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Effects of Antibiotics on Shiga Toxin 2 Production and Bacteriophage Induction by Epidemic *Escherichia coli* O104:H4 Strain

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The role of antibiotics in treatment of enterohemorrhagic *Escherichia coli* (EHEC) infections is controversial because of concerns about triggering hemolytic-uremic syndrome (HUS) by increasing Shiga toxin (Stx) production. During the recent large EHEC O104:H4 outbreak, antibiotic therapy was indicated for some patients. We tested a diverse panel of antibiotics to which the outbreak strain is susceptible to interrogate the effects of subinhibitory antibiotic concentrations on induction of stx_2-harboring bacteriophages, stx_2 transcription, and Stx2 production in this emerging pathogen. Ciprofloxacin significantly increased stx_2-harboring phage induction and Stx2 production in outbreak isolates (P values of <0.001 to <0.05), while fosfomycin, gentamicin, and kanamycin insignificantly influenced them ($P > 0.1$) and chloramphenicol, meropenem, azithromycin, rifaximin, and tigecycline significantly decreased them ($P \leq 0.05$). Ciprofloxacin and chloramphenicol significantly upregulated and downregulated stx_2 transcription, respectively ($P < 0.01$); the other antibiotics had insignificant effects ($P > 0.1$). Meropenem, azithromycin, and rifaximin, which were used for necessary therapeutic or prophylactic interventions during the EHEC O104:H4 outbreak, as well as tigecycline, neither induced stx_2-harboring phages nor increased stx_2 transcription or Stx2 production in the outbreak strain. These antibiotics might represent therapeutic options for patients with EHEC O104:H4 infection if antibiotic treatment is inevitable. We await further analysis of the epidemic to determine if usage of these agents was associated with an altered risk of developing HUS.

Enterohemorrhagic *Escherichia coli* (EHEC) causes diarrhea, bloody diarrhea, and hemolytic-uremic syndrome (HUS), mostly in children (21, 37). The most common EHEC serotype causing human diseases worldwide is O157:H7 (21, 37). In May to July 2011, Germany was afflicted by a massive outbreak caused by a rare EHEC serotype, O104:H4 (6, 13, 25). This outbreak involved >3,800 cases, including 855 patients with HUS and 53 deaths (33), and was the largest and most devastating outbreak of HUS in recorded history. The most likely outbreak vehicle was contaminated sprouts (6). Unusual epidemiological, clinical, and microbiological features of this outbreak were the predominance of adults and women (13, 33), an unprecedented proportion of HUS cases (~22%) (13, 33), severe neurological symptoms (epileptic seizures, pareses, delirium, and coma) in a subset of HUS patients (18), and the hybrid nature of the outbreak strain, which had combined virulence characteristics of EHEC and enteroaggregative *E. coli* (EAEC) (2, 26, 32). The outbreak strain displays an extended-spectrum beta-lactamase phenotype (2) from its CTX-M-15 genotype (32), i.e., it is resistant to all penicillins and cephalosporins and susceptible to carbapenems (ertapenem, imipenem, and meropenem) (2). It is also resistant to trimethoprim-sulfamethoxazole and susceptible to fluoroquinolones (ciprofloxacin) and aminoglycosides (gentamicin and tobramycin) (2).

Antibiotic therapy is generally not recommended for EHEC infections (17, 36, 37, 38) because of no benefit (30, 37), or even harm, in particular an increased risk of HUS development in patients treated with antibiotics during the initial period of diarrhea (1, 10, 36, 37, 38). One plausible mechanism by which antibiotics increase the risk of HUS development is that they increase the production and/or release of Shiga toxin (Stx), the major virulence factor of EHEC involved in the pathogenesis of HUS (21, 37). This occurs via induction of prophages harboring Stx-encoding genes (*stx*) (27, 40), which enhances *stx* transcription, Stx production, and toxin release from the bacterial cells via phage-mediated lysis (22, 27, 40). However, the effects of different antibiotic classes on Stx production *in vitro* differ and are also dependent on the antibiotic concentration and the nature of the EHEC strain (e.g., O group or Stx type) (16, 22, 24, 28, 29, 39). In studies performed with EHEC O157:H7, fluoroquinolones, trimethoprim-sulfamethoxazole, and ampicillin significantly increased Stx2 production (16, 20, 22, 23, 24, 29, 39, 40), whereas macrolides (24, 29, 39), carbapenems (22, 23), aminoglycosides (22, 24), rifampin (31), rifaximin (28), and fosfomycin (22, 24, 40) either had no effect on Stx2 production or suppressed it. These *in vitro* data are in accordance with experiments with animal models (31, 39, 40).

