Transcriptional Responses of *Escherichia coli* to a Small-Molecule Inhibitor of LolCDE, an Essential Component of the Lipoprotein Transport Pathway

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**ABSTRACT**

In Gram-negative bacteria, a dedicated machinery consisting of LolABCDE components targets lipoproteins to the outer membrane. We used a previously identified small-molecule inhibitor of the LolCDE complex of *Escherichia coli* to assess the global transcriptional consequences of interference with lipoprotein transport. Exposure of *E. coli* to the LolCDE inhibitor at concentrations leading to minimal and significant growth inhibition, followed by transcriptome sequencing, identified a small group of genes whose transcript levels were decreased and a larger group whose mRNA levels increased 10- to 100-fold compared to those of untreated cells. The majority of the genes whose mRNA concentrations were reduced were part of the flagellar assembly pathway, which contains an essential lipoprotein component. Most of the genes whose transcript levels were elevated encode proteins involved in selected cell stress pathways. Many of these genes are involved with envelope stress responses induced by the mislocalization of outer membrane lipoproteins. Although several of the genes whose RNAs were induced have previously been shown to be associated with the general perturbation of the cell envelope by antibiotics, a small subset was affected only by LolCDE inhibition. Findings from this work suggest that the efficiency of the Lol system function may be coupled to a specific monitoring system, which could be exploited in the development of reporter constructs suitable for use for screening for additional inhibitors of lipoprotein trafficking.

**IMPORTANCE**

Inhibition of the lipoprotein transport pathway leads to *E. coli* death and subsequent lysis. Early significant changes in the levels of RNA for a subset of genes identified to be associated with some periplasmic and envelope stress responses were observed. Together these findings suggest that disruption of this key pathway can have a severe impact on balanced outer membrane synthesis sufficient to affect viability.

Multiply drug-resistant (MDR) bacterial pathogens pose a serious challenge in clinical medicine. Currently, the options for the treatment of serious infections caused by Gram-negative organisms are narrowing. With the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE), it is clear that new sources of efficacious compounds to address infections caused by Gram-negative bacteria are a necessity (1, 2). The presence of two dissimilar membranes surrounding Gram-negative bacteria, a cytoplasmic membrane and an outer membrane, presents a particular challenge to antibiotic therapy of infections caused by this group of organisms (3–5). Whereas the inner cytoplasmic membrane has properties of a typical lipid bilayer, the outer membrane has an asymmetric character, with a phospholipid-containing inner surface and an outer surface consisting largely of lipopolysaccharide. Proteins of the inner membrane are mostly typical membrane proteins with α-helices and transmembrane loops, whereas the majority of outer membrane proteins have either β-barrel structures or are lipoproteins (4, 6). Various small molecules, including nutrients or antibiotics, that need to reach the cytoplasm often penetrate the outer membrane by diffusion through the hydrophilic channels of β-barrel porins. The antibiotics subsequently traverse the inner membrane primarily through diffusion across the phospholipid bilayer, requiring some degree of lipophilicity and a neutralized charge (zwitterionic properties) (7). Because of the membranes’ orthogonal properties, it has been difficult to identify antibiotics that have the chemical properties needed to penetrate both the outer and inner membranes (7, 8). An additional challenge to the effective eradication of Gram-negative bacteria is the presence of broad-substrate efflux pumps in the periplasm which act to reduce antibiotic concentrations in the bacteria (9).

The unique components of the outer membrane of Gram-negative bacteria are assembled during cell elongation and division. Three outer membrane assembly pathways with components located in each of the membranes and in the periplasm are known to exist in these bacteria: Bam (β-barrel assembly machine), Lpt (lipopolysaccharide transport proteins), and Lol (lipoprotein transport) (10–12). Each of these is essential for the biogenesis of a functional outer membrane. Compromising...
the structure of the outer membrane not only could potentially lead to improved kinetics of penetration of existing antibiotics into Gram-negative bacterial pathogens but also could disrupt the assembly or function of the tripartite efflux pumps.

In *Escherichia coli* there are more than 90 different lipoproteins, with the majority residing in the inner leaflet of the outer membrane (12). The components of the Bam, Lpt, and Lol pathways include essential lipoproteins; therefore, disruption of lipoprotein synthesis leads to an imbalance in outer membrane biogenesis caused by a malfunction in all three systems (12). The lipoprotein transport pathway has five protein components: the LolCDE complex provides the energy for transport, is essential, and resides in the cytoplasmic membrane, while LolA is localized in the periplasm and LolB is an outer membrane lipoprotein (13). The LolCDE complex of *E. coli* has been shown to consist of one copy each of the membrane-spanning subunits LolC and LolE and two copies of the ATPase subunit LolD (14). On the basis of the current model for lipoprotein transport in *E. coli* and likely in all Gram-negative bacteria, the lipoprotein precursors are acylated on the sulphydryl of the cysteine in a consensus lipobox sequence, and following the cleaved signal peptide, the newly created N terminus of the cysteine is also acylated. These reactions are carried out sequentially in the inner membrane by three enzymes, Lgt, LspA, and Lnt (12). Following these covalent modifications, LolCDE catalyzes the release of the lipoproteins destined for the outer membrane from the inner membrane to the periplasmic lipoprotein carrier, LolA (15, 16). LolA in turn transports the lipoprotein across the periplasm to the outer membrane, where LolB accepts the nascent lipoproteins and facilitates their insertion into the outer membrane (17).

Until recently, the only known small molecule capable of interfering with lipoprotein transport was globomycin, an inhibitor of the type II signal peptidase Lsp (18). Employing a general cell wall reporter assay, Nayar et al. reported the discovery of a small-molecule inhibitor of the Lol pathway in *E. coli* (19). This molecule contained a pyrazole core and had a molecular weight of 345.4 (Fig. 1). It exhibited potent activity against efflux-deficient *E. coli* with a MIC of 0.125 to 0.25 μg/ml. This compound was shown to inhibit Lol transport by demonstration of the blocking of the release of Lpp from *E. coli* spheroplasts and by isolation of resistant mutants with gene mutations leading to amino acid changes in either the LolC or LolE component of the lipoprotein transport machinery. Since these two proteins show modest sequence conservation (27% identity), it is conceivable that the compound recognizes a structurally related fold interfering with the function or assembly of the LolCDE complex.

We exploited the activity of the small-molecule inhibitor of the Lol pathway to investigate additional physiological effects of interference with lipoprotein transport in *E. coli*. The impact of the compound on bacterial viability and cell integrity over time was assessed, and the early transcriptional changes brought about by exposure to the compound are described. In addition, we compared the transcriptional levels of several key genes with altered expression levels resulting from inhibition of the Lol pathway with the transcriptional levels in the presence of several antibiotics with different mechanisms of action. Our findings demonstrate that pharmacological inhibition of the Lol pathway results in increased transcription in several envelope stress pathways.

**Materials and Methods**

**Bacterial strains and growth.** For all experiments, *Escherichia coli* BW25113 (rpsL32 ΔlacZ4787 hsdR514 ΔaraBAD)657 Δ(rhaBAD)568 rph-1] with the ΔacrB deletion (CGSC JW0451-2 8609) was employed (20). Use of the ΔacrB strain allowed the use of lower concentrations of the limited supply of the LolCDE inhibitor compound (compound 2), for which the MIC in LB is 0.6 μg/ml. All experiments were performed in LB broth cultures at 37°C with shaking at 200 rpm. For experiments in which compounds or antibiotics were added, the MIC values determined in LB broth microtiter plates with low concentrations of bacterial cells (5 × 10⁶ CFU/ml) (21) were used as guidelines to test bacterial growth at the higher cell densities needed for the RNA extractions. Cells were grown in larger cultures, and compound or antibiotic was added at concentrations below and above the broth microdilution MICs. Growth was monitored using the optical density at 600 nm (OD₆₀₀), and the concentrations that produced reductions in the growth rate were employed for the experiments (see Fig. S1 in the supplemental material).

