Chemical Genetic Interactions for Antibiotics in 

*Escherichia coli*

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

The discovery of penicillin ushered in the era of the mass use of antibiotics in clinical settings. Today the development of antibiotic resistance and lack of discoveries of new antibiotics have created a serious public health concern. Recently, new experimental tools, such as bacterial genome-wide deletion collections, have provided exciting new possibilities for studying biological networks in bacteria that could potentially also be exploited for antibiotic research. In this study, I used the Keio knockout collection of *Escherichia coli* (*E.coli*) strains, along with an in-house collection of hypomorphic alleles of essential genes, to study the effects of chemical perturbations by twenty-two antibiotics and four other chemicals on the biological pathways of *E.coli*. These experiments uncovered a set of mutants hypersensitive to drugs of different classes, information which could potentially be exploited for future antibiotic research. The results also shed light on how different classes of antibiotics behave with respect to their target pathways and the various functional modules with which they are associated.
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Chapter 1
Introduction and Background Information

1.1 Evolution of anti-microbial research

By the late 1800s, as the “germ theory of disease” grew in acceptance following the seminal discoveries of Pasteur and Koch, the systematic search for antibacterial compounds began (Wright 2007). The resulting discovery of salvarsan in 1908 by Paul Ehrlich’s group marked the first organized effort to optimize the biological activity of a compound through systematic chemical modifications, the basis for nearly all modern pharmaceutical research (Drews 2000). Salvarsan was the first organic anti-syphilitic, a great improvement over the previous treatment using mercury.

Efforts to control infections date back at least 3000 years. For example, anecdotal evidence suggests that mouldy soybean curds were used to treat infected tissue by the ancient Chinese (Pathania and Brown 2008). However, antibacterial drug discovery today as a large interdisciplinary endeavour has foundations rooted in basic chemistry. By the late 1800’s, certain fundamentals of chemistry, such as theories on the structure of aromatic organic compounds, paved the way for research on coal-tar derivatives such as dyes (Drews 2000). Dye chemistry research, in turn, had a profound effect on medicine. Paul Ehrlich’s work on the staining of biological tissues with dyes led to the idea of “chemoreceptors”. He also argued later that chemoreceptors on parasites and hosts would be different and could be exploited for therapeutic uses (Drews 2000). This led to the birth of chemotherapy, which, over the last century, has made astronomical progress in therapeutics and medicine.
However, it was the discovery of penicillin by Alexander Fleming in 1928 that shifted the antibiotic discovery paradigm from entirely synthetic compounds to the exploitation of natural products (Fleming 1940; Drews 2000). The fortuitous accident that led to the discovery of penicillin is one which is familiar to most of us. As it happened hundreds of times before Fleming and since, mould growth contaminated agar plates. Fleming, however, that realized the clear zone near the mould where there was an inhibition of bacterial growth and concluded that secretions from the mould prevented nearby bacterial growth. Between the discovery and large scale use of penicillin, there was a large gap of time. It has been suggested that lag of time was due to a) scepticism of the scientific community and b) failure to purify the compound from the mould (Dworkin 2006). However, between 1877 and 1939, a number of antibiotic compounds were described in the scientific literature (Fleming 1940; Drews 2000). It was the fortunate rediscovery of penicillin by Florey and Chain in 1938, however, which ushered in the age of antibiotics that we live in today (Drews 2000).

The success of penicillin led to its mass production, and pharmaceutical companies and scientists began searching for novel compounds with antibacterial activity. This led quickly to the discovery of a number of different classes of antibiotics, including tetracyclines (Duggar, 1948; McCormick et al.1959; Miller et al., 1956), phenicols (Ehrlich et al., 1947) and aminoglycosides (Jones et al.1944) (Figure 1 ). This ‘golden era’ of antibiotic discovery (1945–1960), during which most of the chemical classes of antibiotics now in clinical use were discovered, was followed by the golden decade (1970–1980) of “medicinal chemistry”, wherein extensive and elaborate changes were made to existing chemical scaffolds to increase the efficacy and reduce
Figure 1-Timeline of major discoveries in antibiotic research
resistance to the drugs (Drews 2000). Yet, very few new scaffolds were generated during that era.

From early on, it became clear that the true mechanisms of action of various antibacterials needed to be elucidated in order to facilitate the development of newer drugs and expand the therapeutic arsenal. Early systematic investigations on the modes of action of antibiotics involved searching for direct targets of the drug and the cellular consequences of drug-target interactions (Park and Strominger, 1957; Wisseman et al., 1954). This process often involved examining the biochemical properties of the drug and how modifications that affected drug-target binding correlated with the efficacy of the drug. Soon afterwords, drug uptake and the bacterial cell envelope became a central focus in antibiotic discovery research.

During this time, it also became apparent that antibacterials work by interfering with essential cellular functions. These functions then provided a basis for classification of antibiotics (Walsh, 2000). As is evident in Figure 2, most antibiotics on the market today are classified into a few broad classes, mainly inhibitors of cell wall and membrane biogenesis (e.g., β-lactams, cephalosporins, vancomycin), inhibitors of DNA replication and repair (e.g., quinolones, sulfamethoxazole, trimethoprim), and inhibitors of protein synthesis (e.g., aminoglycosides, phenicols, macrolides, tetracyclines). In addition, antibiotics are also classified into two broad catergories: bacteriostatic or bactericidal. Bacteriostatic agents halt any further reproduction of the bacteria and, if removed, bacteria can continue to reproduce, while bactericidal agents kill the bacteria. The majority of the cell wall biosynthesis inhibitors, DNA replication and repair inhibitors, and aminoglycosides are classified as bactericidal. Inhibitors of the ribosome, with the
exception of the aminoglycosides, typically only arrest cell growth and are classified as bacteriostatic.
Figure 2 - Major classes of antibiotics and their modes of action
1.2 **Inhibitors of bacterial cell wall and membrane biogenesis**

