

Chemical Conditionality: A Genetic Strategy to Probe Organelle Assembly Resource

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Summary

The assembly of the *Escherichia coli* outer membrane (OM) is poorly understood. Although insight into fundamental cellular processes is often obtained from studying mutants, OM-defective mutants have not been very informative because they generally have nonspecific permeability defects. Here we show that toxic small molecules can be used in selections employing strains with permeability defects to create particular chemical conditions that demand specific suppressor mutations. Suppressor phenotypes are correlated with the physical properties of the small molecules, but the mutations are *not* in their target genes. Instead, mutations allow survival by partially restoring membrane impermeability. Using “chemical conditionality,” we identified mutations in *yfgL*, and, here and in the accompanying paper by Wu et al. published in this issue of *Cell* (Wu et al., 2005), we show that YfgL is part of a multiprotein complex involved in the assembly of OM β barrel proteins. We posit that panels of toxic small molecules will be useful for generating chemical conditionalities that enable identification of genes required for organelle assembly in other organisms.

Introduction

The outer membrane (OM) of *Escherichia coli* is an asymmetric bilayer in which the outer leaflet is comprised largely of lipopolysaccharides (LPS) and the inner leaflet is comprised of phospholipids (Figure 1; Smit et al., 1975; Kamio and Nikaido, 1976). β barrel proteins span the bilayer, and lipoproteins are attached to the inner surface by lipid anchors (for recent review, see Nikaido [2003]). This organelle serves as a protective barrier preventing entry of toxic substances, such as detergents and antibiotics, into the cell (Figure 1).

The biogenesis of the OM is not well understood, in part because methods to probe the assembly process have not been developed. Mutations in a large number of genes cause a general “leakiness” that is manifested

as sensitivity to bile salts, for example, but because this mutant phenotype is not distinct, it is difficult to assess whether or not these gene products function directly in the OM assembly process. Here we show that certain types of small molecules can be used to create conditions that enable highly specific phenotypes to emerge in selections for suppressors of mutations that confer “leaky” phenotypes. These highly specific phenotypes, which are defined by the type of toxic small molecules that can or cannot enter and prevent growth of the mutant cell, are conferred by suppressor mutations in particular genes. We have exploited this chemical conditionality to identify genes involved in OM biogenesis, and this, in turn, provides a starting point for dissecting the machinery involved in membrane assembly. We believe that this is a general strategy that can be used for probing the assembly of many biological membranes and organelles.

The work described here was initiated to investigate the mechanism of action of chlorobiphenyl vancomycin (CBPV) and moenomycin (compounds 2 and 4, respectively, Figure 2A), two antibiotics that inhibit bacterial cell wall (peptidoglycan) synthesis. We performed genetic selections to identify the target(s) of these antibiotics by searching for mutations that confer resistance. Because in *E. coli* the OM surrounds the peptidoglycan layer and this membrane is naturally impermeable to these compounds, we used an *E. coli* strain with an OM defect that allows the entry of these antibiotics (Eggert et al., 2001). The increased permeability of the OM of this mutant strain is caused by the *imp4213* allele, an in-frame deletion allele in the *imp* (increased membrane permeability) gene, which encodes a protein essential for OM assembly in *E. coli* (Braun and Silhavy, 2002). Cells carrying this allele are sensitive to antibiotics and detergents (Figure 1; Sampson et al., 1989) and are frequently used to study mechanisms of action of compounds that cannot penetrate the OM of wild-type *E. coli* (for example, see Singh et al. [2003]).

Here we show that the *only* mutations that answer selections for resistance to either CBPV or moenomycin but not vancomycin or erythromycin (Figure 2A) are loss-of-function mutations in *yfgL*. However, we demonstrate that the loss of YfgL suppresses sensitivity to these antibiotics not by altering the drug target but rather by altering the permeability of the OM of *imp4213* cells (Figure 1), since we have found that *yfgL* mutations also suppress sensitivity of *imp4213* cells to bile salts, a detergent that disrupts membrane integrity (compound 9, Figure 2A). Although Imp function is essential in *E. coli* and has been implicated in membrane biogenesis (Braun and Silhavy, 2002), YfgL is not an essential protein in wild-type cells, and nothing was known of its function prior to the work reported here and in the accompanying paper published in this issue of *Cell* (Wu et al., 2005). The genetic interactions between *imp* and *yfgL* that we have uncovered using chemical probes indicate a role for YfgL (a putative OM lipoprotein) in OM biogenesis as well.

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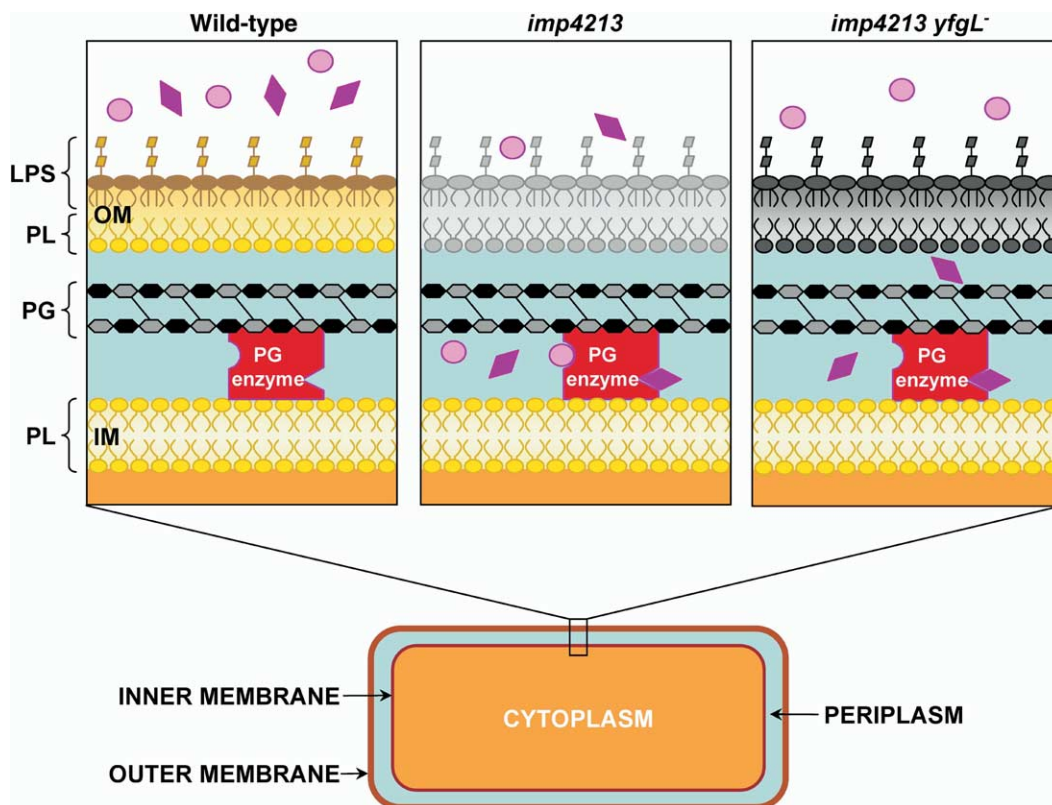


