Enterobactin synthesis and uptake mutants are hypersensitive to an antimicrobial peptide that limits the availability of iron in addition to blocking Holliday junction resolution

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Abstract

The peptide wrwycr inhibits Holliday junction resolution and is a potent antimicrobial. To study the physiological effects of wrwycr treatment on *Escherichia coli* cells, we partially screened the Keio collection of knockout mutants for those with increased sensitivity to wrwycr. Strains lacking part of the ferric-enterobactin (iron-bound siderophore) uptake and utilization system, parts of the enterobactin synthesis pathway, TolC (an outer-membrane channel protein), or Fur (an iron-responsive regulator) were hypersensitive to wrwycr. We provide evidence that the ∆tolC mutant was hypersensitive to wrwycr because it had reduced ability to efflux wrwycr from the cell rather than due to its export of newly-synthesized enterobactin. Deleting *ryhB*, which encodes a small RNA involved in iron regulation, mostly relieved the wrwycr hypersensitivity of the fur and ferric-enterobactin uptake mutants, indicating that the altered regulation of a RyhB-controlled gene was at least partly responsible for the hypersensitivity of these strains. Chelatable iron in the cell, measured by electron paramagnetic resonance spectroscopy, increased dramatically following wrwycr treatment, as did expression of Fur-repressed genes and, to some extent, mutation frequency. These incongruous results suggest that while wrwycr treatment caused accumulation of chelatable iron in the cell, iron was not available to bind to Fur. This is corroborated by the observed induction of the *suf* system, which assembles iron-sulfur clusters in low-iron conditions. Disruption of iron metabolism by wrwycr, added to its effects on DNA repair, may make it a particularly effective antimicrobial in the context of the low-iron environment of a mammalian host.

Introduction

Holliday junctions are DNA structures with four double-stranded arms that serve as intermediates in recombination-dependent repair and rearrangement of DNA in both prokaryotes and eukaryotes. We have identified synthetic hexapeptides that bind Holliday junctions as disulfide-bridged dimers and inhibit Holliday junction resolution (Boldt *et al.*, 2004;
Kepple et al., 2005). One of these peptides, with the sequence wrwycr (we use the D-amino acid form to limit biological degradation), is a potent, broad-spectrum antimicrobial with the ability to inhibit, in vivo, mechanisms of DNA recombination and damage repair that proceed through a Holliday junction intermediate (Gunderson & Segall, 2006; Gunderson et al., 2009; L. Marcusson, D. Medina-Cleghorn, N. Agrawal, P. Gutierrez, and A.M.S., ms. in preparation). Because mammalian host defenses such as gastric acid and the oxidative burst in macrophages induce DNA damage and thus DNA repair in bacterial invaders, use of wrwycr in antimicrobial therapy may increase the efficacy of these natural defense systems. Recently, wrwycr was shown to inhibit the growth of Salmonella enterica serovar Typhimurium inside macrophages (Su et al., 2010) and significantly enhance acid-induced killing of Shiga toxin-producing strains of Escherichia coli associated with hemolytic uremia syndrome (Lino et al., 2011).

While it is known that wrwycr targets Holliday junctions, it is not known how wrwycr enters cells, what other cellular targets it may have, and what defense mechanisms cells employ against wrwycr. To address these questions, we screened a knockout library of Escherichia coli for mutants that are resistant or hypersensitive to wrwycr. We expected mutations that conferred wrwycr resistance to inform us of cellular targets of wrwycr or mechanisms of wrwycr entry into the cell. We expected wrwycr hypersensitive mutants to inform us of wrwycr efflux or other detoxification mechanisms as well as pathways that, when perturbed, increase targets for the peptide. From a set of wrwycr hypersensitive mutants described here, we have discovered that wrwycr also affects the regulation of iron-responsive genes. Incongruously, wrwycr appears to simultaneously increase the measureable amount of desferrioxamine-chelatable iron and decrease the pool of bioavailable iron in the cell. This latter trait makes wrwycr especially detrimental to bacteria in iron-deficient environments or to bacteria that, through mutation, have lost components of their high-affinity iron-uptake system or their iron-responsive regulatory system.
Iron is an essential element for almost all organisms, but can also be toxic. In what is known as Fenton chemistry, iron catalyzes the decomposition of hydrogen peroxide (Fenton, 1894), resulting in hydroxyl radical and hydroxyl anion production (Haber & Willstätter, 1931; Haber & Weiss, 1932; Haber & Weiss, 1934). The hydroxyl radical product can damage DNA, proteins, and lipids (Halliwell & Gutteridge, 1984; Imlay et al., 1988). Because Fenton reactions are catalyzed by free iron, cells regulate the amount of iron they import from their environment. Fur, an iron-responsive transcriptional regulator, plays a key role in this regulation in *E. coli* by repressing transcription of genes encoding iron uptake proteins in the presence of sufficient iron (Bagg & Neilands, 1987). In low-iron conditions, Fur no longer binds to the promoters and transcription of these same genes is derepressed.

An additional layer of iron regulation in *E. coli* is mediated through the small RNA RyhB, which, as a repressor, acts in concert with the RNA-binding protein Hfq to facilitate target transcript degradation by ribonuclease E (Massé et al., 2003). While Fur regulates the amount of incoming iron, RyhB aids degradation of certain transcripts to direct available iron away from non-essential uses (such as iron storage or incorporation into non-essential proteins) in low iron conditions, thus ensuring that the limited amount of iron is put to the most beneficial use (Massé et al., 2005). RyhB has also been shown to increase the levels of some transcripts (Massé et al., 2005), perhaps by preventing their degradation by ribonucleases, and to activate translation of the *shiA* (shikimate transport) transcript by allowing access to the transcript's ribosome-binding site (Prévost et al., 2007). In high-iron conditions, Fur represses transcription of *ryhB* (Massé & Gottesman, 2002; Vassinova & Kozyrev, 2000), leading to an increase in iron storage and a decrease in shikimate uptake.

