$^{-1}$. The active fractions were concentrated and dialyzed with 20 mM Tris-HCl (100 mM NaCl, pH 7.5) up to 5 ml. The fractions were applied in sequence to a gel filtration column (HiLoad 26/600 Superdex 200 pg, GE Healthcare). The target protein was eluted with 20 mM Tris-HCl (100 mM NaCl, pH 7.5) at a flow rate of 2 ml min$^{-1}$. Thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), and conalbumin (75 kDa) from GE Healthcare were used as standards. The eluted protein was concentrated and dialyzed in 50 mM HEPES buffer (pH 7.5) with 10% glycerol. Purification was monitored by SDS-PAGE analysis and protein was determined by using a MicroBCA Protein Assay Kit (Thermo Scientific).

Norfloxacin resistance as shown in broth cultures and a disk assay. Overnight cultures of *E. coli* BL21 (DE3) pLysS / pET-11a-*glnA* in LB medium were transferred into 200 μl of fresh LB medium as a 1% inoculum. Cells were grown at 28°C in 96-well plates with agitation in a microplate reader (Synergy #2, BioTek, Winooski, VT) at 28°C. When the optical density at 600 nm had reached 0.2, IPTG was added at a final concentration of 0.8 mM and
norfloxacin was added at 0, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 μg ml\(^{-1}\). Growth was monitored every 15 min up to 16 h. All tests were repeated in triplicate.

For the disk assay, cells cultured overnight were spread with a sterilized cotton swab on LB agar, with or without 0.8 mM IPTG. A norfloxacin disk (10 μg, Remel, Lenexa, KS) was applied to the middle of each plate. After 15 h at 28°C, the diameter of the clear zone was measured. All tests were repeated in triplicate.

**Enzyme activity analysis and inhibitor screening.** Enzyme activity was analyzed with 0.06 mM substrate, 0.2 mM acetyl-CoA and 5 mM CaCl\(_2\) in 50 μl of 50 mM Tris-HCl buffer with 20 mM NaCl (pH 8.0). One enzyme unit was defined as the amount of enzyme that converts 1 μmole norfloxacin to N-acetylnorfloxacin, with saturated acetyl-CoA, at pH 8.0 at 45°C in 1 h.

To screen potential inhibitors of N-acetylation activity, glutamate, glutamine, NH\(_4\)Cl, MgCl\(_2\), MnCl\(_2\), ATP, and ADP were prepared as 100 mM stock solutions and added to the reaction mixture at final 2 mM concentrations. Activity of the enzyme was expressed as residual activity (%) compared to control reaction mixtures without inhibitors.

**In silico docking analysis.** A 3-D protein structure of the gene, based on the structure of the glutamine synthetase (GS) from *Mycobacterium tuberculosis* (26), was generated using the SWISS-MODEL server (3). PDBsum (http://www.ebi.ac.uk/pdbsum/) provided a quick overview, including schematic diagrams and interaction of GS with ligands (ADP, norfloxacin, and acetyl-CoA). Automated docking of norfloxacin and acetyl-CoA to GS was performed using AutoDock Vina (47). The grid box (Center\(_x\) = -25.368, Center\(_y\) = 83.074, Center\(_z\) = 11.188) was centered in the catalytic active region (size\(_x\) = 58, size\(_y\) = 50, and size\(_z\) = 56). The maximum number of binding modes to generate was 1,000 for all docking analyses. An input pdbqt file of GS, with norfloxacin having the lowest binding energy of −8.0 kcal mol\(^{-1}\), was used.
for the AutoDock docking simulation of acetyl-CoA. Initial attempts to choose the functional binding modes of norfloxacin and acetyl-CoA were guided by (i) the nucleotide ADP binding site and (ii) the distance (< 5 Å) between the N-terminal of the piperazine ring and the S1P of acetyl-CoA. PyMOL (0.99RC6) (http://www.pymol.org/) was used to visualize all structural figures.

RESULTS

Partial purification and identification of protein responsible for norfloxacin N-acetylation. The N-acetylation activity in Microbacterium sp. 4N2-2 increased up to ~7-9 days in OM medium with Casamino acids (Fig. 1), so cell extracts were prepared from cultures grown in this medium for 9 days. Acetyl-CoA (39) was found to be required as an acetyl group donor for N-acetylation by cell extracts and CaCl₂ was found to be required as a cofactor.

