Posttranscriptional Repression of *Escherichia coli* OmpF Protein in Response to Redox Stress: Positive Control of the *micF* Antisense RNA by the *soxRS* Locus

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The *soxRS* regulon is a cornerstone of the adaptive defense systems of *Escherichia coli* against oxidative stress. Unexpectedly, activation of this regulon also enhances bacterial resistance to multiple antibiotics that seem unrelated to oxygen radicals. We previously correlated this multiple antibiotic resistance with a reduced rate of synthesis of the OmpF outer membrane porin that does not affect the OmpC or OmpA porins. Studies presented here, with operon and gene fusions of *ompF* to *lacZ*, show that the *soxRS*-dependent repression of OmpF is achieved posttranscriptionally. We also show posttranscriptional repression of OmpF mediated by the *soxQ1* mutation, which maps to the *marA* locus. These repressions are dependent on the *micF* gene, which encodes a small RNA partially complementary to the 5' end of the *ompF* message. Northern (RNA) blotting experiments show that *micF* transcription is strongly inducible by the superoxide-generating agent paraquat in a manner that depends completely on the *soxRS* locus. The *soxR*-constitutive and *soxQ1* mutations elevate the expression of *micF* in the absence of redox stress. However, the antibiotic resistance mediated by a *soxR*-constitutive mutation was only partially reversed upon deletion of *micF*. The *soxRS* regulon therefore includes other components that contribute to general antibiotic resistance, although the relation of this phenotype to oxidative stress remains to be established.

Enteric bacteria respond to a variety of environmental challenges by coordinately controlling the expression of regulons (groups of genes or operons) and stimulons (groups of regulons) (34). *Escherichia coli* responds to the redox stress imposed by superoxide-generating agents such as paraquat (PQ) or quinones by increasing the expression of ~80 polypeptides (18, 44). A key group of these proteins is controlled by the *soxRS* locus, which exerts positive control on 8 to 10 promoters (19, 42). The elements of the *soxRS* regulon include many components of a multilevel antioxidant defense, with inducible functions for superoxide scavenging, NADPH production, and repair of oxidized DNA, although several of the proteins controlled by *soxRS* remain unidentified (19). We also noticed that *soxRS* decreases the expression of several proteins (19).

Strains bearing constitutive *soxRS* mutations [called *soxR* (Con)] exhibited elevated resistance not only to certain oxidants but also (unexpectedly) to many antibiotics (1, 19). Similarly broad antibiotic resistance occurs in strains with mutations at the *marA* locus, which maps distantly from *soxRS* (7, 15). In a selection for oxidant-resistant strains with *soxR*(Con) mutations, we also isolated a strain bearing a mutation termed *soxQ1*, which maps to the *marA* locus (17). Strains harboring the *soxQ1* allele have elevated expression of some (but not all) *soxRS*-controlled genes and express multiple antibiotic resistance (17). Correlated with this antibiotic resistance in *soxR*(Con) (19) and *soxQ1* (17) strains was a significantly diminished rate of synthesis of the outer membrane protein OmpF, as previously observed for *marA* strains (7). OmpF forms one of the major porin structures in the outer membrane of gram-negative bacteria and is in-

involved in the passive uptake of various compounds from the environment (35).

Expression of the *ompF* gene is under elaborate control. Transcriptional regulation is mediated both positively and negatively by the two-component EnvZ-OmpR system, which responds to changes in the osmolarity of the environment (9). OmpF synthesis can also be regulated posttranscriptionally by the *micF* antisense RNA. *micF* is a small gene located just upstream of the *ompC* gene and is transcribed in the opposite direction to yield a small (4.5S) RNA that is partially complementary to the 5' end of the *ompF* message (33). This RNA interferes with the translation of the *ompF* message (8, 30, 33) in a mechanism that may involve a specific binding protein (2). The role of *micF* in the osmoregulation of OmpF is in doubt (9), but the antisense control does operate in response to increased temperature (3, 8), to salicylate (40), and to mutations at the *marA* locus (7, 25) and in *tolC* (32). We show here that *micF* also mediates the repression of OmpF exerted by the *soxRS* system in response to oxidative stress.