Resistant mutants of BW25113 ΔacrB were selected on LB agar plates with 5 μg/ml (8× MIC) of compound 2. After 20 h of incubation, selected colonies were transferred onto fresh LB plates with 5 μg/ml of compound 2 and grown overnight at 37°C. The growth from these plates was stored in 10% glycerol in LB at −80°C. Genomic DNA was isolated from several clones, and LolC and LolE were amplified. A mutant with a previously reported (19) mutation in LolC (N256K) that confers resistance to the compound was selected for further study. The MIC for this clone was measured in LB with serial dilutions of compound. The MIC increased from 0.6 μg/ml for the parent to 32 μg/ml for the mutant.

**Compound.** Compound 2 (19) was obtained in a powder form from AstraZeneca, Waltham, MA. It was dissolved in dimethyl sulfoxide to obtain a 5-mg/ml stock and kept at −20°C. The MIC for *E. coli* BW25113 was periodically checked to ensure the retention of activity.

**Cell-killing kinetics.** The rate of growth/killing of *E. coli* BW25113 ΔacrB was determined by treating mid-logarithmic-phase cells with a range of increasing concentrations of the LolCDE inhibitor. Samples were removed at timed intervals and serially diluted in 10-fold series, and 100-μl aliquots from each dilution were spread on plates containing LB agar. The plates were incubated for 24 h, and cells were enumerated by counting the colonies. Bacterial lysis was monitored by following the changes in the OD₆₀₀ after periodically taking samples, diluting them into the linear range, and measuring the absorbance on a VWR UV 1600 PC spectrophotometer.

**Transcriptome analysis by RNA-seq.** For transcriptome sequencing (RNA-seq), *E. coli* BW25113 ΔacrB was grown overnight in LB broth with shaking at 37°C. On the next morning, a 1:200 dilution was made in 150 ml of LB, and the bacteria were grown at 37°C with shaking until an OD₆₀₀ of 0.5 was attained. The culture was then split into six portions of 20 ml each that were placed into six flasks, with two flasks being used as biological replicate controls, two replicate flasks receiving 0.3 μg/ml of the LolCDE inhibitor, and two replicate flasks receiving 1.2 μg/ml of the LolCDE inhibitor. After 30 min, 800 μl of culture from each flask was placed directly into prewarmed (65°C) lysis mix-acid phenol solution. Lysis mix consisted of 320 mM sodium acetate, 8% SDS, and 16 mM EDTA (all...
from Ambion, Thermo Fisher) in nuclelease-free water. One hundred micro-

collters of the above-described lysis mix was combined with 700 μl of
acid phenol-chloroform (Ambion) in 2-ml tubes. The cells and lysis mix-

acid phenol were rapidly mixed on a vortex mixer and kept at 65°C with

vortexing for 5 to 10 s every minute for 10 min. After centrifugation for 5

min at 12,000 × g, the upper phase was carefully removed, transferred

into 700 μl of phenol-chloroform-isosamyl alcohol (Ambion), and vor-
texed to mix. This extraction procedure was repeated twice, and cen-

trifugation at 12,000 × g for 5 min was used to separate the phases, with

the upper phase being taken each time. Finally, the upper phase was trans-

ferred to 600 μl (equal volume) of chloroform-isosamyl alcohol and again

centrifuged as described above. The upper phase was removed, and RNA

was precipitated by treatment with 2 volumes of 100% ethanol overnight

at 80°C. On the following morning, the mixture was centrifuged at 18,000 ×
g at 4°C for 10 min. The pellets were washed once with 70% ethanol in

DNase- and RNase-free water (Invitrogen). The pellets were dried under

vacuum in a Savant SpeedVac system for 5 to 10 min. The dried pellets

were resuspended in 30 μl of diethyl pyrocarbonate-treated water (Invit-

rogen). RNA concentrations were determined using a Nanodrop spectro-

photometer (Thermo Fisher). Ribosome integrity numbers (RINs) were

determined with an Agilent Bioanalyzer 2100 instrument and an Agilent

RNA 6000 Nano kit (Santa Clara, CA). The initial RIN for all six RNA

samples was 10. DNase treatment was carried out with a Turbo DNA-free

kit (Ambion). The RINs were again checked after DNase treatment, and

the values ranged from 9.4 to 9.9. The rRNA was depleted using a Ribo

Zero rRNA removal kit for Gram-negative bacteria (Illumina). RNA li-

braries were prepared with an NEBNext ultradirectional RNA library

preparation kit for the Illumina system using NEBNext multiplex oligo-

gene sequencers for Illumina index primer set 1 (New England Biolabs).

The size distribution of the library was tested with an Agilent 2200 TapeStation

high-sensitivity D1000 ScreenTape system. RNA-seq was carried out on

an Illumina HiSeq platform, with the coverage of the six samples ranging

from 18 million to 24 million reads each. Analysis of the data was per-

formed using CLC Bio Genomics Workbench software, with the reads

being mapped to the genome sequence of

E. coli

BW25113. Duplicate sample data were averaged, and the complete comparative data for RNA-

seq are provided in Tables S1 and S2 in the supplemental material. Low-

cutoff in terms of the number of reads per million (22).

qPCR. For determination of RNA levels by quantitative PCR (qPCR),

various concentrations of antibiotic were first tested in cultures grown in

20-ml volumes. The concentrations of antibiotics that were twice the

lowest level required to inhibit growth, determined by measurement of the

optical density (see Fig. S1 in the supplemental material), were selected,

and in a subsequent experiment cells were incubated with those concen-

trations for 30 min. RNA was then prepared from these cultures with the

hot acid phenol procedure as described above. Primers were designed by

use of the GenScript real-time PCR primer design tool. cDNA was syn-

thesized with a SuperScript III first-strand synthesis system for reverse

transcription-PCR (Invitrogen) and random hexamer primers. The qPCR

was carried out using PerfeCTa SYBR Green FastMix (Quanta Biosci-

ences) in a Mastercycler Realplex2 system from Eppendorf. Changes in

transcript levels relative to the levels in the untreated control cultures were

calculated. The growth and exposure for RNA extractions for the qPCR

experiments with 30-min compound exposures were performed twice

(biological replicates) on different days.

RESULTS

Effect of Lol inhibition on growth. Previous work has established

that the inhibitor (compound 2) (Fig. 1) (19) blocks lipoprotein

transport and that resistance to the compound is found in bacte-

rial cells with amino acid substitutions in either LolC or LolE. The

impact on the rate of bacterial growth was not reported, so an

initial step, cell viability was measured over time at several com-

pound concentrations. In the present study, a volume sufficient

for determination of the optical density and cell numbers and for

preparation of RNA was desired. A standard 20-ml-volume cul-

ture in LB was employed for all experiments, and compound 2 was

added at various concentrations, using the MIC for this strain of

0.25 μg/ml as a baseline. As can be seen in Fig. 2A, concentrations

above 0.6 μg/ml resulted in a significant decline in bacterial vi-

ability. The viability experiments were purposely performed at a

bacterial mass that was necessary for the direct, rapid isolation of

RNA from the cultures, without prior centrifugation or other ma-

nipulations to concentrate the cells. Compound 2 (0.3 and 1.2

μg/ml) was added to a culture of

E. coli

ΔacrB at an OD600 of 0.5

(5 × 10^6 CFU/ml) for 30 min prior to RNA isolation by addition of

a portion of the culture to hot acid phenol. These concentra-

tions of the compound were just below and just above the levels

where effects on growth were detected (Fig. 2A). The cultures were

monitored after the 30-min period, and it was noted that the op-
tical density declined at the higher compound concentrations over time (Fig. 2B).