Many bacteria, both environmental and pathogenic species, have specialized systems for taking up iron from their environments, which can be very low in soluble iron (Neilands, 1981). Enterobactin is a siderophore synthesized non-ribosomally in a number of Gram-negative bacteria and in a few Gram-positive bacteria (reviewed in (Raymond et al., 2003)). It is
exported to the extracellular milieu by EntS (Furrer et al., 2002) and TolC (Bleuel et al., 2005) and, once it has bound iron to become ferric enterobactin, is imported by the cell (Figure S1). FepA is an outer membrane protein that transports extracellular ferric enterobactin into the periplasm; the TonB-ExbB-ExbD complex associated with the cytoplasmic membrane provides the energy for the activity of FepA (Guerinot, 1994). FepB binds to ferric enterobactin in the periplasm and directs it to the FepD-FepG cytoplasmic membrane transporter; FepC is an ATPase that assists FepD and FepG in transporting ferric enterobactin into the cytoplasm (Guerinot, 1994). Fes is the ferric enterobactin esterase that degrades ferric enterobactin into three 2,3-dihydroxybenzoyl-L-serine units to release iron, which is then reduced by an unknown mechanism (Brickman & McIntosh, 1992).

In our screen for E. coli mutants having altered sensitivity to wrwycr, we identified several physiologically-related hypersensitive mutants: ΔfepB, ΔfepC, ΔfepD, ΔfepG, Δfes, ΔtolC, and Δfur. In the course of the work presented here, we found ΔentB, ΔentC, and ΔmenF mutants also to be wrwycr hypersensitive. Fur represses expression of the fep genes in high-iron conditions, EntB and EntC are involved in synthesizing enterobactin (Figure S1) in addition to other compounds (Bleuel et al., 2005; Koronakis, 2003), and MenF synthesizes isochorismate for menaquinone production (Daruwala et al., 1996). We present evidence that wrwycr limits iron availability (although it actually increases chelatable iron in the cell) or otherwise alters iron regulation in the cell, causing hyper-repression or hypo-activation of an unidentified gene by RyhB and thus hypersensitivity in the fur and enterobactin synthesis and uptake mutants.

Methods

Strains, media and chemicals

Strains (Supplemental Table S1) were routinely cultured in LB (5 g l⁻¹ NaCl (Davis et al., 1980)) at 37°C with shaking (225 rpm). The medium used for screening the Keio collection, No
Citrate E salts (NCE)/glucose/casamino acids/vitamins (NGCV), was 1X NCE minimal medium base (Davis et al., 1980) with 0.2% glucose, 1% autoclaved casamino acids, and 1X vitamin mix 1 (500X stock of vitamin mix 1 is an autoclaved mixture of 0.025% folic acid, 0.025% pantothenic acid, 0.025% nicotinamide, 0.025% pyridoxal HCl, 0.025% thiamine HCl, 0.0025% riboflavin and 0.05% biotin) and no added iron. The 3-(N-morpholino)propanesulfonate (MOPS)/glucose/casamino acids/vitamins (MGCV) medium was based on a component of a "culture medium for enterobacteria" (Neidhardt et al., 1974) and consisted of 40 mM MOPS, 4 mM N-Tris(hydroxymethyl)-methyl glycine (Tricine) (the 10X MOPS-Tricine mixture was adjusted to pH 7.4 with KOH), 9.5 mM NH₄Cl, 0.276 mM K₂SO₄, 0.5 µM CaCl₂, 0.525 mM MgCl₂, 5 mM NaCl, 1.32 mM K₂HPO₄, 0.2% glucose, 1% casamino acids, and 1X vitamin mix 2 (1000X stock of vitamin mix 2 is a filter-sterilized mixture of 0.05% folic acid, 0.05% pantothenic acid, 0.05% nicotinamide, 0.05% nicotinic acid, 0.05% pyridoxine-HCl, 0.05% thiamine-HCl, 0.005% riboflavin and 0.1% biotin, with NaOH added to assist solubilization). FeCl₃ was added to 2.4 µM or 6 µM (as indicated) for low-iron conditions and up to 150 µM (as indicated) for high-iron conditions. Mueller-Hinton Broth powder was commercially prepared (Becton-Dickinson and Company). Chemicals and media components were purchased from Sigma-Aldrich or Fisher Scientific unless indicated otherwise. See Supplementary Material for details on D-wrwycr, the peptide used in these studies.

**Screening of Keio collection for hypersensitive mutants**

To screen the Keio collection of 3985 mutants (Baba et al., 2006), a 96-place replicator was used to inoculate microtiter wells containing the tolerance-testing medium (100 µl) with overnight cultures. The tolerance-testing medium was NGCV with either DMSO (solvent-only control; final concentration 0.5%) or wrwycr (20 µM with 0.2% final DMSO concentration) added. The inoculated plates were sealed with paraffin film to reduce evaporation, incubated at 30°C and the OD₆₀₀ read at 24 h and 72 h. To factor out the different wrwycr-free growth abilities of
the various mutant strains, the reading for OD in the presence of wrwycr for each time point was divided by the OD for the same strain in the presence of DMSO alone to get a ratio. Typically, mutants with a ratio of <0.3 were deemed to be candidate hypersensitive strains if the majority of strains on the plate had a higher ratio. For verified hypersensitive strains and additional Keio collection strains of interest, the mutated locus was transduced into *E. coli* MG1655 (Table S1) or other desired genetic background using bacteriophage P1 (Miller, 1972). Where indicated, the kanamycin-resistance cassette (FRT-kan′-FRT) was removed via recombination between the flanking FRT sites using Flp recombinase supplied from plasmid pCP20 (Cherepanov & Wackernagel, 1995); the strains were subsequently cured of pCP20 by growth at 42°C. The deletion/insertion site of all listed strains (Table S1) was verified by PCR.

Other strain construction

See Supplementary Material for details on the construction of the ∆*ryhB* mutant and the double mutants tested herein.

Growth assays

Because phosphate, a major component of NCE and thus the NGCV medium, co-precipitates with iron and other cations, a MOPS-based medium (MGCV, above) was used for growth studies involving the addition of iron, except when noted. For wrwycr sensitivity and iron complementation assays, overnight cultures were diluted to 1% in MGCV, or NGCV for the ∆*fur* and ∆*fur* ∆*ryhB* mutants, containing the concentration of wrwycr indicated in figure legends or the same volume of 100% DMSO and FeCl₃ (dissolved in 0.1 N HCl) added at the concentrations indicated in the figure legends or the same volume of 0.1 N HCl in a 96-well plate, the plates sealed with paraffin film, grown at 37°C with 1 minute of agitation every 10 minutes, and periodically assayed for OD₆₀₀ in a VersaMax or SpectraMax plate reader.
(Molecular Devices). The data are presented with the Y-axis on a log₂ scale, as appropriate for cell growth data.