During the German EHEC O104:H4 outbreak, therapeutic or prophylactic administration of antibiotics was necessary for a subset of patients. According to the recommendations of the German Society for Infectious Diseases (15), carbapenems were used for treatment of invasive complications caused by the outbreak strain or by superinfecting microorganisms, azithromycin was used for eradication of nasopharyngeal meningococcal colonization before eculizumab therapy, and rifaximin, an oral nonabsorbable drug, was used for intestinal eradication of EHEC O104 in persistently colonized individuals and for other situations

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TABLE 1 MICs of nine antibiotics for EHEC strains investigated for antibiotic-mediated effects on stx_2-harboring phage induction, stx_2 transcription, and Stx2 production

Antibiotic	MIC (μg/ml)		
	LB226692 (O104:H4)	LB226806 (O104:H4)	EDL933 (O157:H7)
Ciprofloxacin	0.25	0.25	0.06
Meropenem	0.016	0.016	0.016
Azithromycin	8.0	8.0	4.0
Rifaximin	128.0	128.0	64.0
Tigecycline	0.25	0.25	0.125
Fosfomycin	1.0	1.0	2.0
Chloramphenicol	8.0	8.0	8.0
Gentamicin	0.125	0.125	0.125
Kanamycin	0.5	0.5	0.25

without indications for systemic antimicrobial therapy. Because the effects of antibiotics on Stx production in the EHEC O104:H4 outbreak strain are unknown, we investigated a diverse panel of antibiotics for their effects on induction of stx_2-harboring phages and, correspondingly, their ability to affect stx_2 transcription and Stx2 production in this emerging highly virulent pathogen.

MATERIALS AND METHODS

Bacterial strains. EHEC O104:H4 outbreak isolates studied for antibiotic-mediated induction of stx_2-carrying phages, stx_2 transcription, and Stx2 production were LB226692, first characterized and sequenced in our laboratory (2, 26), and an additional HUS isolate, LB226806 (2). Both isolates contain stx_2 in combination with typical EAEC loci (*aggA*, *aggR*, *set1*, *pic*, *aap*, and *aatA*) (2). EHEC O157:H7 strain EDL933 (39) was used for comparison. Twenty additional outbreak isolates tested for susceptibility to the investigated antibiotics were recovered from patients with HUS ($n = 15$) or bloody diarrhea without HUS ($n = 5$) in different parts of Germany.

Antibiotics and antimicrobial susceptibility testing. Ciprofloxacin, meropenem, fosfomycin (all from Sigma-Aldrich, Taufkirchen, Germany), chloramphenicol, gentamicin, and kanamycin (all from AppliChem, Darmstadt, Germany) were purchased from commercial suppliers; azithromycin, tigecycline (both from Pfizer, Groton, CT), and rifaximin (Alfa Wassermann, Bologna, Italy) were provided by their manufacturers. MICs were determined using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (7). For fosfomycin, for which the broth microdilution method is not approved (7), the MIC was determined using the agar dilution method (7) on Mueller-Hinton agar supplemented with 25 μg/ml of glucose-6-phosphate. The MICs (Table 1) were used to calculate subinhibitory concentrations in the range of 1/2 to 1/64 MIC.

