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Nitazoxanide Inhibits Biofilm Production and Hemagglutination by Enteroaggregative *Escherichia coli* Strains by Blocking Assembly of AafA Fimbriae[▽]

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Enteroaggregative *Escherichia coli* (EAEC) strains have emerged as common causes of persistent diarrhea and malnutrition among children and HIV-infected persons. During infection, EAEC typically adheres to the intestinal mucosa via fimbrial adhesins, which results in a characteristic aggregative pattern. In the study described here we investigated whether the broad-spectrum antiparasitic and antidiarrheal drug nitazoxanide (NTZ) might be active against EAEC *in vitro*. While *E. coli* strains were resistant to NTZ in rich Luria-Bertani medium (MIC > 64 μg/ml), the drug was slightly inhibitory in a minimal medium supplemented with glucose (MinA-G medium; MIC, ~32 μg/ml). NTZ also inhibited biofilm production by strain EAEC 042 in both Dulbecco's modified Eagle's medium and MinA-G medium with a 50% inhibitory concentration of ~12 μg/ml. Immunofluorescence and immunoblot analyses with antibody against the major fimbrial subunit AafA of aggregative adherence fimbriae vaariant II (AAF/II) established that the numbers of AAF/II filaments on bacteria grown in the presence of NTZ were dramatically reduced. Comparative quantitative reverse transcription-PCR and reporter gene fusions (*aafA*::*phoA*) indicated that *aafA* expression was unaffected by NTZ, while *aggR* transcript levels and *aggR*::*lacZ* expression were increased ~10- and 2.5-fold, respectively, compared with that for the untreated controls. More generally, NTZ inhibited hemagglutination (HA) of red blood cells by the non-biofilm-producing strain JM221 expressing either AAF/I or type I fimbriae. Our findings suggest that the inhibitory action of NTZ on biofilm formation and HA is likely due to inhibition of fimbrial assembly. Antimicrobial agents that inhibit the assembly or function of fimbrial filaments should be good candidates for the prevention of infection.

Infectious diarrheal diseases are the second highest global cause of morbidity and mortality, and repeated or prolonged episodes of diarrhea can stunt the growth of infected children and impair cognition (10, 17, 34). The World Health Organization has estimated that stunting affects approximately 147 million children in the developing world (4), where every child less than 5 years old suffers an average of three diarrheal episodes per year (21). Due to the morbidity burden of diarrheal disease, especially during early childhood, more effective therapies are expected to save many disability-adjusted life years (11, 17).

Enteroaggregative *Escherichia coli* (EAEC), first identified and described as diarrheagenic *E. coli* in 1987 (29), has emerged as a leading cause of acute and persistent (≥14 days) diarrhea among children, patients with AIDS, and international travelers in developing and industrialized countries (2, 9, 19, 25, 36). Around the world, EAEC accounts for 8 to 32% of acute diarrhea cases among infants and children and 20 to 30% of persistent diarrhea cases (17). Individuals most often contract infection via the fecal-oral route by consuming contaminated food and water or by practicing poor hygiene.

The clinical presentation of EAEC infection often consists of watery diarrhea, at times with the passage of blood and mucus, but some infections are asymptomatic (17, 28). This phenomenon is likely due to differences in both host susceptibility and strain heterogeneity. Patients often experience intestinal inflammation marked by elevated levels of fecal lactoferrin (10, 39), and EAEC infection may perpetuate childhood malnutrition.

The pathogenesis of EAEC is complex and not fully understood, in large part due to the heterogeneity of the strains (32). Generally, EAEC pathogenesis involves three stages: (i) adherence to the intestinal mucosa, mediated by aggregative adherence fimbriae (AAF); (ii) biofilm formation on the surface of host enterocytes; and (iii) the release of EAEC toxins, the elicitation of an inflammatory response, intestinal secretion, and mucosal toxicity, which results in microvillus vesiculation and epithelial cell extrusion (12, 17, 18, 25, 28).

Several virulence factors have been implicated in mucosal adherence and biofilm formation. The most important and best-studied virulence factor is the master transcriptional regulator AggR, whose gene is located on a 60- to 65-MDa pAA plasmid present in many, but not all, strains of EAEC (1, 12, 17, 30). AggR is activated in response to environmental cues such as low levels of sodium, oxygen, and nutrients and a low pH (35) and controls the expression of several plasmid-en-

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coded genes involved in fimbrial biogenesis, notably, *aafA* (GenBank accession no. AF012835), which encodes a major structural subunit of AAF variant II (AAF/II), expressed by pathogenic strain 042 (3, 28). AAF/II fimbriae have been described to be 5 nm in diameter and arranged in semirigid, filamentous bundles (7), and they are thought to mediate adherence to the colonic mucosa and to polystyrene and glass surfaces (26). AggR also controls the expression of other fimbrial genes (e.g., those for AAF/I and AAF/III) that are antigenically different (1, 3), some of which can agglutinate erythrocytes or have other non-biofilm-producing phenotypes (1, 3).

Following adherence, EAEC produces a mucosal biofilm that promotes colonization and resists the penetration of antimicrobials (25). The biofilm of strain 042 consists of thick aggregates of bacteria interspersed with void spaces, similar to other bacterial biofilms and characteristic of the heavy biofilm that forms over the epithelium during infection (37). Biofilm formation is thought to be mediated by AAF or similar structures and to involve two chromosomal genes, *fis* and *yafK*, which are activated by AggR (37). However, the genetic markers that characterize biofilm-producing strains and the clinical relevance of biofilm formation during infection remain unclear.