Figure 1. Chemical Conditionality

The *E. coli* cell (bottom of figure) is composed of four compartments: cytoplasm, inner membrane, periplasm, and outer membrane. The inner membrane, periplasm, and outer membrane are collectively called the cell envelope. While the inner membrane (IM, top left panel) is a phospholipid (PL) bilayer, the inner leaflet of the outer membrane (OM) is composed of PL, but its outer leaflet is composed of lipopolysaccharides (LPS). No membrane proteins are shown for simplicity. The peptidoglycan (PG) layer resides in the periplasm and is a structure essential for viability. The three panels at the top of the figure show the permeability status of the outer membrane in three strains: wild-type (left panel), *imp4213* (center panel), and *imp4213 yfgL⁻* (right panel). The outer membrane (OM) of a wild-type strain (left panel) acts as a barrier that prevents the entry of some antibiotics (represented as diamonds and circles) that inhibit enzymes (PG enzyme) involved in peptidoglycan (PG) biosynthesis. Because neither antibiotic can penetrate the wild-type OM, they cannot reach their target in the periplasm, and therefore the cell survives in their presence. In an *imp4213* strain (center panel), the defective OM (represented by a change in color to light gray, compare to left panel) is not impermeable to either antibiotic. Both antibiotics can pass through the OM into the periplasm where they bind to their target. Since binding of either antibiotic to the PG enzyme inhibits peptidoglycan biosynthesis, the cell dies in the presence of either compound. Assuming that the “circle” antibiotic represents either CBPV or moenomycin, we selected for mutations that confer resistance to either compound in *imp4213* cells. The only mutation that specifically confers resistance to CBPV or moenomycin (circles) but not other antibiotics (diamonds) is a loss-of-function mutation in *yfgL*. In *imp4213 yfgL⁻* (right panel), the loss of YfgL causes a decrease in the permeability of the OM (represented by a change in color to dark gray, compare to center panel). Since the barrier function of the OM in *imp4213 yfgL⁻* has been partially restored, neither CBPV nor moenomycin (circles) can pass through the OM, but the antibiotic represented by a diamond still can. Therefore, *imp4213 yfgL⁻* cells survive in the presence of either CBPV or moenomycin but die in the presence of other antibiotics.

Our work demonstrates that toxic small molecules can be exploited not only to probe their targets but also to identify factors involved in the biogenesis of membranes that surround the compartment where the target for such toxic compounds resides. Our results also indicate that the defects in outer membrane permeability caused by *imp4213* can be corrected to varying degrees. Small chemical probes can be classified in a continuum that is based on the quantity and quality of suppressor that each can elicit when used in selections for resistance. We discuss the chemical basis for this continuum and propose an expanded role for small molecules in non-target-directed chemical genetic analysis of membranes and organelle biogenesis.

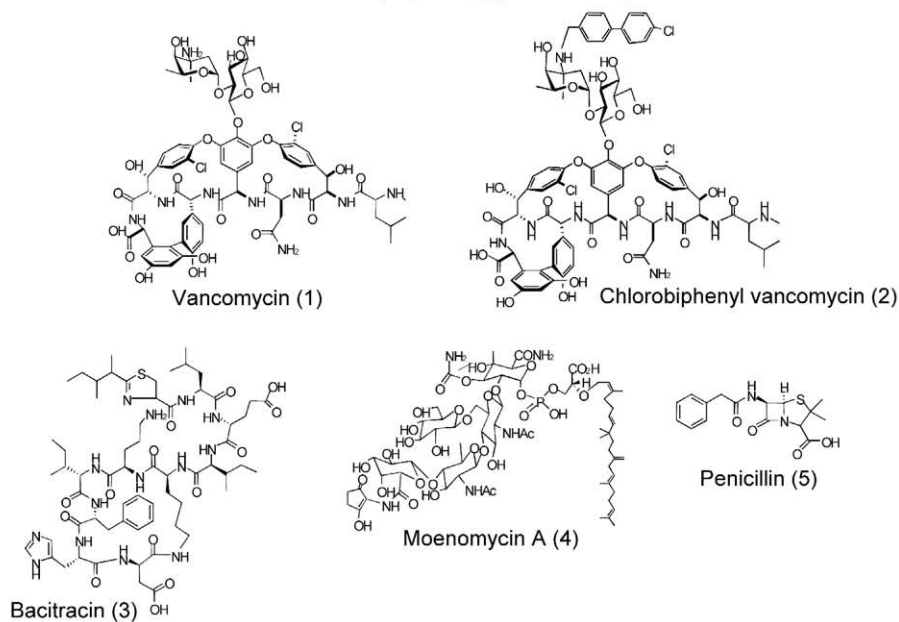
Results

The Remarkable Specificity of CBPV and Moenomycin in *imp4213* Strains

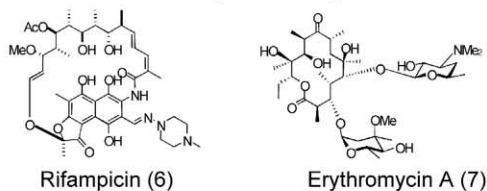
In our preliminary investigation we had selected for spontaneous mutants resistant to CBPV and moenomycin in the *imp4213* background and discovered *yfgL* (Eggert et al., 2001). In order to find additional factors involved in the same pathway as YfgL, and perhaps the direct targets of these inhibitors, we repeated our original selections for spontaneous CBPV- and moenomycin-resistant mutants on a larger scale. Strikingly, the only mutations that we obtained were 34 more independent IS1E element insertions in the *yfgML* locus ori-

A

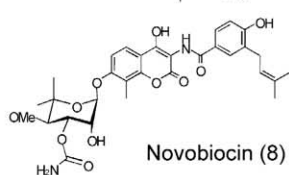
Inhibitors of peptidoglycan biosynthesis



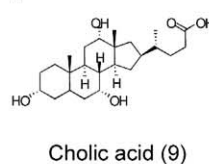
Inhibitors of protein synthesis



Inhibitor of DNA gyrase



Disrupts membrane integrity



B

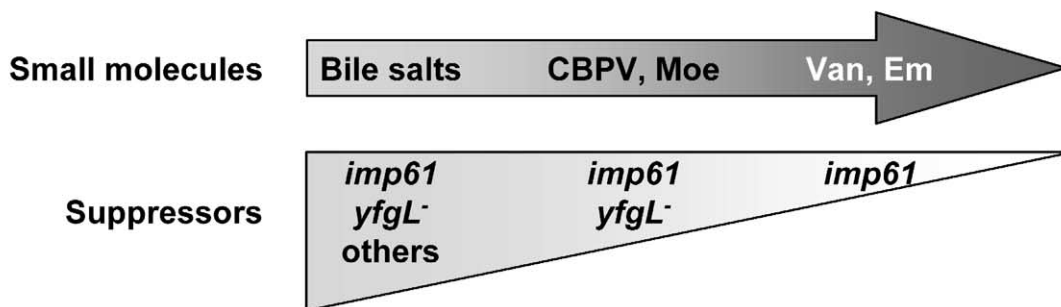


Figure 2. Small Molecules and the Suppressors of *imp4213* that They Elicit

(A) Chemical structure and mechanism of action of the toxic small molecules used in this study. Numbers in parenthesis are used in text to refer to each compound.