Antibiotic induction. In the initial experiment, the strains were grown (37°C, 180 rpm) in 150 ml of Luria-Bertani (LB) broth containing 5 mM $CaCl_2$ until reaching an optical density at 600 nm (OD_{600}) of 0.5. Each culture was divided into 10-ml aliquots, which were supplemented with 1/4 MIC of each of the nine antibiotics (Table 1) (and with 25 μg/ml of glucose-6-phosphate for fosfomycin) or with no antibiotic (control) and then grown (37°C, 180 rpm) for 15 h. After centrifugation, filtered (0.2-μm membrane filter; Corning, Lowell, MA) culture supernatants were used in phage and toxin assays. Subsequently, six antibiotics (ciprofloxacin, meropenem, azithromycin, rifaximin, tigecycline, and chloramphenicol) were also tested in dilution series of subinhibitory concentrations (1/2 to 1/16 MIC) to investigate concentration-dependent effects. Rifaximin, for which a broad range of MICs has been reported (19, 34), was also tested at 1/32 and 1/64 MIC.

Detection of stx_2-carrying phages, stx_2 transcription, and Stx2 production. The presence of stx_2-harboring bacteriophages in 10-fold serial dilutions of culture filtrates was determined using a plaque assay (35) followed by plaque hybridization with a digoxigenin-11-dUTP-labeled stx_2 probe (DIG DNA labeling and detection kit; Roche Diagnostics, Mannheim, Germany). stx_2-carrying phage titers were expressed as numbers of stx_2 PFU per ml.

stx_2 transcription was determined using total bacterial RNA (RNeasy miniprep kit; Qiagen, Hilden, Germany) and one-step quantitative real-time reverse transcription-PCR (3) performed with a CFX96 real-time PCR system (Bio-Rad, Munich, Germany) and a SensiMix SYBR No-ROX one-step kit (Peqlab Biotechnologie, Erlangen, Germany). Primers RT-stx2F (5′-CGACCCCTCTTGAACATA-3′) and RT-stx2R (5′-TAGA-CATCAAGCCCTCGTAT-3′) were used to amplify stx_2, and primers GapA_forward and GapA_reverse (4) amplified the reference gene *gapA* (for normalization). A melting curve analysis to confirm the specificity of the amplification products was performed with continuous fluorescence reading from 65°C to 95°C. Data were analyzed using CFX Manager software, version 1.6 (Bio-Rad).

Stx2 titers were determined using a Vero cell assay (2) and were expressed as numbers of 50% cytotoxic doses (CD_{50}; the highest filtrate dilutions that caused cytotoxicity in 50% of cells after 72 h) per ml. The specific role of Stx2 in Vero cell cytotoxicity was verified by immunodetection of Stx2 in the filtrates, using a reversed passive latex agglutination test (VTEC-RPLA; Denka Seiken, Co., Ltd., Niigata, Japan).

The effects of each antibiotic on stx_2-harboring phage induction, stx_2 transcription, and Stx2 production are expressed as fold increases of the stx_2-harboring phage titer, stx_2 transcription level, and Stx2 titer, respectively, in the antibiotic-treated culture compared to a control, untreated culture (reflecting baseline, spontaneous stx_2-harboring phage induction, stx_2 transcription, and Stx2 production, each defined as 1). The baseline stx_2-carrying phage and Stx2 titers in the control cultures were as follows (range [mean ± standard deviation]): for strain LB226692, stx_2-harboring phage titers of 2.9×10^4 to 8.4×10^4 ($5.63 \times 10^4 \pm 2.24 \times 10^4$) and Stx2 titers of 640 to 1,280 (960 ± 369); for strain LB226806, stx_2-harboring phage titers of 4.5×10^4 to 1×10^5 ($7.15 \times 10^4 \pm 2.25 \times 10^4$) and Stx2 titers of 320 to 1,280 (880 ± 480); and for strain EDL933, stx_2-harboring phage titers of 5.2×10^3 to 7.8×10^4 ($6.55 \times 10^4 \pm 1.1 \times 10^4$) and Stx2 titers of 1,280 to 5,120 (3,520 ± 1,920). For stx_2 transcription, the stx_2/*gapA* mRNA ratio of the control culture of each strain was considered 1.