Nitazoxanide (NTZ) is a broad-spectrum antiparasitic agent that is approved for use by the FDA for the treatment of infections caused by *Cryptosporidium* and *Giardia* species in children and adults (8, 15). The drug also shows broad-spectrum activity *in vitro* against anaerobic bacteria, including *Clostridium difficile* and members of the *Epsilonproteobacteria*, including *Helicobacter pylori* and *Campylobacter jejuni* (16, 38). In these organisms, NTZ is a potent inhibitor of the pyruvate ferredoxin oxidoreductase (16, 27, 38). The anionic nature of the active form of NTZ and its ability to abstract protons from enzymatic reactions may account for the wide range of reactions that have been reported for this therapeutic and may account for the nonspecific improvement of chronic diarrhea in humans (43).

In previous studies aimed at assessing the mutation frequency of NTZ for *E. coli* in a papillation type of assay (LacZ reversions) in which MinA (see below) was the base medium, we noted that NTZ concentrations higher than 15 μg/ml were inhibitory for growth (38). In contrast, no inhibitory action was observed in nutrient-rich Luria-Bertani (LB) medium (MIC > 64 μg/ml), suggesting that NTZ affected some conditionally essential biosynthetic pathway. Given anecdotal reports that NTZ is a nonspecific antidiarrheal drug, we considered the possibility that the drug might affect the fitness of enteric pathogens, much like how prophylaxis with bismuth subsalicylate wards off traveler's diarrhea (6). We have investigated the effect of NTZ on EAEC strain 042, which is often used as a model system for the study of biofilm production. Here we report that NTZ inhibits biofilm production and hemagglutination (HA) at drug concentrations that do not appreciably inhibit growth. Furthermore, our studies reveal that the basis for this inhibition is the inhibition of fimbrial filament formation and not the regulation of *aafA* gene expression. Thus, NTZ, by blocking the aggregative behavior of EAEC, might have efficacy for the treatment of enteric diarrheal diseases.

MATERIALS AND METHODS

Bacterial strains and culture medium. Enteroaggregative *E. coli* strains 042, 17-2, and JM221 were obtained from the collections of the Center for Vaccine Development, University of Maryland School of Medicine. The strains were cultured in Luria-Bertani (LB) medium or a modified chemically defined MinA medium (MinA-G) containing the following (per liter): 1% glucose, 1 mM $MgSO_4$, 1 g $(NH_2)_2SO_4$, 4.5 g KH_2PO_4, 10.5 g K_2HPO_4, and 0.5 g sodium citrate.

Effect of NTZ on bacterial growth and motility. The EAEC strains were grown overnight in MinA-G medium at 37°C with shaking and were used to inoculate fresh MinA-G medium containing a range of NTZ concentrations (0, 5, 10, 15, 20, and 25 μg/ml) to a starting optical density at 600 nm (OD_{600}) of 0.1. Growth was recorded as the absorbance at 600 nm at 30-min intervals over a period of 8 h. MIC testing of all strains was done by microdilution in MinA-G medium (0 to 32 μg/ml). Motility was assessed in LB soft agar (0.3% agar) containing 0 to 25 μg/ml of NTZ. The diameter of outward spreading was measured daily, and the level of inhibition was computed as a percentage of that for the control.

Quantitative biofilm assay. A quantitative biofilm assay was used to determine the effect of NTZ on biofilm formation (41). Strain 042 was grown overnight at 37°C with shaking and was inoculated 1:100 in 200 μl culture medium in Costar 96-well, flat-bottom, polystyrene microtiter plates containing the appropriate concentrations of NTZ. The plates were incubated statically at 37°C in a humidified incubator. At 24 h, bacterial growth was measured by either transferring 100-ml aliquots to another microtiter plate to determine the turbidity or by diluting 100-μl aliquots 1:100 in 9.9 ml phosphate-buffered saline (PBS) for determination of quantitative plate counts on LB medium. Bacterial counts (determined in triplicate) are reported as the numbers of CFU/ml. The biofilm was visualized following staining with 0.5% crystal violet (41), and the absorbance was read at 570 nm with a microplate reader (Molecular Dynamics). All experiments were performed in triplicate, and the results are reported as the means and standard deviations.

The assays were initially performed with and without 25 μg/ml NTZ and five different growth media: Dulbecco's modified Eagle's medium (DMEM), DMEM with 0.4% glucose, LB medium, LB medium with 0.4% glucose, and our chemically defined medium containing 1% glucose (MinA-G medium). *E. coli* DH5α served as a negative control for biofilm formation. The assays were then run with increasing concentrations of NTZ (0, 5, 10, 15, 20, 25 μg/ml) both in the chemically defined medium and in DMEM with 0.4% glucose. Denitro nitazoxanide, a biologically inert compound, was used as a negative control, and dimethyl sulfoxide (DMSO) was used to control for any hydrophobic effects from the addition of NTZ (which was prepared as a 25-mg/ml stock solution in DMSO).

Hemagglutination of red blood cells by whole bacterial cells. HA has previously been shown to correlate with the expression of AAF by various EAEC strains (3). HA assays were performed in 96-well microplates as described elsewhere (1). The bacteria were grown overnight (in MinA-G medium with or without 25 μg/ml NTZ) without shaking and were suspended in PBS and diluted to an OD_{660} of 1.5, 1.0, and 0.5; and 100 ml was mixed with an equal volume of a 3% (vol/vol) sheep erythrocyte suspension containing 1% mannose, except when type I fimbria-producing strains were assayed. For the assay with the latter strains, the bacteria were grown statically in LB medium (with or without NTZ) for 48 h, and HA was determined with guinea pig erythrocytes. The HA reaction mixtures were incubated at 4°C for 20 min. HA was determined visually against the controls that received no NTZ. All assays were prepared in triplicate and repeated twice.