(B) Toxic small molecules can be arranged in a continuum in which there is a correlation between the quantity and quality of suppressors that they elicit in *imp4213* cells. See Results and Discussion sections for a detailed explanation of this continuum.

Table 1. Phenotypes of *imp* and *yfgL* Mutants

Relevant genotypes	Phenotypes ^a			Inhibition Zone (mm) ^b			
	Mac	CBPV	Van	Bac	Nov	Rif	Em
<i>imp4213</i>	S	S	S	18	16	23	17
<i>imp61</i>	R	R	R	10	ND	17	13.5
<i>imp4213 yfgL8</i>	R	R	S	17	14	26	20
<i>imp4213 yfgL::IS1E</i>	R	R	S				
<i>imp*</i>	R	R	R	ND	ND	7.5	ND
<i>imp* yfgL::kan</i>				8	10	17	10
<i>imp* yfgL::IS1E</i>				12	9	19	10

^aSensitivity to bile salts (Mac), CBPV, and vancomycin (Van) was determined by assessing growth on plates containing these compounds as described in [Experimental Procedures](#). Strains that grow in the presence of the compound were categorized as resistant (R), while those that did not grow were categorized as sensitive (S).

^bOvernight cultures of the indicated strains were plated on LB agar using a soft agar overlay. Disks containing antibiotics were placed onto the solidified agar surface. The diameter of the zone of inhibition of growth observed after overnight incubation is shown in mm. The disks used were 6 mm in diameter. Bac refers to bacitracin, Nov to novobiocin, Rif to rifampin, and Em to erythromycin; ND indicates no detectable zone of inhibition.

ented in the opposite direction from the transcription of the *yfgML* operon, similar to those isolated previously ([Eggert et al., 2001](#)). Because this locus seemed to be a hotspot for *IS1E* insertions, we subjected *E. coli imp4213* to chemical mutagenesis with N-methyl-N-nitrosoguanidine (NTG) and then selected for mutants resistant to moenomycin. All of the base substitution mutations obtained were recessive null alleles of *yfgL* or missense alterations of *imp4213* itself. All of the *yfgL* mutants were found to be crossresistant to CBPV but not to vancomycin or to other antibiotics. In contrast, the intragenic suppressors in *imp4213* increased resistance not only to moenomycin and CBPV but to other antibiotics as well ([Table 1](#) and see below). We conclude that the only mutations that answer the selection specifically for resistance to CBPV and moenomycin in the *imp4213* background are those that abolish or greatly reduce production of functional YfgL. It is surprising that the lack of a putative outer membrane lipoprotein (YfgL) selectively confers resistance to a subset of compositionally dissimilar antibiotics while excluding molecules that are structurally similar.

The *yfgL* Mutations Alter OM Permeability

Cells carrying the *imp4213* allele are sensitive not only to antibiotics but also to detergents such as bile salts. Therefore, since MacConkey agar contains bile salts, strains carrying *imp4213* cannot grow on this medium ([Sampson et al., 1989](#)). However, we discovered that *imp4213 yfgL::IS1E* double mutants do grow on MacConkey agar. Thus, *imp4213 yfgL::IS1E* double mutants are resistant to bile salts, CBPV, and moenomycin while remaining sensitive to other antibiotics, such as vancomycin ([Table 1](#)).

As indicated above, selections for resistance to CBPV or moenomycin in the *imp4213* background specifically yield *yfgL* mutants. We next tested whether selection for bile salt resistance was also specific for *yfgL* mutants. By selecting for growth on lactose MacConkey agar, we obtained spontaneous mutants resistant to bile salts at a frequency of ca. 4×10^{-7} . After various independent selections, we determined that 30%–60% of the bile salt-resistant mutants were also CBPV resistant. Mutations that conferred resistance to bile salts only were not linked to either *yfgL* or *imp4213*

and will not be discussed any further. Mutations that conferred resistance to both bile salts and CBPV were in either the *yfgML* locus or in *imp*, and the latter also conferred resistance to vancomycin (see below). All of the mutations that mapped to the *yfgML* locus were recessive loss-of-function alleles, since expression of wild-type *yfgL* in *trans* restored sensitivity to both bile salts and CBPV (data not shown). Thus, like the selections for resistance to CBPV or moenomycin, selections for bile salt resistance yielded intragenic suppressors of *imp4213* and loss-of-function alleles of *yfgL*. However, unlike the former, selections for bile salt resistance also yielded mutations other than those in *imp4213* or *yfgL*. Regardless of the selection employed, the only mutations that specifically give resistance to CBPV and moenomycin in the *imp4213* background are those that abolish or greatly reduce production of functional YfgL.

Although selections for CBPV or moenomycin resistance only yielded one type of mutation in the *yfgL* locus (*IS1E* element insertions, see above), selections for bile salt resistance yielded multiple types of *yfgL* mutations. For example, *yfgL8* is an in-frame 12 base pair deletion (codons 252–255). This allele is a recessive, loss-of-function mutation (data not shown), and it confers the same resistance phenotypes as the *yfgL::IS1E* allele ([Table 1](#)).

Unlike the *yfgL* mutations, the intragenic suppressor mutation *imp61* increases resistance to all the other antibiotics tested ([Table 1](#)). Phenotypically, this mutation resembles the intragenic suppressors of *imp4213* obtained upon selection for CBPV resistance following NTG mutagenesis (see above). Indeed, DNA sequence analysis revealed that the *imp61* mutation (codon 274 was changed from AAC to AUC; N2741 at the amino acid level) was identical to one of the *imp* suppressor alleles obtained by NTG mutagenesis. The other *imp* alleles obtained by NTG mutagenesis contain multiple mutations reflecting the heavy level of mutagenesis employed. In any event, unlike the *yfgL* suppressors, all of the intragenic suppressors in *imp4213* that we have examined suppress or partially suppress all of the *imp* sensitivity phenotypes.

Thus, there is a continuum of suppressor quantity and quality ([Figure 2B](#)). Bile salts are at one end of the

continuum (left in Figure 2B). There are multiple suppressors of bile salt sensitivity, and these suppressors may or may not suppress sensitivity of compounds to the right. At the other end of the continuum is vancomycin or erythromycin. Only intragenic *imp* mutations suppress sensitivity to these compounds, and these suppressors always suppress sensitivity to compounds to the left. The *yfgL* suppressors of CBPV and moenomycin sensitivity lie in the middle of this continuum. They suppress sensitivity to bile salts but not sensitivity to vancomycin or erythromycin.