**Determining dimer:monomer ratio and cell-associated concentration of wrwycr**

Reverse-phase (RP)-HPLC was used to visualize (by absorbance at 280 nm) and separate the monomer and dimer peaks of wrwycr in order to monitor the extent of dimerization of the wrwycr stocks and to determine the amount of cell-associated wrwycr in formic acid cell extracts. For details, see Supplementary Material.

**Quantitative PCR of Fur regulon genes**

Overnight cultures (200 µl) from 3 independent colonies per strain grown in MHB were used to inoculate 20 ml of MHB in 50-ml flasks. After 1 h growth, 10 µM wrwycr or 0.1% DMSO was added to the cultures. After 1.5 h further growth, RNA was extracted and used to synthesize cDNA, which was then subjected to quantitative PCR. See Supplementary Material for details.

**MUG assays for β-galactosidase activity**

Overnight cultures in MGCV supplemented with 6 µM FeCl₃ were subcultured 1:500 in fresh MGCV + 6 µM FeCl₃ and incubated at 37°C until the cultures reached an OD₆₀₀ of ~0.3. Cells were treated in a 96-well microtiter plate by adding 100 µl of the cultures to 100 µl of MGCV + 6 µM FeCl₃ containing 2X of the indicated concentration of wrwycr. See Supplementary Material for details.

**EPR spectroscopy**
Intracellular chelatable iron concentrations were measured by EPR spectroscopy as described elsewhere (Woodmansee & Imlay, 2002). Details are given in the Supplementary Material file.

Determination of mutation frequency using the median method

Sixteen overnight cultures, each inoculated from an independent colony, were subcultured 1:500 in the NGCV media with no added iron and grown to OD$_{600}$ of 0.15, then diluted 1:1 with an equal volume of NGCV media containing either DMSO alone or wrwycr to achieve a final concentration of 8 or 16 uM. Aliquots were taken at time 0 to determine the number of pre-existing mutants, as well as after 3 h or 24 h of treatment. No cultures with pre-existing mutations were used in this determination. Aliquots (2.5 µl) of 10-fold serial dilutions ($10^{-1}$-$10^{-6}$) were spot-plated on LB agar without any additions to determine the viable count. The remaining 190 µl were spread onto LB agar plates supplemented with 50 µg ml$^{-1}$ rifampicin. The number of large rifampicin-resistant colonies was counted and the median number of mutants from all the cultures was divided by the mean total colonies at each time point to obtain the mutation frequency.

Statistical analysis

A one-way analysis of variance with Tukey’s multiple comparison test was performed to compare the total amount and dimer:monomer ratio of cell-associated wrwycr detected by RP-HPLC for all samples. A two-tailed t test was performed to analyze the quantitative PCR and catalase (Supplementary Material) results (Prism version 5.0b for Macintosh; GraphPad Software).

Results

Identification of wrwycr-hypersensitive E. coli mutants
The Keio collection of *Escherichia coli* deletion-insertion mutants was partially screened to find mutations that increased or decreased the tolerance of *E. coli* for the peptide wrwycr (Methods). Here we describe our investigation of a subset of strains that appeared hypersensitive in this screen, a group of mutants related to enterobactin synthesis and transport, namely ΔfepB, ΔfepC, ΔfepD, ΔfepG, and Δfes. FepB (a periplasmic binding protein), FepC, FepD, and FepG are involved in the uptake of the iron-bound siderophore molecule ferric enterobactin across the cytoplasmic membrane, and Fes breaks down imported ferric enterobactin in the cytoplasm to release iron (Guerinot, 1994). We verified the hypersensitivity of these mutants by transducing the mutations into *E. coli* MG1655 and testing the response of the MG1655-based strains to wrwycr compared to that of the MG1655 parent strain. We also measured the effect of two other ferric enterobactin uptake-related mutations transduced from the Keio collection, ΔfepA and ΔtonB, on sensitivity of MG1655 to wrwycr. We found that, in the MG1655 background, ΔfepB, ΔfepC, ΔfepD, ΔfepG, ΔtonB, and Δfes showed no growth (optical density at 600 nm (OD₆₀₀) ratio <0.3, calculated as described in Methods) in 15 μM wrwycr in NGCV medium, which has no added iron other than that introduced by other media constituents or from the glassware, at 30°C even after 72 h incubation. The ΔfepA mutant showed hypersensitivity only at the 24 h and not at the 48 h and 72 h observation points. MG1655 is resistant (OD₆₀₀ ratio >0.8) to 15 μM wrwycr in the same conditions (for comparison, it is sensitive to 25-30 μM wrwycr). We removed the kanamycin-resistance cassette from the MG1655-based strains and followed their growth when challenged with 22 μM wrwycr in MOPS-based MGCV medium (Methods) containing 2.4 μM FeCl₃; this amount of iron was chosen because the enterobactin uptake mutants were unable to grow in lower concentrations of FeCl₃ in MGCV in the absence of wrwycr. This medium contains a low level of phosphate, insufficient to precipitate the added iron, unlike the greater amount of phosphate present in NGCV. All the mutants grew essentially the same as the parent strain in MGCV/2.4 μM FeCl₃ without wrwycr.
but with the DMSO solvent (Figure 1A). In contrast, the growth of the mutants, except $\Delta fepA$ and $\Delta tonB$, in MGCV/2.4 µM FeCl$_3$ with wrwycr was delayed relative to M1655 (open symbols in Figures 1B and 1C), verifying the original phenotype; the $\Delta fepA$ and $\Delta tonB$ mutants showed very little hypersensitivity to wrwycr in this assay (Figure 1B). In all cases, the mutants recover, often with a similar growth rate; we show below that this recovery is likely largely dependent on the TolC-dependent efflux, since the efflux-defective $\Delta tolC$ strain is hypersensitive to wrwycr and loses much of its ability to recover.