Transcriptional effects of Lol inhibition by RNA-seq. The data for genes that were upregulated 5-fold or more by treatment with compound 2 are listed in Table 1; complete transcript-level data are presented in Tables S1 and S2 in the supplemental material. Comparative graphs derived from the transcriptome data for duplicate measurements and control cells versus compound-treated cells are shown in Fig. S2 in the supplemental material. Poorly expressed genes with very low numbers of reads per million (below 16) were removed from the data in the tables (22). An average level of upregulation of RNA in treated cells exceeding 10-fold the average level of regulation in control cells was observed for 38 genes with the lower concentration of the compound and 74 genes with the higher concentration of the compound. In almost all cases, a dose-response relationship in which higher compound concentrations resulted in greater magnitudes of RNA concentration changes could be seen. The majority of RNA species that were upregulated were for genes associated with envelope stress. Several different stress-induced regulons contributed to these changes. In the 30 min of compound 2 treatment, there was no increase in the levels of mRNA for the compound target, LolCDE. The levels of expression of all of the lol genes (lolA, lolB, and lolCDE) were also checked by qPCR (see below).

In terms of decreased levels of RNA in response to pharmacological inhibition of lipoprotein transport, the number of genes for which major reductions in RNA levels were seen was much smaller than the number for which increases in RNA levels were seen. As can be seen in Table 2, the vast majority of genes with reduced levels of RNA expression encode flagellar components. This may be the consequence of disruption of flagellar assembly due to mislocalization of the lipoprotein FlgH, the L-ring subunit of the flagellar basal body. Consequently, failure to assemble the hook/basal body complex and retain the anti-sigma factor FlgM in the cytoplasm would lead to the global repression of flagellar gene expression (23, 24).

Transcriptional impact of antibiotic inhibition on select genes. Next we selected several genes that demonstrated significant upregulation or downregulation and determined the concentrations of mRNA for those genes by quantitative PCR (qPCR). Their transcript levels were also compared to those obtained following treatment of cells with seven other antibiotics with several distinct mechanisms of action, in order to determine if a subset of the genes under study was uniquely regulated by the LolCDE inhibitor or whether these were also affected by antibiotic disruption of other cell physiological properties. Preliminary experiments with high-volume shaking cultures established the minimal concentrations of the various antibiotics that were inhibitory to E. coli ΔacrB growth under conditions analogous to those employed in the RNA-seq experiments with the higher cell numbers and higher concentrations of the LolCDE inhibitor. The E. coli strain was exposed to these compounds at concentrations 2 times the inhibitory levels (along with the LolCDE compound at the higher concentration) for 30 min, conditions identical to those used in the RNA-seq experiments. RNA was extracted, purified, and converted to cDNA for qPCR determinations. Results for individual selected genes are presented in Fig. 3.

We used rpsL, encoding the ribosomal protein S12, as a housekeeping gene whose level of transcription should not vary significantly from that for the control cells to determine the efficiency and variability of the RNA extractions in the different experiment using cells treated with the LolCDE inhibitor or various antibiotics. All of the rpsL transcript levels were within ~2-fold of those for the control cells (Fig. 3). In the presence of the LolCDE inhibitor, several genes associated with cell envelope stress were markedly upregulated, and this result was confirmed by qPCR. The concentrations of mRNA for the genes clkB, katE, ycfT, and ecnB were reproducibly elevated in bacteria treated with the LolCDE inhibitor, and the levels were very similar to the levels observed in the RNA-seq experiments. None of the genes in this group showed alterations in their transcript levels when the bacteria were treated with several other antibiotics. The largest increase in the level of expression of mRNA resulting from LolCDE inhibition was seen for osmB, and this result was confirmed by qPCR. The levels of osmB mRNA were also markedly elevated by polymyxin B treatment. The expression levels of yegS were also elevated by treatment with the LolCDE inhibitor, and additional modest levels of increase were noted with the two β-lactam compounds (imipenem and meropenem), as well as with polymyxin B. A similar pattern emerged with the hypersensitivity-associated gene ycfJ, whose transcript levels were notably higher following treatment with the β-lactams and polymyxin B and were similar to the levels observed with the LolCDE inhibitor. The levels of the mRNAs for the putative toxin-antitoxin pair yngD and yngG were also elevated in the presence of the LolCDE inhibitor, the two β-lactam compounds, and polymyxin B. A similar pattern in response to antibiotic treatment was observed for ivy, which protects cells permeabilized by chemical or physical stresses (25). The transcript levels for CpxP, a protein involved in the regulation of degradation of misfolded proteins, were also increased by the LolCDE inhibitor, polymyxin B, and gentamicin. Similarly, transcripts specifying DegP, which functions in the same degradation pathway as CpxP, were also increased by these treatments, albeit to a lesser extent. Little effect on any of the LolCDE inhibitor-induced mRNA changes was observed following ciprofloxacin exposure; however, when the effect of ciprofloxacin exposure on the SOS gene sulA was tested, it was found to be upregulated by this antibiotic, as expected (26).

Among the genes whose mRNAs showed a decrease when lipoprotein transport to the outer membrane was blocked, one (flhD) was examined by qPCR. In addition to being affected by the LolCDE inhibitor, its concentration was also reduced by treatment with the other antibiotics tested, with the exception of fosfomycin, the inhibitor of MurA, an enzyme involved in the early stages of the peptidoglycan biosynthetic pathway. It appears that a number of different cellular perturbations have the ability to affect the highly orchestrated and hierarchical regulation of flagellar genes.

Comparison of gene expression in the parent strain and resistant mutants. One possibility is that the observed changes in the levels of expression of the stress response genes are not mediated directly by LolCDE inhibition but instead are indirect effects of the compound on the regulatory pathways. To address this possibility, a previously reported compound 2-resistant mutant (19, 27) with a mutation in LolC (N256K) was isolated, and duplicate cultures of the mutant and parent strain were treated with the compound (1.2 μg/ml) for 30 min. Duplicate cultures of untreated samples were also tested. RNA was extracted from all six cultures and processed for qPCR as described above. The levels of
TABLE 1  Genes whose RNA was upregulated >5-fold in cells treated with the LolCDE inhibitor compared with the level of regulation in control cells