Because *yfgL* mutations in the *imp4213* background confer resistance to bile salts, CBPV, and moenomycin, it is likely that the lack of YfgL alters some aspect of the OM permeability defect caused by *imp4213*. Resistance to CBPV and moenomycin requires this particular alteration absolutely. Since this change in permeability does not affect sensitivity of the *imp4213* strain to the closely related molecule vancomycin, we infer that the striking specificity of CBPV relates to some physical property of this molecule, perhaps its increased hydrophobicity. In any event, this change in permeability suggests a functional relationship between Imp and YfgL, and this in turn suggests a role for YfgL in OM biogenesis.

The Levels of Imp and YfgL in Various Mutant Backgrounds

Since low levels of wild-type Imp have been reported to lead to OM permeability defects (Abe et al., 2003), we determined by Western blot analysis whether any of the suppressors affected Imp levels. The *imp4213* mutation is a 23 codon deletion (Braun and Silhavy, 2002), and, therefore, the mutant Imp4213 protein migrated faster than wild-type Imp on an SDS polyacrylamide gel, reflecting this loss of mass (Figure 3A). We found that the levels of the mutant Imp4213 protein were lower than wild-type Imp, and this difference was especially apparent in overnight cultures (Figure 3A). By monitoring Imp4213 levels during cell growth, we see that levels of Imp4213 decrease early in stationary phase (data not shown). We believe that the lower levels of Imp4213 are not due to decreased synthesis, since the levels of SurA, which is cotranscribed with *imp* (Braun and Silhavy, 2002), remain unchanged in *imp4213* cells (data not shown). Instead, the lower levels of Imp4213 are likely the result of a defect in Imp4213 biogenesis.

Western blot analysis showed that the Imp levels expressed from the *imp61* allele were not significantly different from those expressed from *imp4213* during logarithmic phase (data not shown). However, Imp levels were higher in the *imp61* mutant than in *imp4213* in late stationary cultures but still much lower than wild-type (Figure 3B).

Since the *yfgL8* allele is an in-frame deletion, the YfgL mutant protein migrates slightly faster than wild-type on SDS gels (Figure 3B). Although this mutation destroys YfgL function, it does not destabilize the protein, since the levels of the mutant YfgL were the same as wild-type. In addition, during logarithmic growth, Imp4213 levels were not affected by the presence of the *yfgL8* allele (data not shown). However, samples from overnight cultures of the *imp4213 yfgL8* double

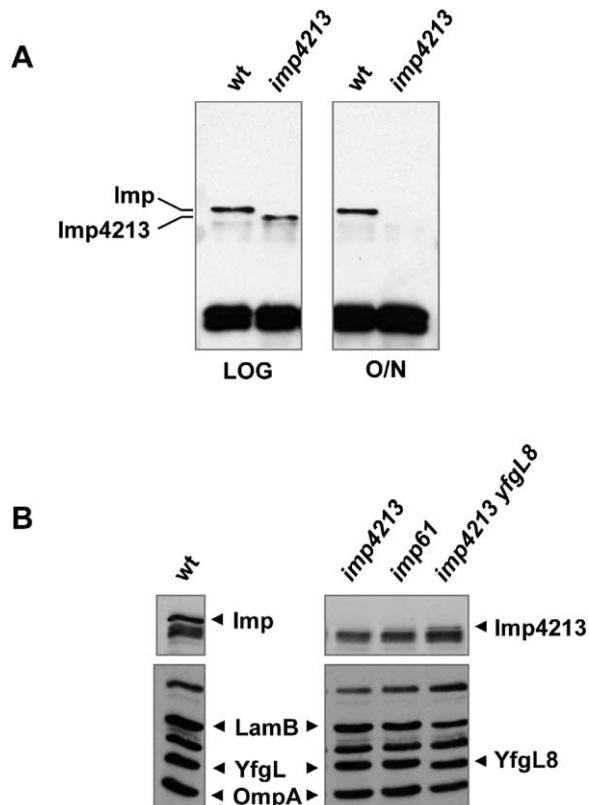


Figure 3. Levels of Outer Membrane Proteins in *imp4213* and Suppressor Strains

(A) Western blot analysis of Imp levels in whole cells that have been grown logarithmically (LOG) or overnight (O/N) in LB. The 23 codon internal deletion in *imp4213* results in decreased levels of the mutant Imp when compared to wild-type (wt).

(B) Western blot analysis of Imp, LamB, YfgL, and OmpA was conducted on overnight cultures. When compared to wild-type (wt), the *imp4213* mutant has reduced levels of Imp but comparable levels of LamB, YfgL, and OmpA, and the intragenic suppressor *imp61* does not change them. The *yfgL8* allele carries a 4 codon internal deletion that does not affect YfgL stability. However, this strain contains reduced levels of LamB and OmpA. This mutation also slightly increases the levels of Imp.

mutant contained increased levels of Imp4213 when compared to the *imp4213* single mutant, although they were still clearly lower than those in the wild-type (Figure 3B).

Recall that *imp61* confers resistance to a broader spectrum of antibiotics than does *yfgL8* (Table 1). Yet *yfgL8* is more effective at stabilizing Imp4213 than *imp61*. Clearly, antibiotic resistance does not correlate with Imp4213 levels, and it is likely that none of the mutations confer resistance by stabilizing this mutant protein. It is also clear that none of the *imp* mutations have any effect on the levels of YfgL (Figure 3B).

The *yfgL* Null Mutations Affect Growth Rate Differently but All Confer Similar Permeability Phenotypes

We observed that, while *imp4213 yfgL::IS1E* strains grow at roughly 25% the rate of their *imp4213* parent, *imp4213 yfgL8* strains grow at roughly 75% the rate of