RNA extraction. EAEC 042 was grown overnight in MinA-G medium and was then diluted to a starting OD_{600} of 0.05 in three 50-ml MinA-G medium cultures with 0, 10, and 25 μg/ml NTZ. The bacterial cells were grown with shaking until the cultures reached an OD_{600} of ~0.4 and were harvested by centrifugation at 4°C. The pellets were resuspended in 200 μl TE (Tris-EDTA) buffer and transferred to polystyrene round-bottom 14-ml tubes, and total RNA was extracted by the *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–hot phenol method, as described previously (15). The RNA was reprecipitated and washed two times with 500 μl 70% ethanol, prepared with diethyl pyrocarbonate (DEPC)-treated water, to remove any remaining salts. The pellets were dried at 37°C and suspended in 100 μl DEPC-water. The RNA concentration was determined with a NanoDrop ND-1000 UV-visible spectrophotometer. The RNA yields were typically in the 1.6- to 3.2-μg/μl range. The residual DNA was removed by treatment with TURBO DNase and was stored at −80°C (15).

RT-PCR. The primers used for real-time quantitative reverse transcription-PCR (qRT-PCR) were designed to yield approximately 150-bp amplicons for the *aggR* and *aafA* virulence genes and for the *rpoS* stringent response gene, which served as an internal control. The primers for the *aggR* gene were as follows: 5′-CTAATTGTACAATCGATGTATACAC-3′ (forward) and 5′-TAATGTAT

CGCTGTTTAATCTG-3′ (reverse). The primers for the *aafA* gene were as follows: 5′-ACTTCATATAGGCCTGGTCGTA-3′ (forward) and 5′-ATTCAC TCTGGCCTCTCCTAGGT-3′ (reverse). The primers for the *rpoS* gene were as follows: 5′-AGTCAGAATACGCTGAAAGTTCATG-3′ (forward) and 5′-AA GGTAAAGCTGAGTCGCGTC-3′ (reverse). SuperScript II reverse transcriptase (Invitrogen) was used to synthesize first-strand cDNA from 1 μg purified RNA with 100-ng random primers (Invitrogen). RNA, random primers, and deoxynucleoside triphosphates (dNTPs; final concentration, 0.5 mM) were mixed and the mixture was heated for 65°C for 5 min, briefly centrifuged, and quickly chilled on ice. To each 20 μl of the reaction mixture was added first-strand buffer, dithiothreitol (final concentration, 0.01 M), and 20 U RNase inhibitor (Applied Biosystems). The contents of the tube were gently mixed and the tubes were incubated at 25°C for 2 min. SuperScript II reverse transcriptase was added, and the reaction mixtures were incubated at 25°C for 10 min and 43°C for 50 min, followed by heat inactivation of the enzyme at 70°C for 15 min. Each reaction mixture was diluted by adding 80 μl DEPC-water.

PCR of the three genes was performed for each of the three cDNA reactions in an iCycler thermal cycler with SYBR green as the detection agent. Each 25-μl reaction mixture consisted of 10 μl of diluted cDNA, 1× PCR buffer (containing 1.5 mM $MgCl_2$), 0.5× Q solution, 4 mM $MgCl_2$, 0.2 mM dNTP mix, 0.05 μg each forward primer and reverse primer, 1.25 U HotStar *Taq* DNA polymerase (Qiagen), and 0.25 μl of SYBR green (diluted 10^{-3}). The reaction mixtures were incubated for 15 min at 95°C to activate the HotStar *Taq* DNA polymerase, followed by 35 amplification cycles of 95°C for 30 s, 58°C for 30 s (optimized by gradient PCR of genomic DNA), and 60°C for 20 s, followed by a final 10-min extension at 60°C. Following the PCR, the temperature was increased in 0.5°C increments every 10 s to generate melting curves of the PCR amplicons. To account for any amplification due to contaminating genomic DNA, PCRs with RNA at the concentration corresponding to the concentration used for cDNA synthesis served as negative controls for each sample. All reactions were performed in triplicate. Repeated trials were conducted to optimize the RNA purification and PCR conditions and to verify the reproducibility of the results.

Reporter gene fusions. Reporter gene fusions were provided by James Nataro (University of Maryland) and included *E. coli* 042 *aggR-lacZ* and *E. coli* 042 *aafA-phoA* (strains 3.4.14 and 2.94) (3) and an undefined promoterless *lacZ* control strain of *E. coli* 042. Strains were grown without shaking in MinA-G medium supplemented with 0, 12, or 25 μg/ml of NTZ for 16 h at 37°C. β-Galactosidase activity was determined spectrophotometrically and is reported in Miller units (24). Alkaline phosphatase activity was also determined spectrophotometrically by using *p*-nitrophenol, as described previously (23). All assays were performed in triplicate in three independent experiments, and the means and standard deviations are reported.