We reasoned that if wrwycr hypersensitivity in the $\Delta fep$ and $\Delta fes$ mutants was caused by hindrance of ferric enterobactin transport across the cytoplasmic membrane rather than hindrance of transport of another compound through the same system, the impairment of enterobactin synthesis would also render *E. coli* hypersensitive to wrwycr. We thus transduced the mutated loci of *entB* and *entC*, two genes whose products are required for enterobactin synthesis, from the corresponding Keio collection strains to the MG1655 background and measured the growth of the resulting strains in wrwycr. While both the enterobactin synthesis mutants tested were hypersensitive to wrwycr, the $\Delta entB$ strain was more hypersensitive than the $\Delta entC$ mutant strain (Figure 1C). These results suggest that enterobactin synthesis is required for wild-type-level resistance to wrwycr but that there is an EntC-independent way of making or taking up the dihydroxybenzoate substrate for enterobactin synthesis under our test conditions. EntC is an isochorismate synthase and *E. coli* encodes another isochorismate synthase, MenF (Daruwala *et al.*, 1996), which is needed to produce menaquinone for electron transport in anaerobic conditions. As our growth assays were done in sealed, predominantly static 96-well microtiter plates with glucose as the carbon source, it is likely that the dissolved oxygen content in the cultures was very low. Therefore, we tested whether MenF could substitute for EntC in enterobactin synthesis in the low-oxygen environment of microtiter plates. A $\Delta menF$ mutant had a hypersensitivity to wrwycr intermediate between that of the wild-type strain and the $\Delta entC$ mutant strain (Figure S2A), while a $\Delta entC \Delta menF$ double mutant was more
sensitive to wrwycr than either of the single mutants (Figure S2A), suggesting that either MenF activity can compensate for the lack of EntC for enterobactin synthesis in our assay conditions or menaquinone synthesis itself is important for resistance to wrwycr in low-oxygen environments.

A ΔtolC mutant is hypersensitive to wrwycr

Another wrwycr-hypersensitive strain identified in the original screen was the ΔtolC mutant. This strain was significantly more sensitive to wrwycr than were the other strains reported here—it did not grow in 10 μM wrwycr even after 48 h (Figure 1D), while the other mutants all grew at least somewhat in 22 μM wrwycr (Figures 1B and 2C). TolC is essential for the export of newly-synthesized enterobactin across the outer membrane (Bleuel et al., 2005), but also assists in the efflux of many other compounds, including antibacterial drugs (reviewed in (Koronakis et al., 2004)). EntS is involved in transport of enterobactin across the cytoplasmic membrane (Furrer et al., 2002) and thus an entS mutant could be expected to have the same phenotype as a tolC mutant. However, the E. coli ΔentS strain was not hypersensitive to wrwycr (Figure S2B). This suggests that enterobactin export by EntS is not essential for the ability of E. coli to withstand wrwycr treatment, likely because entS mutants can export components of enterobactin that are themselves siderophores (Furrer et al., 2002), and that the tolC mutant must have an additional defect that made it hypersensitive to wrwycr. Below, we present evidence that the additional defect is a diminished capacity to export wrwycr.

Added iron reduces the antimicrobial effects of wrwycr, by causing its precipitation

The enterobactin synthesis and uptake mutants described above are deficient in the cell’s high-affinity iron (as ferric enterobactin) uptake system and our initial screening medium, NGCV, had no added iron and the phosphate buffer the medium may have precipitated any inherent iron. Indeed, the addition of iron to NGCV improves the growth of the wild-type strain in
the absence of wrwycr (data not shown). We thus hypothesized that the hypersensitivity of the
enterobactin synthesis, export, and uptake mutants to wrwycr may be due to their limited ability
to get enough iron through their other, lower-affinity iron uptake systems (for a review,
(Guerinot, 1994)). To address this hypothesis, we added exogenous iron to a low-phosphate,
low-iron growth medium, MGCV, and found that the mutants were better able to withstand
wrwycr treatment, evidenced by shorter lag times in the presence of wrwycr (Figure S3).
However, we subsequently found that high concentrations of iron (150 µM and above)
precipitate the peptide in the media (Table S1), thereby lowering the effective concentration of
wrwycr to which cells are exposed (Figure S4). Note that we did not observe any significant
growth differences between cultures supplemented with FeSO₄ versus FeCl₃, either at low (6 or
12 µM) or at high, 120 µM, final concentration (data not shown).

The ΔtolC mutant, but not the ΔfepB mutant, has an increased level of cell-associated
wrwycr relative to the MG1655 parent

Because the entS mutant was not hypersensitive to wrwycr, we hypothesized that the
ΔtolC mutant is wrwycr hypersensitive not because of its involvement in enterobactin export but
rather because of its inability to efflux wrwycr. To investigate if the tolC mutant is defective for
wrwycr efflux, we measured the levels of cell-associated wrwycr in the ΔtolC mutant and in the
parent (MG1655) strains using reversed-phase high-performance liquid chromatography (RP-
HPLC) (Figure 2A). “Cell-associated wrwycr” refers to all the wrwycr that could be extracted by
formic acid from the cells, be it in the cytoplasm, periplasm, chromosome, or in the membranes.
We found that the ΔtolC mutant does accumulate wrwycr to a higher level than the TolC⁺
MG1655 parent strain (Figures 2B and C), suggesting that the ΔtolC mutant is hypersensitive to
wrwycr due at least in part to reduced efflux of wrwycr. In contrast, the ΔfepB mutant’s
hypersensitivity is not due to increased cell-associated wrwycr. Interestingly, the ΔtolC mutant
also showed an increased level of monomer wrwycr relative to dimerized wrwycr (Figure 2D), while the $\Delta$feplmutant has about the same concentration and dimer-to-monomer ratio of cell-associated wrwycr as MG1655 (Figures 2B-2D).

