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Norfloxacin Resistance Mechanism of *E. coli* 11 and *E. coli* 101-Clinical Isolates of *Escherichia coli* in Korea

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E. coli 11 and *E. coli* 101, clinical isolates of *Escherichia coli* were resistant to various quinolones, especially MICs to norfloxacin of both strains were higher than 100 μg/ml. In the presence of carbonyl cyanide *m*-chlorophenylhydrazone, a proton gradient uncoupler, norfloxacin uptake in both strains was increased, suggesting that an efflux system play an important role in the norfloxacin resistance. Outer membrane proteins of the susceptible and resistant strains which could affect the route of norfloxacin entry into cells were different. When quinolone resistance determining region (QRDR) of *gyrA* was amplified using PCR and cut with *Hinf* I, QRDR in the susceptible strain yielded two fragments while QRDRs in *E. coli* 11 and *E. coli* 101 yielded only one uncut fragment. When DNA sequence of QRDR was analyzed, there were two mutations at Ser-83 and Asp-87 in both resistant strains. These residues were changed to Leu-83 and Asn-87, respectively. These results showed that the norfloxacin resistance of *E. coli* 11 and *E. coli* 101 was resulted from multiple changes-an altered DNA gyrase A subunit, a change in route of drug entry, and reduction in quinolone concentration inside cells due to an efflux system.

Key Words : Resistance, Quinolone, Norfloxacin, *Escherichia coli*, Clinical isolate, Antibacterial agents

INTRODUCTION

Fluoroquinolones have been widely used because of their potent antibactericidal effects and a broad spectrum. As they have been widely used, the emergence of quinolone resistant strains became a big problem. Since the resistance to antibacterial agents could be transferred to other bacteria and a resistant strain could be transferred to other country in a short period owing to the development of a transport system, it is very urgent to overcome this problem. However, only a small number of reports about the occurrence of quinolone resistant strains and the resistant mechanism of them had been published in Korea (Lee *et al*, 1992; Lee *et al*, 1993).

Up to the present time, it has been reported that the resistance to quinolone was due to a mutation in DNA gyrase, an alteration in routes of drug entry, or an existence of an efflux system. Because mutations in DNA gyrase was clustered within a narrow region between 199 nucleotide and 318 nucleotide in *gyrA* (Yoshida *et al.*, 1990), this region was called as 'Quinolone Resistance Determining Region' (QRDR). Especially a point mutation at Ser-83 has been known to be related to the quinolone resistance in clinical isolate of *Escherichia coli* (Aoyama *et al.*, 1987; Cullen *et al.*, 1989; Cambau *et al.*, 1993).

We selected two quinolone resistant *E. coli* strains (*E. coli* 11 and *E. coli* 101) for the study of the norfloxacin resistant mechanism, and examined DNA sequence of QRDR in *gyrA*, a profile of outer membrane proteins, norfloxacin uptake, and a possible existence of an efflux system for norfloxacin.

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MATERIALS AND METHODS

Bacterial strains

E. coli TEM-a quinolone susceptible strain was provided from Korea Research Institute of Chemical Technology (KRICT). Clinical isolates of *E. coli* were provided from Je-il Hospital and Korea Cancer Center Hospital, Seoul, Korea. *E. coli* 11 and *E. coli* 101 were selected among quinolone resistant isolates which were grown at 10 μg/ml of ciprofloxacin, ofloxacin, or norfloxacin.

Reagents

[^{3}H]thymidine (specific activity, 50 Ci/mmol), [^{3}H]

TTP (specific activity, 30 Ci/mmol), *Taq* DNA polymerase, and *Hinf* I were purchased from Amersham (Buckinghamshire, England). Media were purchased from Difco (Detroit, IL). Quinolones and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Susceptibility test

MICs were measured using a broth dilution method of Champman and Georgopadakou (1988).

