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### The role of Escherichia coli YrbB in the lethal action of quinolones

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**Objectives:** To explore bacterial cellular factors that protect against the lethal effect of antimicrobial stress as potential targets of antimicrobial potentiators, the role of *Escherichia coli* YrbB in protecting cells from quinolone-mediated cell death was studied.

**Methods:** A set of isogenic strains containing different mutations in stress response genes of *E. coli* was constructed by P1-mediated transduction. The susceptibility of these strains to the lethal action of quinolones was determined by measuring viable colony counts on agar plates after treatment with quinolones under various conditions.

**Results:** A *yrbB* mutation rendered *E. coli* cells more susceptible to the lethal action of quinolones under conditions in which bacteriostatic susceptibility was unaffected. YrbB worked in both lethal pathways of quinolone action. Hydroxyl radical accumulation was required for nalidixic acid-mediated killing; however, in the absence of functional YrbB there was an additional mechanism through which nalidixic acid could kill cells independently of hydroxyl radical action. The *E. coli* chromosomal toxin–antitoxin system ChpB, but not the SOS system, was found to be involved in the hydroxyl radical-independent lethal mechanism. In addition, proteases ClpP and Lon were also involved in the action of YrbB. Besides quinolones, YrbB also played a protective role in cellular responses to other stressors, such as mitomycin *C*, ultraviolet light and hydrogen peroxide.

**Conclusions:** YrbB played a protective role in the lethal action of quinolones through a hydroxyl radicalindependent and toxin-antitoxin-dependent mechanism, which makes it a potential target for antimicrobial enhancement.

Keywords: nalidixic acid, ciprofloxacin, hydroxyl radical, toxin-antitoxin

### Introduction

The emergence of antimicrobial resistance is severely limiting treatment options for many important infectious diseases.<sup>1,2</sup> Traditionally, the problem of antimicrobial resistance has been approached by developing new compounds having increased potency. Such a process becomes increasingly difficult as the more obvious bacterial targets become extensively explored and the more effective compounds become refined for greater activity. More importantly, the development of new compounds is not keeping pace with the emergence of antibiotic-resistant pathogens. Consequently, novel strategies are needed to preserve existing agents. Blocking bacterial resistance mechanisms is one of the approaches that have been explored to enhance the activity of antimicrobial agents with some success. For example, β-lactamase inhibitors extend the utility of β-lactams when delivered as combinations such as amoxicillin/clavulanic acid,<sup>3</sup> and inhibitors of efflux pumps produce synergistic inhibition of growth against tetracycline-resistant Escherichia coli when used in combination with doxycycline.<sup>4</sup> However, such an approach often only works for a distinct antimicrobial class. A more general approach would be to target mechanisms that allow bacteria to survive under adverse conditions. Such an approach would be likely to potentiate multiple antimicrobial agents and would thus provide 'potency enhancers' of antimicrobial agents already in the marketplace.

Bacterial stress response networks may provide a valuable source of targets for antimicrobial enhancement, since stress responses may help bacteria to survive drug treatment when they are activated.<sup>5–11</sup> Efforts have been made to explore targets within bacterial stress response networks for antimicrobial enhancement.<sup>12–14</sup> So far, these studies have focused mainly on bacterial two-component signal transduction systems. In our previous work, a novel screening strategy was used to identify a collection of genes in *E. coli* that are involved in cellular responses to lethal environmental stresses.<sup>15</sup> This diverse set of poorly characterized genes were able to protect *E. coli* cells from the lethal action of quinolones, which makes them potential targets

© The Author 2010. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com of small-molecule enhancers of antimicrobial agents. One of the genes identified, *yrbB*, codes for a protein that is predicted to be a nucleotide-binding protein containing a sulphate transporter and anti-sigma antagonist (STAS) domain.<sup>16</sup> Although YrbB is reported to be a component of the ATP-binding cassette (ABC) transport system MlaABCDEF, which maintains lipid asymmetry in the Gram-negative bacterial outer membrane by preventing surface exposure of phospholipids,<sup>17,18</sup> the exact role of YrbB in protecting bacterial cells under stressful conditions, especially under antimicrobial treatment, remains elusive.