Purification and analysis of fimbrial proteins by SDS-PAGE. A simplified procedure for the mechanical removal of fimbriae was developed by modification of the procedure of Dodd and Eisenstein (5). EAEC 042 was grown in MinA-G medium and LB medium overnight, inoculated 1:100 in 100 ml of the appropriate culture medium in 250-ml flasks with or without 25 μg/ml of NTZ, and grown statically overnight at 37°C. The cultures were spun down at 4,000 rpm for 5 min at 25°C. The pellets were resuspended in 2 ml PBS, vortexed two times for 30 s each time to shear off the flagella and fimbriae, and then centrifuged at 8,000 rpm for 10 min to remove whole cells. The supernatants, which contained fimbrial filaments, were transferred to new tubes, and 8 ml of acetone was added to precipitate the proteins. The solutions were again spun down at 8,000 rpm for 10 min, and the acetone was decanted. The remaining protein pellets were resuspended in 50 μl PBS and 100 μl 3× loading buffer containing 2-mercaptoethanol.

Biofilms were observed at the air-liquid interface in the LB medium culture and at the bottom of the flask in the MinA-G medium culture without NTZ. To analyze the proteins contained in the biofilm, the flasks were rinsed with water to remove the residual bacteria and the biofilms were dissolved in 1 to 2 ml 1% SDS. Fifty microliters of each solution was combined with 100 μl 3× loading buffer in PCR tubes. All five samples were run in a NuPAGE 4 to 12% bis-Tris gel at 120 V and stained with Coomassie brilliant blue or used directly for the transfer of proteins to nitrocellulose.

Immunoblot and immunofluorescence analyses. Ponceau red-stained nitrocellulose was used to visualize the transferred proteins, and the immunoblotting protocols used in this study have been described previously (14). Hyperimmune rabbit serum raised against the AafA fimbrial subunits of EAEC strain 042 was obtained from James Nataro and was used at a dilution of 1/10,000. Alkaline phosphatase-conjugated goat anti-rabbit IgG (1/5,000) was used with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium to identify immunoreactive proteins. For assessment of fimbrial expression by immunofluorescence, the bacteria were briefly heat fixed onto microscope slides and treated for 1 min with 2% paraformaldehyde. Following treatment with anti-AafA serum for 30 min, the slides were washed three times with PBS and stained with rhodamine-conjugated goat anti-rabbit immunoglobulin. The slides were washed three times in PBS, dried, and visualized with a Zeiss epifluorescence microscope.

Statistical analysis. SigmaStat software was used to perform statistical analyses of the data acquired from the biofilm assays and qRT-PCR.

RESULTS

NTZ partly inhibits growth of EAEC 042. We previously reported that the antiparasitic drug NTZ partly inhibits the growth of *E. coli* strain CC104 when it is grown on MinA-G medium but not when it is grown on LB medium (38). MIC testing under static conditions indicated that on LB medium, the MIC was >64 μg/ml, while on MinA-G medium it was ~32 μg/ml. We reasoned that if the human intestine resembled MinA-G medium nutritionally (i.e., is nutrient poor), unlike the nutrient-rich LB medium, then perhaps NTZ might show some efficacy against these organisms (35). To test this hypothesis, we reproduced growth experiments with EAEC strains 042 (AAF/II) and 17-2 (AAF/I), which are typical of the strains that cause persistent diarrhea. As shown in Fig. 1, NTZ and the deacetylated metabolite tizoxanide inhibited the aerobic growth of EAEC 042 and, to a lesser extent, 17-2 in a dose-dependent manner in MinA-G medium (only data for growth with NTZ are shown). While growth was not completely inhibited at concentrations up to 25 μg/ml, we estimated that 50% inhibition could be achieved at NTZ concentrations between 15 and 20 μg/ml for strain 042 and 20 to 25 μg/ml for strain 17-2. Under static conditions, the EAEC strains exhibited a MIC_{50} of ~20 μg/ml. Such concentrations are achievable clinically when doses of 1 g per day are used to treat parasitic infections (8, 13).

NTZ inhibits biofilm formation. In MIC testing performed by microdilution, we noticed that EAEC strain 042 formed a biofilm in the plastic wells in MinA-G medium and that sub-MIC levels of NTZ inhibited biofilm production. It has been suggested that the presence of an EAEC mucosal biofilm correlates with infectivity by promoting persistent colonization and increasing the rates of resistance to antibiotics (25). Both the biofilm formation and aggregative adherence behaviors are attributable to the expression of AAF/II fimbriae (41). To explore the effect of NTZ further, we compared biofilm formation in MinA-G medium with that in DMEM and LB medium. As seen in Fig. 2, EAEC biofilms (those of strain 042 but not those of strains17-2 and JM221) were the densest in MinA-G medium ($OD_{570} > 0.5$) and absent in LB medium ($OD_{570} < 0.01$), consistent with previous findings that demonstrated higher rates of biofilm expression in high-glucose medium (37). Repeated assays in MinA-G medium revealed a dose-dependent inhibition of biofilm formation with NTZ. Inhibition was the most dramatic between 10 and 15 μg/ml, with almost complete inhibition occurring at 20 μg/ml (Fig. 3). Analogous assays with DMEM plus 0.4% glucose yielded comparable results, but the overall biofilm density was lower and there was a steadier decrease in the response to increasing NTZ doses (data not shown). Plate counts from aliquots taken from wells containing each of the drug concentrations tested revealed high bacterial densities ($>10^8$ CFU/ml) in all wells, indicating that the bacteria were in stationary phase by 24 h postinoculation. Thus, the reduction in the level of biofilm formation was not due to the inhibition of growth.