MATERIALS AND METHODS

Strains and cell growth. The *E. coli* strains used in this study are listed in Table 1. The transducing phage P1vir (31) was used for strain constructions. Permanent bacterial stocks were stored in 20% glycerol at -80°C, and working stocks were maintained on LB agar at 4°C for up to 2 weeks. Unless otherwise indicated, cultures were grown at 37°C with shaking at 200 rpm in liquid culture in L or LB broth (31) or in supplemented M9 medium (18). The solid medium for plates was LB broth supplemented with 1.5% agar. L medium contained 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 2 g of glucose per liter.

In strain SM3001, the chromosomal *micF* gene has been

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TABLE 1. Bacterial strains

Strain	Genotype ^a	Source(s) or reference(s)
GC4468	F ⁻ $\Delta(lac)U169$ <i>rpsL</i>	4
JHC1068	GC4468 <i>cfxB1</i> <i>zdd-2207::Tn10Km</i> (60% linked)	17
JHC1069	GC4468 <i>cfxB1</i> <i>zdd-2208::Tn10Km</i> (80% linked)	17
JHC1071	GC4468 <i>soxQ1</i> <i>zdd-2207::Tn10Km</i> (60% linked)	17
JHC1072	GC4468 <i>soxQ1</i> <i>zdd-2208::Tn10Km</i> (80% linked)	17
JHC1092	GC4468 <i>zjc-2204::Tn10Km</i> $\Delta(soxRS-zjc-2205)$	17
JHC1101	GC4468 $\Delta micF1$; P1 transduction from SM3001, selecting Km ^r	This work
JHC1103	JTG936 $\Delta micF1$; P1 transduction from SM3001, selecting Km ^r	This work
JMS150	MC4100 $\Phi(ompF'-lac^+)$ 16-13 (operon fusion); fusion as in strain MH513 (23)	J. M. Slauch and T. Silhavy
JTG936	GC4468 <i>soxR105</i>	19
JTG1052	GC4468 <i>soxR101</i> <i>zjc-2206::Tn10Km</i> (40% linked)	19
MC4100	F ⁻ $\Delta lacU169$ <i>araD</i> <i>rpsL</i> <i>relA</i> <i>thi</i> <i>fibB</i>	22, 30
MH610	MC4100 <i>araD</i> ⁺ $\Phi(ompF'-lacZ^+)$ Hyb16-10 (gene fusion)	22
SM3001	MC4100 $\Delta micF1$	30

^a Tn10Km, an minitransposon containing a kanamycin resistance cassette (17, 19); Km^r, kanamycin resistant.

deleted and replaced by a DNA segment specifying kanamycin resistance ($\Delta micF::kan$) (30). This segment was isolated from transposon Tn5 (30, 39, 46), which contains a bleomycin resistance gene in an operon with the kanamycin resistance gene (13). The strains bearing $\Delta micF::kan$ had abnormally high resistance to bleomycin (5), and this phenotype was exploited for the transduction of $\Delta micF::kan$ into strains already resistant to kanamycin by virtue of Tn10Km insertions (Table 1), which do not affect resistance to bleomycin (5).

Toxicity measurements. Gradient plates of oxidants and antibiotics were prepared as described by Cunningham et al. (10) except that each plate contained 60 ml of solid medium (30 ml each in top and bottom layers). Cell culture and plating was as previously described (19). Confluent growth along the gradient was scored after 18 to 24 h at 37°C.

β -Galactosidase assays. β -Galactosidase was assayed in whole cells grown at 30 or 37°C in L or LB medium after permeabilization of the bacteria with sodium dodecyl sulfate (SDS)-chloroform (31). Where indicated, the cells were first treated with 0.1 to 1.3 mM PQ for 45 min after the cultures had reached an optical density at 600 nm of ≈ 0.1 .