**Transcription of Fur-repressed genes is upregulated in wild-type E. coli in response to wrwycr treatment**

The hypersensitivity of the Fep system mutants to wrwycr suggested the possibility that wrwycr restricts the availability of iron to the bacteria. To determine the availability of intracellular iron during wrwycr treatment using a biological assay, we monitored the expression level of two genes, entB and fhuF, that are repressed in high iron conditions by Fur. Compared to the expression level in MG1655 treated with DMSO, the expression level of entB and fhuF in MG1655 treated with 10 µM wrwycr for 1.5 h was increased an average of 111 and 161 fold, respectively, as measured by quantitative PCR (qPCR) (Figures 3A and 3B). This increased level of expression was nearly as high as that seen in a $\Delta$fur mutant not treated with wrwycr, in which fhuF and entB expression should be completely de-repressed (Figures 3A and 3B). This suggests that wrwycr treatment reduces the amount of bioavailable iron in *E. coli* MG1655 and that the Fur regulon responds accordingly.

We anticipated that entB and fhuF transcript levels in the $\Delta$fur strain would appear insensitive to wrwycr treatment compared to DMSO treatment. Instead, we did see changes (albeit relatively small, just over 3-fold), but entB and fhuF transcript levels responded in opposite directions. For example, while the level of fhuF transcripts in wrwycr-treated $\Delta$fur cultures was about half of that in DMSO-treated $\Delta$fur cultures at 1.5 h, the level of entB transcripts was about 3-fold higher for the same comparison. The $\Delta$fur strain is itself hypersensitive to wrwycr (see below); however, we do not know why wrwycr-treated $\Delta$fur cultures had different levels of entB and fhuF transcripts than did DMSO-treated $\Delta$fur cultures,
or the reason for the differential response of *entB* vs. *fhuF* transcripts in the ∆*fur* background (see Discussion).

**Growth in NGCV medium and wrwycr treatment decrease catalase activity in cells**

Low bioavailable iron levels lead to a decrease in the cellular concentration of the enzymatically-active form of non-essential iron-containing enzymes, such as the catalase hydroperoxidase I (KatG or hemoprotein b-590) (Hubbard *et al*., 1986); thus, catalase activity may report iron availability in the cell. Using a H₂O₂ depletion assay, we quantified the amount of total catalase activity in cell-free extracts of the enterobactin uptake mutant ∆*fepB* and in the MG1655 parent strain after overnight growth in the screening medium, NGCV (see Extended Methods in Supplementary Material). Consistent with reduced levels of available iron in the conditions of our screen, we found that extracts of ∆*fepB* grown in NGCV overnight without wrwycr treatment had 29% of the catalase activity of the parent strain MG1655 in the same conditions (Figure S5A). Treatment of MG1655 in MGCV medium with 10 µM wrwycr for 1.5 h resulted in only 15% of the catalase activity of the DMSO-treated samples (Figure S5B).

**wrwycr treatment increases the chelatable iron in the cell**

The pattern of expression of Fur-regulated genes, observed by qPCR, and the decrease in catalase activity suggested that bioavailable iron levels in the cell are reduced by wrwycr treatment. We used electron paramagnetic resonance (EPR) spectroscopy to measure the amount of desferrioxamine-chelatable iron in wrwycr-treated cells. Surprisingly, EPR spectroscopy revealed that levels of chelatable iron in the cell actually increased 6- or 7-fold after 50 minutes of treatment with 8 or 16 µM wrwycr, respectively, relative to cells treated with the same volume of DMSO (Table 1). Combined with our other findings, this suggests that while wrwycr treatment increases chelatable iron in the cell, this iron is not available to Fur or KatG and that additional iron must be imported to rescue cells from the effects of wrwycr.
A possible source of the excess chelatable iron in wrwycr-treated cells may be wrwycr-related damage to proteins that contain iron-sulfur clusters. Collins and colleagues have suggested that many antibiotics may damage such proteins, causing them to release iron into the cell (Kohanski et al., 2007). We explored this possibility by testing the LacZ levels expressed from the suf operon promoter, which encodes proteins involved in iron-sulfur cluster assembly under iron-limiting or oxidative-stress conditions (Outten et al., 2004). Indeed we observed dose-dependent induction of β-galactosidase activity, which decreased over time at the lower doses of wrwycr tested (Fig. 4A). The suf promoter is activated independently by apo-IscR and OxyR (Yeo et al., 2006). We found that deletion of iscR prevented the rise in wrwycr-dependent LacZ activity, whereas deletion of oxyR did not affect induction (Fig. 4B). This suggests that Fe-S clusters are being damaged and are unavailable to bind to apo-IscR following wrwycr treatment.

Free intracellular iron in conjunction with oxygen has been linked to increased DNA damage, leading to higher mutation frequencies (Imlay et al., 1988). To determine if the additional chelatable iron in wrwycr-treated cells leads to an increased DNA mutation frequency, we treated cultures with either 8 or 16 µM wrwycr for 3h or 24 h and measured the mutation frequency compared to that in untreated cultures. To minimize the effect of exogenous iron, cultures were grown in NGCV without any added iron. After 3 h, there was no difference in mutation frequency in treated versus untreated cultures. After 24 h of treatment, the mutation frequency was less than 1.6 x 10^{-10} cells in untreated cultures, but increased 38 fold to 6.2 x 10^{-9} cells in cultures treated with 8 µM wrwycr, and increased almost 9-fold to 1.4 x 10^{-9} cells in cultures treated with 16 µM wrwycr. The reduced mutation frequency observed after 16 µM wrwycr treatment compared to 8 µM wrwycr treatment may be due to lesser fitness of the mutants and/or a reduced replication rate at the higher peptide concentration.
ryhB overexpression is a cause of the wrwycr hypersensitivity of a ∆fur mutant and the enterobactin uptake mutants

Because of the role Fur plays in regulation of the enterobactin uptake, we tested the sensitivity of the ∆fur mutant to wrwycr, and found that it was hypersensitive to 20 μM wrwycr (Figure 5). When Fur is not bound to iron, as is typical in low-iron media conditions, it does not repress ryhB transcription. In turn, the additional RyhB regulatory RNA molecules produced may decrease the expression of a gene that is important for wrwycr tolerance and/or increase expression of a wrwycr target during growth in the low-iron NGCV and MGCV media. In fact, the hypersensitivity of the ∆fur mutant was alleviated by the deletion of ∆ryhB (Figure 5).