The hallmark of bacterial morphology and first line of defence is the cell envelope, comprising the cell wall plus the cell membranes in bacteria. In gram negative bacteria, the envelope also consists of a periplasmic space containing peptidoglycan between the cell membranes. Figure 3 is a schematic model of the cell envelope of the *E. coli* bacterium. The inner membrane (IM), a phospholipid bilayer whose phospholipid content varies depending on the bacterial species, contains proteins responsible for carrying out fundamental biological processes, including small molecule transport, oxidative phosphorylation, phospholipid biosynthesis and protein translocation (Leverrier, Vertommen et al.). The outer membrane (OM), composed of phospholipids and lipopolysaccharides (LPS), contains specialized β-barrel proteins (OMP) called porins, which allow for passive transport of many ions, sugars and amino acids. The periplasm, located between the outer and inner membranes, contains the peptidoglycan layer, which provides mechanical strength crucial for the cell’s ability to survive various environmental insults; the degree of peptidoglycan cross-linking correlates with the structural integrity of the cell (Holtje 1998). Peptidoglycan biosynthesis, in turn, is the main target for many antibiotics, especially the β-lactams.
Figure 3 - Schematic model of the inner and outer membranes and the periplasmic space, comprising the cell envelope of *E. coli*

1.2.1 β-lactams

β-Lactams are a broad class of antibiotics, which inhibit cell wall biosynthesis. This class of drugs includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems (Lock and Harry, 2008). The primary mode of action of penicillin and other derivatives is to interfere with the synthesis of peptidoglycan. Peptidoglycan biosynthesis occurs in three phases. First is the assembly of disacharride peptide monomer units by enzymes in the cytoplasm or the inner cytoplasmic membrane. Second, the transfer of the monomers across the membrane occurs concurrently with their synthesis. Finally, the cross-linking and binding of the nascent peptidoglycan to the existing cell wall occurs. This last phase is extensively exploited by drugs like the β-lactams, which specifically block transpeptidation reactions between the D-alanine residues of peptidoglycan strands (Tipper and Strominger, 1965; Wise and Park, 1965).

This transpeptidation reaction in the synthesis of peptidoglycan is carried out by penicillin binding proteins (PBP’s). PBPs were initially named after their ability to bind penicillin and, in E.coli, there are as many as seven PBP’s. These proteins localize to different areas of the cell wall, help maintain the peptidoglycan layer of the cell envelope, and are important determinants of cell shape and size during cell growth (Holtje, 1998; Scheffers and Pinho, 2005). Derivatives of penicillin or sub-classes of β-lactams have varying affinities for PBP’s (Tomasz 1979).

The primary cause of cell death by β-lactams was originally thought to be cell lysis due to the breakdown of the cell envelope (Tomasz 1979). It was hypothesized that inhibition of cell wall biosynthesis caused cell death because of increased internal
pressure due to increased growth without accompanying cell wall expansion. This notion was further supported by the finding that active protein synthesis was required for penicillin-mediated cell lysis (Strominger and Tipper 1965; Tipper and Strominger 1965). However, when strains of pneumococci that lacked murein hydrolases (enzymes which digest and make holes in the cell wall) were treated with β-lactams, lysis did not occur, suggesting a more complex mechanism (Tomasz, Albino et al. 1970; Tomasz 1974; Tomasz 1979). Furthermore, it was later discovered that some β-lactams preferentially bound PBP's with roles in cell division (PBP-2 and PBP-3) and induce changes in morphology rather than lysis (Spratt 1975; Spratt and Pardee 1975). These above observations collectively suggest multiple or more complex mechanisms for β-lactam mediated cell death.
1.3 Inhibitors of bacterial DNA replication and repair

DNA replication and repair are crucial, tightly controlled processes in bacteria, as well as in eukaryotes. In *E.coli*, replication is initiated at the origin of replication (oriC) of the circular bacterial chromosome and proceeds outward in a bidirectional manner. Results of various molecular and biochemical experiments with *E.coli* suggest as many as 30 proteins play parts in the replication process, while the number is far greater for eukaryotes. Key replication proteins include DNA gyrase (a type of topoisomerase), which relaxes supercoils in DNA, DNA helicase, which separates the DNA strands during replication, and single-stranded DNA-binding proteins, as well as primase for generating primers needed for lagging strand DNA replication. DNA ligase forms the covalent bond in the lagging strand, while DNA polymerases I-V all play differing roles during the replication and repair processes. The processes of replication and repair in eukaryotic and prokaryotic cells share overall features, but there are significant differences. For example, some of the general differences reflect the cellular localization of replication, recombination and repair. In bacteria, which lack a distinct nucleus, all three processes occur in the bacterial “cytoplasm” but in eukaryotes these processes are restricted to the nucleus. The bacterial genome consists of one circular chromosome with one origin of replication (oriC), highly conserved amongst gram negative bacteria, whereas eukaryotes have multiple chromosomes with multiple origins of replication. In addition, one key difference is that, in *E.coli*, there is only one DNA Pol III which is responsible for synthesizing both the leading and the lagging strand, whereas in eukaryotic cells, there are multiple polymerases. Structural difference between the proteins involved can also be exploited for synthetic antibiotic development, which in turn would reduce human toxicity.
1.3.1 Quinolones

The quinolone antibiotics are a class of broad spectrum antibiotics which were first discovered in 1962 (Lesher et al., 1962). Unlike most antibiotics, which were originally isolated from bacteria or fungi, these drugs are completely synthetic derivatives of nalidixic acid, a byproduct of the synthesis of chloroquine (Rubinstein 2001). Nalidixic acid is rarely used in clinical settings today due to its high toxicity, but second (ciprofloxacin), third (levofloxacin) and fourth (gemifloxacin) generation derivatives, classified on the basis of their chemical structures and qualitative differences in their mechanisms of action, are extensively used (Lu, Zhao et al. 2001; Rubinstein 2001). Quinolones interfere with the normal topology of the bacterial chromosome by irreversibly binding to DNA gyrase (topoisomerase II) or topoisomerase IV (Chen, Malik et al. 1996; Drlica and Zhao 1997; Champoux 2001; Drlica, Malik et al. 2008). Several studies have shown that the preferential binding to one topoisomerase over the other is dependent on the gram negative or gram positive classification of the bacterium (Gellert, Mizuuchi et al. 1976).

DNA gyrase, the primary target in E. coli, which is crucial for transcription, repair and recombination, is responsible for negative supercoils in the DNA (Gellert, Mizuuchi et al. 1976; Wang 1996). It is composed of gyrA and gyrB subunits, which form a complex on DNA substrates (Mizuuchi, O'Dea et al. 1978). Most gyrase inhibitors bind the GyrA subunit, which causes the induction of lesions in the DNA, leading to widespread double-strand breaks and compromising the entire chromosome (Maki, Takiguchi et al. 1992; Chen, Malik et al. 1996). The cascading events following large-scale DNA damage induce the SOS response and cell division arrest (Hanawalt 1966; Drlica, Malik et al. 2008). Irreversible binding of DNA gyrase inhibitors stalls all
replication and repair processes along with transcription and causes cell death (Kreuzer and Cozzarelli 1979; Critchlow, O'Dea et al. 1997; Drlica and Zhao 1997; Couturier, Bahassi el al. 1998; Cox, Goodman et al. 2000). Though much is known about the mechanism of cell death induced by gyrase inhibitors, there are indications that other factors are still at play which are yet to be understood. Because the addition of low concentrations of protein synthesis inhibitors during quinolone treatment reduces the rate of cell death, more investigation into the mechanism of quinolone action is still required (Drlica, Malik et al. 2008).