The fluoroquinolones are broad-spectrum antibacterial agents that are becoming increasingly popular as bacterial resistance erodes the effectiveness of other agents.<sup>19</sup> The quinolones have DNA gyrase and DNA topoisomerase IV as their cellular targets (for reviews, see references<sup>20-24</sup>). The intracellular effects of quinolones arise from the reversible trapping of the enzymes on DNA as complexes, which block the movement of replication forks and transcription complexes and thereby interfere with bacterial growth. The subsequent release of double-strand DNA breaks from the complexes is thought to contribute to cell death.<sup>23</sup> It is proposed that there are two lethal pathways in auinolone-mediated cell death.<sup>23</sup> One pathway, the chloramphenicol-sensitive pathway, requires ongoing protein synthesis to kill E. coli cells, while the other pathway, the chloramphenicol-insensitive pathway, does not. Prototype quinolones, such as nalidixic acid, kill cells only through the chloramphenicol-sensitive pathway. Newer fluoroquinolones, such as ciprofloxacin, kill cells through both pathways. Nevertheless, after decades of study the underlying mechanism of quinolone-mediated lethal action is still poorly understood.

In the present work we studied the role of YrbB in the lethal action of quinolones. The involvement of YrbB in the two lethal pathways of quinolones was examined and the relationship between YrbB and several other bacterial stress response systems was explored. The results provided evidence showing that YrbB played a protective role in both lethal pathways of quinolones, and in the absence of functional YrbB there was a hydroxyl radical-independent and bacterial ChpB toxin – antitoxin (TA) system-dependent mechanism through which nalidixic acid could kill *E. coli* cells. Proteases ClpP and Lon were also found to be involved in the action of YrbB.

### Materials and methods

### Bacterial strains and growth conditions

Bacterial strains were derivatives of *E. coli* K-12 (Table 1). Strain TL11 was a *yrbB*::Tn5 derivative of AB1157 isolated as described.<sup>15</sup> TL67 and TL25 were *yrbB*::Tn5 derivatives of MG1655 and DM4100, respectively, which were constructed from TL11. TL47 (*recA56*) and TL52 (*yrbB*::Tn5 *recA56*) were derivatives of DM4100 constructed from JC10240 (*recA56*, kindly provided by G. Walker, MIT, Cambridge, MA, USA). TL411 (*clpP*::*cat*) and TL412 (*yrbB*::Tn5 *clpP*::*cat*) were derivatives of MG1655 constructed from SG22098 (*clpP*::*cat*, kindly provided by S. Gottesman, National Cancer Institute, Bethesda, MD, USA). TL413 (*lon146*::Tn10) and TL414 (*yrbB*::Tn5 *lon146*::Tn10) were derivatives of MG1655 constructed from GW7501 (*lon146*::Tn10, kindly provided by G. Walker). TL75 (*yrbB*::Tn5 *ΔchpB*), TL83 (*yrbB*::Tn5 *ΔrelBE*) and TL91 (*yrbB*::Tn5 *ΔchpAIK*) were derivatives of MG1655 constructed from their corresponding strains, SC31 (*ΔchpB*), SC34 (*ΔrelBE*) and SC38 (*ΔchpAIK*) (kindly provided by K. Gerdes, University of Newcastle, Newcastle, UK), respectively.

Table 1.	Bacterial	strains
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Strain	Relevant genotype	Source or reference
DM4100	wild-type	K. Drlica
MG1655	wild-type	K. Gerdes
TL11	yrbB::Tn5°	15
TL67	yrbB::Tn5 <sup>b</sup>	this work
TL25	yrbB::Tn5 <sup>c</sup>	this work
JC10240	recA56 srl::Tn10	G. Walker
TL47	recA56 srl::Tn10 <sup>c</sup>	this work
TL52	yrbB::Tn5 recA56 srl::Tn10 <sup>c</sup>	this work
SG22098	clpP::cat	S. Gottesman
TL411	clpP::cat <sup>b</sup>	this work
TL412	yrbB::Tn5 clpP::cat <sup>b</sup>	this work
GW7501	lon146::Tn10	G. Walker
TL413	<i>lon146</i> ::Tn10 <sup>b</sup>	this work
TL414	yrbB::Tn5 lon146::Tn10 <sup>b</sup>	this work
SC31	$\Delta chpB$	K. Gerdes
SC34	$\Delta relBE$	K. Gerdes
SC38	$\Delta$ chpAIK	K. Gerdes
TL75	yrbB::Tn5 $\Delta chpB^{b}$	this work
TL83	yrbB::Tn5 $\Delta$ relBE <sup>b</sup>	this work
TL91	yrbB::Tn5 ΔchpAIK <sup>b</sup>	this work

<sup>a</sup>AB1157 background.