To identify the responsible protein, selected bands containing partially purified protein (~50-55 kDa) were excised from an SDS-PAGE gel and analyzed by MS/MS. Eight proteins were considered to be possibly responsible for N-acetylation of norfloxacin, but glutamine synthetase (GS) was considered the most likely because N-acetylation activity in Microbacterium sp. 4N2-2 is governed by the nitrogen source and is strongly enhanced by Casamino acids (25). Expression of GS is also enhanced by Casamino acids in Bacillus subtilis (24). Casamino acids lacks glutamine (the product of GS, which causes feedback inhibition), but glutamate (the substrate) represents 8.4-15.9% of the total amino nitrogen (BD Bionutrients™ Technical Manual, BD Science).
To understand the relation between GS and norfloxacin N-acetylation, cultures of *Microbacterium* sp. 4N2-2 were grown with various nitrogen sources. Production of N-acetylnorfloxacin was found in media with Casamino acids or glutamate, but not in media containing glutamine or ammonium (Fig. 1).

Cloning of gene encoding N-acetyltransferase. The gene encoding GS (*glnA*) was cloned for heterologous expression and then characterized biochemically to reveal any unexpected functions of the protein. In the absence of a genome database for *Microbacterium* sp. 4N2-2, degenerate PCR primers were used for cloning. Genes encoding GS (with the ORF numbers from annotated genomes) from *Microbacterium testaceum* StLB037 (MTES3453), *Clavibacter michiganensis* subsp. *sepedonicus* (CMS1619), *Leifsonia xyli* CTCB07 (LXX10080), *Arthrobacter phenanthrenivorans* sphe3 (Asphe15760), *Renibacterium salmoninarum* ATCC33209 (RSal2447), and *Mycobacterium smegmatis* mc²155 (MSMEG4290) were aligned and used for designing degenerate primers. The forward primer (nucleotides 145-173 of MTES3453), and the reverse primer (nucleotides 1,183-1,212 of MTES3453) were used to generate an amplicon (1.1 kbp) that was used subsequently to obtain sequence information for the middle region of *glnA*. Two other primers (a reverse primer for upstream and a forward primer for downstream), designed from the sequence information obtained, were used to amplify regions outward from the middle fragment of *glnA*. The fragments were assembled manually to give a full nucleotide sequence for *glnA* of *Microbacterium* sp. 4N2-2.

The cloned gene sequence, which was deposited in GenBank (JX901058), shared 86, 82, 81, 73, 73 and 65% deduced amino acid sequence identities, respectively, with the strains used for primer design, whereas GS from *E. coli* K12 shared only 54% identity with the deduced
protein. Two other GSs of *M. testaceum* StLB037 (MTES3446 and MTES1058) shared 36 and 30% identities, respectively, with the deduced protein.

**Comparison of MS/MS data with the sequence of GS.** After the cloning of *glnA*, peptide information for the GS of *Microbacterium* sp. 4N2-2 was sought in the MS/MS spectra measured previously during partial purification of the proteins. One suggested peptide sequence, based on the *M. testaceum* StLB037 genome, was IPITGSNPK. The corresponding deduced amino acid sequence of GS of *Microbacterium* sp. 4N2-2 was IPLTGSNPK; the MS/MS spectrum of this peptide was exactly identified (*p* ≤ 0.001) in the data. This implied that GS from the cell extract of *Microbacterium* sp. 4N2-2 was most likely the protein responsible for N-acetylation.

**Heterologous expression of GS and activity analysis.** Based on the sequence obtained, an expression construct (pET-11a-*glnA*) was prepared and expressed in *E. coli* BL21 (DE3) pLysS. Cells from IPTG-induced and noninduced cultures of *E. coli*, containing either pET-11a-*glnA* or pET-11a, were harvested and disrupted. GS, which was expressed only in the IPTG-induced cells with pET-11a-*glnA*, had the predicted size of ~53 kDa (Fig. 2A). Cell extracts from these cultures showed high activity for converting norfloxacin to N-acetyl-norfloxacin, whereas the low activity (background) in cell extracts from the controls presumably originated from *E. coli* enzymes.

**Contribution of GS to norfloxacin resistance.** Because *Microbacterium* sp. 4N2-2 grew well with a norfloxacin concentration of more than 70 μg ml⁻¹, the contribution of GS to norfloxacin resistance was not evaluated directly in this strain. A norfloxacin-sensitive strain, *E. coli* BL21 (DE3) pLysS, was used to show the role of this enzyme in norfloxacin resistance. In the conditions used for this assay, the enzyme was active in the soluble fraction.
In broth cultures of *E. coli* BL21 (DE3) pLysS pET-11a-glnA without IPTG, 0.5 μg ml\(^{-1}\) of norfloxacin inhibited growth (Fig. 3). Induced cells expressing heterologous GS did not grow as well as noninduced cells in the absence of norfloxacin, indicating that there was a metabolic load due to the heterologously expressed protein. After induction of GS, the induced cells grew in the presence of 0.5 and even 1.0 μg ml\(^{-1}\) norfloxacin at a rate similar to induced cultures without norfloxacin. However, growth of both induced and noninduced cultures was inhibited by 2.0 μg ml\(^{-1}\) norfloxacin (Fig. 3).