Statistical analysis. The experiments were performed three (stx_2 transcription) or four (stx_2-harboring phage induction and Stx2 production) times, and results are expressed as means ± standard deviations for the repeated experiments. The differences in stx_2-harboring phage induction, stx_2 transcription, and Stx2 production caused by the different antibiotics were analyzed using unpaired Student's *t* test. *P* values of ≤0.05 were considered significant.

RESULTS

Effects of subinhibitory antibiotic concentrations on induction of stx_2-harboring bacteriophages and Stx2 production. The antibiotics were initially investigated for their effects on stx_2-harboring phage induction and Stx2 production at 1/4 MIC. Ciprofloxacin, an inducer of stx_2-harboring phages in EHEC O157:H7 (40), significantly increased stx_2-harboring phage titers and Stx2 titers in both EHEC O104:H4 outbreak isolates and EHEC O157:H7 strain EDL933 ($P < 0.001$) (Table 2). This demonstrated that each of the outbreak isolates contains an inducible stx_2-harboring bacteriophage. Meropenem, azithromycin, fosfomycin, gentamicin, and kanamycin at 1/4 MIC had only slight, and statistically insignificant, effects on the baseline stx_2-harboring phage titers and Stx2 titers in all strains (Table 2). In contrast, rifaximin, tigecycline, and chloramphenicol significantly decreased the phage and/or Stx2 titer for both outbreak isolates ($P \leq 0.05$) (Table 2).

FIG 1 Effects of various subinhibitory concentrations of ciprofloxacin (CIP), meropenem (MEM), azithromycin (AZN), rifaximin (RIF), tigecycline (TGC), and chloramphenicol (CHL) on stx_2-harboring phage induction in EHEC O104:H4 outbreak isolates LB226692 (A) and LB226806 (B) and EHEC O157:H7 strain EDL933 (C). Data show fold increases in stx_2-harboring phage titers (indicating stx_2-harboring phage induction) compared to those of antibiotic-free controls (defined as 1) and are means ± standard deviations for four independent experiments. All antibiotics were tested at 1/2 to 1/16 MIC, and rifaximin was also tested at 1/32 and 1/64 MIC. *, $P < 0.001$; #, $P \leq 0.05$ (unpaired Student's *t* test).

For EHEC O157:H7 strain EDL933, decreases in the phage and toxin titers mediated by the last three antibiotics did not reach statistical significance (Table 2).

The six antibiotics that most profoundly changed stx_2-harboring phage induction and/or Stx2 production in the outbreak isolates at 1/4 MIC and/or were proposed as therapeutic options during the EHEC O104:H4 outbreak (15) were tested for their effects on phage titers (Fig. 1) and toxin titers (Fig. 2) at a broader range of subinhibitory concentrations. Ciprofloxacin significantly increased both stx_2-harboring phage and Stx2 titers (*P* value of <0.001 or <0.05) at all subinhibitory concentrations (1/2 to 1/16 MIC), in a dose-dependent manner (Fig. 1 and 2). Chloramphenicol caused a concentration-dependent decrease in stx_2-harboring

TABLE 2 Effects of antibiotics at 1/4 MIC on induction of stx_2-harboring bacteriophages and Stx2 production[a]