| Gene | Fold upregulation at compound 2 concn of b (b): | Product | Predicted function(s) and/or comment(s) |
|------|-------------------------------------------------|---------|----------------------------------------|
| osmB | 114.75 at 0.3 μg/ml, 194.64 at 1.2 μg/ml | Lipoprotein | Putative membrane protein, osmotic adaptation |
| ycfJ | 102.84 at 0.3 μg/ml, 172.38 at 1.2 μg/ml | Predicted protein | |
| bdm | 88.28 at 0.3 μg/ml, 138.58 at 1.2 μg/ml | Biofilm-dependent modulation protein | The RcsCDB His-Asp phosphorelay positively regulates Bdm (biofilm-dependent modulation) |
| wcaE | 72.43 at 0.3 μg/ml, 135.85 at 1.2 μg/ml | Predicted glycosyl transferase | Resistance to acid and to thermal stress |
| wza | 46.80 at 0.3 μg/ml, 93.05 at 1.2 μg/ml | Capsular polysaccharide translocon | Resistance to acid and to thermal stress |
| wzb | 45.10 at 0.3 μg/ml, 95.34 at 1.2 μg/ml | Protein-tyrosine phosphatase | Resistance to acid and to thermal stress |
| ypeC | 41.97 at 0.3 μg/ml, 59.57 at 1.2 μg/ml | Putative periplasmic protein | |
| ypfG | 37.65 at 0.3 μg/ml, 44.18 at 1.2 μg/ml | Predicted protein | |
| yaiY | 37.19 at 0.3 μg/ml, 50.51 at 1.2 μg/ml | Predicted inner membrane protein | |
| ygaC | 34.03 at 0.3 μg/ml, 46.72 at 1.2 μg/ml | Predicted protein | |
| spy | 31.52 at 0.3 μg/ml, 60.63 at 1.2 μg/ml | Envelope stress-induced periplasmic protein | |
| yjbJ | 28.22 at 0.3 μg/ml, 57.10 at 1.2 μg/ml | Predicted stress response protein | |
| ydeI | 28.06 at 0.3 μg/ml, 66.02 at 1.2 μg/ml | Conserved protein | |
| yghA | 25.59 at 0.3 μg/ml, 57.66 at 1.2 μg/ml | Predicted glutathionylspermidine synthase, NAD(P)-binding Rossmann fold domain | |
| osmY | 25.29 at 0.3 μg/ml, 52.53 at 1.2 μg/ml | Periplasmic protein | |
| ygbG | 25.16 at 0.3 μg/ml, 53.38 at 1.2 μg/ml | Predicted protein | |
| ymgG | 24.63 at 0.3 μg/ml, 26.53 at 1.2 μg/ml | Predicted protein | |
| mliC | 24.44 at 0.3 μg/ml, 36.51 at 1.2 μg/ml | Predicted lipoprotein | |
| yfbF | 24.28 at 0.3 μg/ml, 45.69 at 1.2 μg/ml | Predicted lipoprotein | |
| iiv | 22.68 at 0.3 μg/ml, 37.01 at 1.2 μg/ml | Inhibitor of vertebrate c-type lysozyme | |
| ymgD | 19.43 at 0.3 μg/ml, 24.26 at 1.2 μg/ml | Predicted protein | |
| yjkA | 18.30 at 0.3 μg/ml, 35.85 at 1.2 μg/ml | Predicted protein | |
| yhp| 17.60 at 0.3 μg/ml, 33.10 at 1.2 μg/ml | Cytoplasmic | |
| wzc | 17.35 at 0.3 μg/ml, 44.98 at 1.2 μg/ml | Protein-tyrosine kinase | |
| yncJ | 16.53 at 0.3 μg/ml, 33.64 at 1.2 μg/ml | Predicted protein | |
| ydeG | 15.59 at 0.3 μg/ml, 30.16 at 1.2 μg/ml | Conserved protein | |
| yhe | 15.38 at 0.3 μg/ml, 32.49 at 1.2 μg/ml | Predicted protein | |
| rcsA | 15.21 at 0.3 μg/ml, 22.95 at 1.2 μg/ml | DNA-binding transcriptional activator; the coregulator is RcsB | |
| ybgS | 15.06 at 0.3 μg/ml, 32.04 at 1.2 μg/ml | Conserved protein | |
| yebE | 14.40 at 0.3 μg/ml, 31.47 at 1.2 μg/ml | Conserved protein | |
| cpxP | 12.57 at 0.3 μg/ml, 20.10 at 1.2 μg/ml | Periplasmic protein that combats stress | |
| clsB | 12.32 at 0.3 μg/ml, 24.47 at 1.2 μg/ml | Cardiolipin synthase 2 | |
| yegS | 12.15 at 0.3 μg/ml, 18.37 at 1.2 μg/ml | Conserved protein | |
| wcaF | 12.12 at 0.3 μg/ml, 25.69 at 1.2 μg/ml | Predicted acyl transferase | |
| hslF | 11.29 at 0.3 μg/ml, 12.86 at 1.2 μg/ml | Heat-inducible protein | |
| ysl | 11.13 at 0.3 μg/ml, 16.62 at 1.2 μg/ml | Predicted lipoprotein | |
| ysaB | 11.11 at 0.3 μg/ml, 11.23 at 1.2 μg/ml | Predicted protein | |
| wcaA | 11.06 at 0.3 μg/ml, 25.98 at 1.2 μg/ml | Predicted glycosyl transferase | |
| ygdP | 9.52 at 0.3 μg/ml, 10.62 at 1.2 μg/ml | Conserved protein | |
| ycfT | 9.33 at 0.3 μg/ml, 16.52 at 1.2 μg/ml | Predicted inner membrane protein | |
| gmd | 9.20 at 0.3 μg/ml, 22.66 at 1.2 μg/ml | GDP-β-mannose dehydratase, NAD(P) binding | |
| osmA | 8.91 at 0.3 μg/ml, 16.55 at 1.2 μg/ml | Osmotically inducible, stress-inducible membrane protein | |
| dgcZ | 8.84 at 0.3 μg/ml, 15.60 at 1.2 μg/ml | Diguanylate cyclase | |

(Continued on following page)
### TABLE 1 (Continued)