RNA extraction and Northern (RNA) blot analysis. Overnight cultures in LB broth at 37°C were diluted 100-fold into 150 ml of fresh medium and incubated until the cell density reached an optical density at 600 nm of 0.3. Where indicated, PQ was added to a final concentration of 1.3 mM, and all cultures were incubated for an additional 45 min, at which time sodium azide was added to a final concentration of 20 mM. The cells were then chilled on ice and pelleted by centrifugation. The RNA was extracted from the cells obtained from 40-ml aliquots of culture, usually in triplicate for each strain and treatment, as described by Coyer et al. (8). The concentration of total RNA in the samples was determined spectrophotometrically. RNA samples (50 μ g) were denatured and separated on agarose gels and then transferred overnight to Nytran filters for Northern blotting as described by Coyer et al. (8). The probe for *micF* RNA was a 5' ³²P-labeled synthetic 54-mer oligodeoxynucleotide (5×10^7 cpm) complementary to the central portion of 4.5S *micF* RNA (positions 11 to 64) (8).

RESULTS

Multiple antibiotic resistance and underexpression of OmpF in *soxR*(Con) and *soxQ1* strains. Previous work demonstrated that *soxR*(Con) (19), *soxQ1*, and *cfxB1* (17, 26) mutants

exhibit elevated resistance both to redox agents, such as menadione, and to the antibiotics chloramphenicol and nalidixic acid. We examined whether this increased resistance extends to other agents seemingly unrelated to oxidative stress. As shown in Table 2, the *soxR105* (constitutive), *soxQ1*, and *cfxB1* mutations also provide considerably increased resistance to ampicillin and tetracycline. A similar pattern of antibiotic resistance has been found for four other *soxR*(Con) alleles (*soxR101* to *soxR104*) (5) and cotransduces with all of the other *soxR*(Con) (19) and *soxQ1* phenotypes (17). These antibiotics are structurally unrelated, have different targets within the cell, and inhibit cell growth by different mechanisms.

Such multiple antibiotic resistance is similar to that exhibited by *marA1* mutants (14), in which decreased expression of the outer membrane porin OmpF plays a key role (7). Two-dimensional polyacrylamide gel electrophoretic analysis of pulse-labeled cellular proteins previously indicated that the rate of OmpF synthesis (but not the synthesis of OmpC or OmpA) was decreased in *soxR*(Con) and *soxQ1* strains (17, 19). In the present work, we found that the decreased synthesis of OmpF in these strains was accompanied by a significant reduction in the steady-state amount of the protein in the outer membrane (5), as shown by SDS-polyacrylamide gel electrophoresis (7). Densitometric analysis of such a gel yielded OmpF/OmpC ratios as follows: wild-type cells, 0.97; *soxR101* cells, 0.31; *soxR105* cells, 0.26; $\Delta soxRS$ cells, 0.83; *soxQ1* cells, 0.06; and *cfxB1* cells, 0.07.

Posttranscriptional regulation of *ompF* dependent on the

TABLE 2. Antibiotic resistances in *soxR*(Con), *soxQ1*, and *cfxB1* strains

Strain	Growth (% of gradient) ^a			
	Nal	Cml	Amp	Tet
GC4468 (wild type)	47	23	25	20
JTG936 (<i>soxR105</i>)	96	55	45	36
JHC1071 (<i>soxQ1</i>)	75	75	79	56
JHC1068 (<i>cfxB1</i>)	97	100	91	85

^a The measurements were repeated at least three times; values from a representative experiment are shown. Amounts (weight added per plate; see Materials and Methods) of the antibiotics were as follows: nalidixic acid (Nal), 400 μ g; chloramphenicol (Cml), 700 μ g; ampicillin (Amp), 1 mg; and tetracycline (Tet), 30 μ g.

