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Effects of disruption of heat shock genes on susceptibility of *Escherichia coli* to fluoroquinolones

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Abstract

Background: It is well known that expression of certain bacterial genes responds rapidly to such stimuli as exposure to toxic chemicals and physical agents. It is generally believed that the proteins encoded in these genes are important for successful survival of the organism under the hostile conditions. Analogously, the proteins induced in bacterial cells exposed to antibiotics are believed to affect the organisms' susceptibility to these agents.

Results: We demonstrated that *Escherichia coli* cells exposed to levofloxacin (LVFX), a fluoroquinolone (FQ), induce the syntheses of heat shock proteins and RecA. To examine whether the heat shock proteins affect the bactericidal action of FQs, we constructed *E. coli* strains with mutations in various heat shock genes and tested their susceptibility to FQs. Mutations in *dnaK*, *groEL*, and *lon* increased this susceptibility; the *lon* mutant exhibited the greatest effects. The increased susceptibility of the *lon* mutant was corroborated by experiments in which the gene encoding the cell division inhibitor, SulA, was subsequently disrupted. SulA is induced by the SOS response and degraded by the Lon protease. The findings suggest that the hypersusceptibility of the *lon* mutant to FQs could be due to abnormally high levels of SulA protein resulting from the depletion of Lon and the continuous induction of the SOS response in the presence of FQs.

Conclusion: The present results show that the bactericidal action of FQs is moderately affected by the DnaK and GroEL chaperones and strongly affected by the Lon protease. FQs have contributed successfully to the treatment of various bacterial infections, but their widespread use and often misuse, coupled with emerging resistance, have gradually compromised their utility. Our results suggest that agents capable of inhibiting the Lon protease have potential for combination therapy with FQs.

Background

FQs are broad-spectrum agents applicable to a range of Gram-positive and Gram-negative infections, and they have good oral absorbability [6]. Because of these advantages, FQs have been widely used against a variety of bacterial infections for about two decades. They target the