<sup>b</sup>MG1655 background.

<sup>c</sup>DM4100 background.

All strains were constructed by using P1-mediated transduction according to a standard procedure.<sup>25</sup> Bacterial cells were grown at 37°C either in Luria–Bertani (LB) broth or on LB agar plates.<sup>25</sup>

### Antibacterial agents

All chemicals were from Sigma-Aldrich Corp. (St Louis, MO, USA) unless otherwise indicated. Ciprofloxacin was obtained from Bayer Corp. (West Haven, CT, USA). Stock solutions of quinolones were prepared by dissolving in 0.1 M NaOH to yield a final concentration of 10000 mg/L. Chloramphenicol, 2,2'-bipyridyl, mitomycin C and tetracycline were dissolved in 100%, 70%, 70% and 50% ethanol, respectively. Mitomycin C was freshly prepared before use. Other compounds were dissolved in distilled water and stored as concentrated stock solutions at  $-80^\circ$ C.

### Susceptibility testing

Susceptibility to the bacteriostatic action of antimicrobial agents was measured as the MIC<sub>99</sub>, which was defined as the minimal concentration of antimicrobial agent that inhibited growth of 99% of the input cells. MIC<sub>99</sub> was determined by applying 10  $\mu$ L of serial dilutions of mid-log phase cultures [optical density at 600 nm (OD<sub>600</sub>)=0.3-0.5] in triplicate to LB agar plates containing various concentrations of antimicrobial agents. Colonies were counted after overnight incubation. The fraction of colonies recovered (relative to the cfu per mL on drug-free plates) was plotted against drug concentration, and MIC<sub>99</sub> was determined by interpolation.

To measure susceptibility to the lethal action of antimicrobial agents, mid-log phase cells ( $OD_{600}=0.3-0.5$ ) were split into 1 mL aliquots in test tubes, and various concentrations of antimicrobial agents were added. After incubation for 2 h with shaking, cells were diluted in LB broth,

which eliminated drug carryover, and 10  $\mu$ L aliquots from the dilutions were spotted in triplicate on drug-free LB agar plates. Colonies were counted after overnight incubation. Susceptibility was expressed as percentage survival relative to the cfu per mL at the time of drug addition. The effect of blocking protein synthesis on susceptibility was measured by pre-treating cells with 20 mg/L chloramphenicol for 10 min followed by quinolone treatment at various concentrations. The effect of hydroxyl radical accumulation on susceptibility was assessed as described previously<sup>26,27</sup> by pre-treating cells with 100 mM thiourea plus 0.25 mM 2,2'-bipyridyl for 10 min followed by quinolone treatment at various concentrations.

Susceptibility of cells to UV irradiation was tested by applying serial dilutions of mid-log phase cultures to agar plates that were irradiated with an Ultraviolet Crosslinker CL-1000 (UVP) at various doses in a dark room. The plates were then covered with aluminium foil and incubated overnight. For H<sub>2</sub>O<sub>2</sub>, mid-log phase cells were treated with various concentrations of H<sub>2</sub>O<sub>2</sub> (cells were resuspended in 0.9% saline before treatment) for 15 min. Serial dilutions were then prepared, and 10  $\mu$ L aliquots from the dilutions were spotted in triplicate on plates and incubated overnight. Susceptibility of cells to these stressors was expressed as percentage survival of treated cells relative to that of untreated cells determined at the time of treatment.