1.4 Inhibitors of protein synthesis in *E.coli* (ribosome inhibitors)

Protein synthesis in bacteria is a complex and highly conserved process with three distinct phases: initiation, elongation and termination (Kohanski, Dwyer et al.). Initiation involves assembly of the components of ribosomal subunits (50S and 30S subunits), the mRNA, the first aminoacyl tRNA and the intitation factors to form the initiation complex (Kohanski, Dwyer et al.). Growth of the peptide chain during the elongation phase involves addition of amino acids to the carboxyl end of the growing chain and is aided by three elongation factors (EF-Ts, EF-Tu and EF-G). Peptide bond formation between the amino acids is catalyzed by ribosomal RNA acting as a ribozyme (Noller 1991).

Inhibitors of protein synthesis are a large group of antibiotics and are usually divided into two subclasses, inhibitors of the 50S subunit and inhibitors of the 30S subunit. Macrolides like erythromycin derivatives and amphenicols (chloramphenicol) inhibit the 50S ribosomal subunit, while the 30S is inhibited by the tetracycline and
aminocyclitol (aminoglycoside) families of drugs (Brock and Brock 1959; Weisblum and Davies 1968; Chopra and Roberts 2001).

1.4.1 Inhibitors of the 50S ribosomal subunit

Inhibitors of the 50S subunit include the oxazolidinones and amphenicols (chloramphenicol), which inhibit the peptidyl tranferase, and the macrolides (erythromycin and derivatives), lincosamides, and streptogramins, all of which interfere with the transpeptidation/ translocation steps (Katz and Ashley 2005; Mukhtar and Wright 2005). Working models for the mechanisms of action of the 50S subunit inhibitors include blocking the association of the peptidyl tRNA with the ribosome to varying degrees and blockage of the peptidyltranferase reaction by steric hindrance, resulting in the eventual dissociation of the peptidyl tRNA (Menninger and Otto 1982; Vannuffel and Cocito 1996)

1.4.2 Inhibitors of the 30S ribosomal subunit

Two major subclasses of 30S ribosomal subunit inhibitors are the aminoglycoside and tetracycline families of antibiotics. Tetracyclines block the binding of charged tRNAs to the ribosomes and are generally bacteriostatic against gram-negative bacteria (Chopra and Roberts 2001). Naturally derived aminoglycosides are among the few classes of antibiotics that are broadly bactericidal (Kohanski, Dwyer et al.). The binding of aminoglycosides to the 16S rRNA causes enough conformational change to result in mismatching of tRNAs with the mRNA codons and subsequent protein mistranslation (Weisblum and Davies 1968). Treatment with aminoglycosides does not result in immediate halting of protein synthesis but rather causes the insertion of mistranslated proteins into the membrane, which subsequently induces a myriad of cascading events that lead to cell death.
1.5 *E. coli* as a model organism and experimental workhorse

*E. coli* has been extensively studied as a model organism since Lederberg and Tatum first described bacterial conjugation in 1946 (Muller and Grossniklaus; Lederberg and Tatum 1946). It played an integral part in the development of phage genetics and the isolation of the first DNA polymerase (Bessman, Lehman et al. 1958; Lehman, Bessman et al. 1958). This bacterium has also played a central role in our efforts to understand metabolic pathways, mechanisms of chemotaxis, concepts of transcriptional regulation, DNA replication and repair and pathogenesis (Jacob and Monod 1961; Schaechter 2001; Vladimirov and Sourjik 2009). Cultivated strains (e.g. *E. coli* K12) are well-adapted to the laboratory environment and are used as molecular tools for cloning purposes, including cloning genes from other organisms (Muller and Grossniklaus).

A commonly used *E. coli* K12 strain (Blattner, Plunkett et al. 1997) has 4453 genes, of which 4297 are predicted to code for proteins. Only 307 (7%) are essential for cell viability under normal laboratory growth conditions (Riley, Abe et al. 2006). Databases such as COG, EcoCyc and STRING contain annotations for many of the genes. COGs, or Cluster of Orthologous Groups, is a method of classifying proteins by comparing the protein sequences of completely sequenced genomes with each cluster containing proteins of paralogous proteins from at least three lineages (Tatusov, Koonin et al. 1997). COGs are subsequently classified into functional categories as described by Table 1.
| A | RNA processing and modification |
| B | Chromatin structure and dynamics |
| C | Energy production and conversion |
| D | Cell cycle control and mitosis |
| E | Amino acid metabolism and transport |
| F | Nucleotide metabolism and transport |
| G | Carbohydrate metabolism and transport |
| H | Coenzyme metabolism |
| I | Lipid metabolism |
| J | Translation |
| K | Transcription |
| L | Replication and repair |
| M | Cell wall/membrane/envelope biogenesis |
| N | Cell motility |
| O | Post-translational modification, protein turnover, chaperone functions |
| P | Inorganic ion transport and metabolism |
| Q | Secondary structure |
| T | Signal transduction |
| U | Intracellular trafficking and secretion |
| Y | Nuclear structure |
| Z | Cytoskeleton |
| R | General functional prediction only |
| S | Function unknown |