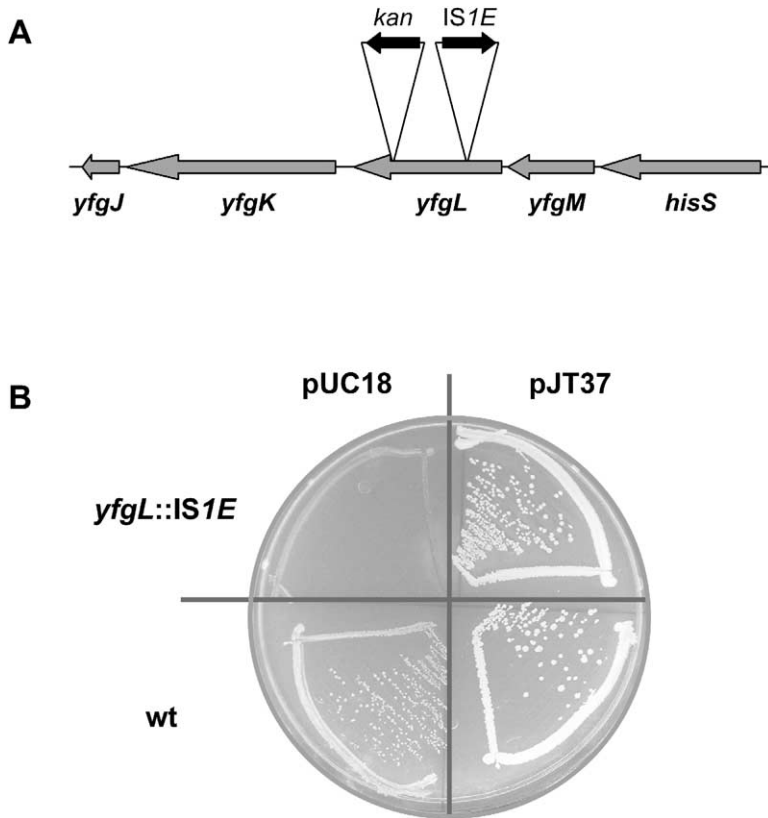


Figure 4. Polar Effects of *yfgL::IS1E* on *yfgK* Expression

(A) The *hisSyfgMLKJ* genes are predicted to constitute an operon (Bockhorst et al., 2003). All the *IS1E* insertions in *yfgL* and *yfgM* isolated in our selections are oriented in the same direction, with the *insA* ORF transcribed in opposite direction from the *hisS* operon, causing lower expression of the downstream genes. The *kan* cassette in the *yfgL::kan* allele is transcribed in the same direction as the *hisS* operon, and its promoter drives the expression of downstream genes. (B) Strains carrying the *yfgL::IS1E* allele grow more slowly than wild-type (wt). Introduction of pJT37, a pUC18-based plasmid expressing YfgK under the control of the derepressed *lac* promoter, rescues the growth defect. Cells were grown on LB agar containing 50 mg/ml of ampicillin.

the *imp4213* single mutant. We confirmed that polarity (i.e., decreased expression of downstream genes) caused by the *IS1E* insertion is responsible for the pronounced difference in their growth rates. As Figure 4A shows, *yfgK* and *yfgJ* are located immediately downstream of *yfgL* and transcribed in the same direction, which is opposite to that of the only ORF (*insA*) in the *IS1E* element. Since the *yfgL8* allele is a 12 base pair in-frame deletion in *yfgL* and the mutant protein is made at wild-type levels, it is not polar. In contrast, the *yfgL::IS1E* allele would be expected to decrease *yfgKJ* transcription if they are in an operon with *yfgML* (Figure 4A). Since YfgK (also known as EngA and Der) is an essential GTPase (Hwang and Inouye, 2001), it seemed likely that a decrease in the expression of *yfgK* due to polarity could result in slower growth.

To assess the effects of polarity without complications owing to the *imp4213* allele, we moved the various *yfgL* mutant alleles into an *imp+* background. Although the *yfgL8* allele did not affect growth, the *yfgL::IS1E* allele caused severe growth defects. This slow growth phenotype was rescued by pJT37, a pUC18-based plasmid encoding YfgK only under the control of the derepressed *lac* promoter (Tan et al., 2002), but it was not rescued by the control plasmid pUC18 (Figure 4B). This result demonstrates that *yfgMLK* are cotranscribed and that the growth defects observed with the IS insertions are due to polarity. This agrees with the prediction that *hisSyfgMLKJ* genes constitute an operon (Bockhorst et al., 2003).

In addition, we obtained a strain carrying a Tn5KAN-

I-SceI insertion in *yfgL* (*yfgL::kan*), where the transcription of the kanamycin resistance cassette is in the same orientation as that of *yfgMLKJ* (Figure 4A), from the *E. coli* Genome Project at the University of Wisconsin-Madison (insertion is located at position 653). This strain grew at the same rate as wild-type. We suspect that, in this strain, expression of the downstream genes *yfgKJ* is being driven by the same promoter that controls *kan* expression.

Recall that both the polar and nonpolar *yfgL* alleles (except for *yfgL::kan*, see below) confer resistance to bile salts and CBPV in the *imp4213* background. Indeed, the drug sensitivity profiles of both types of mutations are identical. Therefore, it is the lack of functional YfgL that is responsible for antibiotic resistance in this strain. The different growth rates observed with various *yfgL* alleles do not alter these phenotypes.

Genetic Interactions between *imp* and *yfgL*

As noted above, our data show that the lack of YfgL decreases the OM permeability of *imp4213* cells, causing increased resistance to bile salts, CBPV, and moenomycin. Paradoxically, in an *imp+* background, cells carrying any of the *yfgL* mutant alleles exhibit increased OM permeability; they are more sensitive to antibiotics such as bacitracin, CBPV, novobiocin, and erythromycin than their wild-type parents (Table 1). Yet, in combination, *imp4213* and *yfgL* confer resistance to bile salts, CBPV, and moenomycin (Table 1). Thus, we have a situation in which, individually, *yfgL* and *imp4213* both confer sensitivity to a variety of antibiotics, but the

Table 2. Phenotypes of the Various *yfgL* Alleles in the *imp*⁺ and *imp4213* Backgrounds

Relevant genotype	Relevant phenotypes	<i>yfgL</i> Alleles		
		<i>yfgL::IS1E</i>	<i>yfgL8</i>	<i>yfgL::kan</i>
<i>imp</i> ⁺	<i>yfgK</i> expression	Reduced	Wild-type	Wild-type ^e
	Growth defect ^a	Severe	None	None
	CBPV sensitivity ^b	S	S	S
<i>imp4213</i>	Conditional synthetic lethality	No	Yes ^d	Yes
	CBPV sensitivity ^c	R	R ^d	N/A ^f

^aDetermined in LB medium.

^bA sensitive (S) strain is one that, in a disk diffusion assay for CBPV, shows a zone of inhibition of growth larger than its *yfgL*⁺ parent.

^cA resistant (R) strain is one able to grow on the LB agar containing 4 μg/ml CBPV.

^dThe original *imp4213 yfgL8* isolate carries an uncharacterized mutation that suppresses the synthetic lethal phenotype. This isolate was used to determine sensitivity to CBPV.

^eThe expression of *yfgK* is probably under the control of the promoter of the *kan* cassette.

^fN/A, not applicable.

double mutants are resistant. Since the double mutants exhibit phenotypes different from either singly mutant parent, it is a synthetic phenotype.

We have found a second synthetic phenotype with *Imp* and *YfgL*. Although neither *imp4213* nor any of the *yfgL* null mutations are lethal by themselves, both the *yfgL::kan* and *yfgL8* alleles are lethal in combination with *imp4213* (Table 2). We first noticed this synthetic lethality when attempting to construct the *yfgL::kan imp4213* double mutant. Using P1 transduction followed by selection in rich media, we were unable to introduce either allele into the corresponding single mutant background. We know that *imp4213* cells deficient in *YfgL* are viable; that is how the *yfgL* mutations were identified. Moreover, we can overexpress *YfgK* when we introduce pJT37 into *imp4213* cells, since overproduced *YfgK* can be simply detected by Coomassie blue staining after electrophoresis of cell extracts (data not shown). Therefore, the synthetic lethality that we find between the *imp4213* and *yfgL::kan* alleles cannot be explained by proposing either a toxicity caused by upregulation of *yfgK* or the lack of functional *YfgL*.