FIG. 1. Effects of *micF* on expression of *ompF::lacZ* operon and gene fusions. Overnight cultures were diluted 200-fold into L broth at 37°C, and β -galactosidase was measured after 2 h. (A) *ompF::lacZ* operon fusion strains with the *soxR*⁺ (wild type [w.t.]; JMS150), *soxR101*, Δ *soxRS*, *soxQ1*, or *cfxB1* allele; (B) *ompF::lacZ* gene fusion strains with the wild-type (w.t.; MH610), *soxR101*, Δ *soxRS*, *soxQ1*, or *cfxB1* allele. The *soxR101*, Δ *soxRS*, *soxQ1*, *cfxB1*, and Δ *micF1* alleles were transduced from JTG1052, JHC1092, JHC1072, JHC1069, and SM3001, respectively. Open bars, *micF*⁺; shaded bars, Δ *micF1*.

***soxRS* locus.** The expression of OmpF may be controlled either transcriptionally (as by the EnvZ-OmpR system [9]) or posttranscriptionally via the *micF* antisense RNA (3, 8, 30, 33). The level of control at which *soxRS* and *soxQ1* operate was tested by using gene and operon fusions.

Various alleles of *soxR*, or the *soxQ1* or *cfxB1* mutation, were transduced into strains JMS150 (Table 1) and MH610 (22), which bear chromosomal *ompF::lacZ* operon (promoter) and gene (protein) fusions, respectively. In L medium at 37°C, expression from the operon fusions was only slightly reduced by the *soxR101* allele and was reduced ~2-fold by the *soxQ1* and *cfxB1* mutations (Fig. 1A). In contrast, the same mutations reduced expression from the gene fusions approximately 3-, 10-, and 20-fold, respectively (Fig. 1B). The quantitative effects of the *soxR101* and *soxQ1* mutations on the *ompF* gene fusion paralleled their effects on the steady-state OmpF protein levels cited above. These experiments indicate that the modulation of *ompF* expression by the *soxR*(Con), *soxQ1*, and *cfxB1* mutations occurs primarily posttranscriptionally.

It seemed likely that *ompF* would also be regulated posttranscriptionally in *soxR*⁺ cells in response to oxidative

stress. Indeed, exposure to the superoxide-generating agent PQ strongly repressed β -galactosidase expression from the *ompF::lacZ* gene fusion but had hardly any effect on the operon fusion (Fig. 2). This PQ-mediated repression was abolished by deletion of the *soxRS* locus (Fig. 2B). Thus, OmpF repression in response to redox stress occurs posttranscriptionally and under the control of *soxRS*.

Control of *micF*. The posttranscriptional regulation of OmpF suggested the involvement of the *micF* antisense RNA. The modulation of *micF* expression was confirmed directly in Northern blots probed with a synthetic oligonucleotide complementary to the central portion of 4.5S *micF* RNA (3). In wild-type bacteria under our conditions, the *micF* transcript was barely detectable but could be strongly induced by PQ (Fig. 3). Induction of *micF* by PQ was abolished by deletion of *soxRS* (Fig. 3) but was unaffected by deletion of the *marA/soxQ/cfxB* locus (5). The *soxR101* and *soxQ1* strains constitutively overexpressed the *micF* RNA (Fig. 3), as did a *cfxB1* mutant (5). Densitometric quantitation of the radioactivity (8) bound to the blot shown in Fig. 3 demonstrated a >10-fold induction by PQ in wild-type *E. coli* and 2- to 4-fold constitutive increases in the *soxR101* and

FIG. 2. Effects of PQ on expression of *ompF::lacZ* operon and gene fusions. Saturated cultures were diluted 100-fold into LB medium at 37°C. Where indicated, PQ was added to a final concentration of 100 μ M. (A) *ompF::lacZ* operon fusion strains with wild-type (w.t.; JMS150), Δ *soxRS*, Δ *micF1*, and Δ *soxRS* Δ *micF1* alleles; (B) *ompF::lacZ* protein fusion strains with wild-type (w.t.; MH610), Δ *soxRS*, Δ *micF1*, and Δ *soxRS* Δ *micF1* alleles. Δ *soxRS* and Δ *micF1* alleles were transduced from JHC1092 and SM3001, respectively. Open bars, untreated samples; shaded bars, PQ-treated samples.