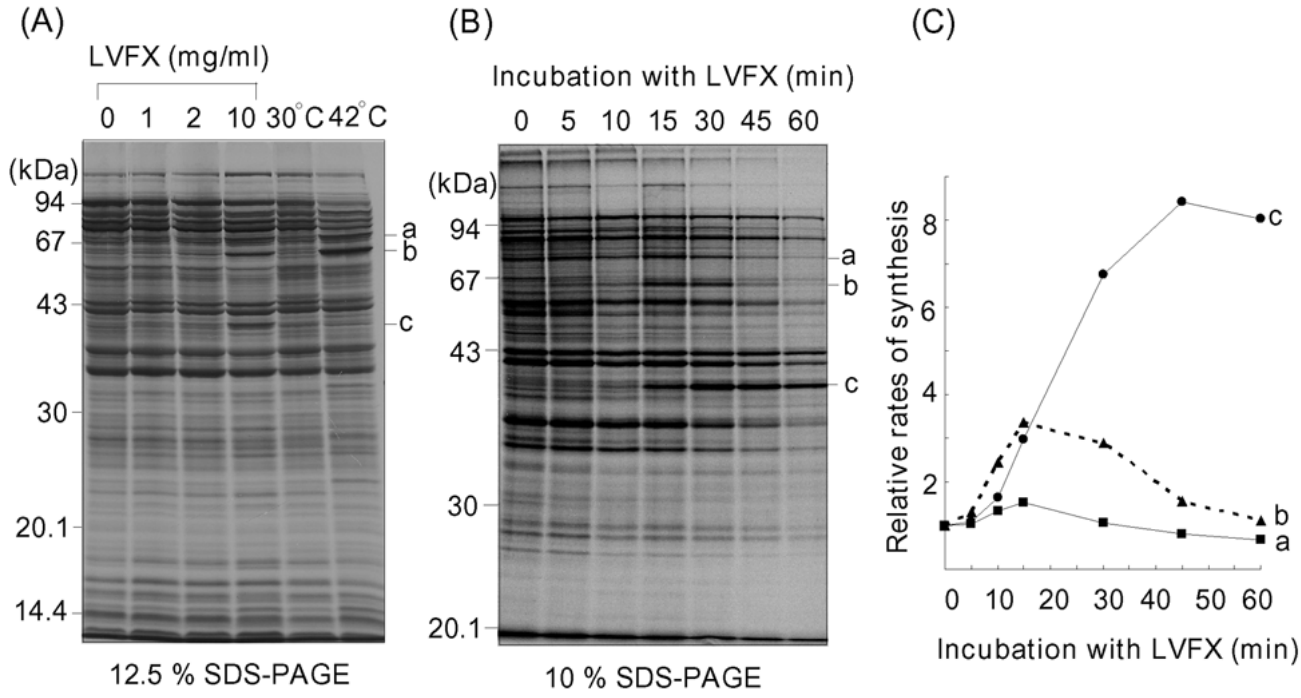

Figure 1

Proteins synthesized in *E. coli* exposed to LVFX. (A) Exponentially-growing cells were incubated with 1, 2, or 10 μg/ml of LVFX for 10 min and then labeled with [^{35}S]-methionine and cysteine for 1 min at 30°C. Heat shock culture was prepared as described in Methods. (B) After incubation with 10 μg/ml of LVFX, aliquots (100 μl) of the cultures were taken at indicated times and labeled for 1 min at 30°C. Equal amounts of acid-precipitable counts were applied to each lane and the proteins were separated by SDS-PAGE. The gels were dried and then exposed to films (A and B). The protein bands alphabetized in (B) were quantified using a BAS2000A image analyzer and the relative rates of synthesis are shown in (C).

type II topoisomerases, DNA gyrase and topoisomerase IV, which are essential for controlling the topological state of DNA during replication and transcription [12].

Bacteria are known to respond to unfavorable conditions, e.g., exposure to toxic chemicals and physical agents, nutrient limitation, or sudden increase in growth temperature, by rapid expression of regulons related to the heat shock, SOS, and oxidative stress responses. DNA damage by UV irradiation, or treatment with naldixic acid, induce both the SOS and heat shock responses [9,19]. Puromycin has been reported to induce first the SOS and secondly the heat shock responses; hydrogen peroxide induces the oxidative stress and SOS responses; and $CdCl_2$ strongly induces all three stress responses. Protein induction by these responses is widely believed to be important for the organism's survival under hostile conditions. Analogously, the proteins induced when bacterial cells are exposed to antibiotics may affect the susceptibility of the organisms to these agents.

The aim of present study was to identify the bacterial responses affecting the bactericidal action of FQs. We analyzed the proteins induced in *Escherichia coli* by exposure to FQs, then examined the susceptibilities to these agents of *E. coli* strains with mutations in the genes encoding these proteins.

Results and Discussion

Analysis of protein synthesis in E. coli exposed to levofloxacin

Figure 1(A) shows a profile of proteins in *E. coli* cells that were pulse-labeled after incubation for 10 min with different concentrations of LVFX. The results show a large increase in synthesis of 70 kDa (a), 60 kDa (b), and 40 kDa proteins (c) at 10 μg/ml LVFX. These proteins were also induced by lower concentrations of LVFX, but increasing concentrations increased the rate of synthesis. Comparison of the protein profiles of LVFX-treated, temperature-shifted (42°C) and unshifted (30°C) bacteria indicates that the 70 kDa (a) and 60 kDa (b) bands may