As noted above, *yfgL::kan* does not affect growth in otherwise wild-type cells (Table 2). Because the various *yfgL* alleles affect growth differently, we considered the possibility that this might play a role in the synthetic lethality observed with the *imp4213* and *yfgL::kan* alleles. In other words, synthetic lethality is prevented in *yfgL::IS1E imp4213* strains because growth is compromised by downregulation of the essential gene *yfgK*.

To address this possibility, we tested whether we could introduce the *yfgL::kan* allele into *imp4213* cells if we slowed their growth. To this end, we performed P1 transductions using *imp4213* as recipient and selecting for *yfgL::kan* on either rich (Luria-Bertani, LB) or minimal medium conditions. Although we were unable to obtain transductants when selecting on LB agar, transductants did appear on minimal glycerol agar. Furthermore, these transductants could only be propagated in minimal medium. Similarly, if the *imp4213* recipient also carried a *pstS* null mutation, which is known to slow growth (Ruiz and Silhavy, 2003), we could then introduce the *yfgL::kan* allele by P1 transduction even if selection was performed on LB medium.

We conclude that the synthetic lethality of *yfgL* null mutations and *imp4213* is conditional; it is only evidenced in rapidly growing cells.

As stated above, the *imp4213* and *yfgL8* alleles also exhibit this conditionally synthetic lethal phenotype. The original *imp4213 yfgL8* double mutant was obtained in a selection for mutants resistant to bile salts. This original isolate contains an uncharacterized suppressor mutation that allows the double mutant to survive. We know this because this isolate can be used as an *yfgL8* donor in a transduction cross if the recipient strain is *imp*⁺ but not if the recipient carries *imp4213*. We suggest that this uncharacterized suppressor slows growth sufficiently to allow the double mutant to survive. Indeed, this uncharacterized mutation can also suppress the synthetic lethality between the *imp4213* and *yfgL::kan* alleles, since we can introduce the *yfgL::kan* allele into the original *imp4213 yfgL8* double mutant even if the selection was performed on LB media. Moreover, we can introduce the *yfgL8* allele into an *imp4213* strain if selection is done on minimal glycerol agar. Therefore, the only *yfgL* mutations that can be moved by transduction into the *imp4213* strain if the selection is performed on rich media are the polar *yfgL::IS1E* insertions. This likely explains why these mutations appeared so frequently in our selections for resistance to CBPV or moenomycin, which were done on LB agar. In hindsight, we inadvertently demanded them because they simultaneously remove *YfgL* to confer antibiotic resistance and suppress synthetic lethality by reducing *YfgK* levels to slow cell growth.

The results presented above indicate that *yfgL* null mutations and *imp4213* cannot exist in the same strain unless growth is slowed by either downregulating *yfgK* expression or using minimal glycerol medium, or if some additional unidentified suppressor is present (Table 2). We conclude that a *yfgL* null allele and *imp4213* are a conditionally lethal synthetic pair.

Taken together, the synthetic phenotypes documented in this section argue strongly for an interaction (direct or indirect) between *Imp* and *YfgL*.

The *yfgL* Mutations Decrease the Levels of OM Proteins

imp⁺ *yfgL* mutants have decreased levels of OM β barrel proteins such as LamB and OmpA (Figure 5A). Since

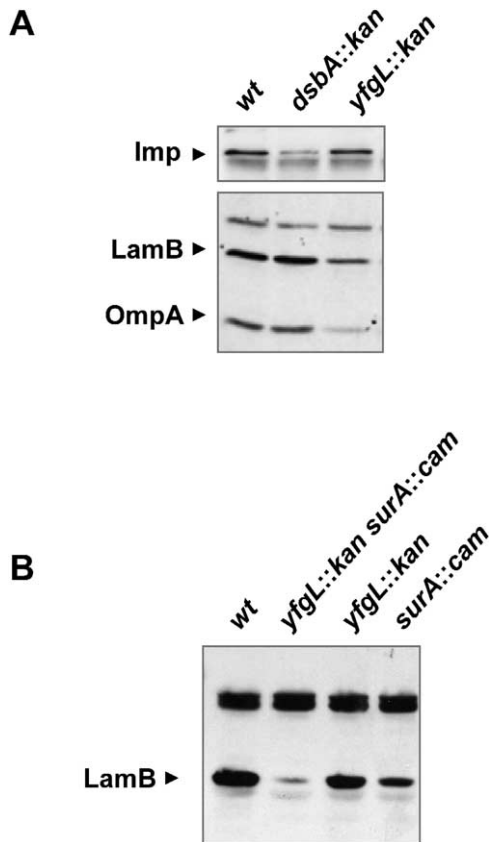


Figure 5. Cells Lacking YfgL Contain Reduced Levels of Outer Membrane β Barrel Proteins

(A) Logarithmically growing cells were subjected to Western blot analysis for Imp, LamB, and OmpA. A strain carrying the *yfgL::kan* allele had decreased levels of LamB and OmpA when compared to wild-type (wt) but had a similar amount of Imp. On the contrary, a *dsbA* mutant contains wild-type levels of LamB and OmpA but reduced levels of Imp, since DsbA participates in Imp assembly (Braun and Silhavy, 2002; Kadokura et al., 2004).

(B) Overnight cultures were subjected to Western blot analysis for LamB. Cells lacking either SurA or YfgL contain reduced levels of the outer membrane protein LamB. The levels of LamB are reduced even further in the double mutant.

LamB and OmpA are not coregulated, we suspect these decreased levels in the *yfgL* mutants reflect an assembly defect. This defect, however, is not as severe as those found in cells lacking SurA, a periplasmic chaperone with peptidyl-proline isomerase activity (Lazar and Kolter, 1996; Rouviere and Gross, 1996). Because the assembly defects caused by *surA* and *yfgL* are additive (Figure 5B), i.e., they are worse in the double mutant than in either of the singly mutant parents, we believe that SurA and YfgL affect different steps of OM biogenesis.

Together, these data argue a role for YfgL in OM biogenesis.