Antibiotic[b]	LB226692 (O104:H4)				LB226806 (O104:H4)				EDL933 (O157:H7)			
	stx_2-harboring phage titer	*P* value	Stx2 titer	*P* value	stx_2-harboring phage titer	*P* value	Stx2 titer	*P* value	stx_2-harboring phage titer	*P* value	Stx2 titer	*P* value
CIP	**$6.4 \times 10^4 \pm 7.5 \times 10^3$[c]**	**<0.001**	**128.0 ± 0[c]**	**<0.001**	**$1.4 \times 10^5 \pm 9.3 \times 10^3$[c]**	**<0.001**	**128.0 ± 0[c]**	**<0.001**	**$6 \times 10^3 \pm 8.8 \times 10^2$[c]**	**<0.001**	**128.0 ± 0[c]**	**<0.001**
MEM	0.98 ± 0.2	0.68	0.67 ± 0.18	0.28	0.95 ± 0.10	0.68	0.51 ± 0.09	0.13	1.0 ± 0.1	0.79	0.50 ± 0.06	0.12
AZM	0.72 ± 0.1	0.43	0.50 ± 0	0.12	0.51 ± 0.11	0.11	0.54 ± 0.13	0.14	0.63 ± 0.09	0.22	0.59 ± 0.04	0.19
RIF	**0.12 ± 0.01[d]**	**0.011**	**0.13 ± 0[d]**	**0.012**	0.39 ± 0.04	0.062	**0.25 ± 0.06[d]**	**0.025**	0.61 ± 0.11	0.21	0.93 ± 0.28	0.65
TGC	**0.21 ± 0.02[d]**	**0.05**	0.58 ± 0.1	0.17	**0.16 ± 0.04[d]**	**0.014**	**0.29 ± 0.11[d]**	**0.031**	0.85 ± 0.12	0.52	0.50 ± 0.14	0.12
FOF	0.97 ± 0.22	0.71	1.2 ± 0.42	0.95	0.96 ± 0.26	0.72	0.75 ± 0.23	0.37	1.8 ± 0.48	0.45	0.87 ± 0.28	0.54
CHL	**0.25 ± 0.06[d]**	**0.024**	**0.13 ± 0.02[d]**	**0.012**	**0.13 ± 0.03[d]**	**0.012**	**0.04 ± 0.02[d]**	**0.0068**	0.61 ± 0.09	0.21	0.55 ± 0.06	0.15
GEN	0.57 ± 0.12	0.17	0.83 ± 0.18	0.53	1.3 ± 0.23	0.89	0.67 ± 0.18	0.29	2.4 ± 0.12	0.24	1.0 ± 0.18	0.96
KAN	1.56 ± 0.42	0.37	0.83 ± 0.18	0.51	0.48 ± 0.09	0.21	0.58 ± 0.18	0.33	1.0 ± 0.11	0.99	0.83 ± 0.18	0.51

[a] Fold increases of stx_2-harboring phage titers (indicating stx_2-harboring phage induction) and Stx2 titers (indicating Stx2 production) in antibiotic-treated cultures compared to those in control, untreated cultures (defined as 1) are presented (for the actual stx_2-harboring phage and Stx2 titers in the control cultures, see Materials and Methods). Data are means ± standard deviations for four independent experiments. *P* values (by unpaired Student's *t* test) of ≤0.05 are statistically significant (data shown in bold).

[b] CIP, ciprofloxacin; MEM, meropenem; AZM, azithromycin; RIF, rifaximin; TGC, tigecycline; FOF, fosfomycin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin.

[c] Significantly increased ($P < 0.001$) compared to control.

[d] Significantly decreased ($P \leq 0.05$) compared to control.