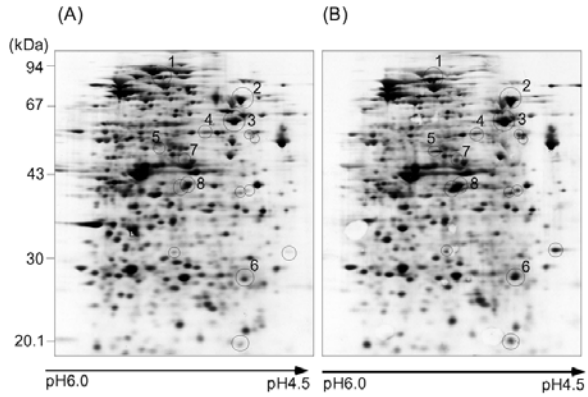

Figure 2
Two-dimensional gel electrophoresis patterns of proteins synthesized in *E. coli* exposed to LVFX. Bacterial cells were exposed to 10 μg/ml of LVFX for 15 min and then pulse-labeled for 1 min. A portion of the protein samples from untreated cells (A) or LVFX-treated cells (B) was subjected to two-dimensional gel electrophoresis using 10 % SDS-polyacrylamide gel as the second dimension. Spots enclosed in circles represent the proteins induced during exposure to LVFX. The proteins numbered are interpreted in the text.

be heat shock proteins. Figures 1(B) and 1(C) show the time-course of synthesis of these proteins after incubation with 10 μg/ml LVFX for 60 min. Synthesis of the 70 kDa and 60 kDa proteins appears to have accelerated for 15 min and then stopped. In contrast, induction of the 40 kDa protein continued up to 45 min after exposure to LVFX, suggesting that the induction mechanism is different from that for the 70 and 60 kDa proteins.

The proteins were separated on two-dimensional gels to facilitate identification (Figure 2). Visual scanning of the autoradiogram showed that synthesis of at least 16 proteins was markedly elevated in bacterial cells exposed to LVFX compared with untreated cells. The spots numbered on the gel are proteins that could be identified by mass spectrometric analysis. Among these, heat shock proteins are identified as follows: ClpB (spot 1), DnaK (spot 2), GroEL (spot 3), HtpG (spot 4), HslU (spot 5) and GrpE (spot 6). Enhanced production of the small heat shock proteins GroES, IbpA, and IbpB was also observed when 12.5 % SDS-PAGE was used as the second dimension (results not shown). Phosphogluconate dehydrogenase (spot 7) and phosphoglycerate kinase (spot 8) were also identified.

The 40 kDa protein showing the most marked induction in Figure 1 does not appear on the two-dimensional gel (Figure 2). We therefore fractionated the proteins from LVFX treated *E. coli* cells along with those from control cells. As shown in Figure 3, the 40 kDa protein seems to be largely associated with high salt-soluble proteins (lanes 6 and 8). A portion of the high salt-soluble fraction was separated by two-dimensional gel electrophoresis; the spot corresponding to the LVFX-induced 40 kDa protein