Assay of norfloxacin concentration inside cells

Bacterial cells at log phase were collected with centrifugation, resuspended in new LB making A_{530} to 10, and further incubated for 30 min at 37°C. Norfloxacin was added to the cells and the reaction mixture was incubated for 5 min at 37°C, then carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to this. The reaction was continued for 10 min and stopped with centrifugation after 200 µl of the reaction mixture was loaded on 500 µl of cold silicon oil in a 1.5 ml microcentrifuge tube. The bottom layer which containing cells was cut with a cutter, transferred into a new tube, and cells were suspended in 10 mM phosphate buffer (pH 7.0) with vigorous skaking. Norfloxacin was extracted from cells with boiling in a boiling water bath for 7 min and assayed at 279 nm for excitation and 443 nm for emission in a Fluoresence Spectrophotometer (Kontrons SFM25, Switzland) after cells were removed with centrifugation at 10,000×g for 5 min.

Isolation of outer membrane proteins

Bacterial cells were grown overnight at 37°C, collected with centrifugation, and resuspended in a buffer containing 10 mM phosphate (pH 7.0), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were broken in a bead beater (bead diameter=0.1 mm, Biospec Products, U.S.A.) with 20 times of 20 seconds burst and 20 seconds rest at 4°C. Unbroken cells were removed with centrifugation at 10,000×g (Sorvall RC5B, U.S.A.) for 5 min. The supernatant was centrifuged at 100,000×g for 1 hour in a ultracentrifuge (Beckman L8-60M, U.S.A.). The pellet was treated with sarkosyl (final conc., 1%) for 30 min to remove the inner membrane parts and washed with 10 mM phosphate buffer (pH 7.0) by centrifugation at 100,000×g for 1 hour. Pelleted outer membrane parts were treated with SDS (final conc., 1%) and boiled for 10 min to extract proteins.

Polyacrylamide gel electrophoresis of outer membrane proteins

Outer membrane proteins isolated as described above were electrophoresed using a gel with 3.75% acrylamide for stacking and 12.5% acrylamide for resolving in a mini protein II gel electrophoresis kit (Bio-Rad, U.S.A.) following a method of Laemmli (1970). After electrophoresis, proteins were visualized with 0.1% Coomassie brilliant blue R250 and analyzed with Bioprofil (Vilberlourmat, France).

In vitro DNA synthesis in permeabilized cells

In vitro DNA synthesis was assayed using permeabilized cells as described elsewhere (Lee, *et al.*, 1992; Lee, *et al.*, 1993). Bacterial cells at log phase were harvested with centrifugation at 10,000×g for 10 min and suspended in one tenth volume of cells with buffer A [10 mM Tris-HCl (pH 7.8), 50 mM NH_4Cl, 10 mM $(CH_3COO)_2Mg \cdot 4H_2O$, 7 mM β-mercaptoethanol]. Cells were treated with toluene (final conc., 2%) and shaken for 10 min in an ice bath. The reaction mixture was diluted with three volumes of buffer B [0.1 mM Tris-HCl (pH 7.4), 6.2 mM NH_4Cl, 19 mM $(CH_3COO)_2Mg \cdot 4H_2O$]. Permeabilized cells were added to the reaction mixture containing 40 µM of dNTP, 0.2 mM of NADH, and norfloxacin. After 5 min incubation at 37°C, 0.1 µCi of [^{3}H]dTTP was added to the reaction mixture and incubated for 10 min. The reaction was stopped by adding 1 ml of cold 10% TCA. The acid insoluble precipitates were collected on a GF/C filter (Sigma Chemical Co., MO) using a vacuum manifold (Hoefer, U.S.A.). Filters were presoaked in 0.05% thymidine solution to prevent nonspecific binding of [^{3}H]thymidine. After filters were washed with 4 ml each of 5% TCA, 0.1 N HCl, and 95% ethanol and dried in the air. The amount of radioactivity remaining on a filter was measured in a liquid scintillation counter (Packard TRI-CARB 4530, U.S.A.).