| Gene  | Fold upregulation at compound 2 concn of[^c] | Product[^a] | Predicted function(s) and/or comment(s) |
|-------|---------------------------------------------|-------------|----------------------------------------|
| degP  | 8.66                                        | Serine endoprotease (protease Do), membrane associated | Predicted to be required for global protein degradation |
| osmF  | 8.55                                        | Predicted periplasm-localized binding component of an ABC superfamily transporter | Putative transporter |
| katE  | 8.50                                        | Hydroperoxidase HPII (catalase) | Enzyme, detoxification |
| ybbO  | 8.43                                        | Predicted intracellular protease | hbbO mutant is highly sensitive to oxidative, thermal, UV, and pH stresses |
| yauX  | 8.38                                        | Predicted protein | RpoS stress induced |
| osmE  | 8.38                                        | DNA-binding transcriptional activator | Regulator of global regulatory functions |
| yjaE  | 8.17                                        | Predicted membrane protein | Stress response protein |
| yfdC  | 8.04                                        | Predicted inner membrane protein | Putative transporter, not classified |
| ygdR  | 7.78                                        | Predicted protein | Rcs induces the gene in response to cell wall damage (peptidoglycan) |
| atsB  | 7.77                                        | Trehalose-6-phosphate phosphatase, biosynthetic | Enzyme, osmotic adaptation |
| rhaS  | 7.63                                        | 30S ribosomal subunit protein S22 | Structural component of ribosomal proteins |
| ybaY  | 7.57                                        | Predicted outer membrane lipoprotein | Lipoprotein with unknown function |
| ydhS  | 7.31                                        | Conserved protein with FAD/NAD(P)-binding domain | Putative oxidoreductase |
| ybdK  | 7.18                                        | Gamma-glutamylcysteine ligase | A weak gamma-glutamylcysteine ligase |
| ybiO  | 7.09                                        | Predicted mechanosensitive channel | Putative transporter with unknown function |
| ymdF  | 7.02                                        | Conserved protein | |
| fbaB  | 6.82                                        | Fructose bisphosphate aldolase class I | Putative enzyme, not classified |
| ybdR  | 6.78                                        | Predicted oxidoreductase, Zn dependent and NAD(P) binding | EcnAB form a linked toxin-antitoxin addiction module, entericidin A; antidote to lipoprotein entericidin B |
| ecbB  | 6.66                                        | Cell envelope bacteriolytic lipoprotein | |
| lolP  | 6.56                                        | Predicted peptidase | Enzyme, degradation of proteins, peptides, and glycopeptides |
| yliI  | 6.22                                        | Predicted dehydrogenase | Putative enzyme, not classified |
| rhsB  | 6.08                                        | rhsB element core protein RshB | Open reading frame with transposon-related functions |
| yceB  | 6.06                                        | Predicted lipoprotein | Lipoprotein |
| dppB  | 5.95                                        | Dipptide transporter, membrane component of ABC superfamily | Transport, protein and peptide secretion |
| tomB  | 5.91                                        | Toxin overexpression modulator | Induced during biofilm formation |
| drrA  | 5.73                                        | Regulatory, antisense RNA | Regulatory RNA, regulates transcriptional silencing by H-NS protein, enhances translation of RpoS antisense RNA |
| ytkK  | 5.70                                        | Conserved protein | Resistance to desiccation, colanic acid biosynthesis (M antigen), resistance to acid and to thermal stress |
| wcaC  | 5.70                                        | Predicted glycosyl transferase | |
| yuhA  | 5.59                                        | Conserved protein | Putative transporter, not classified |
| elA B | 5.56                                        | Conserved protein | GDP-fucose synthase is a bifunctional enzyme, catalyzes the two-step synthesis of GDP-fucose |
| ydeF  | 5.55                                        | Conserved protein | Converts UDP-glucose to UDP-glucuronic acid for, colanic acid biosynthesis |
| ydcT  | 5.54                                        | Spermidine/putrescine transporter | Putative regulator, not classified |
| wcaG  | 5.44                                        | 14.19 | Transport, protein and peptide secretion |
| ycaC  | 5.41                                        | Predicted hydrolase | Overexpression of the ybbEFGH operon alters colony morphology |
| ugd  | 5.37                                        | UDP-glucose-6-dehydrogenase | Transport, protein and peptide secretion |
| raiA  | 5.37                                        | Cold shock protein associated with 30S ribosomal subunit | |
| dppC  | 5.20                                        | Dipptide transporter, membrane component of ABC superfamily | |
| yjBH  | 5.16                                        | Predicted porin | |
| dppD  | 5.10                                        | Dipptide transporter, ATP-binding component of ABC superfamily | |
| ybjP  | 5.07                                        | Predicted lipoprotein | Putative enzyme, not classified |
| ycfJ  | 5.06                                        | Predicted cytochrome b_{601} | Putative enzyme, not classified |

[^a]: Genes are listed in order of their fold upregulation at compound 2 concn of b.
[^b]: Concentrations of compound 2 in ug/ml.
[^c]: Predicted function(s) and/or comment(s)
expression of selected upregulated genes involved in each of the stress pathways, as well as those of the downregulated flagellar genes, were assessed. As can be seen in Fig. 4, the mutant with the LolC mutation resistant to the compound no longer exhibited upregulation of the representative genes involved in the three stress pathways, nor was flagellar gene expression downregulated in this strain. In contrast, the treated parent strain exhibited changes in the levels of expression of the selected genes.

**DISCUSSION**

Earlier work (19) established that the compound used in the present study, compound 2, stimulated peptidoglycan damage, result-

| Gene | 0.3 µg/ml | 1.2 µg/ml | Predicted function(s) and/or comment(s) |
|------|-----------|-----------|----------------------------------------|
| **narK** | 5.03 | 27.62 | Nitrate/nitrite transporter |
| **otsA** | 4.93 | 8.81 | Trehalose-6-phosphate synthase |
| **sfb** | 4.89 | 6.88 | Sulfate transporter subunit, periplasm-localized binding component of the ABC superfamily |
| **yngE** | 4.86 | 11.78 | Predicted inner membrane protein |
| **yiaG** | 4.77 | 8.29 | Predicted transcriptional regulator |
| **yicS** | 4.70 | 8.04 | Periplasm-localized binding component of an ABC superfamily predicted spermidine/putrescine transporter |
| **pxoB** | 4.68 | 9.33 | Pyruvate dehydrogenase (pyruvate oxidase), thiamine dependent, FAD binding |
| **ygiB** | 4.60 | 10.03 | Conserved protein |
| **yeaG** | 4.56 | 9.52 | Conserved protein with nucleotide triphosphate hydrolase domain |
| **dxr** | 4.48 | 6.16 | 1-Deoxy-D-xylulose 5-phosphate reductoisomerase |
| **msyB** | 4.45 | 8.10 | Predicted protein |
| **ascB** | 4.44 | 6.50 | Cryptic 6-phospho-beta-glucoisidase |
| **yhbN** | 4.43 | 9.14 | Conserved inner membrane protein |
| **cysN** | 4.42 | 8.40 | Sulfate adenylyltransferase, subunit 1 |
| **cysD** | 4.38 | 6.56 | Sulfate adenylyltransferase, subunit 2 |
| **cysA** | 4.27 | 6.83 | Sulfate/thiosulfate transporter subunit, ATP-binding component of ABC superfamily |
| **csiD** | 4.26 | 6.39 | Predicted protein |
| **ybbA** | 4.23 | 6.56 | Predicted transporter subunit, ATP-binding component of ABC superfamily |
| **dacC** | 4.23 | 6.41 | d-Alanyl–d-alanine carboxypeptidase (penicillin-binding protein 6a) |
| **ynfD** | 4.19 | 6.85 | Predicted protein |
| **ycaP** | 4.17 | 7.00 | Conserved inner membrane protein |
| **ygaU** | 4.13 | 8.01 | Predicted protein |
| **rcnB** | 4.12 | 4.87 | Predicted protein |
| **dps** | 4.11 | 8.30 | Fe-binding and storage protein |
| **acrD** | 4.10 | 6.31 | Aminoglycoside/multidrug efflux system |
| **yodD** | 4.09 | 6.76 | Predicted protein |
| **yeaG** | 4.09 | 8.00 | Predicted protein |
| **dppA** | 4.08 | 6.27 | Periplasm-localized binding component of an ABC superfamily dipeptide transporter |
| **ldtD** | 3.99 | 8.17 | Outer membrane lipoprotein (lipocalin) |
| **dhpF** | 3.99 | 6.63 | ATP-binding component of an ABC superfamily |
| **ldhD** | 3.95 | 7.72 | d-Ala–d-Ala transporter |
| **yeaD** | 3.91 | 6.49 | Predicted transporter subunit, ATP-binding component of ABC superfamily |
| **yfcG** | 3.90 | 7.27 | Glutathione S-transferase |
| **yebV** | 3.89 | 6.18 | Predicted protein |

* Gene designations are from the *Escherichia coli* BW25113 genome sequence.
* Fold upregulation over the control values at the two compound concentrations. All values are averages from two RNA-seq determinations for each condition with two biological replicates.
* Gene product from EcoGene (http://www.ecogene.org).
ing in activation of the *ampC* promoter, and also led to inhibition of the lipoprotein outer membrane transport pathway in *E. coli*, among other effects. Mutants resistant to the action of the compound were selected, and amino acid changes associated with resistance were found to reside in two subunits of the LolCDE complex. Therefore, LolC and LolE likely represent the direct targets of the Lol pathway inhibitor. These mutations individually resulted in a large increase in the MIC of the compound and led to cross-resistance to another small molecule also recently identified to be a LolCDE inhibitor (27). Our goal in the present study was to characterize the immediate transcriptional responses of *E. coli* to inhibition of LolCDE by the compound. This was accomplished by performing RNA-seq analysis with two different concentrations of the compound, a concentration just above the concentration that affected cell growth (measured by determination of both the OD600 and the number of CFU per milliliter). These results were compared to those obtained with control cells, which received no compound. While RNA-seq can measure only transcript levels, the compound and antibiotics tested likely directly or indirectly affected the transcriptional regulation of particular genes; however, we cannot exclude the possibility that the effect was posttranscriptional at the level of mRNA stability.