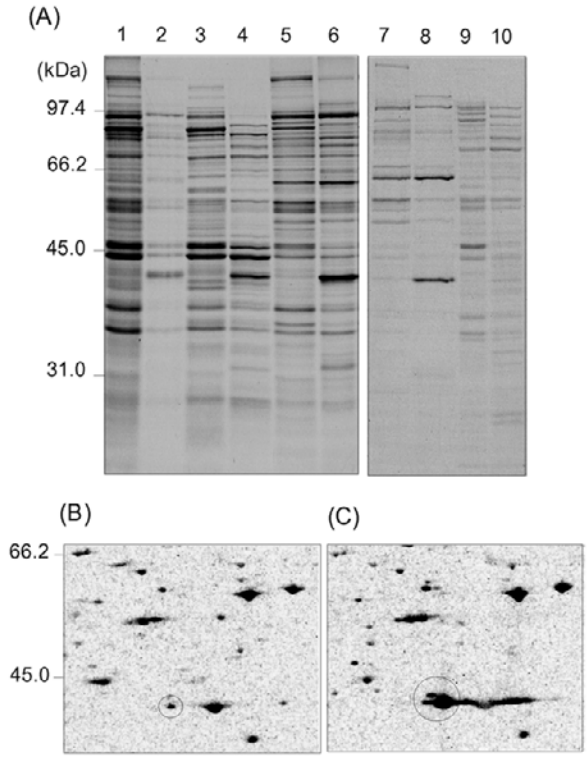

Figure 3
Fractionation of proteins synthesized in *E. coli* exposed to LVFX. Proteins from cultures labeled in the presence of 10 μg/ml LVFX were fractionated as described in Methods and separated by SDS-PAGE (A). The protein samples from untreated cells were run in lanes 1, 3, 5, 7 and 9. The samples from LVFX-treated cells were in lanes 2, 4, 6, 8 and 10. Lanes contain protein fractions as follows: lanes 1 and 2, whole cell lysates; lanes 3 and 4, cytoplasmic and periplasmic protein fractions; lanes 5 and 6, membrane-associated protein fractions. The membrane-associated proteins were solubilized in 1 M NaCl and then dialyzed described in Methods. After centrifugation, the pellets were solubilized in lysis buffer and applied in lanes 7 and 8. The supernatants were applied in lanes 9 and 10. Portions of the protein samples applied in lanes 7 and 8 were also subjected to two-dimensional gel electrophoresis and the results are shown in (B) and (C), respectively.

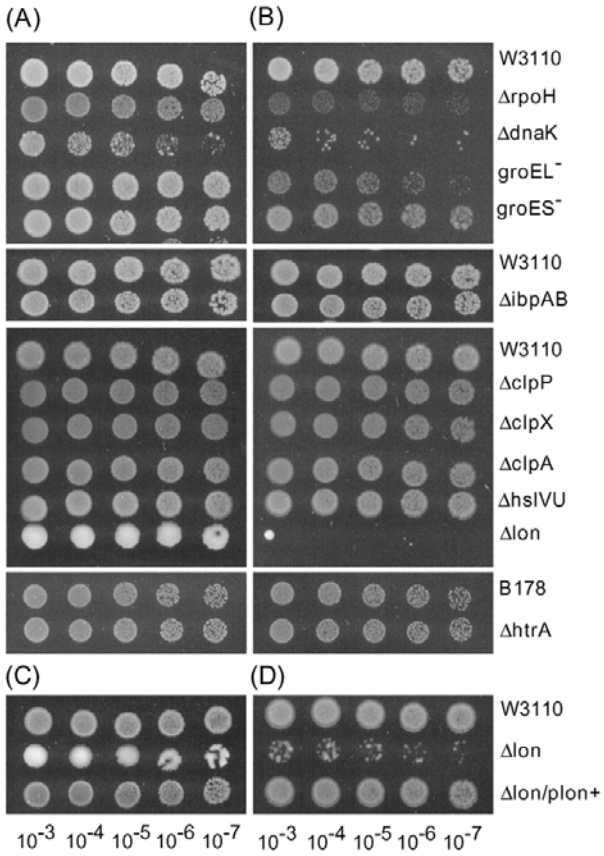

Figure 4
Susceptibility of chaperone- or protease-deficient mutants of *E. coli* to LVFX. Bacterial cultures were diluted from 10^{-3} to 10^{-7} and then spotted on to agar plates prepared by the standard agar doubling dilution method. (A) and (C) are the results with drug-free agar plates. (B) and (D) are the results with agar plates containing 0.0125 μg/ml LVFX. Abbreviation: p*lon*+, a complementing plasmid carrying the intact *lon* gene [15].

was subjected to mass spectrometric analysis and identified as RecA. RecA is usually cytosolic in *E. coli* and binds to single-stranded regions of damaged DNA [14]. Detection of a large amount of RecA protein in the high salt-soluble fraction suggests that when it is synthesized in abnormally large amounts, it aggregates with various denatured and misfolded proteins.

In *E. coli*, the expression of heat shock genes is positively regulated at the transcriptional level by the heat shock specific sigma subunit of RNA polymerase, σ^{32}. In addition, the heat shock response is negatively autoregulated by the DnaK chaperone machine that interferes with the efficient binding of σ^{32} to the RNA polymerase core, turning off the response [2,4,16]. Heat-induced aggregation of proteins induces the expression of the heat shock regulon through the titration of the DnaK chaperone by the aggregates [4,16]. Therefore, in these experiments, the induction of the *E. coli* heat shock regulon could have been triggered by protein aggregates accumulated after incubation with LVFX.