Discussion

Small molecules that interfere with cellular processes have been invaluable tools in biology. For decades,

factors involved in essential cellular functions that are targets for such small molecules have been identified using genetic screens and selections. For example, selection for rifampicin resistance was used to identify the gene for a subunit of RNA polymerase in *E. coli* (Tocchini-Valentini et al., 1968; Ezekiel and Hutchins, 1968). We set out to identify the direct target of a glycolipid derivative of vancomycin and discovered that challenging *E. coli imp4213* with this molecule elicited a remarkably specific response. After repeated selections, only one solution to the challenge, other than intragenic *imp* suppressors, emerged: loss-of-function mutations in a gene called *yfgL*. Because the lack of YfgL also confers resistance to another transglycosylation inhibitor (moenomycin) but not to any other antibiotic examined, we thought initially that YfgL might somehow sense the status of the peptidoglycan (Egger et al., 2001). However, results presented here suggest another role for YfgL. First, removing YfgL in a wild-type background confers sensitivity to CPBV and moenomycin, as well as other unrelated antibiotics with different structures and mechanisms, suggesting a general defect in OM permeability. Second, the absence of YfgL in the *imp4213* strain confers resistance not only to CPBV and moenomycin but also to bile salts. Thus, rather than identifying the direct target of CPBV, we have discovered a gene, *yfgL*, that appears to be involved in maintaining OM integrity in *E. coli*.

It is not known how the OM is assembled and maintained, although a few genes essential for the process, including *imp*, have been identified recently (Braun and Silhavy, 2002; Voulhoux et al., 2003; Narita et al., 2004). Cells depleted of Imp appear to mislocalize newly synthesized OM proteins and lipids (Braun and Silhavy, 2002). However, in depletion experiments, it is sometimes difficult to distinguish the primary defect from secondary consequences that occur as cells die. In *Neisseria*, where LPS is not an essential component, *imp* is not an essential gene, and cells lacking Imp have defects in LPS assembly in the OM (Bos et al., 2004). They suggest that *E. coli imp4213* cells likely have defects in LPS assembly, and we agree. Indeed, neither we nor others (Sampson et al., 1989) can detect changes in OM protein levels in *E. coli imp4213* cells, and this is consistent with the proposal that this allele more likely affects the lipid composition of the OM. However, we do not agree with their suggestion that *imp4213* cells simply have less LPS in their OM. Their simple model cannot explain why some of the phenotypes of *imp4213* cells are recessive, while others are dominant (Sampson et al., 1989). The recessive phenotypes are likely the consequence of a loss of function and may well be due to less LPS, but dominance requires gain of function and implies that the mutant Imp4213 protein is actively causing some additional OM alterations.

Nevertheless, the partial loss of Imp function in cells carrying *imp4213* and their wild-type levels of OM proteins support the idea that the OM of *imp4213* cells are defective in LPS, and the permeability defects likely result from phospholipids that have been translocated to the outer leaflet to fill the void (Nikaido, 2003). On the other hand, YfgL is needed to maintain wild-type levels of OM β barrel proteins. YfgL may act directly as a nonessential component of the protein assembly

machinery and/or indirectly by regulating the various machineries that function to assemble the OM. Given our findings, we propose the different activities of Imp and YfgL must be coordinated in order to maintain the proper balance between LPS, phospholipids, and proteins. Shifting this balance, by compromising either Imp or YfgL, therefore results in increased OM permeability. If the LPS component is altered in *imp4213* cells, the lack of YfgL might then restore the balance sufficiently to correct some (bile salt and CBPV sensitivity) but not all permeability defects. Thus, both mutations deleteriously alter the OM composition, but they do so by different mechanisms.

This proposal offers a satisfying explanation for the conditional synthetic lethality of the *imp4213* and *yfgL* null alleles; cells cannot tolerate both defects in OM biogenesis unless growth is compromised. Such strict conditions for cell viability and suppression might also explain why only loss of function of YfgL and, in particular, why only specific types of *yfgL* mutations answered our selection. Further evidence for a role for YfgL in OM biogenesis is presented in the accompanying paper (Wu et al., 2005). The function of nonessential genes is often difficult to ascertain without the ability to measure obvious phenotypes. Given the subtle defects of a *yfgL* mutant, it would have been difficult to identify YfgL as a player in OM biogenesis without the use of CBPV and the bile salts, i.e., chemical conditionality.

Our results demonstrate a continuum of suppressor quality and quantity (Figure 2B). All of the compounds we employ in our selections must cross the OM permeability barrier to act. The *imp4213* mutation disrupts the barrier and allows this to occur. All of the suppressors confer resistance by restoring, to varying extents, barrier quality. The inverse relationship between quality and quantity reflects the number of genes that can be mutated to confer a suppressor phenotype. Restoring sensitivity to bile salts is relatively easy, and there are multiple genetic targets. Restoring resistance to vancomycin or erythromycin is difficult, and only specific intragenic mutations in *imp* answer these selections.

Since resistance reflects changes in permeability, the molecules we employ must be specific probes of barrier quality, and the specificity of these probes must reflect the different physical properties of these molecules. We do not yet understand the physical basis of the specificity. However, CBPV and moenomycin have amphipathic physical characteristics, a property they share with the bile salts but not with vancomycin. The chlorobiphenyl modification of vancomycin allowed us to discover the continuum. In a similar vein, we think it should be possible to expand the continuum further by chemically altering the physical properties of these molecules.

The use of chemical probes in biology is most often conceived in terms of a one molecule-one protein match (Schreiber, 2003). Phenotypic alterations of a cellular response to a particular small molecule are typically thought to result from mutations in the gene encoding the target of the molecule or a closely related protein (Shah et al., 1997). Here we have shown that antibiotics can elicit highly specific genetic phenotypes that do not involve mutations in the genes for their di-

rect targets but rather in genes that affect their ability to reach their targets. Antibiotic action is a multistep process: (1) passage of the antibiotic across the membrane(s) to reach the cellular compartment where its target resides, (2) interaction of the antibiotic with its target, and (3) downstream events that lead to cell death or growth inhibition. Since membranes surround cellular compartments and since resistance to an antibiotic can be accomplished by preventing its entry into the cellular compartment where its target is located, antibiotics can function as probes of membrane integrity. In *E. coli*, the machinery involved in peptidoglycan biosynthesis is protected by an OM permeability barrier. We used a series of compounds with targets inside the cell to identify genes required to assemble the OM. We have shown that this membrane can be rendered generally permeable to a range of small molecules by individual mutations in several genes, including *imp* and *yfgL*, or it can be rendered *selectively permeable* through combinations of mutations. Selective permeability can only be revealed through the use of an appropriate set of small molecule probes that create chemically conditional phenotypes. The mutations that confer both general and selective permeability provide insight into the machinery involved in assembling the OM.

It is possible to chemically modify many bioactive small molecules to produce derivatives having varying physical properties. Assuming that the composition of other biological membranes (or organelles) is also tightly controlled by the action of some molecular machine, it should be possible to shed light on the components of the machine through the deliberate use of sets of small molecules having targets inside the membrane (or organelle) of interest.

Experimental Procedures

Growth Conditions

Luria-Bertani (LB), lactose MacConkey agar, and M63 glycerol or glucose minimal agar media were prepared as described previously (Silhavy et al., 1984). All liquid cultures were grown under aeration at 37°C, and their growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). Tetracycline and kanamycin were used at a concentration of 25 µg/ml. Chloramphenicol was used at a concentration of 15 µg/ml for strains carrying the *imp4213* allele and of 20 µg/ml for those carrying the *imp* wild-type allele.