Restriction fragment length polymorphism (RFLP)

A fragment of 258 base pairs containing one *Hinf* I site at Ser-83 was amplified with PCR. The reaction mixture contained 1 µg of template DNA and 100 pmol each of primer 1 (5'-ATTGTTGGCCGTGCGCTGCCAGAT-3', corresponding to positions +85 to +109 nt in *gyrA*) and primer 2 (5'-TCGCCGTCGATAGAACCGAAGTTA-3', complementary to positions +319 to +343 nt). PCR was carried out in 100 µl of a reaction mixture containing 200 µM dNTP mixture, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) Triton X-100, and 5 U of *Taq* DNA polymerase. To prevent evaporation, 100 µl of mineral oil was layered on top. PCR was carried out with an initial denaturation at 94°C for 4 min and 25 cycles of 94°C for 1 min, 68°C for 2 min, 72°C for 2 min, and a final elongation step at 72°C for 10 min in a thermocycler (EquiBio, Belgium). PCR products were confirmed with electroph-

oresis using a 8% polyacrylamide gel in TBE buffer and purified directly from the reaction mixture using DNA purification system (Wizard PCR Preps; Promega, Madison, WI), and used for RFLP. Purified PCR product was digested with *Hinf* I for 1 hour at 37°C and electrophoresed in a 8% polyacrylamide gel in TBE and visualized with ethidium bromide.

DNA sequencing

Quinolone resistant determining region (QRDR) of DNA gyrase subunit A (*gyr*A) was amplified with PCR. The condition for PCR was identical with that for RFLP. Enzymatic extension reactions were performed using ABI PRISM Dye Terminator Cycle Sequencing Core Kit (Perkin Elmer Co., U.S.A.) with AmpliTaq DNA polymerase on GeneAmp PCR systems (Perkin Elmer Co., U.S.A.), and sequences were analyzed with ABI 373 DNA sequencer (Perkin Elmer Co., U.S.A.).

RESULTS

MICs of *E. coli* TEM, *E. coli* 11 and *E. coli* 101

Among quinolone resistant clinical isolates, two strains (*E. coli* 11 and *E. coli* 101) were selected for this study because of their high MICs to ciprofloxacin, ofloxacin, and norfloxacin (Table 1.).

Table 1. MICs of *E. coli* TEM, *E. coli* 11, and *E. coli* 101

	ciprofloxacin (μg/ml)	norfloxacin (μg/ml)	ofloxacin (μg/ml)
E. coli TEM	0.05	0.5	0.4
E. coli 11	200	100	100
E. coli 101	50	100	25

*MICs were determined with a broth dilution method (Champman & Georgopapadakou, 1988).

SDS denaturing polyacrylamide gel electrophoresis of outer membrane proteins

As shown in Fig. 1, even though there were no significant changes in major proteins with 25 kD, 35 kD, and 38 kD, protein profiles of the resistant strains were different from that of *E. coli* TEM. A protein with 19 kD disappeared in *E. coli* 11 while increased remarkably in *E. coli* 101, and proteins with 57 kD, 52 kD, and 50 kD newly appeared in the resistant strains.

The effect of CCCP on norfloxacin concentration inside cells

Norfloxacin concentration inside cells was assayed

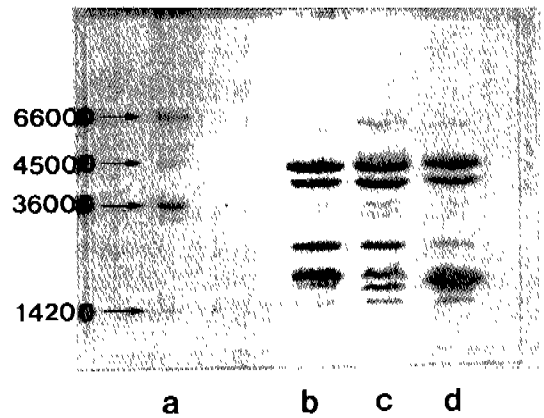

Fig. 1. SDS denaturing polyacrylamide gel electrophoresis of outer membrane proteins. Outer membrane proteins were isolated from each strain and electrophoresed in a 12.5% SDS-denaturing polyacrylamide gel. Lane a, marker protein; phosphorylase b (97,400); serum albumin (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); trypsin inhibitor (21,500); lysozyme (14,400); lane b, *E. coli* TEM; lane c, *E. coli* 11; lane d, *E. coli* 101