Antibiotic susceptibility of E. coli strains with mutations in heat shock genes

The heat shock proteins comprise chaperones such as the DnaK/DnaJ/GrpE, GroEL/GroES and IbpA/IbpB systems, and ATP-dependent proteases such as Lon, ClpXP and HslVU. Whereas the chaperones are abundantly synthesized, the levels of the proteases are relatively low even under inducing conditions. In these experiments, proteases were not present in sufficient amounts to identify by mass spectrometry in LVFX-treated cells. However, all the heat shock genes in one regulon under the control of σ^{32} would be simultaneously induced in cells exposed to LVFX.

To examine the involvement of heat shock proteins in the susceptibility of *E. coli* to FQs, we constructed strains with mutations in various heat shock genes and then tested their susceptibility to LVFX (Figure 4A and 4B). Among the chaperone mutants, Δ*dnaK* and *groEL*⁻ exhibited increased susceptibility. However, a mutation in the small chaperone, IbpAB, did not affect susceptibility. Among the protease mutants, Δ*clpP*, Δ*clpX*, Δ*clpA*, Δ*hslVU* and Δ*htrA* all showed the same susceptibility to LVFX as the wild type. In contrast, Δ*lon* showed a greatly increased susceptibility. This hypersusceptibility of the Δ*lon* mutant was corroborated by providing a functional *lon* using a low copy-number-vector (Figure 4C and 4D). The results suggest that the hypersensitivity of the strain to LVFX is largely due to the disruption of *lon*. Δ*lon* showed a similarly increased sensitivity to other FQs such as ciprofloxacin, sparfloxacin and sitafloxacin (data not shown). As expected, a mutation in *rpoH*, which encodes σ^{32}, also resulted in increased susceptibility of *E. coli* to LVFX.

Chaperones and proteases are essential for de novo folding and quality control of proteins, acting by preventing protein aggregation and by refolding or degrading misfolded proteins. Therefore, the increased susceptibility of the chaperone and *lon* mutants can partly be explained by the disruption of the quality control system in *E. coli* cytosol.

Effect of sulA-disruption on the hypersusceptibility of the Δlon mutant to fluoroquinolones

The Lon protease degrades SulA, which inhibits separation by binding to FtsZ, a key cell division protein [1].

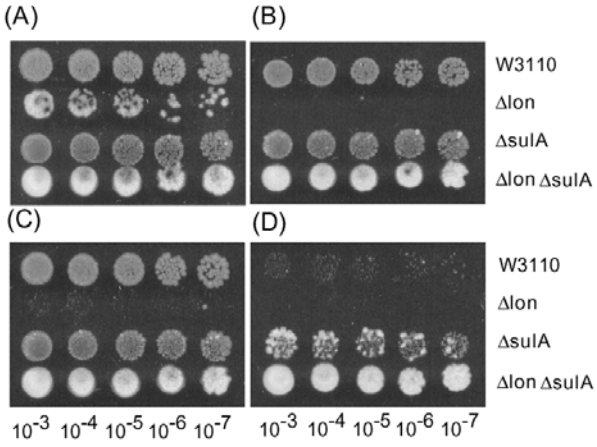

Figure 5
Susceptibility of *E. coli* Δ*lon* and Δ*sulA* double mutant to LVFX. (A), (B), (C) and (D) show the results of spot testing with agar plates containing 0 μg/ml, 0.0125 μg/ml, 0.025 μg/ml and 0.05 μg/ml LVFX, respectively.

SulA is a SOS response protein induced by DNA damage after UV-irradiation and quinolone-treatment [23]. We examined the possibility that the hypersensitivity of the Δ*lon* mutant is due to the accumulation of SulA protein. The *sulA* gene was disrupted in both wild-type and Δ*lon*-mutant cells and then the resultant double mutants were compared for susceptibility to LVFX. As shown in Figure 5, the hypersusceptibility of Δ*lon* was suppressed by the subsequent disruption of *sulA*, suggesting that the increased sensitivity is due to the inhibition of cell division by abnormal accumulation of SulA. It therefore appears that in Δ*lon*, depletion of the Lon protease and continuous induction of the SOS response in the presence of LVFX result in abnormally high levels of the cell division inhibitor SulA. Complementation analysis also indicates that continuous induction of SulA by FQs contributes to quinolone killing. This hypothesis is supported by the finding that the Δ*sulA* mutant is less sensitive to LVFX than the wild type (Figure 5). Moreover, disruption of *lon* did not affect the susceptibility of bacteria to antibiotics other than quinolones, such as ampicillin, cephalothin, streptomycin, polymixin-B or rifampicin (our unpublished data).