Table 1 - COG categories
However, there is a lack of experimental data supporting biological functions for nearly 40% of the genes in *E. coli* (Riley, Abe et al. 2006). The large number of non-essential genes suggests buffering in the laboratory conditions tested, as is evident from genome-wide genetic interaction experiments done on *E. coli* and other organisms such as yeast and worms (Tong, Evangelista et al. 2001; Tong, Lesage et al. 2004; Lehner, Crombie et al. 2006; Tong and Boone 2006; Butland, Babu et al. 2008; Typas, Nichols et al. 2008). Interestingly, recent work by Hillenmeyer et al. (2010) showed that 97% of yeast genes are essential for optimal growth in at least one of the conditions tested (Hillenmeyer, Ericson et al.). It is well established that genes do not function in isolation but instead are components of molecular pathways, which, in turn, are components of interconnected networks that modulate all major biological functions such as cellular behavior and responses to stress. Experimental elucidation of these networks is important for our understanding of basic biological functions of the organism. Given the prominent role that *E. coli* plays in antibacterial drug discovery, our lack of understanding of cellular pathways and networks also acts as a barrier for further drug development. Because of high rates of resistance to existing drugs, it has become increasingly important to use new genomic tools to expand our knowledge of the global molecular organizations of bacterial species. An important advance in this regard was the development of the Keio collection of *E. coli* K12 strains containing individual deletions of 3985 non-essential genes, each marked by the insertion of a kanamycin resistance cassette (Baba, Ara et al. 2006)
1.6 Systems biology approach to elucidate molecular mechanisms

Systems biology, a field of research that studies the interactions between components of biological systems and how the interactions modulate the behaviour of the system, has been gaining momentum for over a decade. However, the notion of network biology is not new, and it can be argued that it truly began with the landmark research of Francois Jacob and Jacques Monod on the *lac* operon in *Escherichia coli* (Jacob and Monod, 1961) and of Mark Ptashne on bacteriophage lambda's lytic/lysogenic switch (Ptashne, 1967). Their work led to the notion that genes, proteins and other biomolecules work together in complex networks (Jacob and Monod 1961) giving rise to the concept of systems biology. Today this approach involves studying interactions among components of biological systems and how these interactions modulate the behaviour of these systems.

Recent advances in genomics, such as the sequencing of key model organism genomes and high throughput technology development, have propelled the fields of molecular and systems biology. New tools like genome-wide deletion sets, over-expression arrays, microarrays and protein arrays are being exploited to study biological systems. Tong et al. (2001) first developed Synthetic Genetic Arrays (SGA), a high-throughput, automated, plate-based pinning method that mates α and β yeast strains for assaying genetic interactions between genes in *Saccharomyces cerevisiae*. The premise behind SGA, as illustrated in Figure 4, is that, whereas each of the mutations or deletions maybe non-lethal on its own, double mutants would give rise to synthetic lethal (SL), synthetic sick (SS), or suppressing phenotypes if the genes work in the
same or compensatory pathways to perform an essential function (Tong, Evangelista et al. 2001). The concept of synthetic lethality was, however, first described by C.B. Bridges in 1922 (Bridges, CB, 1922).

Figure 4- Redundancy in parallel pathways for essential processes. Deletions of genes in pathways are marked by X. If genes in parallel pathways which combine to perform an essential function are deleted, the resulting phenotype is either synthetic sick or synthetic lethal. The last panel demonstrates the same fundamental principle except that, instead of deleting both genes, one gene product is inhibited by a drug.
Since then, SGA technology has been applied to restricted sets of genes in epistatic miniarray profile (E-MAP) studies by Krogan et al. (Schuldiner, Collins et al. 2005) and has been used on a much larger scale by Costanza et al (2011). SGA methods have also been developed for analysis of the fission yeast *Saccharomyces pombe* (Baryshnikova, Costanzo et al.; Roguev, Wiren et al. 2007). Similar SGA technology based on the bacterial conjugation system, named eSGA, was developed to examine genetic interactions in *E.coli* (Butland, Babu et al. 2008; Typas, Nichols et al. 2008). In principle, the relatively “simple” biological circuits of *E.coli* should enable one to perform such genetic and phenotypic research with relative ease.

Phenotype-based screening can also be done utilizing small molecules instead of mutations as tools to interrogate biological pathways (Figure 4). This use of small molecule chemicals instead of genetics to modulate protein function has been termed ‘chemical genetics’ (Schreiber 1998). This method offers certain advantages in comparison with genetics, because the effects of drugs can be observed in a dose-dependent manner and the levels of inhibition of essential pathways can be varied, whereas gene deletions create an all or nothing effect. In principle, this method of perturbing pathways can also complement pure genetic approaches.

Pioneering studies in chemical genetics were carried out with the budding yeast *Saccharomyces cerevisiae* (Sturgeon, Kemmer et al. 2006). Among the earliest studies were those done by Giaever and colleagues, who were originally involved in the creation of the bar-coded yeast deletion collection and subsequent studies functionally profiling the yeast genome using this collection (Birrell, Giaever et al. 2001; Birrell, Brown et al. 2002; Giaever, Chu et al. 2002; Steinmetz, Scharfe et al. 2002). This group also developed a novel technique known as haploinsufficiency profiling (HIP), which
took chemical genetics a step further (Giaever, Shoemaker et al. 1999; Giaever, Flaherty et al. 2004). In the HIP assay, a pool of heterozygous deletion strains (one copy of the gene deleted in yeast), uniquely bar-coded for the identities of the deleted genes, is grown in the presence of a drug of interest. The drug further inhibits the remaining gene product, resulting in a lethal phenotype. After treatment, the genomic DNA is isolated, PCR amplified and hybridized to microarrays. Strains that are hypersensitive to the drug become less abundant during the growth in the presence of the drug. The proteins which are encoded by the genes deleted in the strains that "drop out" are candidate targets of the drugs.

As a proof of principle, this group tested 10 chemically diverse compounds with known targets against the complete bar-coded yeast heterozygous deletion collection (Giaever, Flaherty et al. 2004). They were able to identify previously known interactions, including established targets of certain drugs. For example, in their experiments using methotrexate, they were able to not only identify its target, DFR1, which encodes dihydrofolate reductase, but also several other genes in the folic acid biosynthesis pathway. In their methotrexate screens, they also identified previously functionally uncharacterized genes, which at first glance did not seem to participate directly in the drug target pathway, but were later predicted to be involved in the drug’s transport (Giaever, Flaherty et al. 2004). Their work demonstrated that it was possible to use this method to not only identify targets of drugs but also reveal novel interactions and new links between drugs and the pathways they perturb.

Genome-wide chemical genetic studies have also been done in bacteria. Li et al. (2004) used a multicopy suppression method to identify the cellular targets and possible resistance mechanisms for novel antimicrobials identified through high-throughput
screening. High-copy or multicopy suppression involves the creation of a plasmid-encoded genomic library followed by a screen for clones that have a suppressor phenotype. This method was used previously to identify genes that are capable of suppressing the activities of antibacterials, antifungals and even antiparasitics (Chen and Bishai 1998; Delling, Raymond et al. 1998; Launhardt, Hinnen et al. 1998; Cotrim, Garrity et al. 1999; Miura, Kaneko et al. 1999; Calabrese, Bille et al. 2000; Apfel, Locher et al. 2001; Tsukahara, Hata et al. 2003). These studies showed that the use of protein overexpression for chemical screens can identify proteins that contribute to resistance (mostly efflux pumps) and/or putative targets (whose overexpression enables the cell to survive under treatment conditions).