### Results

#### YrbB protects cells under quinolone treatment

To study the role of YrbB in the lethal action of auinolones, we constructed a *yrbB*::Tn5 mutant on the MG1655 background from its corresponding strain isolated in previous work<sup>15</sup> by P1-mediated transduction. The yrbB::Tn5 transductant (TL67) showed no growth defect when compared with its parental strain, MG1655 (data not shown), suggesting that the function of YrbB was dispensable under no-stress conditions. However, the yrbB mutation rendered the cells over 100 times more susceptible than the wild-type strain MG1655 when treated with various concentrations of nalidixic acid (Figure 1a, filled symbols), indicating that YrbB played a protective role in the lethal action of nalidixic acid. Since bacteriostatic action (arising from reversible formation of DNA gyrase-DNAquinolone complexes on the chromosome) and lethal action (arising from irreversible release of DNA breaks from constraint in complexes) are distinct events in quinolone action,<sup>23</sup> we also measured the effect of the yrbB mutation on the bacteriostatic action of nalidixic acid to examine whether YrbB was involved in processes before the formation of DNA gyrase-DNA-guinolone complexes or in subsequent processes. The results showed that the bacteriostatic activity (measured as MIC<sub>99</sub>) of nalidixic acid against the mutant was in the same range as that against the parental strain, MG1655 (Table 2). Thus, the hypersusceptibility of the yrbB mutant was not likely to be caused by either increased uptake or enhanced formation of complexes. It was more likely to be due to defects in cellular processes occurring after complex formation.

### YrbB functions in both lethal pathways of quinolone action

Since the above experiments suggested that YrbB worked in cellular processes occurring after the formation of the DNA gyrase-DNA-quinolone complex, we then examined the role of YrbB in the two proposed lethal pathways of



**Figure 1.** Hypersusceptibility of the *yrbB* mutant to (a) nalidixic acid and (b) ciprofloxacin. The wild-type strain and the *yrbB* mutant were treated with various concentrations of quinolones for 2 h at 37°C. Serial dilutions were then prepared, and 10  $\mu$ L aliquots from the dilutions were spotted in triplicate on plates and incubated at 37°C overnight. Susceptibility was expressed as percentage survival relative to the cfu per mL at the time of drug addition. The effect of blocking protein synthesis on susceptibility was measured by pre-treating cells with chloramphenicol (CHL) 20 mg/L for 10 min followed by quinolone treatment at various concentrations. The graphs represent averages from three independent experiments.

quinolones, the chloramphenicol-sensitive pathway and the chloramphenicol-insensitive pathway.<sup>23</sup> The observed protective effect of YrbB against nalidixic acid-mediated cell death indicated that YrbB worked in the chloramphenicol-sensitive pathway, since nalidixic acid is a prototype quinolone that kills *E. coli* cells only through this pathway. Susceptibility measurement with cells pre-treated with chloramphenicol showed that chloramphenicol could fully protect both the wild-type strain and the *yrbB* mutant from nalidixic acid-mediated killing (Figure 1a, open symbols), which further confirmed that ongoing protein synthesis was required for the chloramphenicol-sensitive pathway.

While the prototype quinolones, such as nalidixic acid, have only one lethal pathway, newer fluoroquinolones, such as ciprofloxacin, can kill cells in both the presence and the absence of protein synthesis, the latter through a pathway called the chloramphenicol-insensitive pathway.<sup>23</sup> To examine whether

	MIC <sub>99</sub> (mg/L)	
Strain	nalidixic acid	ciprofloxacin
DM4100	$5.1 \pm 0.1$	0.0056±0.0012
MG1655	5.2±0.4	$0.0061 \pm 0.0017$
TL67	4.3±0.2	$0.0057 \pm 0.0020$
TL25	$3.5 \pm 0.1$	$0.0050 \pm 0.0015$
TL47	$1.4 \pm 0.1$	$0.0013 \pm 0.0003$
TL52	$1.5 \pm 0.1$	$0.0013 \pm 0.0002$
TL411	$6.0 \pm 0.6$	$0.0116 \pm 0.0006$
TL412	4.5±0.7	$0.0104 \pm 0.0023$
TL413	$1.4 \pm 0.1$	$0.0190 \pm 0.0001$
TL414	$2.7 \pm 0.1$	$0.0028 \pm 0.0001$
SC31	6.0±0.3	$0.0057 \pm 0.0022$
SC34	$5.5 \pm 0.1$	$0.0067 \pm 0.0014$
SC38	$5.5 \pm 0.3$	$0.0062 \pm 0.0007$
TL75	$5.3 \pm 0.5$	$0.0072 \pm 0.0004$
TL83	$5.5 \pm 0.9$	$0.0065 \pm 0.0009$
TL91	4.2 <u>±</u> 0.2	$0.0066 \pm 0.0003$

 Table 2. Susceptibility of mutants to the bacteriostatic action of quinolones

Values shown are means of three independent experiments with standard deviations.