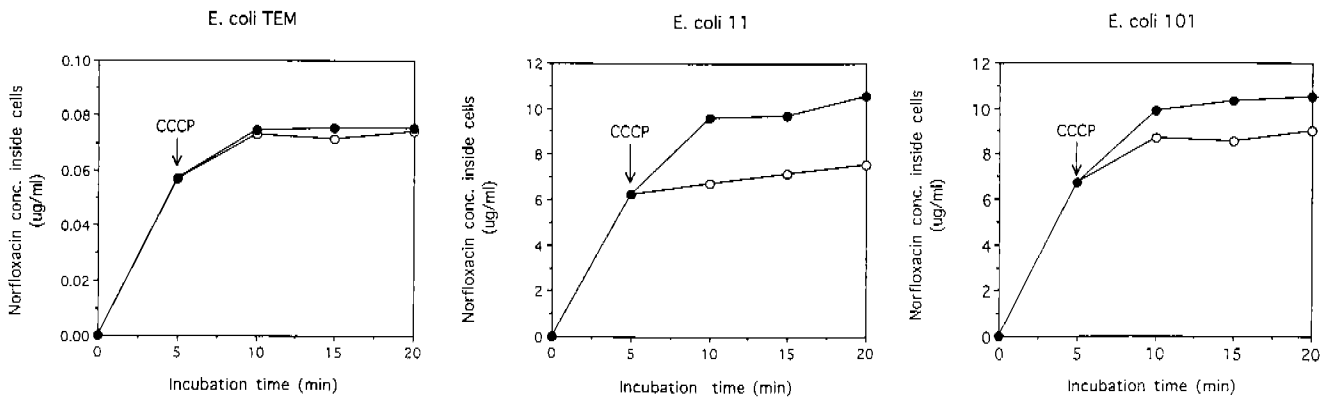

Fig. 2. The effect of CCCP on norfloxacin concentration inside cells. Cells in log phase were incubated with norfloxacin (0.3 μg/ml for the susceptible strain and 400 μg/ml for the resistant strains) and CCCP (2 μM for the susceptible strain and 25 μM for the resistant strains) was added to the cells at the time indicated. At various time intervals, cells were taken from the reaction mixture. Norfloxacin was extracted from cells with boiling and assayed in a fluorescence spectrophotometer. ○, with norfloxacin; ●, with norfloxacin and CCCP

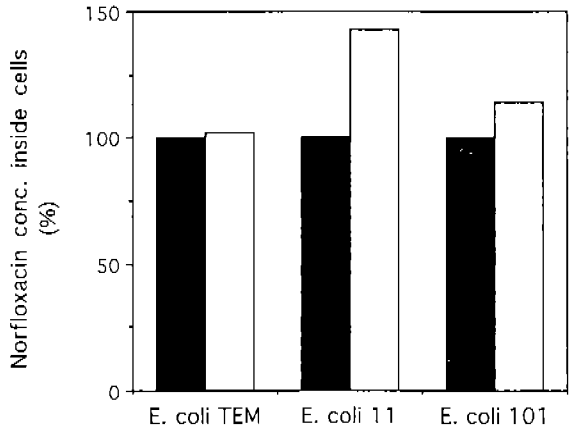

Fig. 3. Norfloxacin concentration inside cells after the addition of CCCP. Norfloxacin concentrations at 10 min after the addition of CCCP in Fig. 3. were replotted. ■, without norfloxacin; □, with norfloxacin

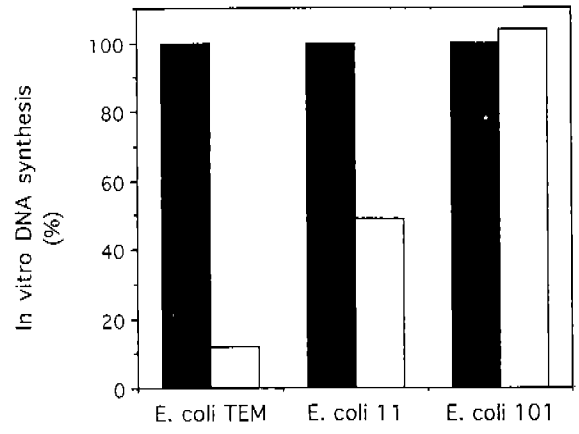

Fig. 4. The effect of norfloxacin on in vitro DNA synthesis. Cells were permeabilized with toluene and incubated with norfloxacin (1 μg/ml for *E. coli* TEM, 50 μg/ml for *E. coli* 11 and *E. coli* 101) at 37°C for 5 minutes. [^{3}H]TTP (specific activity, 30 μCi/mmol) was added to the permeabilized cells and the radioactivity in the acid insoluble precipitates was measured in a liquid scintillation counter. The amount of DNA synthesized in the absence of norfloxacin was considered as 100%. ■, without norfloxacin; □, with norfloxacin