FIG. 3. Analysis of *micF* RNA levels. PQ was added where indicated to a final concentration of 1.3 mM, and incubation continued for 45 min. Each lane contained 50 μ g of independently isolated RNA; triplicate independent samples were loaded in adjacent lanes for all strains and conditions except for *soxQ1* and the PQ-treated wild type, which were duplicates. After electrophoresis and blotting, the filter was probed with a synthetic oligonucleotide (8), washed, and autoradiographed. Wild type, GC4468; Δ *soxR*, JHC1092; *soxR101*, JTG1052; *soxQ1*, JHC1072, NA, untreated cells.

soxQ1 strains. Thus, *micF* responds strongly to redox stress under the strict control of *soxRS*.

Functional role of *micF*. We next tested whether the redox regulation of *ompF* was due to the *soxRS*-dependent induction of the *micF* antisense RNA. Deletion of *micF* did not significantly affect *ompF* transcription monitored with the *ompF::lacZ* operon fusion (Fig. 1A and 2A). In contrast, the PQ-induced repression of the *ompF::lacZ* gene fusion was negated by the Δ *micF* mutation and was not further changed by the additional deletion of *soxRS* (Fig. 2B). Similarly, the repression of the *ompF::lacZ* gene fusion by the various mutations was abolished [*soxR*(Con)] or decreased (*soxQ1* or *cfxB1*) upon deletion of *micF* (Fig. 1).

It is noteworthy that the *soxQ1* and *cfxB1* mutations exert both a modest effect on the *ompF* operon fusion independent of *micF* (Fig. 1A) and a stronger effect on the gene fusion (Fig. 1B). When the transcriptional effects of *soxQ1* and *cfxB1* (~2-fold) are taken into account, the posttranscriptional repression (an additional 5- to 10-fold) caused by these mutations seems to depend entirely on *micF*. Thus, the *marA/soxQ/cfxB* locus may exert complex control over *micF*, with both transcriptional and posttranscriptional components.

The multiple antibiotic resistance of a strain bearing the *soxR105* (constitutive) allele was only partially dependent on *micF*. Introduction of the Δ *micF* mutation into this strain diminished the cellular resistance to all four antibiotics tested (Table 3). However, significant residual resistance to all the antibiotics remained in JHC1103 [*soxR*(Con) Δ *micF*] compared with JHC1101 (*soxR*⁺ Δ *micF*) (Table 3), which points to other *soxRS*-regulated mechanisms that counteract the toxicity of antibiotics.

TABLE 3. Antibiotic resistance in *soxR* and *micF* strains

Strain	Growth (% of gradient) ^a			
	Nal	Cml	Amp	Tet
GC4468 (<i>soxR</i> ⁺ <i>micF</i> ⁺)	53	27	31	17
JHC1101 (<i>soxR</i> ⁺ Δ <i>micF</i>)	43	27	23	13
JTG936 [<i>soxR</i> (Con) <i>micF</i> ⁺]	95	48	53	21
JHC1103 (<i>soxR</i> (Con) Δ <i>micF</i>)	85	41	33	17

^a The measurements were repeated at least four times; values from a representative experiment are shown. The amounts added to the gradient plates and abbreviations are as described in the footnote to Table 2 except for tetracycline, of which 50 μ g was used.

The Δ *micF1* mutation also lowered the resistance of wild-type bacteria to nalidixic acid, ampicillin, and tetracycline (Table 3). Although Δ *micF1* can also increase the expression of OmpC (30), this increase is not seen in high-osmolarity media such as that used here to measure antibiotic resistance. Thus, OmpF repression via *micF* may also play a role in the antibiotic resistance of wild-type cells in the absence of redox stress agents such as PQ.

DISCUSSION

The *soxRS*-dependent repression of OmpF synthesis diminishes the level of this protein in the *E. coli* outer membrane. This change contributes to cellular resistance to diverse antibiotics. Promoter and protein fusions of *ompF* to *lacZ* demonstrate that the redox-inducible, *soxRS*-dependent depletion of OmpF is posttranscriptional. Such post-transcriptional repression of OmpF synthesis is also caused by the *soxQ1* and *cfxB1* mutations. The repression depended in all cases on the *micF* gene, which we have now shown is under the inducible transcriptional control of *soxRS*.