YrbB also worked in the chloramphenicol-insensitive pathway, we measured the susceptibility of the mutant to ciprofloxacin. As with nalidixic acid, the yrbB mutant was more susceptible than the wild-type strain to the lethal action (Figure 1b, filled symbols) but not to the bacteriostatic action (Table 2) of ciprofloxacin, indicating that the variation in guinolone structure did not affect the function of YrbB. When chloramphenicol was used to block the chloramphenicol-sensitive pathway, the survival rates of both the wild-type strain and the yrbB mutant were much higher than those without chloramphenicol (Figure 1b, open symbols). This was expected because only the chloramphenicol-insensitive pathway contributed to cell death. However, the yrbB mutant remained more susceptible than the wild-type strain in the presence of chloramphenicol, suggesting that YrbB also functioned in the chloramphenicol-insensitive pathway of quinolone action.

### There is an additional hydroxyl radical-independent mechanism through which nalidixic acid kills cells in the absence of YrbB

It is reported that reactive oxygen species (ROS) participate in quinolone-mediated cell death,<sup>28,29</sup> and that treatment of cells with thiourea or 2,2'-bipyridyl, which interferes with the accumulation of hydroxyl radicals, reduces the lethal effect of quinolones.<sup>26,27,29</sup> Since hydroxyl radical action contributes to quinolone-mediated cell death occurring via the chloramphenicol-sensitive lethal pathway but not via the chloramphenicol-insensitive pathway,<sup>27</sup> and since our previous experiments suggested that YrbB worked in the chloramphenicol-sensitive pathway, the effect of hydroxyl radical accumulation on

the susceptibility of cells in the absence of functional YrbB was an intriguing question. In these experiments, cells were pre-treated with thiourea plus 2,2'-bipyridyl to block the accumulation of hydroxyl radicals, and then the susceptibility of cells to nalidixic acid was measured. The results showed that thiourea plus 2,2'-bipyridyl could fully protect the wild-type strain from nalidixic acid-mediated killing (Figure 2, open circles), indicating that hydroxyl radical accumulation was required for the lethal action of nalidixic acid in the wild-type strain. However, thiourea plus 2,2'-bipyridyl only partially blocked the lethal action of nalidixic acid in the yrbB mutant (Figure 2, open triangles), which suggested that when YrbB was not present there was an additional mechanism through which nalidixic acid could kill cells independently of hydroxyl radical action.

## YrbB function is related to that of the ChpB TA system and proteases ClpP and Lon

DNA damage caused by quinolones could have several consequences. One is activation of DNA-repairing systems, such as the SOS regulon, to repair the damage. Another, in the case of a failed DNA-repairing attempt, is the stimulation of programmed cell death (PCD) to eliminate the damaged cells. In order to explore cellular factors that might be involved in the hydroxyl radical-independent lethal mechanism, we examined the relationship between YrbB and the SOS pathway or the PCD pathway by combining the *yrbB* mutation with mutations in these pathways and then measuring the susceptibility of the double mutants. The idea is that if the two genes are in the same pathway, the effects of the mutations on susceptibility would not be additive.

To examine the relationship between YrbB and the SOS pathway, a *recA56* mutation was introduced into the *yrbB* mutant by P1-mediated transduction. Susceptibility



**Figure 2.** The effect of thiourea plus 2,2'-bipyridyl (Thio) on the hypersusceptibility of the *yrbB* mutant to nalidixic acid. The wild-type strain and the *yrbB* mutant were treated with various concentrations of nalidixic acid as described in the legend to Figure 1. The effect of blocking hydroxyl radical accumulation on susceptibility was measured by pre-treating cells with 100 mM thiourea plus 0.25 mM 2,2'-bipyridyl for 10 min followed by nalidixic acid treatment at various concentrations. The graph represents averages from three independent experiments.

measurement with the *yrbB*::Tn5 *recA56* double mutant showed that though both the *recA* and the *yrbB* mutation made cells hypersusceptible to nalidixic acid, their effects were additive (Figure 3a). Thus, the function of YrbB was not related to the cellular processes of repairing DNA damage.