after CCCP was added to cells. If there were a proton gradient dependent efflux system for norfloxacin, the addition of CCCP would increase the norfloxacin concentration inside cells. As soon as CCCP was added to cells, norfloxacin concentrations in *E. coli* 11 and *E. coli* 101 started to increase (Fig. 2.). After 10 min, norfloxacin concentration increased upto 143% in *E. coli* 11 and 114% in *E. coli* 101 compared to those without CCCP (Fig. 3.). Since the sensitivities of *E. coli* TEM and the resistant strains to CCCP were very different, CCCP concentration was 2 μM for *E. coli* TEM and 25 μM for *E. coli* 11 and *E. coli* 101 which inhibited 50% of DNA synthesis in each strain (data not shown).

The effect of norfloxacin on *in vitro* DNA synthesis

To observe the inhibitory activity of norfloxacin on DNA synthesis without any effects from an uptake barrier and an efflux system, DNA synthesis in permeabilized cells was assayed. Considering the amount of DNA synthesized in the absence of norfloxacin as 100%, *in vitro* DNA synthesis in each strains were 12% in *E. coli* TEM, 49% in *E. coli* 101, and 104% in *E. coli* 101 in the presence of norfloxacin (Fig. 4.)

Restriction fragment length polymorphism of QRDR

Since a change in Ser-83 was known to be responsible for the quinolone resistance, QRDR with one *Hinf* I site at Ser-83 (from 85 nt to 343 nt) was amplified and cut with *Hinf* I. If there were a mutation at Ser-83, this fragment would not be cut with *Hinf* I yielding one fragment instead of two fragments. As shown in Fig. 5, QRDR of *E. coli* TEM was cut with *Hinf* I as expected while QRDRs of *E. coli* 11 and *E. coli* 101 were not cut with *Hinf* I yielding one fragment.

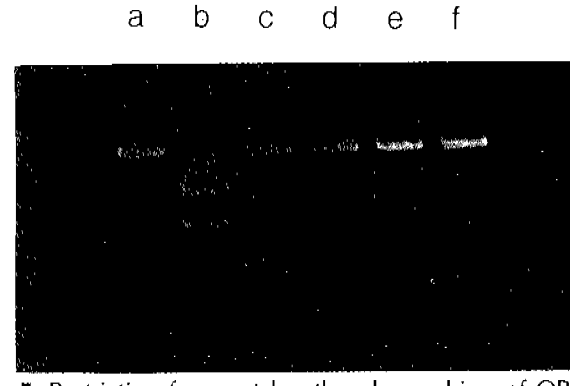

Fig. 5. Restriction fragment length polymorphism of QRDR. QRDR of *gyrA* in each strain was amplified using PCR with primers specific to this region and digested with *Hinf* I. Lane a, PCR product of *E. coli* TEM; lane c, PCR product of *E. coli* 11; lane e, PCR product of *E. coli* 101; lane b, *Hinf* I digested products of the susceptible *E. coli*; lane d, Hinf I digested products of *E. coli* 11; lane f, *Hinf* I digested products of *E. coli* 101

DNA sequencing of QRDR

DNA sequencing of QRDR in *E. coli* 11 and *E. coli* 101 revealed two mutations at Ser-83 (TCG) and Asp-87(GAC) in both strains. These were changed to Leu-83 (TTG) and Asn-87 (AAC) in both resistant strains. Besides these two missense mutations, *E. coli* 11 had two other silent mutations at Arg-91 (CGC) and Tyr-100 (TAT) which were changed to CGT and TAC, respectively.