For their study, Li et al. (2004) first screened a hyperpermeable strain of E.coli for sensitivity to over 8000 small molecules, which led to the identification of some 49 compounds with antimicrobial activities. Subsequently, suppression screens were performed with these lead compounds. Their work revealed that the most common suppressors were multidrug efflux pumps, and they concluded that most of these compounds were susceptible to efflux from the cell. However, it was found that two of the lead compounds targeted the product of the gene folA (Li, Zolli-Juran et al. 2004), and these were subsequently proven to be competitive inhibitors in the folic acid biosynthesis pathway. Their proof-of-principle study suggested that their method is one which could be generalized for a variety of other model organisms. Though this method has many merits, one problem is the large number of efflux pumps identified in the screens, questioning the merit of screening such a large number of compounds. In this particular case, there is also the issue of using a hyperpermeable strain for initial screening, as clinical pathogens are not in most cases hyperpermeable. Finally, one
might also argue that the use of protein overexpression could cause multiple
downstream effects in the cell which cannot be accounted for. Nevertheless this method
offers much promise, especially in combination with other large-scale validation
methods.

Recently, other groups, notably those of Miller, Kohanski, Tavazoie and Gross,
have made use of genome-wide deletion arrays to perform chemical genetic screens in
*E.coli*. Their results have given us certain insights into the antimicrobial drug discovery
process (See Chapter 4 for a more detailed discussion). Although our current level of
knowledge of prokaryotic genomic architecture is quite extensive, it is nowhere near
complete. A systems level approach using chemical genetics could be important for
filling in some of the remaining gaps in our knowledge.
1.7 Antibiotic resistance

Few new antibiotics are being developed, and the existing arsenal of antibiotics will be insufficient for treating infectious diseases over the long term (Bax, Mullan et al. 2000; Coates, Hu et al. 2002; Barrett and Barrett 2003). Resistance is an inevitable byproduct of use of antibiotics. A recent study suggested that there were only 6 antibacterials out of more than 500 drugs in the late stages of clinical testing (Fernandes 2006). At the same time, infection rates are on the rise for certain diseases, and our ability to predict their course and nature is insufficient (e.g. for the recent outbreak of SARS and unexpected links between ulcer and H. Pylori) (Brown and Wright 2005).

As microorganisms continue to evolve and adapt, they also acquire multiple resistance mechanisms, and many have developed resistance to every major class of antibiotics in clinical use (Sefton 2002). The current strategy in antimicrobial research of modifying the structures of existing antibiotics already in clinical use is clearly failing short. In light of the alarming rise in resistance rates and failing drugs, the pharmaceutical industry needs to focus on developing new classes of drugs with novel mechanisms of action. In recent years, new tools such as genome-wide deletion collections, synthetic genetic arrays and new-generation sequencing technologies have offered a plethora of potential new molecular targets. An alternative possibility is the targeting of multiple pathways, each non-essential on its own, whose combined disruption is lethal for the bacterial cell.
1.8 Rationale

Chemical genetics offers a way to generate specific chemical or drug profiles, which can be used in conjunction with other information on genes and pathways to identify biological pathways being perturbed by various drugs. In this study, as outlined in Figure 5, I used the Keio collection of *E. coli* K12 deletion strains, along with a smaller collection of potentially hypomorphic alleles of essential genes, to screen an array of commonly used antibiotics and several other chemicals (Table2). This allowed me to create chemical-genetic profiles for each antibiotic, which not only gave us insights into the molecular mechanisms of the drugs but also into the molecular mechanisms of biological pathways. This may help the design of better antibiotics that target various pathways or combinations of drugs that can reduce rates of drug resistance.
Figure 5-Experimental pipeline for chemical genomic study of E. coli. The Keio collection in 348 format is pinned onto LB rich media plates containing drugs and appropriate no-drug control plates. Digital images are taken and colony sizes are generated using an in-house colony imager. Log ratio scores are then generated and further analysis is performed to find significant hits. Refer to materials and methods section for more details.
Chapter 2
Materials and Methods