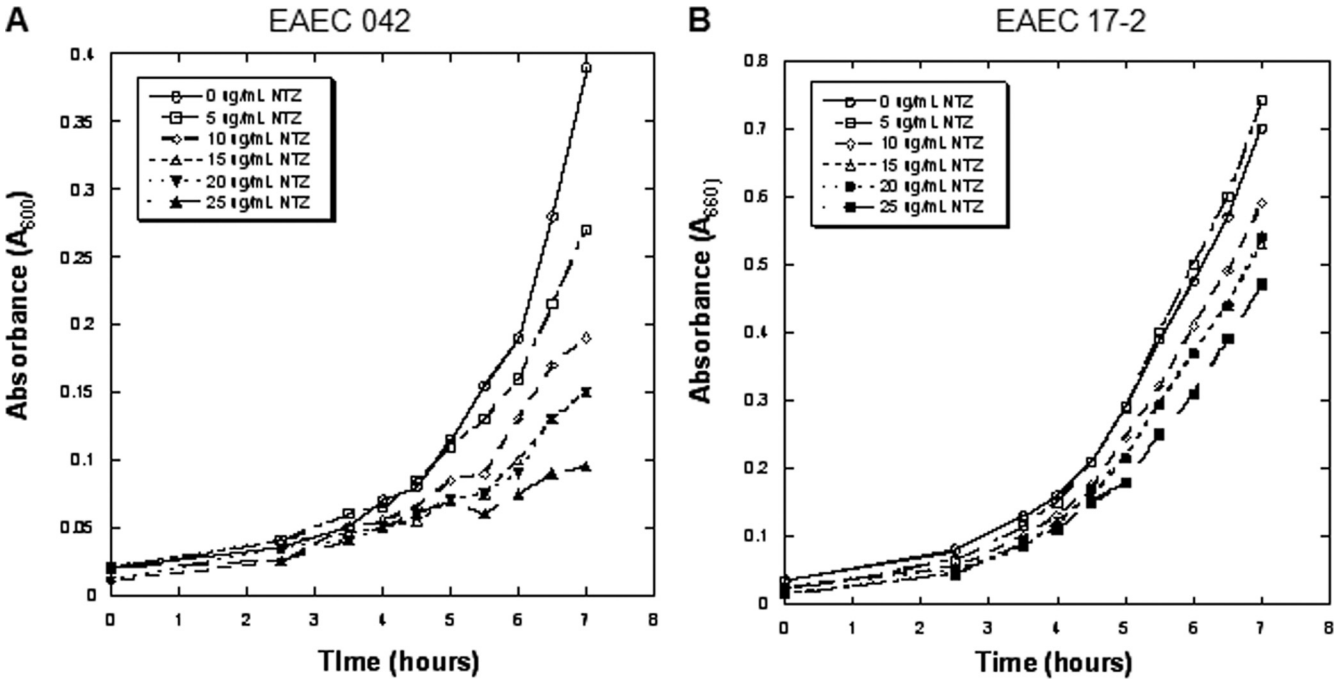

FIG. 1. Bacterial growth in MinA-G medium. AAF/II EAEC strain 042 (A) and AAF/I EAEC strain 17-2 (B) were grown in liquid culture supplemented with NTZ at the concentrations indicated in the keys.

NTZ inhibits hemagglutination. Both AAF/II and AAF/I fimbria-producing strains hemagglutinate erythrocytes, and this was confirmed with sheep erythrocytes. As seen in Table 1, NTZ inhibited HA by both strains of EAEC, suggesting a common inhibitory mechanism. Moreover, similar concentrations of NTZ (20 μg/ml) inhibited HA by EAEC strain JM221 grown statically in LB medium, which promotes the expression of type I fimbriae and which represses AAF. The distinction was further demonstrated by the ability of JM221 grown in LB medium (but not in MinA-G medium) to hemagglutinate guinea pig erythrocytes but not sheep erythrocytes. The inhibitory action of NTZ on the production of type I fimbriae in LB medium (MIC > 64 μg/ml) supports the likelihood of a specific target rather than a nonspecific growth-related mechanism.

NTZ inhibits fimbrial filament assembly. Both the aggregative adherence phenotype and the biofilm formation by EAEC 042 are attributable to the production of AAF and not to the production of polysaccharides (30). While both EAEC strains produce AAF, only fimbriae from strain 042 adhere to plastic.

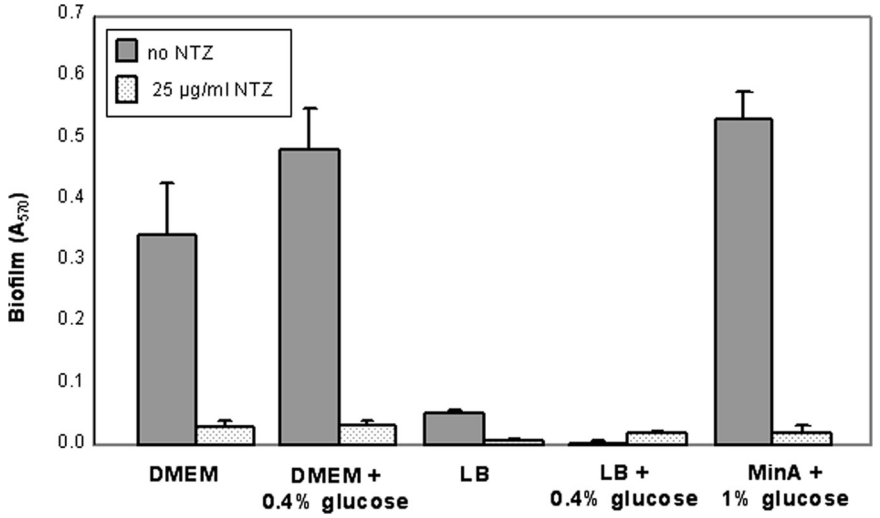

FIG. 2. Effect of medium composition on biofilm production. Biofilm accumulation was determined in DMEM and LB medium (with or without 0.4% glucose) and MinA-G medium (with 1% glucose) with EAEC strain 042 in the presence or the absence of 25 μg/ml NTZ. Biofilm accumulation was quantified by the crystal violet assay. All assays were performed in triplicate.