The norfloxacin disk diffusion assay also supported the idea that GS contributed to norfloxacin resistance. The diameter of the inhibition zone for *E. coli* cells without *glnA* was not significantly altered by IPTG, but *E. coli* cells with *glnA* showed a ~3 mm smaller inhibition zone diameter (statistically significant, \(p<0.05\)) in IPTG-induced cultures than in noninduced cultures (Fig. 4).

**Purification of heterologously expressed GS.** GS was precipitated with 55% ammonium sulfate and then sequentially purified with an anion exchange column (Table 1). The molecular mass of GS, calculated as 53.32 kDa from its deduced amino acid sequence, coincided with the approximate size shown by SDS-PAGE (Fig. 2B). Gel filtration indicated a protein complex with an approximate size of 640 kDa, implying that, like other GSs (29), this GS may form a homododecamer.

**Characterization of GS.** Using GS purified by ammonium sulfate precipitation and anion exchange chromatography, optimum conditions for norfloxacin N-acetylation were evaluated. The optimum pH and temperature were determined to be 8.0 and 37-55°C, respectively. Acetyl-CoA at 0.2 mM was required as an acetyl group donor and calcium ion at 5.0 mM for enzyme activation. With 0.2 mM acetyl-CoA, there was ~5% abiotic background in
total production of N-acetylnorfloxacin at pH 8.0. Apparent $K_m$ and $V_{max}$ values were obtained from Lineweaver-Burk plots; with saturated acetyl Co-A, the $K_m$ was 2,208 µM and $V_{max}$ was 13.2 mU [mg protein]$^{-1}$.

Glutamate, ammonia and glutamine did not inhibit norfloxacin $N$-acetylation by the purified enzyme, but ATP and ADP caused 48% and 39% inhibition, respectively (Fig. 5). This indicated that norfloxacin does not bind to the pocket for substrates and products, but instead to the ATP and ADP binding site (8, 26). Mg$^{2+}$ and Mn$^{2+}$, which may activate GS for its usual substrates and coordinate the substrates at the active site (26, 27), instead caused 48% and 54% inhibition, respectively (Fig. 5).

**In silico structural analysis.** The 3-D structural model of GS from *Microbacterium* sp. strain 4N-2 (Fig. 6A) was constructed using the crystal structure (Protein Data Bank code 2BVC) of the GS from *Mycobacterium tuberculosis* (26) as a template. The rms deviation ($C_\alpha$) of the GS model (474 amino acids) superimposed on the 2BVC structure (486 amino acids) was 0.79 Å, in which 471 amino acid residues were aligned with 63.4% sequence identity. Fig. 6A shows a biologically relevant dodecamer model with two hexameric rings.

In GS, the active site between subunits has a cleft ~14 Å in length, ~9 Å in width, and ~11 Å in height. In the GS: ADP complex, ADP is placed in almost the same way in the cleft, as shown in the 2BVC structure (Fig. 6B). The AutoDock docking simulation of norfloxacin to GS proposed 504 possible binding solutions. About 16 of them were located at the binding site of ADP, in which the reaction center (-N) of the piperazine ring lies appropriately in the cleft. Fig. 6B shows the five binding modes with the lowest binding energy (~ $-8.00$ kcal mol$^{-1}$), all of which have similar conformation and location in a cleft of GS.
Fig. 6C shows 10 potential functional binding modes of acetyl-CoA with the lowest binding energies of ~ $-6.00 \text{ kcal mol}^{-1}$ from 687 possible binding solutions. As revealed in a close-up of the binding modes of acetyl-CoA (Fig. 6C), despite its dispersed binding positions, most of the functional acetyl group moieties resided in the bottom of a cleft bound to the piperazine ring of norfloxacin.