Specific regulation of *micF* without an accompanying effect on the adjacent *ompC* gene has now been seen in several cases. Previous reports connected this kind of regulation with mutations at the *marA* locus (7, 25) and with the temperature-dependent expression of *micF* mediated by an unknown regulator (3). The redox induction of *micF* now defines an independent system for the regulation of OmpF protein expression in which both the controlling elements (encoded by the *soxRS* locus) and the inducing stimulus (provided by superoxide-generating agents) are known (36).

A two-stage model for gene activation in the *soxRS* system has been confirmed genetically (36, 45) and biochemically (36). In this system, the SoxR protein induces the *soxS* gene in response to redox stress, and the SoxS protein activates the various regulon genes (1). Biochemical evidence now links SoxS and the *micF* gene directly, in that in vitro experiments demonstrate binding of the purified SoxS protein to the promoter region of *micF* (28). Like many of the other *soxRS*-regulated genes, *micF* is therefore multiply controlled as part of both *soxRS* and other regulons (11). A fundamental question remains as to how and whether these different regulatory systems are coordinated at a single promoter.

The importance of OmpF suppression as part of a complex cellular response to oxidative stress remains obscure. For example, the elimination of OmpF by an *ompF::Tn5* mutation in two different strain backgrounds did not increase the resistance of *E. coli* to the superoxide-generating agent menadione (16). It remains possible that a protective effect of eliminating OmpF was offset by increased expression of OmpC in those experiments or that repression of OmpF protects against other redox stress agents not yet tested.

Alternatively, the *soxRS* regulon might have evolved to exploit a redox signal that is generated by many environmental toxins (xenobiotics) but is not necessarily responsible for the toxicity of those agents. The observation that the *soxRS* system is activated by a rather restricted range of redox agents (36) undercuts this idea of a generalized xenobiotic response. However, there are reports of oxygen radicals formed by tetracycline (37), β -lactam antibiotics (38), chloramphenicol (41), and nalidixic acid acting as a photosensitizer (43). The hypersensitivity to chloramphenicol (19) and to nalidixic acid (1) of Δ *soxRS* strains compared with wild-type cells is consistent with the possibility that these antibiotics induce the regulon. In this connection,

nalidixic acid has been reported to induce superoxide dismutase (23) and to deplete OmpF from the outer membrane (12). Transcriptional induction of the *marA* locus in response to chloramphenicol and tetracycline was also reported recently (20).

The *saxRS*-dependent antibiotic resistance, as well as that conferred by the *marA1* mutation (7), cannot be explained fully by the *micF*-mediated repression of OmpF. The available evidence points to the existence of separate antibiotic resistance mechanisms activated by *saxRS* or by mutations at *marA/saxQ/cfxB*. George and Levy demonstrated a *marA*-dependent efflux system that excretes tetracycline in an energy-dependent process (14); active efflux of the synthetic quinolone norfloxacin was also observed (6). We have not yet tested whether the *saxRS* regulon activates such an export system for antibiotics or agents of oxidative stress. Still other resistance mechanisms are suggested by the postsynthetic modification of the ribosomal protein S6 (24), which is increased under *saxRS* control (19) and in *saxQ1* and *cfxB1* mutants (17). Some of the unidentified proteins and genes of the *saxRS* regulon may also contribute to the resistances of *E. coli* to xenobiotics.

ACKNOWLEDGMENTS

We thank S. Cohen and S. B. Levy (Tufts University Medical School) for strain SM3001 and for discussions, Nick Delihias (SUNY Stony Brook) for the *micF* probe and practical advice, and J. M. Schlauch and T. Silhavy (Princeton University) for the *ompF::lac* strains and advice.

This work was supported by NIH grant CA37831 and Dreyfus Foundation Teacher-Scholar fellowship TS-87-5, both to B.D.

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