Treatment with the LolCDE inhibitor led to a loss of bacterial viability and cell lysis, similar to the results seen previously following LolCDE depletion (28). No increase in the level of the transcripts of genes encoding the subunits of the actual target of compound 2, the LolCDE complex, was seen when *E. coli* was treated with the compound. There was, however, a modest 3.5-fold increase in lolA transcript levels at the higher concentration of the inhibitor, a result also seen in past experiments employing LolCDE depletion (28). Very large changes in the concentrations of mRNA for many genes that respond to periplasmic and cell envelope stress were observed following treatment with compound 2. In the case of chemical inhibition of the LolCDE function, the stress responses (29, 30) could be predominantly due to signaling controlled by three regulatory systems: the two-component histidine kinase and response regulators CpxA/R (31–33), the three-component Rcs system (34–37), and the rpoS (σ^E^)-mediated stress responses (38–41). The extracytoplasmic response is controlled by at least two partially overlapping signal transduction systems, the Cpx two-component system and the σ^E^-mediated system. Neither rpoE-associated (42–44) nor rpoH-associated (45, 46) genes were upregulated by the LolCDE inhibitor compound under the conditions tested. The 30-min exposure to the inhibitor (approximately 1.5 cell generations) left the transcript levels of the σ^E^-induced β-barrel pathway chaperone genes *skp, jkpA, and sur*, as well as the levels of RNA for several known rpoE regulon genes, such as *bamD, bamB, rpoD, dsbC, yeaY, and yaeI*, unchanged (43, 47). Surprisingly, although the β-barrel system includes several lipoproteins (BamB and the essential lipoprotein BamD) associated with its function, no changes in the levels of their respective mRNAs or the levels of the transcripts of genes encoding other lipoproteins of the Bam pathway, BamC or BamE, were observed (3, 6). Longer exposure to the compound and prolonged inhibition may be necessary to elicit changes. The rpoH (σ^E^)-controlled genes are primarily induced by protein misfolding in association with heat shock. The genes known to be controlled by σ^E^ include *dnaK, dnak, dnaJ, and grpE*, the mRNAs for which also do not show altered levels during LolCDE inhibition (46). Likewise, no effect on *mdtA, mdtB, mdtC, mdtD, or tolC*, associated with the BaeSR system, was observed (48).

Among the genes induced in response to the LolCDE inhibitor compound were *yseJ, yaqE, and cpxP*, as well as *spy*, which encodes the periplasmic chaperone and which is regulated by the CpxA/R stress response (32, 33, 49, 50). The general stress response sigma factor σ^E^ regulates *osmY* (encoding a periplasmic stress protein [51, 52]), *poxB, yidS*, and *katE*, and these were among the genes for which the mRNA levels were elevated by exposure of cells to

| TABLE 2 Genes whose RNA was downregulated >5-fold in cells treated with the LolCDE inhibitor compared with the level of regulation in control cells |
|----------------|---------------------------------|---------------------------------|
| Gene^a         | Fold downregulation at          | Product^c                       | Predicted function                  |
|                | compound 2 concn of g/ml        |                                 |                                  |
|                | 0.3 µg/ml                      | 1.2 µg/ml                       |                                  |
| flhD           | −4.17                          | −11.89                          | DNA-binding transcriptional dual regulator with FlhC | Regulator, surface structures |
| fltE           | −4.11                          | −15.86                          | Flagellar basal body component     | Structural component, surface structures |
| flF            | −4.03                          | −21.47                          | Flagellar basal body MS ring and collar protein | Structural component, surface structures |
| flic           | −3.84                          | −13.37                          | DNA-binding transcriptional dual regulator with FlhD | Regulator, surface structures |
| ihfG           | −3.81                          | −25.97                          | Predicted defective phage integrase (pseudogene) | Structural component, surface structures |
| flG            | −3.76                          | −16.01                          | Flagellar motor switching and energizing component | Structural component, surface structures |
| fliH           | −3.48                          | −14.28                          | Flagellar biosynthesis protein     | Transport, surface structures |
| fliI           | −3.35                          | −12.52                          | Flagellum-specific ATP synthase    | Enzyme, surface structures |
| fliJ           | −3.29                          | −9.77                           | Flagellar export apparatus chaperone | Structural component, surface structures |
| ompF           | −3.19                          | −12.49                          | Outer membrane porin 1a (La)      | Membrane, outer membrane constituents |
| flaA           | −3.16                          | −7.68                           | RNA polymerase, sigma 28 (σ^F^) factor | σ^F^ factor, surface structures |
| yciX           | −3.16                          | −12.44                          | Predicted protein                 |                                  |
| fliG           | −3.10                          | −6.25                           | Flagellar component of cell-proximal portion of basal body rod | Structural component, surface structures |
| fliK           | −3.08                          | −8.02                           | Flagellar hook-length control protein | Structural component, surface structures |
| yeeR           | −3.04                          | −9.26                           | Predicted protein                 |                                  |

^a Gene designations are from the *Escherichia coli* BW25113 genome sequence.

^b Fold downregulation over the control values at the two compound concentrations. All values are averages from two RNA-seq determinations for each condition with two biological replicates.

^c Gene product from EcoGene ([http://www.ecogene.org](http://www.ecogene.org)).
the LolCDE inhibitor (39). Other σ^5_2 stress-related protein-encoding genes whose RNA levels were observed to be increased were _otsA_ and _otsB_, whose gene products synthesize trehalose in response to osmotic stress (41, 53). The levels of expression of RNA for a number of genes associated with the Rcs phosphorelay system, including _osmB_, the Rcs regulator _rcsA_, and the genes for the colanic acid biosynthetic pathway, including _wcaA_, _wcaB_, _wcaC_, _wcaD_, _wcaE_, _wza_, _wzb_, and _wzc_ (54–57), were also found to be increased. Also upregulated by the Rcs system was _ivy_, which protects peptidoglycan when the outer membrane is permeabilized (58).

Several of the RNAs responding to lipoprotein transport inhibition encode lipoproteins: the previously mentioned OsmB, an osmotic stress protein whose expression is controlled by both σ^5_2 and Rcs (54). Other lipoproteins include genes encoding OsmC (a stress-induced lipoprotein), YgdL, MliC, YajL, YbaY, YceB, YbjP,
and YjbF (a lipoprotein also regulated by Rcs) (34, 35, 37). Therefore, the commonality of all of these genes is their response to signaling pathways via regulators sensing periplasmic misfolding or perturbation of the outer membrane caused by a defect in lipoprotein trafficking.

Many fewer large-magnitude changes in the levels of expression genes showing decreases in their transcript levels following interference with lipoprotein trafficking were found. The levels of expression of virtually the entire set of mRNAs for genes associated with flagellum formation were markedly lower, and these genes comprised the group with the largest negative changes in expression (59, 60). This could be explained as a direct consequence of failure to assemble the flagellar ring due to mislocalization of the lipoprotein FlgH, which depends on the Lol pathway for its outer membrane targeting. Other genes whose transcript levels were also lower included ompF, previously shown to be controlled by CpxA/R (61). It is unclear why ompF responds differently from other CpxA/R-regulated genes that are adversely affected by LolCDE inhibition.