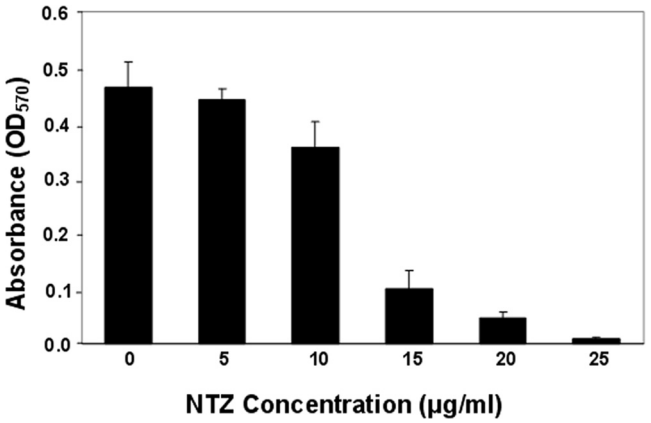

FIG. 3. Dose-dependent inhibition of biofilm formation. EAEC strain 042 was grown in MinA-G medium supplemented with the indicated concentrations of NTZ. All assays were performed in triplicate.

Thus, the inhibition of biofilm formation and HA by NTZ must be due to the absence of surface filaments on the EAEC strains. To distinguish between fimbrial gene expression and assembly, we first determined if fimbriae were indeed absent from NTZ-treated EAEC 042 bacteria by immunoblotting of whole bacteria subjected to vortexing to shear off the filaments. As seen in Fig. 4A (protein staining) and B (immunoblotting), intact filaments were obtained from bacteria grown statically in MinA-G medium without NTZ (supernatants). Fimbrial subunits were also detected in cell extracts that would represent both monomers and filaments and in biofilm scrapings from plastic wells that would represent filaments. In contrast, filaments obtained by vortexing (Fig. 4B, supernatants) of bacteria grown in LB medium were much less abundant, as confirmed previously (35, 37), although fimbrial subunits were detected in cellular extracts and to a much lesser extent from surface ring material extracted from the wells of microtiter dishes. At 20 μg/ml, NTZ inhibited biofilm formation in MinA-G medium and fimbrial filaments were nearly absent from vortexed whole cells (i.e., the filaments were observed at much lower levels than those for bacteria grown in LB medium). No fimbrial subunits were detected in cellular extracts, which might suggest that NTZ affects gene expression. While they are not depicted, the results of indirect immunofluorescent assays with whole cells were consistent with the results of the immunoblotting assay. These findings suggest that NTZ affects the production of AafA filaments either by repressing *aafA* gene expression or by inhibiting some step in AafA assembly.

NTZ does not affect *aafA* expression. To determine whether NTZ affected *aafA* gene expression, real-time qRT-PCR was used to monitor the *aggR* and *aafA* transcript levels in bacteria grown with gentle shaking in MinA-G medium with and without NTZ. The stationary-phase sigma factor gene *rpoS* served as an internal control in the qRT-PCR assays. As seen in Fig. 5, *aafA* expression was unaffected by NTZ: a Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks ($\alpha = 0.05$) found no statistically significant difference in the levels of *aafA* expression ($P = 0.086$) or *rpoS* expression ($P = 0.086$) in the presence or the absence of NTZ. However, the drug caused a 10-fold increase in the level of expression of *aggR* ($P = 0.002$, one-way ANOVA). The NTZ-induced increase in the level of *aggR* expression could also be demonstrated with *lacZ* reporter fusions, as depicted in Fig. 6A. β-Galactosidase assays showed a similar increase in the level of *aggR* gene expression (~2.5-fold), with cells growing statically in the presence of 12 μg/ml NTZ and with a slight decrease in growth occurring with cells grown in the presence of 25 μg/ml. In contrast, the level of *aafA* expression, as monitored by PhoA activity, was consistent with the qRT-PCR findings that NTZ treatment did not alter the level of *aafA* gene expression compared to that achieved by the controls not treated with NTZ (Fig. 6B). The qRT-PCR and reporter fusion assay results indicate that NTZ does not affect *aafA* gene expression. The upregulation of *argR* and not *aafA* might suggest a requirement for additional regulatory factors for the activation of *aafA*.

NTZ does not affect bacterial motility. Since disulfide bond formation is required for fimbrial assembly into filaments (20, 33), we considered the possibility that DsbA, a periplasmic protein that catalyzes this reaction, might be a potential target of the action of NTZ on the basis of previous reports of NTZ binding to related enzymes (13). Since the DsbA function is also required for the assembly and function of flagella, we examined NTZ-treated bacteria for motility. Wet mounts of bacteria grown in MinA-G medium with and without NTZ showed that the bacteria retained their motility, as viewed by phase-contrast microscopy, and that NTZ at concentrations that inhibited biofilm formation had no effect on swarming motility in soft agar chemotaxis assays (data not presented). NTZ concentrations at the MIC did affect swarming, but this was attributed to the inhibition of bacterial growth. The motility results are consistent with the findings of *aafA*::*phoA* fusion studies (DsbA is required to activate PhoA) and indicate that NTZ is not an inhibitor of DsbA function.