chpAIK (also called mazEF), chpB, relBE and several other TA loci on the *E. coli* chromosome are reported to be responsible for bacterial PCD under various stress conditions, including antibiotic treatment (for reviews, see references<sup>30-32</sup>). Therefore, PCD pathways might be involved in the additional hydroxyl radical-independent mechanism we found in the *yrbB* mutant. When the *yrbB* mutation was combined with mutations in genes of the PCD pathways, we could not draw any conclusion on whether YrbB worked in the PCD pathways or not by examining



**Figure 3.** Susceptibility of double mutants to nalidixic acid. The wild-type strain, the *yrbB*::Tn5 mutant, the *recA56* (a),  $\Delta chpAIK$  (b),  $\Delta relBE$  (c),  $\Delta chpB$  (d), *clpP*::cat (e) or *lon*::Tn10 (f) mutant, and the *yrbB*::Tn5 *recA56* (a), *yrbB*::Tn5  $\Delta chpAIK$  (b), *yrbB*::Tn5  $\Delta chpB$  (d), *yrbB*::Tn5  $\Delta chpB$  (d),

the additive effects of combinations of these genes because the mutations in TA genes did not cause hypersusceptibility to nalidixic acid (Figure 3b-d, open squares); however, the *chpB* mutation showed a substantial antagonistic effect on the hypersusceptibility of the *yrbB* mutant (Figure 3d, open triangles), implying that there was a relationship between the function of YrbB and that of ChpB.

Since the ATP-dependent serine proteases ClpP and Lon play important roles in the activation of some chromosomal TA systems of *E. coli* by degrading the antitoxins,<sup>33,34</sup> we also examined the relationship between YrbB and the proteases ClpP and Lon. When the *vrbB* mutation was combined with a *clpP* or a lon mutation, we found that the clpP mutation also antagonized the hypersusceptibility of the yrbB mutant (Figure 3e, open triangles). The antagonistic effect of the clpP mutation on the hypersusceptibility of the yrbB mutant fitted in the scheme in which the TA system ChpB was involved in the hypersusceptibility of the *yrbB* mutant, since the deficiency in ClpP might hamper the activation of ChpB and thereby caused less killing. As regards the *yrbB-lon* combination, neither an antagonistic effect nor an additive effect was observed (Figure 3f, open trianales). Therefore, Lon protease might still be involved in the cellular action of YrbB, though not by degrading antitoxins. Furthermore, the clpP and lon mutants both showed lower survival than the wild-type strain, which suggested that their cellular functions other than degrading antitoxins were involved in protecting cells from nalidixic acid treatment. This was consistent with studies showing that a deficiency of Lon protease activity lowers bacterial survival at high concentrations of nalidixic acid, and that Lon plays a role in reducing the number of DNA breaks caused by quinolone treatment.35

We postulated that nalidixic acid kills cells through two mechanisms: one depends on hydroxyl radicals and the other does not. The latter mechanism only exists when functional YrbB is absent. This would explain why the chpB mutation only partially antagonized the hypersusceptibility of the yrbB mutant, since the hydroxyl radical-dependent mechanism contributed to the killing. If the ChpB TA system is the only cellular factor involved in the hydroxyl radical-independent mechanism, the chpB mutation should be able to fully abolish the hypersusceptibility of the yrbB mutant in the presence of thiourea plus 2,2'-bipyridyl, a combination that prevents hydroxyl radical accumulation and thereby blocks the hydroxyl radical-dependent mechanism. The susceptibility measurement with the *yrbB chpB* double mutant in the presence of thiourea plus 2,2'-bipyridyl showed exactly that (Figure 4a, open triangles). Thus, YrbB and ChpB worked together in the hydroxyl radical-independent mechanism of quinolone action, in which YrbB prevented the ChpB TA system from reacting or overreacting to nalidixic acid-mediated DNA damage to kill cells. The involvement of ClpP in the hydroxyl radical-independent mechanism was also examined, and, as expected, an antagonistic effect was observed (Figure 4b, open triangles). However, the clpP mutation could not fully abolish the hypersusceptibility of the yrbB mutant, which was consistent with our previous observation that ClpP was able to provide protection for cells through other mechanism(s). As regards the combination of the yrbB and the lon mutations, neither an antagonistic effect nor an additive effect was observed (Figure 4c, open triangles), suggesting that the involvement of Lon protease in the action of YrbB was not affected by hydroxyl radical accumulation.