2.1 Drugs, strains and media

All drugs listed in Table 2 were purchased from Sigma and stock solutions were made in water or ethanol in 10mM concentrations. *E. coli* non-essential single gene deletion mutants were obtained from the Keio deletion collection (Baba, Ara et al. 2006). In brief, Baba et al replaced the open reading frames with kanamycin cassettes using the lamda red recombinase system. Strains of *E. coli* containing potentially hypomorphic SPA-tagged (sequential peptide affinity purification tag) essential genes were described previously in work from our lab (Zeghouf, Li et al. 2004; Butland, Babu et al. 2008; Hu, Janga et al. 2009). In short, the lamda red recombinase system was used to add the SPA-tag to the C-terminal of a target protein. All experiments utilized Luria Bertani (LB) medium plates in the presence and absence of drugs.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Target Cellular Process</th>
<th>Target Genes</th>
<th>High Conc</th>
<th>Low Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td></td>
<td>pbpA</td>
<td>32µM</td>
<td>16µM</td>
</tr>
<tr>
<td>Cyclodextrine</td>
<td></td>
<td></td>
<td>25µg/mL</td>
<td>12.5µg/mL</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>Cell and membrane biogenesis inhibitors</td>
<td>pbpA and other penicillin binding proteins</td>
<td>120µM</td>
<td>80µM</td>
</tr>
<tr>
<td>Phosphorylcydicin/P</td>
<td></td>
<td></td>
<td>32µM</td>
<td>16µM</td>
</tr>
<tr>
<td>Phosphoxygenicinin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>Binds to d-alanyl-d-alanine residues of</td>
<td>150ug</td>
<td>100ug</td>
</tr>
<tr>
<td></td>
<td>DNA gyrase inhibitors</td>
<td>peptidoglycan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>gyrA, parC, acrB</td>
<td>1µM</td>
<td>0.5µM</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td></td>
<td>gyrA, parC, acrB</td>
<td>20µM</td>
<td>10µM</td>
</tr>
<tr>
<td>Pefloxacin</td>
<td></td>
<td>gyrA, parC, acrB</td>
<td>0.5µM</td>
<td>0.25µM</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Inhibitor of folic acid biosynthesis</td>
<td>folA</td>
<td>5ug</td>
<td>1ug</td>
</tr>
<tr>
<td>Amikacin</td>
<td></td>
<td>rpsL</td>
<td>2.5 µg/ml</td>
<td>5µg/ml</td>
</tr>
<tr>
<td>Azithromycin</td>
<td></td>
<td>rplV, rplD</td>
<td>8µM</td>
<td>5µM</td>
</tr>
<tr>
<td>Doxycycline</td>
<td></td>
<td>rpsL, rpsD, rplU</td>
<td>10µM</td>
<td>7µM</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td>rplV, rplD</td>
<td>40µg/ml</td>
<td>20µg/ml</td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td>rpsL</td>
<td>1µg/ml</td>
<td>0.5µg/mL</td>
</tr>
<tr>
<td>Hygromycin</td>
<td>Inhibitor of Protein Synthesis</td>
<td>rpsL</td>
<td>100ug</td>
<td>50ug</td>
</tr>
<tr>
<td>Rifampicin/Rifampin</td>
<td></td>
<td>rpoB, rpoC</td>
<td>20µM</td>
<td>10µM</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td></td>
<td>rpsL</td>
<td>50µM</td>
<td>40µM</td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
<td>rpsL</td>
<td>5µM</td>
<td>1µM</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>rpsL</td>
<td>5µg/mL</td>
<td>2.5µg/mL</td>
</tr>
<tr>
<td>Tobramycin</td>
<td></td>
<td>rpsL</td>
<td>10µM</td>
<td>5µM</td>
</tr>
<tr>
<td>Neomycin</td>
<td></td>
<td>rpsL</td>
<td>80µg/ml</td>
<td>40µg/ml</td>
</tr>
<tr>
<td>MMS</td>
<td>No known target in E.coli but implicated in replication and repair</td>
<td></td>
<td>0.0025%</td>
<td>0.001%</td>
</tr>
<tr>
<td>CP2/Promazine</td>
<td>No known target in E.coli</td>
<td></td>
<td>0.4mM</td>
<td>0.275mM</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>No known target in E.coli</td>
<td></td>
<td>0.8mM</td>
<td>0.4mM</td>
</tr>
<tr>
<td>Imipramine</td>
<td>No known target in E.coli</td>
<td></td>
<td>1.2mM</td>
<td>1mM</td>
</tr>
</tbody>
</table>

Table 2- Drugs screened in this study
2.2 Drug concentration optimization and screening

The "Keio" collection of *E. coli* deletion strains and strains containing potentially hypomorphic SPA-tagged essential genes were assayed for their sensitivity to 26 drugs representing the various major classes of antibiotics: inhibitors of cell wall and membrane biogenesis, inhibition of protein synthesis, and inhibition of DNA replication and repair. Also screened were the DNA damaging agent MMS and three functionally related common antipsychotic drugs. Strains were pinned onto LB plates in the absence or presence of drugs. Appropriate drug concentrations were determined by pinning randomly selected plates of the Keio collection (25 percent of the collection) onto plates containing various concentrations of a given drug lower than the recommended MIC as outlined in Figure 6. The two lowest concentrations (arbitrarily call “high” and “low”) at which sick or lethal phenotypes were observed were chosen for full genome-wide screens.

Screens were performed using the replica-pinning RoToR-HDA benchtop robot (Singer Instruments), and reproducible observations were ensured by pinning each mutant strain four times. The plates were incubated at 32°C for 17-24 hours and images were taken with a high resolution digital camera (Canon Powershot A640, 10 Megapixels).
The lowest concentration of a given drug resulting in at least some sick or lethal phenotypes was used along with the next higher concentration to screen the entire strain collection.

Figure 6-Optimization of drug concentrations
2.3 Imaging and score generation

Colony sizes were generated by using an in-house automated image processing system (Butland, Babu et al. 2008). A calibrated software algorithm (in Java) was used which identified colonies arrayed in grid format and determined the fitness as a function of growth rate. The raw scores were normalized to each plate as a whole and corrected for biases such as plate edge effects and inter-plate variation effects. Replicate scores for each mutant were collapsed into one score by using the median value for each mutant in each condition. For each screen condition, a Q/R ratio was calculated, where Q is the median size of a mutant colony in LB medium supplemented with a drug and R is the median size of that mutant in LB medium without drug across all screens. This was then normalized to the median Q/R ratio of the pool for that particular screen. Final log ratio scores for each mutant were generated by taking the log₂ of the normalized Q/R ratio. Synthetic sickness/lethality (aggravating interactions) were defined as negative log ratio scores, whereas positive scores corresponded to alleviating interactions. Scores not statistically different from zero were deemed neutral.

2.4 Functional enrichment and statistical analysis

For each drug at each concentration, we assembled two sets of genes that corresponded to aggravating and alleviating effects on the growth of the *E. coli* colonies containing mutations in these genes.

As described above, the growth was measured by using the log-ratio score ('score', hereafter). We defined score-cutoffs for aggravating and alleviating effects by identifying an aggravating (or alleviating) set of genes that is maximally enriched with
respect to one or more COG categories. For clarity, I describe below the generation of aggravating gene lists. Alleviating gene lists were generated in a similar manner.

For each drug we generated two lists of genes ranked according to their log-ratio scores in each of the two screening concentrations. Each list was then subjected to an enrichment procedure in which the top 'K' genes that exhibited the most statistically significant enrichment of COG classes were selected.

The enrichment analyses were done as follows. Each gene was annotated according to its COG classes. Classes R (General Functional Prediction only) and S (Unknown Function) were discarded due to poor annotations for those genes. For a set of K genes, starting from the top of a list, the expected and observed distributions of COG classes were computed using the COG annotations for the whole *E. coli* genome and the annotations of the K genes in the set. The expected number of genes for a given COG category is defined as the (#of genes in that category/genome size) x K. The observed number of genes for each COG category is the number of genes in the set (the set of K genes) that belonged to that category.

We used the chi-square ($\chi^2$) statistical test to reject the null hypothesis that the distribution of observed COG classes was the same as the expected distribution of classes under a random selection of genes. In order to maintain test accuracy, COG classes with an expected number of genes that was smaller than 6 were discarded from the test. The chi-square test provides a p-value that defines the likelihood of the null hypothesis. For each ranked list, we evaluated lists of top ranked genes beginning at K=50 and continuing to K=250 in increments of 10 genes. We then selected the list of K genes that yielded the lowest p-value.
Finally, we merged the two lists generated for each drug in a similar manner. We started with the list that yielded the lower p-value and continuously added to it genes from the top of the other list as long as the COG enrichment continued to improve (i.e., the p-value decreased). For each drug, we were left with a statistically significant and functionally enriched set of genes.