Effect of the lon mutation in fluoroquinolone-resistant mutants

Bacterial quinolone resistance is usually due to mutations in the genes for targets of these agents: DNA gyrase encoded by *gyrA* and *gyrB*, and topoisomerase IV encoded by *parC* and *parE*. In several species of *Enterobacteriaceae*, decreased susceptibility or resistance to FQs is associated with specific point mutations in *gyrA* [21,22]. An additional mutation in *parC* results in greater resistance [10,20]. We examined whether the Δ*lon* mutation affects the resistance to LVFX in *E. coli gyrA* and *parC* mutants.

In Figure 6, strain CS5086 has a *gyrA* mutation (Ser83 to Leu). To construct a *gyrA* and *parC* double mutant, CS5086 was initially treated with mini F-*parC1* (Gly78 to Asp, Val253 to Ile). Then the chromosomal *parC* gene of the resultant strain was replaced with *par*C::Cm by P1 transduction; *lon* was subsequently disrupted in both the *gyrA* and the *gyrA*, *parC1* mutants. As shown in Figure 6, Δ*lon* decreased the resistance of both *gyrA* and *gyrA*, *parC1* to LVFX.

Effect of recA mutation on susceptibility of E. coli to fluoroquinolone

Since one of the proteins strongly induced by LVFX is RecA (Figure 1 and Figure 3), the role of RecA in the bactericidal action of the agent was examined by susceptibility testing of a *E. coli recA* mutant along with the isogenic wild type. Figure 7 shows that the *recA* mutation increases

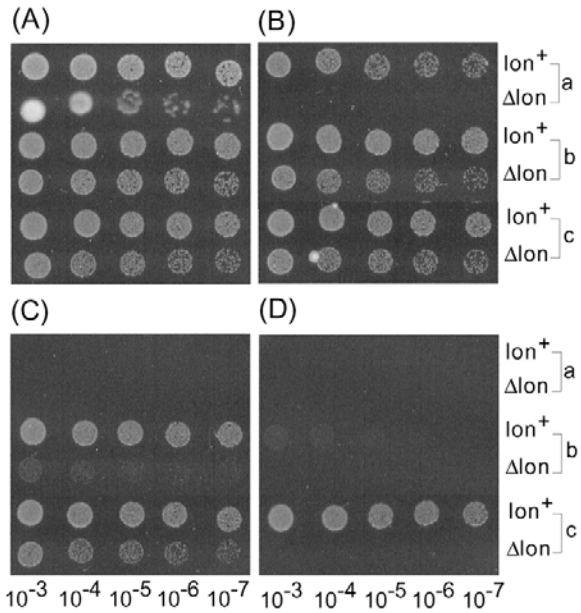

Figure 6
Levels of LVFX-resistance of *E. coli gyrA*, Δ*lon* and *gyrA, parC*, Δ*lon* mutants. (A), (B), (C) and (D) show the results of spot testing with the agar plates containing 0 μg/ml, 0.0125 μg/ml, 0.025 μg/ml, and 0.10 μg/ml LVFX, respectively. Bacterial strains used are as follows: a, W3110 (*lon*+) and CS5140 (Δ*lon*); b, CS5086 (*lon*+) and CS5216 (Δ*lon*); c, CS5217 (*lon*+) and CS5218 (Δ*lon*).