DISCUSSION

The norfloxacin resistance of *E. coli* 11 and *E. coli* 101 could be a result from a change in DNA gyrase, a decreased permeability, an efflux system, or all of these. Since norfloxacin is a hydrophilic quinolone and transported through outer membrane proteins (Champman and Georgapapadakou, 1988), changes in outer membrane proteins could exert an effect on norfloxacin permeability. In both resistant strains, the amount of major outer membrane proteins were not changed compared to those in *E. coli* TEM. However, there were significant changes in other proteins. Some proteins (57 kD, 52 kD and 50 kD) were newly appeared in the resistant strains, especially a protein with 19 kD was significantly increased in *E. coli* 101. These differences might affect the norfloxacin permeability into these resistant cells. A protein which appeared in both resistant strains but not in *E. coli* TEM might be involved in an efflux system like a protein with 50 kD in *Pseudomonas aeruginosa*. In *P. aeruginosa*, a protein with 50 kD was reported to be related to an efflux system (Li, *et al.*, 1995). Norfloxacin concentration inside cells could be decreased via an efflux system as well as decreased permeability. Both resistant strains were found to have a proton gradient dependent efflux system which was inhibited with CCCP (Fig. 2 and 3). One thing to be careful about using CCCP was that each strain was diffrently sensitive to CCCP. Especially *E. coli* TEM was very sensitive to CCCP compared to the resistant strains (data not shown). That's why we used different concentrations of CCCP for each strains.

To find out a change in DNA gyrase, we used three different methods - assay of *in vitro* DNA synthesis, RFLP and a direct DNA sequencing. If there were a change in DNA gyrase, its sensitivity to norfloxacin would be changed. To assay the sensitivity of DNA gyrase to norfloxacin while avoiding the effects from a difference in permeability or an efflux system, *in vitro* DNA systhesis in permeabilized cells were observed. *In vitro* DNA systhesis in both resistant strains were not sensitive to 50 μg/ml norfloxacin while that in *E. coli* TEM was inhibited even at 1 μg/ml norfloxacin. This result suggested a change in DNA gyrase. To confirm this result, DNA sequence of QRDR in DNA gyrase was checked with RFLP and direct DNA sequencing. Ser-83 in QRDR in *E. coli* was known to be related to the quinolone resistance (Yoshida, *et al*, 1988; Cullen, *et al.*, 1989; Hallet, *et al.*, 1991; Heisig, *et al.*, 1993). Since Ser-83 composes *Hinf* I restriction site, cutting a fragment with *Hinf* I which had only one *Hinf* I site at Ser-83 would be a good tool to show a change at Ser-83. If *Hinf* I treatment produced only one band, there should be a change at Ser-83. On the contrary, if two bands were produced, no change must be occurred. A change in Ser-83 in *E. coli* 11 and *E. coli* 101 was confirmed with DNA sequencing. There was a missense mutation at Asn-87 in addition to the one at Ser-83 in both resistant strains. Ser-83 was changed to Leu and Asn-87 was changed to Asn in both resistant strains. There were reports that Ser-83 was changed to Leu, Trp, Gly, and Ala (Yoshida, *et al.*, 1988; Hallet, *et al.*, 1991; Heisig, *et al.*, 1993) and Asp-87 was changed to Gly and Asn (Hallet, *et al.*, 1991; Heisig, *et al.*, 1993). Two other silent mutations were found in *E. coli* 11. Compared to the clinical isolates found in other countries, these isolates had only two mutations. *In vitro* DNA synthesis in *E. coli* 101 was less affected by norfloxacin than *E. coli* 11. From this result we expected that more mutations occurred in QRDR of *E. coli* 101 than that in *E. coli* 11, but the sequencing data showed the same amino acid changes in both strains, so it could be suggested that there are another mutations in non-QRDR region.

We don't know yet whether quinolone caused these mutations or a treatment with quinolone selected these mutants because of their resistance. Since a change at Ser-83 was found in almost all quinolone resistant strains including *E. coli* 11 and *E. coli* 101, a detection of a change at Ser-83 in a clinical isolate would help to screen a quinolone resistant strain. Since a change in Ser-83 could be easily and quickly detected with PCR and RFLP, we suggested PCR from 85 nt to 343 nt in QRDR and RFLP of this fragment with *Hinf* I as a quick screening method for the detection of quinolone resistant *E. coli*.

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