To further test the hypothesis that a RyhB-regulated gene is important for resistance to wrwycr, we introduced a ryhB deletion into the ∆fepB, ∆fepC, ∆fepD, ∆fepG, and ∆fes mutants to relieve repression of RyhB targets in low-iron media and assayed growth in the presence of wrwycr. As shown in Figure 6, all the double mutants except the ∆fepB ∆ryhB strain had near wild-type resistance to wrwycr, while the tested RyhB+ single enterobactin uptake mutants showed their characteristic wrwycr hypersensitivity; in some experiments (data not shown), the ∆fepB ∆ryhB strain had near wild-type resistance to wrwycr, in contrast to the results shown in Figure 6. We thus conclude that at least one RyhB-regulated gene is important for wrwycr resistance in the enterobactin-uptake mutants. However, deletion of ryhB in the wild-type strain does not significantly increase its resistance to wrwycr, thus the altered expression of ryhB due to its reduced repression by Fur in the iron-limited conditions following wrwycr treatment is not the sole cause of the sensitivity of the wild-type strain to wrwycr.

Discussion

In this work, we showed that E. coli mutants defective in enterobactin biosynthesis or ferric enterobactin uptake across the cytoplasmic membrane were hypersensitive to a synthetic antimicrobial peptide, wrwycr. We infer from these results that the ability to take up ferric
enterobactin or another compound that uses this same transport system is important for *E. coli* to withstand wrrwycr treatment. Mutants lacking FepA or TonB, which are responsible for ferric enterobactin transport across the outer membrane, were not significantly hypersensitive to wrrwycr. Our recent analysis of the *E. coli* proteome found that FepA protein levels decreased upon wrrwycr treatment (J.E.R, I.R. Booth, and A.M.S., unpublished data). Since FepA levels are reduced in the wild-type strain by wrrwycr treatment, it is not surprising that the absence of FepA in the ∆fepA mutant did not significantly increase sensitivity of *E. coli* to wrrwycr. Ferric enterobactin may cross the outer membrane more easily in wrrwycr-treated cells because the peptide increases the permeability of cells, as evidenced by greater fluorescence of lipophilic probes such as NPN (Jonnalagadda, 2009).

In support of ferric enterobactin limitation causing wrrwycr hypersensitivity, we found that mutants lacking either EntB or EntC were also hypersensitive to wrrwycr, although less so than some of the uptake mutants (perhaps the reason they were not identified in the original screen). The ∆entC strain was less hypersensitive to wrrwycr than was the ∆entB strain, but a ∆menF ∆entC strain was more sensitive than the ∆entC MenF+ strain. This suggests either that the second isochorismate synthase in *E. coli*, MenF (Daruwala *et al.*, 1996), can make isochorismate for the synthesis of enterobactin in the absence of EntC, and/or that menaquinone synthesis by MenF is itself important for resistance to wrrwycr. Indeed, a ∆menF EntC+ strain was hypersensitive to wrrwycr. The literature suggests that MenF cannot compensate for the lack of EntC for enterobactin synthesis unless the corresponding genes are over-expressed *in trans*, as most of the isochorismate synthesized by MenF and EntC is thought to be channeled to the next enzyme in their respective pathways, MenD or EntB (Buss *et al.*, 2001). Sufficient isochorismate may escape the menaquinone pathway to make dihydroxybenzoate (and therefore enterobactin) in the absence of EntC in the semi-anaerobic conditions of the microtiter plate assays.
*E. coli* entS and tolC mutants are known to excrete little if any enterobactin but they do excrete monomer, dimer, and trimer 2,3-dihydrobenzoylserine molecules (Bleuel *et al.*, 2005; Furrer *et al.*, 2002). These Fes-mediated enterobactin breakdown products are siderophores in their own right (in their iron-bound state they are taken back into the cell by the Fiu and Cir receptors) and thus entS and tolC mutants can achieve comparable growth to that of enterobactin-excreting strains (Hantke, 1990). As such, it is not surprising that the ∆entS mutant tested here was not hypersensitive to wrwycr. To explain the hypersensitivity of the ∆tolC mutant to wrwycr, we propose that TolC is responsible for efflux of wrwycr. Supporting this hypothesis, the ∆tolC mutant had higher levels of cell-associated wrwycr as well as a reduced ratio of dimer to monomer wrwycr compared to MG1655 and the ∆fepB mutant. In the ∆tolC mutant, wrwycr may enter and remain in the cytoplasm long enough for a greater fraction to become reduced to the monomer form. Exactly how wrwycr enters bacteria to achieve the observed steady-state level of cell-associated wrwycr is unknown.

Why is the ability to harvest ferric enterobactin protective against wrwycr in a low-iron medium? We hypothesized that wrwycr treatment reduced bioavailable iron in the cell, necessitating increased iron uptake by the ferric enterobactin transporter. Supporting this hypothesis, after 1.5 h of wrwycr treatment the transcript level of the Fur:Fe$^{2+}$-repressed genes *entB* and *fhuF* increased and catalase activity decreased. These results are consistent with wrwycr treatment reducing the amount of bioavailable iron. In contrast, direct measurement of chelatable iron in the cell indicated that wrwycr treatment actually *increased* chelatable iron, perhaps by damaging iron-sulfur cluster proteins, as has been proposed for other antibiotics (*e.g.*, (Gu & Imlay, 2011; Kohanski *et al.*, 2007)). The induction of *suf* operon promoter activity by wrwycr treatment agrees with this idea, and implicates IscR (and not OxyR) as being necessary for this induction. A possible explanation for the apparent conflict between wrwycr’s induction of genes that promote iron uptake and the cells’ perceived shortage of iron is that the chelatable iron is not in fact available to bind to Fur or KatG and to rescue wrwycr-treated cells.
The increased iron uptake in the $\Delta fur$ mutant should logically have provided protection against wrwycr due to derepression of genes encoding iron-uptake systems, and thus the $\Delta fur$ mutant should not have been hypersensitive to wrwycr, in conflict with our findings. However, perhaps the iron concentration of the test medium was not sufficient to counteract wrwycr challenge. Moreover, RyhB levels would have increased in the $\Delta fur$ mutant, decreasing expression of RyhB-repressed genes and increasing expression of RyhB-activated genes. Deletion of $ryhB$ mostly, but not completely, relieved the wrwycr hypersensitivity of the tested $fep$ and $fes$ mutants, indicating either that a RyhB-repressed function is critical for withstanding wrwycr treatment, or that a RyhB-activated function somehow promotes the inhibitory effects of wrwycr. Deletion of the $shiA$ gene, known to be activated by RyhB, does not render either wild type or $\Delta fepB$ cells more sensitive than the ShiA$^+$ isogenic strains (data not shown). Future studies will be aimed at identifying the RyhB-targeted transcript(s) that affect(s) wrwycr sensitivity. As the altered regulation by RyhB does not fully explain the hypersensitivity of the enterobactin uptake mutants, wrwycr-dependent iron limitation probably plays a role in addition to the altered iron regulation.