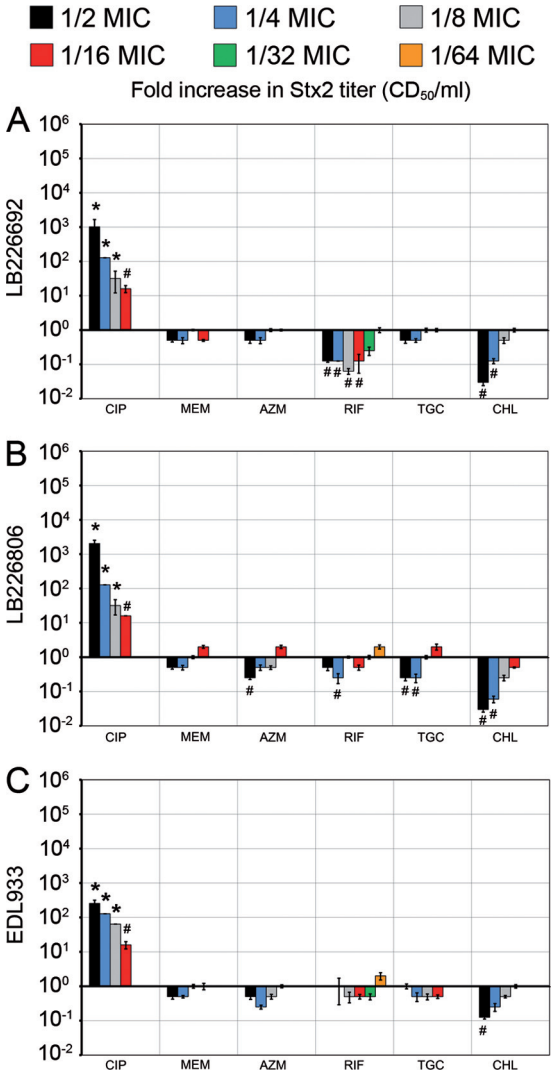

FIG 2 Effects of various subinhibitory concentrations of ciprofloxacin (CIP), meropenem (MEM), azithromycin (AZN), rifaximin (RIF), tigecycline (TGC), and chloramphenicol (CHL) on Stx2 production in EHEC O104:H4 outbreak isolates LB226692 (A) and LB226806 (B) and EHEC O157:H7 strain EDL933 (C). Data show fold increases in Stx2 titers (indicating Stx2 production) compared to those of antibiotic-free controls (defined as 1) and are means ± standard deviations for four independent experiments. All antibiotics were tested at 1/2 to 1/16 MIC, and rifaximin was also tested at 1/32 and 1/64 MIC. *, $P < 0.001$; #, $P \leq 0.05$ (unpaired Student's *t* test).

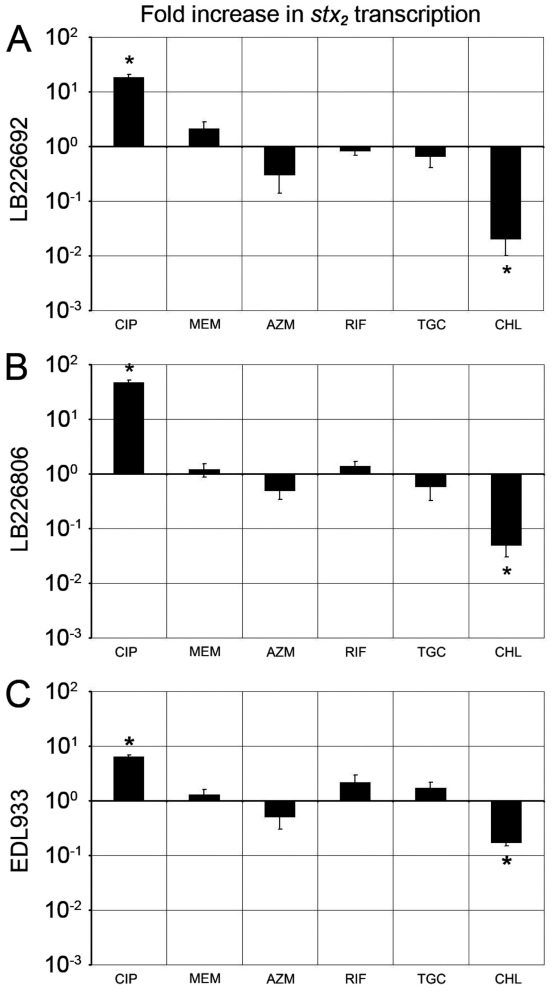

FIG 3 Effects of ciprofloxacin (CIP), meropenem (MEM), azithromycin (AZN), rifaximin (RIF), tigecycline (TGC), and chloramphenicol (CHL) at 1/2 MIC on stx_2 transcription in EHEC O104:H4 outbreak isolates LB226692 (A) and LB226806 (B) and EHEC O157:H7 strain EDL933 (C). Data show fold increases in stx_2 transcription compared to that in antibiotic-free cultures and are means ± standard deviations for three independent experiments. *, $P < 0.01$ (unpaired Student's *t* test).