In order to independently evaluate the significance of the selected genes and their corresponding COG classes we also computed the probability of randomly generating a greater difference between the expected and observed numbers of genes in each COG class. For each class where the observed number of expected genes was greater/lesser than the expected number of genes, we used the binomial theorem to calculate the probability of a random event that yields an observed number of genes that is equal to or greater/lesser than the actual number of observations. These probabilities helped to identify COG classes that are independently enriched (or deprived) without the correlation between COG classes within the context of the chi-square test.
Chapter 3
Results

3.1 Screening and data analysis

The flow chart for our screening procedure is shown in Figure 5. We screened the Keio collection of strains containing deletions of the non-essential genes along with our collection of SPA-tagged (sequential peptide affinity purification tag) strains containing potentially hypomorphic alleles of the essential genes against all three classes of antibiotics and a small set of other chemicals listed in Table 1. Each drug was tested at two different concentrations, and an in-house colony imaging system was utilized to generate log ratio scores based on the phenotypic fitness of each mutant growing in the presence of a drug compared with the fitness of the same mutant growing in the absence of the drug (see Chapter 2 for more details). Twenty-six full-genome screens were successful out of 57 drugs tested, each at two drug concentrations. I was then left with over 200,000 data points for the 26 different drug screens, each at two different concentrations. Most of the data points were normally distributed with neutral growth or no phenotypic change in the presence of drug compared to the no drug control. Small subsets of mutants exhibited significant phenotypic changes in the presence of a drug compared with the no drug controls. Mutants hypersensitive to drugs were deemed as “aggravating” and resistant ones as “alleviating”.

To further examine the subsets of mutants that showed phenotypic changes in the presence of drug treatments, we employed a data analysis method that involved generating a statistically significant cut-off based on the enrichment of particular
Clusters of Orthologous Groups of proteins (COG categories) (Tatusov, Koonin et al. 1997; Tatusov, Natale et al. 2001)) (for details about the analysis and cut-offs, see Chapter 2). For each drug screen at each concentration, we first ranked the genes from the most sensitive (aggravating) phenotype to the most alleviating phenotype according to their log ratio scores. Aggravating and alleviating gene sets were then analyzed separately. We generated distributions of the COG categories for these gene sets and compared them to the statistically expected distribution for *E. coli*. Statistical tests ($\chi^2$) were performed at each cut-off to generate a p-value for the overall distribution of the COG categories. We then took the top ‘K’ genes that exhibited the most significant enrichment of a set of COG classes. Figure 7 shows a graph of the p-values generated for each selection of the top ‘K’ genes causing hypersensitivity to the cell wall drug Cycloserine at low concentration.
Figure 7- P-values at various cut-offs. This example is for mutants hypersensitive to the cell wall drug cycloserine screened at low concentration. The red box represents the optimal p-value of $1.29 \times 10^{-18}$. 

We sometimes found that the two concentrations of a particular drug showed different enrichment profiles for certain COG categories. Figure 8 shows an example of this phenomenon for screens with hygromycin, an aminoglycoside which kills by inhibiting protein synthesis. At low drug concentration, the most enriched COG category (E) for aggravating interactions contains genes involved in amino acid metabolism and transport, whereas the screen at a two-fold higher drug concentration also enriched COG category M containing genes involved in cell wall and membrane biogenesis.

Hygromycin Screen

**Low Concentration**
Genes involved in amino acid metabolism and transport: E

**High Concentration**
Genes involved in amino acid metabolism and transport: E and also genes involved in cell wall and membrane biogenesis: M

Figure 8- Different enrichment profiles at different concentrations of a single antibiotic
To narrow the scope of our analysis, we then examined the union of the genes identified by the two concentrations of a drug in the expectation that a union set could be identified with better COG enrichment (i.e. lower p-value). We devised the union sets by starting with the screen that had the lower p-value for each drug and then adding to that list the top hits from the screen at the other drug concentration. As new genes were added, p-values were calculated on the basis of COG category enrichment, thereby generating a new optimal p-value and COG distribution for each drug. I then found that the union set resulted in lower p-values (more statically significant) in nearly all cases, as shown in Figure 9. Indeed, P < 0.05 was achieved for nearly all screens. For this reason, the genes in the union sets were used for further analyses. Tables 2 and 3 summarize the COG categories enriched for the aggravating and alleviating hits from each screen. It is particularly striking that category C (energy production and conversion) is enriched among the alleviating hits for 17/24 screens, and this will be discussed in more detail in Chapter 4. The COG categories enriched among the aggravating hits for the various screens are discussed below.
Figure 9- Distribution of optimal p-values across all screens. Aggravating and alleviating gene sets were analyzed separately. The boxed-in panel represents the distributions for the union. The table below the bar plots indicates the number of screens that generated gene sets with p<0.05 for COG category enrichment (see text).
<table>
<thead>
<tr>
<th>Drug</th>
<th>Low</th>
<th>High</th>
<th>Union</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>K,M,O,P</td>
<td>E,H,M,N</td>
<td>E,H,I,M,N</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>L,M</td>
<td>T,K</td>
<td>C,K,T</td>
</tr>
<tr>
<td>CPZ</td>
<td>C,K,M,N,T</td>
<td>C,T,O,M</td>
<td>C,K,M,N,T</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>J,L,M</td>
<td>E,L,M</td>
<td>E,F,M,J,O</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>H,J,M</td>
<td>H,J,M</td>
<td>H,J,M</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>L,M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>L,M</td>
<td>E,G</td>
<td>L,M</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>K,T</td>
<td>F,G</td>
<td>K,T</td>
</tr>
<tr>
<td>Hygromycin</td>
<td>E</td>
<td>E,M</td>
<td>E,M</td>
</tr>
<tr>
<td>IMPH</td>
<td>G,M</td>
<td>K,M</td>
<td>G,M</td>
</tr>
<tr>
<td>MMS</td>
<td>L,M</td>
<td>K,L</td>
<td>K,L</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>F,I,J,L,M</td>
<td>K,L,M</td>
<td>F,I,J,L,M</td>
</tr>
<tr>
<td>Phosphomycin</td>
<td>L,M</td>
<td>C,G</td>
<td>C,L,O</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>E,L,M</td>
<td>G,L,M</td>
<td>E,G,L,M</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>K,L,U</td>
<td>J,M</td>
<td>J,M</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>E</td>
<td>E,M,P</td>
<td>E</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>J,L,M</td>
<td>J,K,L,M</td>
<td>F,H,J,L,K</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>G,J,K</td>
<td>G,L</td>
<td>F,J</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>H,J,M</td>
<td>H,J,L,M</td>
<td>H,J,L,M</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>K,M,T</td>
<td>C,K,L,M</td>
<td>K,M,T</td>
</tr>
</tbody>
</table>