To determine if any of the responses observed were also induced by exposure to other antibiotics with different mechanisms of action, bacteria were exposed to several different antibiotics, and their mRNAs were analyzed by qPCR. Compared to a group of selected genes from the RNA-seq experiment, it was found that the transcript levels for four of the genes tested were elevated only by exposure to the LolCDE inhibitor. One of these genes was ecnB, coding for the bacteriocidal toxin part of a toxin-antitoxin pair which has been implicated in osm-2-regulated osmolarity stress and bacteriolysis (62, 63). Interestingly, the gene for the companion antitoxin, ecnA, was not upregulated in this case; this should lead to cell death. Another gene was katE, which encodes a hydroperoxidase also regulated by osm-2 and which is induced in response to oxidative stress (64, 65). The clsB gene, which encodes one of three car-
diolipin synthases in *E. coli* (66), and *ycfT*, which encodes an inner membrane protein implicated in biofilm control and which is located immediately adjacent to and divergent from the genes encoding LolCDE, were also highly responsive to LolCDE inhibition. Among the group of antibiotics tested by qPCR at the inhibitory concentrations determined in this study, only the LolCDE inhibitor had an impact on the transcript levels of these four genes, although the level of transcription of *ycfT* mRNA also displayed a modest elevation with polymyxin B. The apparent specificity of these responses to pharmacological inhibition of LolCDE merits further future characterization of the genes for these proteins as possible candidates for use in cell-based reporter screens to find additional Lol pathway inhibitors. Other tested genes, such as *ycfJ*, implicated in biofilm formation, were also affected by polymyxin B and, to a lesser extent, by the two β-lactam antibiotics imipenem and meropenem, all of which have impacts on the bacterial cell envelope. The levels of both *ymgD* and *ymgG* mRNAs were elevated by the same compounds as well. The same was true of the expression of *ivy*, encoding another periplasmic protein that acts to protect peptidoglycan from hydrolysis by lysozyme and is induced by outer membrane permeabilization (67).

The question as to whether the responses are a direct result of LolCDE inhibition by the compound was addressed by isolating a resistant LolC mutant with the N256K mutation and repeating the compound exposure at the higher concentration employed for RNA-seq and qPCR with this mutant and identically treated parental strain *E. coli* BW25113 ΔacrB. The results obtained with cells of these strains were compared with those obtained with cells of the untreated parent. Quantitative PCR of representative genes involved in the different pathways clearly demonstrated that the transcript expression effects observed were through the inhibition of LolCDE by compound 2.
The purpose of this study was to examine the responses of *E. coli* to treatment with a compound that inhibits the function of LolCDE. Direct inhibition of LolCDE function led to a loss of cell viability and, ultimately, cell lysis. The blockade of lipoprotein transport was found to lead to compound dose-dependent levels of induction of selected cell stress pathways. Many of these are envelope stress responses that have previously been observed under various cell stimuli, such as disturbances in metabolism, periplasmic protein misfolding, or overproduction of lipoprotein NlpE (32, 68). Of interest, a small subset of the RNAs induced in response to LolCDE inhibition at the inhibitory concentrations tested in this study appeared to be unique rather than general responses to antibiotics with different mechanisms of action. These responses could be further defined for the design of refined cell-based reporter screens for Lol pathway inhibitors.

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REFERENCES
1. Viale P, Giannella M, Tedeschi S, Lewis R. 2015. Treatment of MDR-Gram negative infections in the 21st century: a never ending threat for clinicians. Curr Opin Pharmacol 24:30–37. http://dx.doi.org/10.1016/j.coph.2015.07.001.
2. Kollef MH, Golan Y, Micek ST, Shorr AF, Restrepo MI. 2011. Appraising contemporary strategies to combat multidrug resistant gram-negative bacterial infections—proceedings and data from the Gram-Negative Resistance Summit. Clin Infect Dis 53(Suppl 2):S33–S55. http://dx.doi.org/10.1093/cid/cir475.
3. Bos MP, Robert V, Tommassen J. 2007. Biogenesis of the gram-negative bacterial outer membrane. Annu Rev Microbiol 61:191–214. http://dx.doi.org/10.1146/annurev.micro.61.080706.093245.
4. Tokuda H. 2009. Biogenesis of outer membranes in Gram-negative bacteria. Biosci Biotechnol Biochem 73:465–473. http://dx.doi.org/10.1271/ bbb.80778.
5. Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold
Lorenz et al.

13. 16. 21. 22.

25.

11. 14. Mizutani Okuda

17. Chilcott

19. Fl, Ahyong V, Betegon M, DeRisi JL.

26. Power EG, Phillips I. 1992. Induction of the SOS gene (umuC) by 4-quinolone antibacterial drugs. J Med Microbiol 36:78–82. http://dx.doi.org/10.1099/00222615-36-2-78.

23. McLeod SM, Fleming PR, MacCormack K, McLaughlin RE, Whiteaker JD, Narita S-I, Mori M, Tokuda H, Miller AA. 2015. Small-molecule inhibitors of Gram-negative lipoprotein trafficking discovered by phenotypic screening. J Bacteriol 197:1075–1082. http://dx.doi.org/10.1128/JB.02352-14.

17. Ito, Y, Kanamaru K, Taniguchi N, Miyamoto S, Tokuda H. 15. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev 28: 337–418. http://dx.doi.org/10.1128/CMR.00117-14.

Rolla compiled software.

2015. Outer membrane protein biogenesis in Gram-negative bacteria. Philos Trans R Soc Lond B Biol Sci 370:20150023. http://dx.doi.org/10.1098/rstb.2015.0023.

5. 6. O’Shea

7. O’Shea

9. 0.0023.

11. 14. Mizutani Okuda

17. Chilcott

19. Fl, Ahyong V, Betegon M, DeRisi JL.

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23. McLeod SM, Fleming PR, MacCormack K, McLaughlin RE, Whiteaker JD, Narita S-I, Mori M, Tokuda H, Miller AA. 2015. Small-molecule inhibitors of Gram-negative lipoprotein trafficking discovered by phenotypic screening. J Bacteriol 197:1075–1082. http://dx.doi.org/10.1128/JB.02352-14.

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23. McLeod SM, Fleming PR, MacCormack K, McLaughlin RE, Whiteaker JD, Narita S-I, Mori M, Tokuda H, Miller AA. 2015. Small-molecule inhibitors of Gram-negative lipoprotein trafficking discovered by phenotypic screening. J Bacteriol 197:1075–1082. http://dx.doi.org/10.1128/JB.02352-14.

17. Ito, Y, Kanamaru K, Taniguchi N, Miyamoto S, Tokuda H. 15. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev 28: 337–418. http://dx.doi.org/10.1128/CMR.00117-14.

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2015. Outer membrane protein biogenesis in Gram-negative bacteria. Philos Trans R Soc Lond B Biol Sci 370:20150023. http://dx.doi.org/10.1098/rstb.2015.0023.

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23. McLeod SM, Fleming PR, MacCormack K, McLaughlin RE, Whiteaker JD, Narita S-I, Mori M, Tokuda H, Miller AA. 2015. Small-molecule inhibitors of Gram-negative lipoprotein trafficking discovered by phenotypic screening. J Bacteriol 197:1075–1082. http://dx.doi.org/10.1128/JB.02352-14.