phage and Stx2 titers in the range of 1/2 to 1/8 MIC; the most pronounced and statistically significant suppressive effects ($P < 0.05$) of chloramphenicol were regularly observed at 1/2 MIC (Fig. 1 and 2). The remaining four antibiotics (meropenem, azithromycin, rifaximin, and tigecycline) showed no clear concentration-dependent effects on stx_2-harboring phage and Stx2 titers. For EHEC O157:H7 strain EDL933, none of these antibiotics at any concentration significantly changed the baseline phage titers ($P = 0.27$ to 0.92) (Fig. 1) and toxin titers ($P = 0.11$ to 0.89) (Fig. 2). In contrast, with the EHEC O104:H4 outbreak isolates, meropenem, rifaximin, and tigecycline (both isolates), as well as azithromycin (LB226806), significantly decreased stx_2-harboring phage titers ($P \leq 0.05$), regularly at 1/2 MIC and sometimes also at lower concentrations (Fig. 1). Phage titer decreases were usually accompanied by Stx2 titer decreases, though the latter attained statistical significance less often (Fig. 2). None of these four antibiotics at any concentration significantly increased the phage titer (Fig. 1) or toxin titer (Fig. 2) of the outbreak isolates.

Effects of subinhibitory antibiotic concentrations on stx_2 transcription. The above six antibiotics at 1/2 MIC, which most considerably influenced stx_2-harboring phage induction (Fig. 1) and Stx2 production (Fig. 2), were tested for their effects on stx_2 transcription. Ciprofloxacin significantly increased ($P < 0.01$) and chloramphenicol significantly decreased ($P < 0.01$) stx_2 transcription in the EHEC O104:H4 outbreak isolates and strain EDL933 (Fig. 3). Meropenem, azithromycin, rifaximin, and tigecycline had only slight effects ($P = 0.39$ to 0.72), and none of them significantly upregulated stx_2 transcription in any strain (Fig. 3).

Susceptibilities of outbreak isolates to the investigated antibiotics. Each of 20 additionally tested EHEC O104:H4 outbreak isolates was susceptible to ciprofloxacin, meropenem, fosfomycin,

chloramphenicol, gentamicin, and kanamycin according to CLSI guidelines (8) and to tigecycline according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (11) guidelines (no CLSI breakpoints are available for this antibiotic), as were isolates LB226692 and LB226806. The MICs for azithromycin and rifaximin, for which there are no interpretative categories for *E. coli*, were in the ranges reported previously for other EHEC strains (19, 24).

DISCUSSION

The necessity of using antibiotic therapy for a subset of patients during the recent German *E. coli* O104:H4 outbreak prompted us to investigate a panel of antibiotics to which the outbreak strain is susceptible for their effects on induction of stx_2-harboring phages, stx_2 transcription, and Stx2 production in this pathogen. Except for ciprofloxacin (32), no other antibiotics have previously been investigated thusly. We were particularly interested in those antibiotics that had been proposed for indicated use during the O104:H4 outbreak (15) based on their inability to induce stx_2-harboring phages and Stx2 production in EHEC O157:H7 (23, 24, 28, 39), i.e., azithromycin, rifaximin, and meropenem (though another carbapenem, imipenem, not meropenem itself, was investigated with EHEC O157:H7 in this respect) (23). We also included tigecycline because of its broad activity against Gram-positive and Gram-negative bacteria (14) and because, to our knowledge, its effects on induction of *stx*-harboring phages and Stx production have not yet been studied. We used various subinhibitory antibiotic concentrations, the methodology commonly applied to investigate effects of antibiotics on Stx production and/or *stx*-harboring phage induction in other EHEC strains (16, 23, 24, 28, 39), which allows determination of the concentration dependence of the effects studied. In contrast, therapeutic concentrations rapidly inhibit or kill bacteria *in vitro*, thus hampering unbiased measurement of antibiotic effects.