**DISCUSSION**

Many bacterial enzymes that $N$-acetylate various substrates use acetyl-CoA as an acetyl donor (38, 42, 45). In bacteria as well as humans, $N$-acylation may be involved in detoxification of drugs (30, 38, 42, 43), including fluoroquinolones (39). The activity of GS in the Actinomycetales is regulated by transcriptional regulation and post-translational adenylylation (2, 9, 35). The peptide sequence of GS was found in MS/MS spectra from the partial isolation of proteins of *Microbacterium* sp. 4N2-2, and it exactly matched the deduced protein sequence for GS. When it was heterologously expressed in *E. coli* and purified, GS also showed norfloxacin $N$-acetylation activity.

Although $N$-acetylation of antibiotics by GS has not been previously reported, there are reports of $N$-acylation of proteins by GS. A so-called “moon lighting property” of GS in *Mycobacterium* spp. is the $N$-acylation of glutathione S-transferase, with a polyphenolic acetate as an acetyl group donor (5, 14). The activity of *Microbacterium* sp. 4N2-2 GS for norfloxacin $N$-acylation was not as high as that for glutamine synthesis, nor was it as high as the activities of other $N$-acyltransferases (5, 14, 39), as shown by the $K_m$ and $V_{max}$ values. The $K_m$ for norfloxacin was similar to the $K_m$ for glutamate, but it was more than 10-fold higher than the $K_m$
values reported for ATP and NH4Cl with the GS of *Nocardia asteroides* (33). High \( K_m \) and low \( V_{max} \) may be explained by an accidental substrate-enzyme relationship. Thus, the contribution of GS to resistance, which is effective only for low norfloxacin concentrations, may be understood from the high \( K_m \) and low \( V_{max} \).

Together with the biochemical observations, including (i) the low enzyme activity, (ii) acetyl-CoA as an acetyl group donor, (iii) the relatively high \( K_m \) for norfloxacin, (iv) the inhibition by ATP and ADP, and (v) the \( Ca^{2+} \) requirement, the structural model also provides insights into the \( N \)-acetylation activity of GS. This enzyme not only requires acetyl-CoA and \( Ca^{2+} \) for \( N \)-acetylation of norfloxacin, but it also has a structural environment suitable for functional binding of the substrates. When the predicted binding was examined with respect to the active site of the enzyme, the geometry of the active site seemed least likely to be problematic for binding. Moreover, with norfloxacin bound in the active site, the piperazine ring would orient toward the open space, which simultaneously could accept the acetyl group of acetyl-CoA. As revealed in the docking analysis of the complex, when GS bound norfloxacin with the lowest binding energy (\(-8.0 \text{ kcal mol}^{-1}\)), most of the acetyl group of the bound acetyl-CoA was located in the bottom of a cleft, placing the substrate in an ideal position for functional group donation. The substrate binding modes and geometry of the active site support the idea that GS can join norfloxacin and acetyl-CoA together in the active site. The \( N \)-terminal of the piperazine ring lies near the reactive S1P atom of acetyl-CoA, which is an appropriate position for functional group transfer. Therefore, the relatively low \( N \)-acetylation activity of GS suggests that the reaction depends on the precisely coordinated functional binding of the substrates, norfloxacin and acetyl-CoA, and \( Ca^{2+} \).
The GS of *Microbacterium* sp. 4N2-2 appears to belong to a family of highly conserved bacterial GSs (17). Other bacterial GSs that acetylate fluoroquinolones may also exist, considering that GS is conserved in actinobacteria with high structural homology (17, 26) and that N-acetylation activity for norfloxacin is found in several *Mycobacterium* spp. (1). This idea also is supported by the protein N-acetylation function of GS of *M. smegmatis* (14).

The study of various GSs for N-acetylation activity, and enzyme engineering based on models of protein-ligand binding, will provide insight into the N-acetylation function of GS. Moreover, because N-acetylation is an important detoxification mechanism for bioactive molecules (11, 36), GSs of environmental bacteria may be able to modify various chemicals, including antibiotics.

In nutrient-limited and biofilm bacteria, the starvation response mediated by guanosine pentaphosphate is associated with tolerance to antibiotics, including fluoroquinolones (32). Expression of GSs of *E. coli*, *Bacillus subtilis* and *Mycobacterium* spp. is also enhanced by the stringent response of nutrient starvation, especially ammonium (4, 15, 46). This implies that in oligotrophic sites or biofilms a bacterial strain with a GS similar to *Microbacterium* sp. 4N2-2 might show fluoroquinolone resistance. Most environmental sites have low-level (ng l\(^{-1}\)) contamination by fluoroquinolones (7, 13, 37), so activation of GS by nutrient nitrogen limitation could contribute to fluoroquinolone resistance.