**Figure 4.** The effect of thiourea plus 2,2'-bipyridyl (Thio) on the hypersusceptibility of double mutants to nalidixic acid. The wild-type strain, the *yrbB*::Tn5 mutant, the  $\Delta chpB$  (a), *clpP*::cat (b) or *lon*::Tn10 (c) mutant, and the *yrbB*::Tn5  $\Delta chpB$  (a), *yrbB*::Tn5 *clpP*::cat (b) or *yrbB*::Tn5 *lon*::Tn10 (c) double mutant were treated with various concentrations of nalidixic acid as described in the legends to Figure 1 and Figure 2. The graphs represent averages from three independent experiments.

### YrbB also provides protection for cells against other stressors

Although the exact cellular function of YrbB remains unknown, we believe that it is involved in the bacterial cellular responses

to adverse conditions. Since guinolones cause DNA damage, we examined in more detail the susceptibility of the *vrbB* mutant to several environmental stressors that cause damage to DNA, which includes mitomycin C, UV irradiation and  $H_2O_2$ . When the susceptibility of the yrbB mutant to mitomycin C was measured, the mutant had lower survival than the wild-type strain (Figure 5a), indicating that YrbB was involved in protecting cells from mitomycin C-mediated killing. The yrbB mutant was also more susceptible to UV irradiation than its parental strain (Figure 5b). This kind of cross-susceptibility to UV and mitomycin C was also reported in other studies.<sup>36,37</sup> Since part of the lethal mechanism of auinolones is thought to involve oxidative stress,  $^{26-28}$  we then examined the lethal effects of H<sub>2</sub>O<sub>2</sub> on the mutants. The *yrbB* mutant again showed hypersusceptibility to  $H_2O_2$  (Figure 5c). Collectively, these data suggested that YrbB was also involved in cellular responses to other stressors.

### Discussion

In order to adapt or simply survive under adverse conditions, bacteria have evolved elaborate systems that are able to sense and respond to environmental stimuli. YrbB is one of the products of a genetic search by us for proteins involved in the quinolone stress response, which might play an important role in cellular processes for adapting to environmental variations and maintaining cellular homeostasis. In the present work, we characterized a *yrbB* mutant of *E. coli* that was hypersusceptible to the lethal action of quinolones. The work confirmed the role of YrbB in protecting cells from adverse conditions.

The mutation in *yrbB* rendered cells hypersusceptible to the lethal action of auinolones: however, the bacteriostatic susceptibility was not affected. Therefore, YrbB likely worked in cellular processes occurring after complex formation. Our susceptibility measurements showed that YrbB provided protection for cells against guinolone-mediated death in both the chloramphenicolsensitive and the chloramphenicol-insensitive pathways. A newly synthesized suicide protein is supposed to be involved in the chloramphenicol-sensitive pathway.<sup>23</sup> This protein might be an inhibitor of the DNA damage repairing system, since the mechanism of quinolone action is to form DNA gyrase-DNAquinolone complexes and cause lesions on the bacterial chromosome. Another possible role of the suicide protein is to act as a trigger of cellular processes that kill cells when cellular damage is too severe to be repaired. As regards the chloramphenicol-insensitive pathway, subunit dissociation of DNA gyrase is supposed to play a role in cell death.<sup>23</sup> Although plenty of evidence supports the proposed pathways, the exact underlying mechanism remains unknown. How YrbB achieves protection in pathways with distinct mechanisms is currently not understood.

The observation that inhibitors of hydroxyl radical accumulation only partially blocked the lethal action of nalidixic acid in the *yrbB* mutant was intriguing, since ROS are reported to participate in quinolone-mediated cell death.<sup>28,29</sup> We postulated that an additional hydroxyl radical-independent mechanism exists in the lethal action of nalidixic acid, which is blocked by a functional YrbB. The finding that a deficiency in one of the TA systems, ChpB, antagonized the hypersusceptibility caused by the *yrbB* mutation was in agreement with published reports