Among the nine antibiotics tested, only ciprofloxacin, an agent previously shown to induce *stx*-harboring phages in EHEC O157:H7 via triggering of the SOS response (22, 40), significantly increased stx_2-harboring phage induction, stx_2 transcription, and Stx2 production in EHEC O104:H4 outbreak isolates. These effects of ciprofloxacin are in accordance with the structural similarities between the stx_2-harboring phage of EHEC O104:H4 and the stx_2-harboring phage 933W of EHEC O157:H7 strain EDL933 reported by Rasko et al. (32). These authors also showed a significant (~80-fold) ciprofloxacin-mediated increase in stx_2 transcription in the EHEC O104:H4 outbreak strain, but no attempts were made to demonstrate increases in the stx_2-harboring phage titer and the amount of biologically active Stx2 as shown in our study. Because of its unfavorable effects on phage induction and Stx production, which may increase the risk of development of HUS, ciprofloxacin should not be used in patients with diarrhea if an EHEC etiology cannot be excluded. The administration of ciprofloxacin to some patients with diarrhea at the beginning of the EHEC O104:H4 outbreak (our unpublished data), before the EHEC etiology was determined, was due mainly to the predominant involvement of adults, which did not lead to a suspicion of an EHEC-mediated outbreak because EHEC infections typically affect children (21, 37). However, no data are yet available from the German outbreak about the impact of ciprofloxacin treatment on the clinical outcome of infection, in particular the progression of infection to HUS. Similarly, in a study from Denmark, where four of five patients with HUS caused by the German EHEC O104:H4 outbreak strain received ciprofloxacin upon first presenting with diarrhea to their physicians (9), no analysis determined any association between this antibiotic treatment and development of HUS.

The crucial finding in our study is that none of the antibiotics proposed for urgent therapeutic or prophylactic use during the EHEC O104:H4 outbreak (15), specifically meropenem, azithromycin, and rifaximin, induced stx_2-harboring phages or increased stx_2 transcription or Stx2 production in the outbreak isolates in an *in vitro* system. Indeed, these antibiotics, regularly at 1/2 MIC but also at lower concentrations, significantly decreased one or more of these processes. The same holds true for tigecycline. However, despite these promising *in vitro* results, it is premature to advocate, based solely on these data, the use of these antibiotics for treatment of patients with EHEC O104:H4 infection. Further studies using animal models are necessary to determine whether or not the favorable effects on stx_2-harboring phages and Stx2 production observed *in vitro* can be confirmed *in vivo*. Moreover, other effects of these antibiotics, e.g., the extent to which they reduce the normal intestinal flora or their influence on intestinal adherence of the pathogen, might modulate the course of EHEC infection. Indeed, reduced adherence to epithelial cells after their pretreatment with rifaximin has been reported for EAEC (5). Finally, thorough analyses of clinical outcomes of patients who were administered these antibiotics during the outbreak are necessary for ultimate evaluation of their potential usefulness for treatment of humans infected with EHEC O104:H4 in cases where antibiotic therapy is inevitable. A recent study demonstrated that treatment with azithromycin significantly decreased frequency of long-term carriage of the EHEC O104:H4 outbreak strain (27a).

Rifaximin, which is licensed in many countries to treat traveler's diarrhea (34), might be considered an option, in contrast to ciprofloxacin, for treatment of diarrhea of unclear etiology because it does not induce *stx*-harboring phages and Stx production (28; this study) and is thus supposed to have no adverse effect on systemic progression of infection in cases of unrecognized EHEC infection. Although chloramphenicol is not used extensively anymore in the developed world (12), the significant suppressive effects of this antibiotic on stx_2-harboring phage induction, stx_2 transcription, and Stx2 production in all tested strains in our study are important findings which should be kept in mind.

In conclusion, we demonstrated that an antibiotic-mediated effect on stx_2-harboring phage induction is the critical process through which Stx2 production is modulated in EHEC O104:H4. Several antibiotics decrease or do not influence baseline levels of stx_2-harboring phage induction, stx_2 transcription, and Stx2 production in the EHEC O104:H4 outbreak strain and might therefore be potentially therapeutically useful in indicated cases. These data are the first step in the investigation of the effects of antibiotics on the outbreak strain regarding their potential benefit or harm to patients. We await further analysis of the outbreak to determine if usage of these agents was associated with a reduced risk of developing HUS.

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