Bacterial Strains

For strain construction and genetic mapping, P1 transduction was used to introduce alleles into the appropriate strains as described previously (Silhavy et al., 1984). All strains carrying the *imp4213* allele were derived from NR698. NR698 was constructed by introducing the *imp4213* allele of BE100 (Eggert et al., 2001) into MC4100 (Casadaban, 1976) as follows. A *carB::Tn10* allele, which is ca. 20%–25% linked to the *imp* locus, was introduced into BE100 by P1 transduction. Tetracycline-resistant transductants were screened for the presence of the *imp4213* allele by their inability to grow on MacConkey agar. One such transductant (NR685) was used as a donor in a transduction cross with MC4100, where we selected for tetracycline-resistant transductants. By screening for their inability to grow on MacConkey agar, we determined that ca. 22% of transductants carried the *imp4213* allele. One such transductant (NR693) was then used as the recipient in the following transduction. Because *carB* null mutants cannot grow on minimal media (Gigot et al., 1980), we selected for the replacement of the *carB::Tn10* allele with the wild-type *carB* allele from MC4100 by

selecting for growth on M63 minimal glucose media. A *carB*⁺ transductant that retained the *imp4213* allele (i.e., cannot grow on MacConkey agar) was named NR698.

NR698 was used as the starting strain in the selections for CBPV and moenomycin-resistant mutants. In the selection for spontaneous bile salt-resistant mutants, we used either NR698 or NR701, which is a derivative of NR698 that carries the *yfhS::Tn10* allele. The *yfhS::Tn10* allele allows us to easily identify mutations linked to the *yfgL* locus. The *imp61* and *yfgL8* alleles were isolated as suppressors of bile salt sensitivity of NR698 and NR701, respectively.

The *yfgL::IS1E* allele from an isolate of BE103 carrying the *yfhS::Tn10* allele (Eggert et al., 2001) was introduced into MC4100 by cotransduction with the *yfhS::Tn10* allele, and the new strain was named NR452-2. One transductant that was *yfhS::Tn10* and *yfgL*⁺ was named NR453-2. The *yfgL::Tn5KAN-I-Scel* (referred to as *yfgL::kan*) allele was derived from strain FB22443, which we obtained from the *E. coli* Genome Project at the University of Wisconsin-Madison (<http://www.genome.wisc.edu/functional/tmmutagenesis.htm>). We constructed strain NR721 by introducing the *yfgL::kan* allele into MC4100 by P1 transduction. The *yfgL::kan* allele of NR721 was replaced with the *yfgL8* allele by cotransduction with the *yfhS::Tn10* of the original NR701 bile salt resistant isolate. The resulting strain was named NR722. A *surA::cam* allele (from our laboratory collection) was introduced into MC4100 and NR721 to create NR740 and NR742, respectively. A *dsbA::kan* allele (from our laboratory collection) was introduced into MC4100 to create NR713.

To map mutations in the *yfgL* locus, we used the linked *yfhS::Tn10* and *guaA::cam* alleles. To map mutations in the *imp* locus, we used the linked *carB::Tn10*, *leuO::Tn10*, and *apaG::cam* alleles.

Selections

Spontaneous CBPV-resistant mutants were selected as previously described (Eggert et al., 2001). To select for bile salt-resistant mutants, we plated 0.1 ml of overnight LB broth cultures of NR701 onto lactose MacConkey agar. Spontaneous bile salt-resistant mutants appeared after overnight incubation at 37°C. Moenomycin resistant mutants were obtained by plating onto LB agar containing 0.1 µg/ml moenomycin. Strain NR698 was mutagenized with NTG as previously described (Silhavy et al., 1984). Mutations were identified by DNA sequencing after genetic mapping. For DNA sequencing, the allele of interest was amplified by PCR directly from chromosomal DNA, and products were purified using the QIAquick PCR purification kit (Qiagen). DNA sequencing was done by the Princeton University Department of Molecular Biology Synthesis and Sequencing Facility using primers specific to the gene of interest.

Compound Sensitivity

Bile salt sensitivity was assessed by the ability of the strain to grow or not on lactose MacConkey agar. The sensitivity to CBPV and vancomycin of strains carrying the *imp4213* allele was determined by assessing growth on LB agar containing either compound at a final concentration of 4 µg/ml CBPV or 2 µg/ml vancomycin. The sensitivity to CBPV of strains carrying the *imp*⁺ allele was determined by disk diffusion assay as described below using 6 mm filter paper disks (Schleicher & Schuell) impregnated with 150 µg of CBPV. BBL Sensi-Discs Antimicrobial Susceptibility Test Discs (BBL) were used to test strain sensitivity to bacitracin, novobiocin, erythromycin, and rifampin. Disks containing the aforementioned compounds were used in disks diffusion assays as follows. A 0.1 ml inoculum taken from overnight LB broth cultures was mixed with 3 ml of molten LB top agar and poured over an LB agar plate. The disks containing antibiotics were placed on top of the LB top agar, and the plates were incubated overnight at 37°C. The diameter of the zone of inhibition of growth around each disk was recorded in millimeters.

Protein Analysis

For protein analysis, 1 ml samples were taken either from logarithmic (OD₆₀₀ of 0.5–0.6) or overnight LB broth cultures. Samples were

centrifuged (16,000 × g for 5 min), and to standardize samples, pellets were resuspended in a volume (in milliliters) of SDS sample buffer equal to OD₆₀₀/6. Samples were boiled for 10 min, and equal volumes were subjected to electrophoresis in 10% polyacrylamide gels containing SDS as described by Laemmli (1970). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell), and Western blot analysis was performed as previously described (Gibson and Silhavy, 1999). When appropriate, polyclonal sera raised against Imp and YfgL (from our laboratory collection, see below) were used as primary antibodies at a dilution of 1:7000. These antisera also recognize OmpA. Polyclonal antiserum raised against denatured LamB (from our laboratory collection) was used at a dilution of 1:10,000. Donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham Pharmacia Biotech) was used as secondary antibody at a 1:6000 dilution. For visualization of bands, the ECL antibody detection kit (Amersham Pharmacia Biotech) and Biomax film (Kodak) were used.

YfgL Antiserum

An antibody raised in a rabbit against a peptide (NAc-CDSSGF QTEPVAADGKLLIQAKDGTVYSITR-COOH) containing the last 30 amino acids of YfgL was obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA).

Acknowledgments

We thank members of the Silhavy and Kahne labs, particularly Juliana Malinverni, for their helpful suggestions and Susan DiRenzo for all her assistance. We also thank J.C. Bardwell for giving us pJT37. This work was supported by grants GM66174 (D.K.) and GM34821 (T.J.S.) from the National Institute of General Medical Sciences.

Received: November 19, 2004

Revised: February 3, 2005

Accepted: February 10, 2005

Published: April 21, 2005

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