DISCUSSION

In this study, we found that the antiparasitic drug nitazoxanide and its deacetylated metabolite, tizoxanide, inhibit biofilm production by EAEC strain 042 and HA by both biofilm-producing and non-biofilm-producing strains when the strains

TABLE 1. Hemagglutination assay results

Bacterial strain	NTZ	HA of the following at the indicated bacterial OD_{660}[a]:					
		Sheep erythrocytes[b]			Guinea pig erythrocytes[c]		
		1.5	1.0	0.5	1.5	1.0	0.5
EAEC 042	−	+++	++	+			
EAEC 042	+	+	−	−			
EAEC JM221	−	+++	++	−	+++	++	++
EAEC JM221	+	++	−	−	+	−	−

[a] +++, strong hemagglutination reaction; +, weak hemagglutination reaction; −, no hemagglutination reaction.

[b] The bacteria were grown overnight in MinA-G medium supplemented (+) or not supplemented (−) with 20 μg/ml of NTZ. Hemagglutination assays were performed in 96-well microplates in triplicate. Bacteria grown in LB broth do not hemagglutinate sheep erythrocytes, indicating the absence of AAF.

[c] For determination of hemagglutination by type 1 fimbriae, the bacteria were grown statically in LB broth for 48 h in the presence (+) or the absence (−) of NTZ (20 μg/ml).

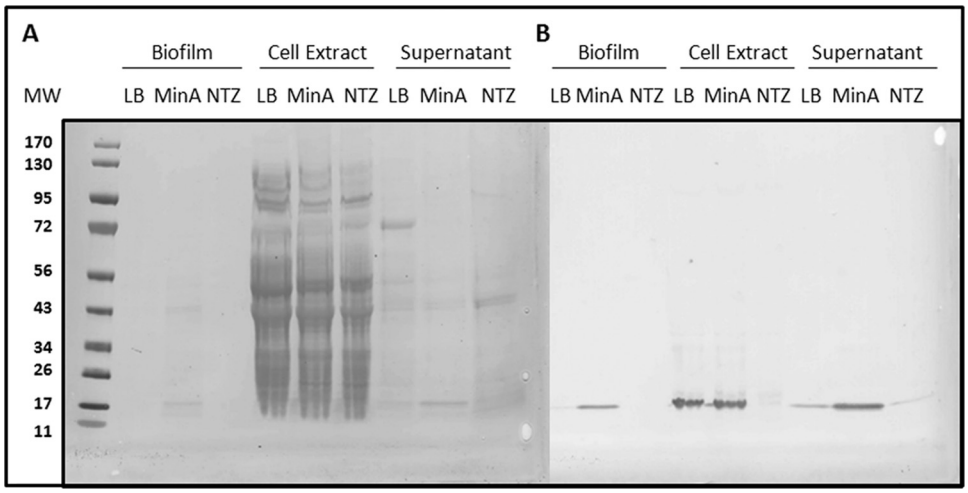

FIG. 4. Inhibition of pilin assembly by NTZ. Pilin was collected from whole cells following vortexing and centrifugation, followed by trichloroacetic acid precipitation, as described in the text. Ponceau red staining (A) and immunoblot analysis (B) of SDS-polyacrylamide gels of extracts prepared from bacteria grown statically in MinA-G medium, MinA-G medium plus NTZ (20 μg/ml), and LB medium were performed. MW, molecular weight (in thousands).

are cultured under conditions that promote biofilm formation and that are permissive for bacterial growth. MinA-G medium was generally superior to DMEM with glucose in promoting biofilm production, and little biofilm was produced in nutritionally rich LB medium. Since biofilm formation is due to the production of AAF, we showed by immunoblot and immunofluorescence assays that the abundance of intact fimbrial filaments recovered from NTZ-treated bacteria was dramatically reduced over that recovered from the control bacteria, suggesting that NTZ inhibited filament biogenesis. The AafA levels in cell extracts from NTZ-treated bacteria were undetectable, consistent with the findings from previous studies that unassembled monomers are rapidly degraded (18). Gene expression studies (qRT-PCR and reporter gene fusion assays) with EAEC 042 bacteria treated with NTZ at the 50% inhibitory concentration showed no change in the level of expression of the *aafA* gene over the level of expression by the controls, while the level of expression of its regulator, *aggR*, was increased severalfold. The upregulation of *aggR* might reflect feedback signaling associated with environmental conditions permissive for biofilm formation resulting from drug action, whereas the absence of increased levels of synthesis of AafA subunits might indicate negative feedback from aborted AafA assembly and protein turnover possibly mediated by extracytoplasmic sigma factors. Alternatively, regulatory elements in addition to AggR are required to activate *aafA* gene expression. While the inhibitory effect of NTZ on EAEC growth in MinA-G medium likely results from the cumulative effects on multiple biosynthetic targets, the profound effect on the assembly of AAF, a recognized virulence determinant, might be expected to affect colonization and the severity of disease. Further *in vivo* studies with animal models would be required to determine if the loss of aggregative adherence is sufficient to reduce the level of infection, the persistence of the bacteria, and the severity of diarrhea.

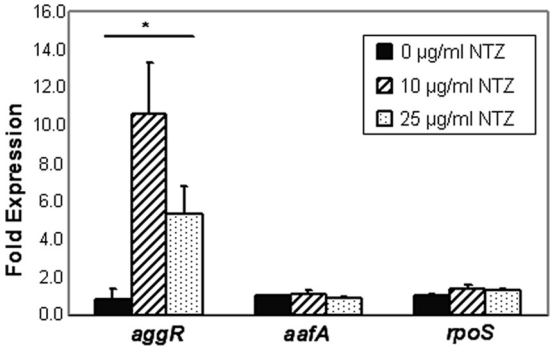

FIG. 5. qRT-PCR of *aggR*, *aafA*, and *rpoS*. Total RNA was prepared from EAEC strain 042 grown in MinA-G medium with the indicated concentrations of NTZ. The assays were performed in triplicate, and the relative fold expression is indexed to that of *aggR*, which was made equal to 1 in the absence of drug.