A surprising result is the discordant transcription response to wrwycr of $entB$ and $fhuF$ in the $\Delta fur$ strain. One possibility for the observed differential response may be the additional positive regulation of $entB$ by CRP-cAMP (Zhang et al., 2005). It is intriguing that in Salmonella, CRP-activated genes are induced about 2 fold in a $\Delta fur$ strain by the presence of the iron chelating agent dipyridyl, similarly to our observed induction of $entB$ transcription in the presence of wrwycr (Campoy et al., 2002). The same study showed that the concentration of cAMP was higher in a $\Delta fur$ mutant compared to wild type in iron-limiting conditions in the presence of dipyridyl. We thus note the parallel between the effects of dipyridyl and wrwycr on $entB$ expression (and perhaps, by inference, on cAMP levels) in the $\Delta fur$ mutant, although the media used in the Campoy study was LB rather than the MHB media we used in our own qPCR experiments.
The observed Fur-regulon derepression in response to wrwycr indicates lower levels of bioavailable iron levels, but how does wrwycr treatment elicit this outcome? The most straightforward explanation would be that wrwycr chelates iron, albeit weakly. However, we have been unable to detect any evidence for such an activity using either a chrome azurol S colorimetric assay (Schwyn & Neilands, 1987), or by monitoring the spectrophotometric or fluorometric profile of wrwycr in the presence of iron. We may not have found the appropriate conditions for testing binding of wrwycr to iron. Alternatively, instead of binding iron directly, wrwycr may indirectly sequester iron by binding a siderophore and causing its aggregation, for example, but we have no clues for the identity of such a molecule. Our results could also formally be explained by wrwycr binding to Fur and KatG directly, competing for iron binding intracellularly (see below). We will test these hypotheses in the future.

It is unclear what the relationship is between the different activities of peptide wrwycr in interfering with DNA repair and accumulating intracellular DNA damage and the iron-restricting activities described here. At least two DNA glycosylases involved in base excision repair, endonuclease III (Nth) and MutY protein, and the DinG helicase, induced by DNA damage, contain [4Fe-4S]^{2+}-type iron-sulfur centers (Boal et al., 2005; Cunningham et al., 1989; Lukianova & David, 2005; Ren et al., 2009). Impairment of the activity of MutY and Nth by wrwycr due to damage to their iron-sulfur centers may be one explanation for wrwycr-dependent increase in the mutation frequency in iron-limited conditions (NGCV media). Another explanation may be the presence of greater concentrations of intracellular iron after wrwycr treatment. Our best current hypothesis is that the effects of wrwycr on DNA repair (Gunderson et al., 2009) and on iron availability are independent, albeit potentially synergistic.

In summary, the antimicrobial properties of wrwycr are potentiated in low-iron conditions. Enterobactin has been thought not to be an important virulence factor because mammalian neutrophils produce siderocalin (lipocalin), which binds enterobactin in serum (Goetz et al., 2002). However, mammalian antimicrobial strategies include the export of iron from
macrophages when they are infected with intracellular pathogens such as *Salmonella*, *Mycobacteria* and others (Forbes & Gros, 2001; Nairz *et al.*, 2007). Moreover, several of the effects of *wrwycr* on iron metabolism described herein, including the accumulation of chelatable but likely unavailable iron in cells, are independent of enterobactin, and thus *wrwycr* almost certainly puts a strain on all high-affinity iron uptake systems. This could make it or similar molecules particularly effective against pathogens in the low-iron conditions of their hosts.