17. Ito, Y, Kanamaru K, Taniguchi N, Miyamoto S, Tokuda H. 15. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev 28: 337–418. http://dx.doi.org/10.1128/CMR.00117-14.

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2015. Outer membrane protein biogenesis in Gram-negative bacteria. Philos Trans R Soc Lond B Biol Sci 370:20150023. http://dx.doi.org/10.1098/rstb.2015.0023.

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23. McLeod SM, Fleming PR, MacCormack K, McLaughlin RE, Whiteaker JD, Narita S-I, Mori M, Tokuda H, Miller AA. 2015. Small-molecule inhibitors of Gram-negative lipoprotein trafficking discovered by phenotypic screening. J Bacteriol 197:1075–1082. http://dx.doi.org/10.1128/JB.02352-14.

17. Ito, Y, Kanamaru K, Taniguchi N, Miyamoto S, Tokuda H. 15. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev 28: 337–418. http://dx.doi.org/10.1128/CMR.00117-14.

Rolla compiled software.

2015. Outer membrane protein biogenesis in Gram-negative bacteria. Philos Trans R Soc Lond B Biol Sci 370:20150023. http://dx.doi.org/10.1098/rstb.2015.0023.

5. 6. O’Shea

7. O’Shea

9. 0.0023.
six lipoproteins in the σE regulon. J Bacteriol 187:4552–4561. http://dx.doi.org/10.1128/JB.187.13.4552-4561.2005.
45. Carruthers MD, Minion C. 2009. Transcriptome analysis of Escherichia coli O157:H7 EDL933 during heat shock. FEMS Microbiol Lett 295:96–102. http://dx.doi.org/10.1111/j.1574-6968.2009.01587.x.
46. Guisbert E, Herman C, Lu CZ, Gross CA. 2004. A chaperone network controls the heat shock response in E. coli. Genes Dev 18:2812–2821. http://dx.doi.org/10.1101/gad.1219204.
47. Sklar JG, Wu T, Kahne D, Silhavy TJ. 2007. Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in Escherichia coli. Genes Dev 21:2473–2484. http://dx.doi.org/10.1101/gad.1581007.
48. Nishino K, Honda T, Yamaguchi A. 2005. Genome-wide analyses of Escherichia coli gene expression responsive to the BaeSR two-component regulatory system. J Bacteriol 187:1763–1772. http://dx.doi.org/10.1128 /JB.187.5.1763-1772.2005.
49. Hagenmaier S, Sterhof Y-D, Henning U. 1997. A new periplasmic protein of Escherichia coli which is synthesized in spheroplasts but not in intact cells. J Bacteriol 179:2073–2076.
50. Quan S, Koldewey P, Tapley T, Kirsch N, Ruane KM, Pfizenmaier J, Shi R, Hofmann S, Foit L, Ren G, Jakob U, Xu Z, Cygler M, Bardwell JC. 2011. Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. Nat Struct Mol Biol 18:262–269. http://dx.doi.org/10.1038/nsmb.2016.
51. Yim HH, Brems RL, Villarejo M. 1992. OsmY, a new hyperosmotically inducible gene, encodes a periplasmic chaperone. J Bacteriol 174:3637–3644.
52. Yim HH, Brems RL, Villarejo M. 1994. Molecular characterization of the promoter of osmY, an rpoS-dependent gene. J Bacteriol 176:100–107.
53. Giaever HM, Styrvold OB, Kaasen I, Strem AR. 1988. Biochemical and genetic characterization of osmoregulatory trehalose synthesis in Escherichia coli. J Bacteriol 170:2841–2849.
54. Jung JU, Gutierrez C, Martin F, Ardourel M, Villarejo M. 1990. Transcription of osmB, a gene encoding an Escherichia coli lipoprotein, is regulated by dual signals: osmotic stress and stationary phase. J Biol Chem 265:10574–10581.
55. Ionescu M, Belkin S. 2009. Overproduction of exopolysaccharides by an Escherichia coli K-12 rpoS mutant in response to osmotic stress. Appl Environ Microbiol 75:483–492. http://dx.doi.org/10.1128/AEM.01616-08.
56. Ferrieres I, Aslam SN, Cooper RM, Clarke DJ. 2007. The yphEFGH locus in Escherichia coli K-12 is an operon encoding proteins involved in exopolysaccharide production. Microbiology 153:1070–1080. http://dx.doi.org /10.1099/mic.0.2006.002907-0.
57. Navasa N, Rodríguez-Aparicio I, Ferrero MA, Montegudo-Mera A, Martinez-Blanco H. 2013. Polysialic and colanic acids metabolism in Escherichia coli K92 is regulated by RcsA and RcsB. Biosci Rep 33:e00038. http://dx.doi.org/10.1042/BSR20130018.
58. Callewaert I, Masschalck B, Deckers D, Nakimbuge D, Atanassova M, Aertsens A, Michiels CW. 2005. Purification of Ivy, a lysozyme inhibitor from Escherichia coli, and characterisation of its specificity for various lysozymes. Enzyme Microb Technol 37:205–211. http://dx.doi.org/10 .1016/j.enzmitec.2005.03.001.
59. Chevance FF, Hughes KT. 2008. Coordinating assembly of a bacterial macromolecular machine. Nat Rev Microbiol 6:455–465. http://dx.doi.org/10.1038/nrmicro1887.
60. Fitzgerald DM, Bonocora RP, Wade JT. 2014. Comprehensive mapping of the Escherichia coli flagellar regulatory network. PLoS Genet 10: e1004649. http://dx.doi.org/10.1371/journal.pgen.1004649.
61. Batchelor E, Walthers D, Kenney LJ, Goulain M. 2005. The Escherichia coli CpxA-CpxR envelope stress response system regulates expression of porins ompF and ompC. J Bacteriol 187:5723–5731. http://dx.doi.org /10.1128/JB.187.16.5723-5731.2005.
62. Bishop RE, Leskiw BK, Hodges RS, Kay CM, Weiner JH. 1998. The entericidin locus of Escherichia coli and its implications for programmed bacterial cell death. J Mol Biol 280:583–596. http://dx.doi.org/10.1006/jmbo.1998.1894.
63. Schwelhorn HE, Audia JP, Wei L, Chang L. 1998. Identification of conserved, RpoS-dependent stationary-phase genes of Escherichia coli. J Bacteriol 180:6283–6291.
64. Loewen PC. 1984. Isolation of catalase-deficient Escherichia coli mutants and genetic mapping of katE, a locus that affects catalase activity. J Bacteriol 157:622–626.
65. Tanaka K, Handel K, Loewen PC, Takahashi H. 1997. Identification and analysis of the rpoS-dependent promoter of katE encoding catalase HPII in Escherichia coli. Biochim Biophys Acta 1352:161–166. http://dx.doi.org /10.1016/S0167-4781(97)00044-4.
66. Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CR, Guan Z. 2012. Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci U S A 109: 16504–16509. http://dx.doi.org/10.1073/pnas.1212797109.
67. Callewaert I, Vanoirbeek KGA, Lurquin I, Michiels CW, Aertsens A. 2009. The Rcs two-component system regulates expression of lysozyme inhibitors and is induced by exposure to lysozyme. J Bacteriol 191:1979–1981. http://dx.doi.org/10.1128/JB.01549-08.
68. DiGiuseppe PA, Silhavy TJ. 2003. Signal detection and target gene induction by the CpxRA two-component system. J Bacteriol 185:2432–2440. http://dx.doi.org/10.1128/JB.185.8.2432-2440.2003.