This report describes the modification of a fluoroquinolone by an enzyme from an environmental bacterium. The approach used in this research, including selective isolation of the bacterium, enzyme activity analysis, protein isolation and characterization, and structure modeling, represents a useful strategy for enzyme identification and characterization in studies of biotransformation and antibiotic resistance.
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The views presented in this article do not necessarily reflect those of the Food and Drug Administration.

REFERENCES


Mycobacterium tuberculosis GlnE promoter and its response to nitrogen availability.
Microbiology 152:2727–2734.

arylamine N-acetyltransferase genes from Mycobacterium smegmatis and Mycobacterium


2007. Cloning and molecular characterization of three arylamine N-acetyltransferase genes
from Bacillus anthracis: identification of unusual enzymatic properties and their contribution

39. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K,
Hooper DC. 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common


41. Schmitz F-J, Jones ME, Hofmann B, Hansen B, Scheuring S, Lückefahr M, Fluit A,
and gyrB mutations in 116 unrelated isolates of Staphylococcus aureus and effects of


Table 1. Purification of recombinant glutamine synthetase from *E. coli* $^a$

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (mU)</th>
<th>Specific activity (mU/mg)</th>
<th>Yield (%)</th>
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</thead>
<tbody>
<tr>
<td>Crude cell extract</td>
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<td>85.9</td>
<td>0.42</td>
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<td>precipitation</td>
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<td>Anion exchange</td>
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</table>

$^a$ Activities were determined with acetyl-CoA and CaCl$_2$. 
FIGURE LEGENDS

Fig. 1. Production of N-acetylnorfloxacin by *Microbacterium* sp. 4N2-2. The nitrogen sources were Casamino acids (■), glutamate (□), glutamine (▲) and ammonium chloride (△).

Fig. 2. SDS-PAGE gels showing (A) heterologous expression of *Microbacterium* sp. 4N2-2 glutamine synthetase in the soluble fraction of *E. coli* BL21 (DE3) pLysS / pET-11a-glnA with IPTG induction; and (B) purification of glutamine synthetase of *Microbacterium* sp. 4N2-2 from heterologous overexpression.

Fig. 3. Decreased sensitivity to norfloxacin (0 to 2 μg ml⁻¹) due to induction of glutamine synthetase in *E. coli* BL21 (DE3) pLysS / pET-11a-glnA. IPTG-induced (■) and noninduced (□) liquid cultures were used to monitor norfloxacin sensitivity by induction of glutamine synthetase in the *E. coli* BL21 (DE3) pLysS host.

Fig. 4. Norfloxacin disk assay of non-induced (gray bars) and IPTG-induced (white bars) cultures of *E. coli* BL21 (DE3) pLysS containing plasmids with or without the glnA insert encoding glutamine synthetase. The assay was used to monitor changes in norfloxacin sensitivity due to expression of glutamine synthetase. In the cells with the glnA insert, the reduced diameter of the clear zones of inhibition due to induction of glutamine synthetase was statistically significant (p<0.05).
Fig. 5. Inhibition of N-acetylation activity of purified glutamine synthetase from *Microbacterium* sp. 4N2-2 by substrates and cofactors (2 mM of each).

Fig. 6. Model of *Microbacterium* sp. 4N2-2 glutamine synthetase structure and potential binding modes of norfloxacin and acetyl-CoA. (A) Top and side view of glutamine synthetase complex. The dimeric active site is indicated by a white square on the surface representation. (B and C) Electrostatic surface diagrams of the substrate-binding pocket, colored by charge, and the potential binding modes of norfloxacin (B, five potential positions) and acetyl-CoA (C, ten potential positions).
Fig. 1
<table>
<thead>
<tr>
<th>Glutamine (kDa)</th>
<th>200.0</th>
<th>116.3</th>
<th>97.4</th>
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<tr>
<td><strong>A</strong> Protein ladder</td>
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<td>0 mM IPTG</td>
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<td>0.8 mM IPTG</td>
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<td>Glutamine synthetase 53.32 kDa</td>
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</table>

Fig. 2
Fig. 3
Fig. 4

Diameter of Clear zone (cm)

IPTG - + - +

E. coli BL21 (DE3) pLysS containing pET-11a

E. coli BL21 (DE3) pLysS containing pET-11a-gluA
Fig. 5

Glutamine synthetase substrates and cofactors

Acetylation activity (%)

- Glutamine
- Glutamate
- NH$_4$Cl
- ATP
- ADP
- MgCl$_2$
- MnCl$_2$

Without inhibitor
Fig. 6

A
Active site

B
ADP Norfloxacin

C
Acetyl-CoA