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References


Table 1. Intracellular concentrations of chelatable iron after treatment of MG1655 with wrwycr, measured by EPR spectroscopy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chelatable intracellular iron (µM) +/-SEM</th>
<th>Fold difference with respect to DMSO treated cells</th>
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<tbody>
<tr>
<td>DMSO</td>
<td>34.2 +/-5.5</td>
<td>1</td>
</tr>
<tr>
<td>8 µM wrwycr</td>
<td>201.7 +/-17.2</td>
<td>5.9</td>
</tr>
<tr>
<td>16 µM wrwycr</td>
<td>238.2 +/-31</td>
<td>7</td>
</tr>
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Figure 1: *E. coli* enterobactin synthesis, export, and uptake mutants in MGCV medium are hypersensitive to wrwycr. (A) The mutants grow similarly to the parent (MG1655) in DMSO (dotted lines) in “low” iron conditions (2.4 µM FeCl₃ added), as indicated by a square and arrow. For comparison purposes, the OD₆₀₀ of MG1655 (G652) grown in 22 µM wrwycr and either 150 µM FeCl₃ (filled squares) or 2.4 µM FeCl₃ (open squares) is also shown. Because the strains were split into two plates for this analysis, the data for the two separate sets of MG1655
comparison wells on each plate are shown. (B-D) Four individual colonies of (B) MG1655 (G652, black squares), ΔfepB (EDT1343, blue diamonds), ΔfepD (EDT1344, green circles), Δfes (EDT1345, orange upward-pointing triangles), ΔfepA (EDT1574, purple hexagons), and ΔtonB (EDT1688, red downward-pointing triangles) or (C) MG1655 (G652, black squares), ΔfepC (EDT1686, purple downward-pointing triangles), ΔfepG (EDT1687, blue upward-pointing triangles), ΔentB (EDT1746, green circles), and ΔentC (EDT1747, red diamonds) or (D) MG1655 (G652, black circles and squares) and ΔtolC (EDT1350, brown triangles and diamonds) were grown overnight in MGCV medium (2.4 µM FeCl₃), then diluted to 1% in fresh MGCV medium with 22 µM wrwycr (all data on panels B and C), 5 µM wrwycr (circles and triangles, panel D), 10 µM wrwycr (squares and diamonds, panel D), or DMSO (dashed lines and open symbols, panel D) and 2.4 µM FeCl₃. The cultures (100 µl total) were grown in microtiter plates at 37°C. Each data point represents the mean OD₆₀₀ of the four individual cultures tested and error bars represent the standard error of the mean. Growth measurements shown in B and C were recorded simultaneously but are presented in two panels for clarity.
Figure 2: The *E. coli* ∆tolC mutant, but not the ∆fepB mutant, has an increased level of cell-associated wrwycr and a lower ratio of dimer to monomer wrwycr relative to the MG1655 parent strain, as measured in an RP-HPLC assay. (A) Overlay of typical RP-HPLC traces of formic acid extracts from *E. coli* MG1655 (G652) treated with wrwycr (solid line) or DMSO (short-dashed line) and of DMSO in formic acid (long-dashed line) to show identification of cell-dependent peaks and monomer and dimer wrwycr, as indicated. (B-D) Log-phase MG1655 (G652, squares), ∆tolC (EDT1311, triangles), or ∆fepB (EDT1316, diamonds) cells were treated with 10 µM wrwycr for 1.5 h, after which cell-associated peptides were extracted using formic acid. The
formic acid extracts were analyzed by RP-HPLC and the detected monomer and dimer peak areas were normalized to (B) the area of a cell-dependent peak, the area of which correlated with the number of DMSO-treated cells subjected to formic acid extraction (data not shown), or to (C) the OD₆₀₀ of the cultures at the time of formic acid extraction. Graphed are (B, C) the normalized total peptide amounts expressed as monomer equivalents and (D) dimer to monomer ratios. For B-D, each data point represents the result for an independent culture and the horizontal lines indicate the means of the samples. Data points are from (B) 6, (C) 3, or (D) 5 separate experiments with 3 independent cultures each. A one-way analysis of variance with Tukey’s multiple comparison test determined that the difference in the means (horizontal bars) within each panel (B-D) is statistically significant (p>0.05) in bars labeled with a different lowercase letter, but not in those labeled with the same letter.
Figure 3: Quantification of relative transcript levels of (A) *entB* and (B) *fhuF* in *E. coli* MG1655 (G652, squares) or ∆fur (EDT1566, triangles) treated with 0.1% DMSO (closed squares), 10 µM wrwycr (open squares), 0.082-0.09% DMSO (closed triangles), or 8.2-9 µM wrwycr (open triangles) for 1.5 h in MHB. Total cellular RNA was harvested from the cultures at 1.5 h, used as a template for cDNA, the cDNA used in a quantitative PCR, and the results for each sample adjusted according to the amount of *polA* cDNA detected for that sample. Horizontal bars indicate the mean of the results for six independent cultures, which were treated and harvested over two separate days (three on each day), and numbers above the bars for MG1655 treated with DMSO provide the mean ± the standard error of the mean for those samples. Two-tailed t tests indicated that the difference in the means with different lowercase letters in each panel is statistically significant (p<0.05; specific p values are provided in each panel for the comparison of MG1655 treated with DMSO or wrwycr).
Figure 4: Peptide wrwycr treatment activates the Suf operon through IscR. (A) Treatment with wrwycr induces a promoter fusion of psuf::lacZ; induction was followed over time using the β-galactosidase substrate 4 methyl umbelliferyl β-D-galactoside, (MUG). Suf promoter activity
expression takes longer to return to the level in untreated cultures with higher doses of wrwycr.

(B) Suf operon activation by wrwycr is dependent upon the IscR activator. Three reporter strains, one lacking IscR, another lacking OxyR, and their isogenic wild-type strain, were assayed after 60 min of wrwycr treatment. (A-B) H$_2$O$_2$ was used as a positive control to induce the Suf operon. DMSO, the solvent of the wrwycr was used as the negative control. The data are expressed as the average of three independent colonies and shown with the standard deviation. Cells were grown and treated in MGCV medium. * = DMSO treatment at a concentration of 0.16%.
Figure 5: The hypersensitivity to wrwycr of an *E. coli* Δfur mutant is corrected by an added mutation, ΔryhB. Three individual colonies of MG1655 (G652, black squares), Δfur (EDT1566, blue diamonds), ΔryhB (EDT1571, green circles), and Δfur ΔryhB (EDT1712, red triangles) were grown overnight in NGCV medium with no added iron, then diluted to 1% in fresh NGCV medium with 0.2% DMSO (closed symbols) or 20 µM wrwycr (open symbols). The cultures (100 µl total) were grown in microtiter plates at 37°C. Each data point represents the mean OD<sub>600</sub> of the three individual cultures tested and error bars represent the standard error of the mean.
Figure 6: Deletion of *ryhB* reduces the hypersensitivity to wrwycr of the enterobactin uptake mutants. Open symbols are RyhB⁺ and closed symbols are RyhB⁻. Graphed is the mean OD₆₀₀ of three independent cultures of (A) *E. coli* MG1655 (G652, open black squares), ∆*ryhB* (EDT1571, closed black squares), ∆*fepC* (EDT1686, open purple upward-pointing triangles), ∆*fepC ∆ryhB* (EDT1700, closed purple upward-pointing triangles), ∆*fepG* (EDT1687, open blue hexagons), and ∆*fepG ∆ryhB* (EDT1701, closed blue hexagons) or (B) *E. coli* MG1655 (G652, open black squares), ∆*ryhB* (EDT1571, closed black squares), ∆*fepB* (EDT1343, open blue diamonds), ∆*fepB ∆ryhB* (EDT1683, closed blue diamonds), ∆*fepD* (EDT1344, open green circles), ∆*fepD ∆ryhB* (EDT1684, closed green circles), and ∆*fes* (EDT1345, open orange triangles), ∆*fes ∆ryhB* (EDT1685, closed orange triangles) measured during growth in MGCV with 20 μM wrwycr at 37°C. Error bars represent standard error of the mean. Growth measurements shown in panels A and B were recorded simultaneously but are presented in two panels for clarity. All strains grow equally well in DMSO (data not shown).