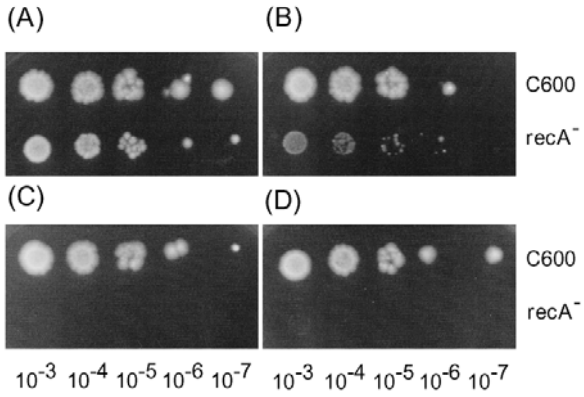

Figure 7
Susceptibility of *recA* mutant to LVFX. (A), (B), (C), and (D) show the results of spot testing with the agar plates containing 0 μg/ml, 0.0032 μg/ml, 0.0063 μg/ml, and 0.0125 μg/ml of LVFX, respectively.

the susceptibility of *E. coli* to LVFX. RecA positively regulates the SOS response, which includes amplification of RecA, UvrA/UvrB (excision repair enzyme), and SulA (cell division inhibitor). Although the continuous induction of the SOS response by quinolones is harmful to wild type cells, synthesis of RecA and UvrA/UvrB, which repair quinolone-induced DNA damage, are prerequisites for withstanding the bactericidal action of quinolones.

Conclusion

The present results show that the DnaK and GroEL chaperones have moderate effects on the antimicrobial activity of FQs. These chaperones might contribute to quinolone resistance because they sequester the aggregates that accumulate in cells exposed to FQs. Lon protease markedly affects the bactericidal action of FQs, as indicated by the hypersusceptibility of the *lon* mutant. This increased susceptibility is corroborated by the effects of subsequent disruption of the gene encoding SulA. SulA protein is induced by the SOS response and degraded by Lon. Collectively, the results suggest that the hypersusceptibility of the *lon* mutant to FQs could be due to abnormal accumulation of SulA, which is depeleted by Lon, and the continuous induction of the SOS response.

FQs have contributed successfully to the treatment of various bacterial infections because of their broad-spectrum, potent antimicrobial activity and ease of oral administration. However, their widespread use and often misuse, coupled with emerging resistance, have gradually compromised their utility. The present work suggests that agents capable of inhibiting the Lon protease have potential for combination therapy with FQs.

Methods

Bacterial strains and plasmids

Bacterial strains used are shown in Table 1. Protease mutant alleles were introduced by P1 transduction into a W3110 background, generating strains CS5137 (*clpP*::Cm), CS5138 (*clpX*::Km), CS5139 (*clpA*::Km), CS5168 (*hslV*::Tc) or CS5140 (*lon*::miniTn*10*). For these transductions, P1 phages were propagated on MC4100 derivatives carrying the corresponding mutations [7,17]. Strains CS5213 (*sulA*::Tn*5*) and CS5314 (*ibpA*::Km) were constructed by transduction of W3110 with P1 phage propagated in strains GC2597 [3] and OW6/*ibpA*::Km [8], respectively. To construct strain CS5217, CS5086 cells were initially transformed with plasmid pJK282-*parC1* and then the *parC* gene was inactivated by P1-mediated transduction with DNS5101CR [10] as a donor. The *lon* gene of strain CS5217 was inactivated by P1 transduction, resulting in strain CS5218.

Antibiotics

Levofloxacin was kindly provided by Daiichi Pharmaceutical Co. Ltd. [Tokyo, Japan].

Labeling of proteins producing in bacteria exposed to LVFX or heat shock

Bacterial cells grown in M9-medium [17] to mid-exponential growth phase were inoculated with LVFX and incubated at 30°C. Cells were labeled with 3.7 Mbq/ml of [^{35}S]-Met and Cys (Protein labeling mix, >37Tbq/mmol, Amersham, Buckinghamshire, U.K.) for 1 min, mixed with TCA (final concentration 5%) and chilled on ice for 15 min. After centrifugation at 16000 g for 2 min, the pellets were washed with acetone and resuspended in sample buffer [18]. The labeling of the heat shock proteins was performed as follows: bacterial cells in mid-exponential growth phase were transferred from 30°C to 42°C, incubated for 10 min and then labeled for 5 min at 42°C.