**Figure 5.** Hypersusceptibility of the *yrbB* mutant to various stressors. Susceptibility of the wild-type strain and the *yrbB*::Tn5 mutant to UV irradiation was tested by applying serial dilutions of mid-log phase cultures (OD<sub>600</sub> 0.3–0.5) to agar plates that were irradiated with an Ultraviolet Crosslinker CL-1000 (UVP) at various doses in a dark room. The plates were then covered with aluminium foil and incubated at  $37^{\circ}$ C overnight. For H<sub>2</sub>O<sub>2</sub>, mid-log phase cells were treated with various concentrations of H<sub>2</sub>O<sub>2</sub> (cells were resuspended in 0.9% saline before treatment) for 15 min. Serial dilutions were then prepared, and 10 µL aliquots from the dilutions were spotted in triplicate on plates and incubated at  $37^{\circ}$ C overnight. Susceptibility of cells to these stressors was expressed as percentage survival of treated cells relative to that of untreated cells determined at the time of treatment. The graphs represent averages from three independent experiments.

showing that some antibiotics act by triggering the bacterial chromosomal TA module to cause the bacterium to commit suicide, <sup>38,39</sup> and a report that the TA system MazEF-mediated cell death was found to be ROS independent when cells were treated with DNA damaging agents.<sup>40</sup> Our model is that YrbB acts as a down-regulator of toxin action to prevent overreaction of the TA system to a variety of stresses. In the absence of YrbB as a safety valve, bacteria would undergo an enhanced PCD process that would eliminate the damaged cells; such a process might have caused the vrbB mutant to be hypersusceptible to guinolones in the present study. The antagonistic effect of a deficiency in protease ClpP on the hypersusceptibility of the *vrbB* mutant was in agreement with its role in the activation of TA systems. However, as far as we know, there is no evidence showing that ClpP is involved in the degradation of ChpB antitoxin. Finally, the hypersusceptibility of the clpP and lon mutants could be explained by their other cellular roles under stressful conditions. For example, Lon protease is known to degrade damaged proteins and proteins produced in excess;<sup>41</sup> it also serves as a chaperone;<sup>41,42</sup> and, most relevantly, it may facilitate processing of DNA lesions generated by trapping of DNA avrase on the bacterial chromosome.<sup>35</sup>

YrbB is predicted to be a nucleotide-binding protein that contains an STAS domain,<sup>16</sup> which is found in the C-terminal cytoplasmic part of anion transporters from eukaryotes and many bacteria, as well as in the bacterial anti-sigma factor antagonists (ASAs). yrbB is located in an operon, called *mlaFEDCB*, that codes for an ABC transport system.<sup>18</sup> Since *yrbB* resides downstream of all the other genes in the operon, and since expressing YrbB from a plasmid was able to restore the susceptibility of the mutant to the level of the wild-type strain (data not shown), it is unlikely that the hypersusceptibility was caused by a polar effect of Tn5 insertion. Although YrbB is proposed to be a component of the ABC transport system,<sup>18</sup> the actual function of YrbB remains unknown. If the only function of YrbB is involvement in maintaining the lipid asymmetry of the bacterial outer membrane, as reported,<sup>18</sup> it is puzzling how it affected cellular processes that are seemingly not directly related to membrane function. YrbB played a protective role in cellular responses to stressors other than quinolones, such as mitomycin C, UV and  $H_2O_2$ , indicating that the protective effect of YrbB was not restricted to guinolones. Therefore, YrbB might play broader roles than previously thought when cells are under stressful conditions. Such a characteristic makes YrbB an ideal target for antimicrobial enhancement.

Several lines of evidence may provide clues for further study of the mechanism of YrbB action. First, it is reported that one of the TA systems, MazEF-mediated cell death, is a population phenomenon that depends on the density of the bacterial culture and depends on a quorum-sensing factor called extracellular death factor (EDF).<sup>40,43</sup> Second, both the EDF response and EDF production are dependent on ClpAP (the protease ClpP using ClpA as the ATPase component), the protease which is responsible for MazE degradation,<sup>44</sup> but not Lon.<sup>45</sup> Third, although activation of the MazEF TA system leads to inhibition of synthesis of most *E. coli* proteins, it still permits the synthesis of a small group of proteins that may function as agents of cell death independently of ROS.<sup>40</sup> Further study of the role of YrbB in these processes would provide insights on how bacteria respond to adverse environmental variations. Bacterial stress response networks may represent a new source of targets that not only allow enhancement of antimicrobial action but also modulate virulence expression. Proteins such as YrbB are candidates in stress response networks for the development of small-molecule potentiators. Studying the underlying mechanisms of action of these protective proteins during antimicrobial treatment would serve as a starting point for identifying novel targets for antimicrobial enhancement.

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### Transparency declarations

None to declare.

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