The AAF group comprises plasmid-encoded type IV bundle filaments that utilize the periplasmic chaperone-membrane usher system for monomer assembly into filaments (1). Other required activities include disulfide bond formation in AafA monomers by DsbA and filament dispersal on the surface by an outer membrane dispersin protein (20, 40). The DsbA thiol: disulfide oxidoreductase plays an essential role in facilitating the formation of disulfide linkages in many extracellular proteins, including fimbrial antigens and flagellin, in which this bond stabilizes the major structural subunits that are required for assembly (33, 42). While DsbA represents an attractive target for the inhibitory action of NTZ, the drug had no effect on bacterial motility or PhoA activity, which are dependent on the DsbA function (42). Our studies suggest that NTZ inhibits fimbrial filament assembly at a later step, perhaps by inhibiting chaperone or usher functions, as noted for the PilD chaperone in urogenic *E. coli* (1, 31, 33). This mechanism is further supported by our finding that NTZ also inhibits HA by EAEC strains that do not adhere to plastic and by EAEC strains expressing type 1 fimbriae. The term pilicide has been coined for small-molecule inhibitors of filament biogenesis (31). Sev-

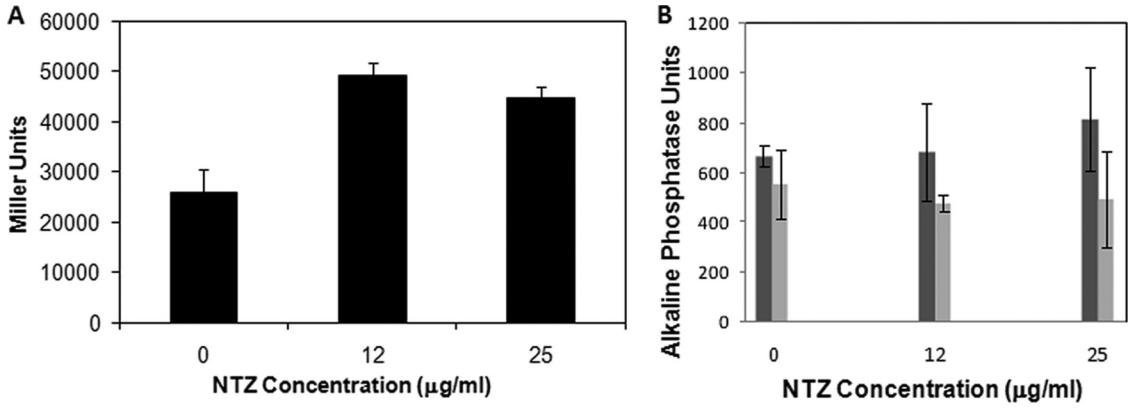

FIG. 6. Expression of reporter fusions. (A) β-Galactosidase activity of *aggR*::*lacZ* fusion in response to different concentrations of NTZ; (B) PhoA activity of *aafA*::*phoA* fusions in response to different concentrations of NTZ.

eral classes of inhibitors have been found to interact with the immunoglobulin-like PapD chaperone of the uropathogenic *E. coli* P-pilus system to block the chaperone-usher function (31). Our studies do not rule out the possibility that NTZ interferes with other steps in the process, such as the secretion or processing of prepilin or perhaps the assembly of pilin monomers. The anionic form of NTZ appears to be required for biological activity, as the denitro form of NTZ does not inhibit biofilm formation. Further study is required to identify the underlying molecular mechanisms of the action of NTZ.

The human large intestine is a complex ecosystem composed of large numbers of bacteria competing for a selected and limited nutrient supply mostly composed of short-chain fatty acids, organic acids, and complex polysaccharides and noticeably absent in sugars (22). Most gut flora are nonadherent and metabolically adapted to the available nutrient menu. Enteric pathogens, on the other hand, tend to become directly adherent via fimbriae or adherent as part of their invasion of epithelial tissue, but regardless of the strategy, their action tends to promote inflammation, which through serum leakage provides amino acids and sugars, a more growth permissive diet. In the case of EAEC, infection is believed to result from the initial attachment by aggregative adhesins, the displacement of the resident flora, the elaboration of various toxins that induce inflammation, and the production of a biofilm, which promotes clonal persistence by the organisms (28). Pilicides, by inhibiting microbial adherence to gut epithelial cells, should dramatically reduce the colonization efficiency. In this regard, NTZ should have efficacy against enteric pathogens whose infection strategy is dependent on adherence through fimbrial adhesins.

We demonstrate here that NTZ, an FDA-approved antidiarrheal drug, inhibits biofilm formation by impairing a key step in the pathogenesis of EAEC and that the use of NTZ offers a novel approach to combating an increasingly prevalent enteric pathogen that is highly and variably resistant to many antibiotics. NTZ does not kill EAEC but inhibits the filament assembly associated with mucosal biofilm formation, which plays a critical role in helping the bacteria to colonize the intestine and compromise the host's immune defense. NTZ could serve as a single agent that knocks out the three primary enteric pathogens that cause repeated or prolonged diarrheal illness among children in the developing world and could significantly allay the burden of diarrhea-associated morbidity (34).

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