Resolution and quantitative estimation of proteins

The radiolabeled proteins were separated by SDS-polyacrylamide gel electrophoresis as described previously [18]. The radioactivities incorporated into proteins were quantified using a BAS2000A photoimager (Fuji Film Co. [Tokyo, Japan])

Identification of proteins

Proteins were separated by two-dimensional gel electrophoresis and the protein spots of interest were excised from the gels and subjected to matrix-assisted laser desorption ionization-mass spectrometry according to the procedure described previously [15].

Table 1: *Escherichia coli* strains used in this study

Strain	Relevant characteristics	Reference/ source
W3110	F-λ- *rph1*	Our collection
MC4100	F- *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Our collection
B178	W3110 *galE*	5
C600 Sm	F- *lacY leu str supE thi thr tonA*	
C600 Sm *recA*	C600 Sm *recA1*	
CS5137	W3110 *clpP*::Cm	This study
CS5138	W3110 *clpX*::Km	This study
CS5139	W3110 *clpA*::Km	This study
CS5168	W3110 *hslV*::Tc	This study
CS5140	W3110 *lon*::miniTn*10*	This study
BB1553	MC4100 *dnaK*::*cat sidB1*	2
BB2395	MC4100 *lon146*::miniTn*10*	17
BB7222	Same as in MC4100 but *araD*+	17
BB7224	BB7222 *rpoH*::Km *zhf*::Tn*10 suhX1401*	17
NRK117	MC4100 *groEL44 zie*::Tn*10*	11
NRK233	MC4100 *groES619 zie*::Tn*10*	11
CG1023	B178 *htrA*::miniTn*10*	13
CS5086	CSH2 *gyrA*	Our collection
CS5213	W3110 *sulA*::Tn*5*	This study
CS5214	CS5213 *lon*::miniTn*10*	This study
CS5216	CS5086 *lon*::miniTn*10*	This study
CS5217	CS5086 *parC*::Cm harboring pJK282-*parC1*	This study
CS5218	CS5217 *lon*::miniTn*10*	This study
CS5314	W3110 *ibpA*::Km	This study

Fractionation of Proteins

Aliquots (10 ml) of cultures were rapidly cooled in an ice-water bath and centrifuged for 10 min at 5000 g to harvest the cells. Pellets were washed with saline and resuspended in 50 μl of buffer A (50 mM Tris-acetate, pH 7.4, 5 mM EDTA, 20 % sucrose, 1 μg/ml lysozyme) and incubated for 30 min on ice. Cell lysis was performed by addition of 450 μl of buffer B [50 mM Tris-acetate, pH 7.4, 5 mM EDTA, protease inhibitor cocktail (SIGMA [St. Louis, Mo.]) and mixing, followed by sonication with a Branson Sonifier 200. Intact cells were removed by centrifugation at 5000 g for 10 min at 4°C. The supernatant was used as a cytoplasmic and periplasmic protein fraction. The pellet containing membrane-associated and -integral proteins was resuspended in 80 μl of buffer B containing 1 M NaCl and centrifuged at 100000 g for 1 h at 4°C. The supernatant, containing membrane-associated proteins, was dialyzed against buffer C (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 10 % Glycerol, 0.1 mM DTT).

Susceptibility testing

Bacterial susceptibility to antibiotics was determined by the twofold standard agar dilution method, except that bacterial cultures were diluted from 10^{-3} to 10^{-7} in buffered saline containing 0.01 % of gelatine.

Author's contributions

YY analyzed the LVFX-related proteins, constructed some of mutants, and performed the susceptibility testing. TT constructed some of mutants and conducted the experiments with them. AT conducted the mass spectrometric analysis of the LVFX-related proteins. MM identified RecA protein by mass spectrometric analysis. TY opened the project here and prepared the submitted manuscript.

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