Table 3- Enriched COG Categories for Drug Screens (Aggravating Hits) (for description of COGS please refer to Table 1 on page 16)
<table>
<thead>
<tr>
<th><strong>Drug</strong></th>
<th><strong>Low</strong></th>
<th><strong>High</strong></th>
<th><strong>Union</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>C,P</td>
<td>C,P</td>
<td>C</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>J,T,C</td>
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<td>C,K,T</td>
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<td>Azithromycin</td>
<td>C,P</td>
<td>G,H,J,P</td>
<td>C,P</td>
</tr>
<tr>
<td>CPZ</td>
<td>E,G,J</td>
<td>E,G</td>
<td>F,G</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>L,J</td>
<td>C,G</td>
<td>G,C</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>C,E,H,J,L,O</td>
<td>C,L</td>
<td>C,L</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>K,T</td>
<td>C,G</td>
<td>K,T</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>J,O</td>
<td>C,G</td>
<td>F,I,J,O</td>
</tr>
<tr>
<td>Gentamycin</td>
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<td>C,E</td>
<td>C,E</td>
</tr>
<tr>
<td>Hygromycin</td>
<td>C,N,U</td>
<td>C,K,L,T</td>
<td>C,M,T,U</td>
</tr>
<tr>
<td>IMPH</td>
<td>C,J</td>
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<td>MMS</td>
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<td>G,M</td>
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<td>Nalidixic Acid</td>
<td>H,P,U</td>
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<td>C,H,P,U</td>
</tr>
<tr>
<td>Perfloxacin</td>
<td>C,K</td>
<td>C,K,T</td>
<td>C,K,T</td>
</tr>
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<td>Rifampicin</td>
<td>E,H,M</td>
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<td>E,H,J,M</td>
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<tr>
<td>Spectinomycin</td>
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<td>C,L</td>
<td>C</td>
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<tr>
<td>Streptomycin</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
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<td>Tetracycline</td>
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<td>N,K,M</td>
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<td>Tobramycin</td>
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<td>C,K</td>
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<td>Vancomycin</td>
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<td>M</td>
<td>C,M,O,T</td>
</tr>
</tbody>
</table>

Table 4- Enriched COG Categories for Drug Screens (Alleviating Hits)

(For description of COGS please refer to Table 1 on page 16)
3.2 Cell wall and membrane biogenesis

The cell wall and membranes are the first line of defense for bacteria, and an assault to this outer boundary can be quite detrimental to the cell. Among the cell and membrane drugs screened were the frequently used β-lactams along with fosfomycin, cycloserine and vancomycin. These drugs primarily inhibit the peptidoglycan biosynthesis pathway. Peptidoglycan, or murein, is a polymer consisting of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues with peptide chains of three to five amino acids attached to the N-acetylmuramic acid (Russell 1996). This forms a mesh-like structure around the inner membrane. Assembly of disacharride peptide monomer units by enzymes in the cytoplasm or the inner cytoplasmic membrane and transfer of the monomers across the membrane are targeted by only two known antibiotics. Cross-linking and binding of the nascent peptidoglycan to the existing cell wall is targeted by many drugs, including the β-lactams (Russell 1996).

The COG categories enriched in screens with cell wall drugs are summarized in Tables 2 and 3. Among the top aggravating hits from my screens with the cell wall antibiotics are genes that are associated with the drug target pathways. For example, in the case of cycloserine, which targets the products of two genes, ddlA and alr, responsible for making precursor products of the peptidoglycan biosynthesis pathway Feng and Barletta 2003; Imming, Sinning et al. 2006), we find significant enrichment of the COG category M (cell wall and membrane biogenesis) for aggravating hits as shown in Figure 10.
Figure 10 - Distribution of COG classes at optimum union p-value for aggravating hits for the cell wall inhibitor cycloserine. The two most enriched categories are COG classes M (cell wall and membrane biogenesis) and J (translation). For description of COGS please refer to Table 1 on page 16. Expected is the number of hits expected based on the population of annotated members of that particular category. Observed is what is seen in my data set.
Upon closer inspection, I found that genes in category M that showed strong aggravating interactions included genes from the *mur* operon, which are directly in the peptidoglycan biosynthesis pathway (Figure 11). We also identified hypersensitive mutants from related pathways, such as those involved in LPS biosynthesis.
Figure 11 - Overlay of top aggravating hits from the M category of the cycloserine screen onto the peptidoglycan pathway.
Mutations in a surprisingly large subset of genes annotated as being in the J (translation) category of COGs also caused high sensitivity to cycloserine. These genes are illustrated in Figure 12. Among the translation mutants causing hypersensitivity to cycloserine were components of the general translation machinery and many in aminoacyl tRNA synthetases. Most of the genes in the translation category are essential and were only present in our screens as SPA-tagged alleles. It is hard to assess the level of attenuation in these mutants, but compromised translational machinery can lead to slower growth rates and can be highly deleterious for the cell. We do not observe significant growth defects for translation mutants in the absence of the drugs, but the mutants are severely sick or dead in the presence of cycloserine, presumably due to the introduction of an additional stress. Our findings are roughly in agreement with the recent finding by another group showing that mistranslation of membrane proteins can cause cell death in the case of aminoglycoside treatment (Kohanski, Dwyer et al. 2008). It is therefore possible to imagine that compromised translational machinery (hypomorphs in our case) causing mistranslation or lower levels of membrane proteins along with an additional perturbation of the membrane caused by cycloserine treatment could be highly detrimental to the cell.
Figure 12-Hits from cycloserine screens involving genes related to the translation machinery. Also shown in this figure are physical interactions among these proteins and other interactions inferred on the basis of genomic context (i.e. genomic neighborhood, co-occurrence etc in the databases).
In my screens with amoxicillin and penicillin, both of which target penicillin binding proteins, I also observed enrichment for aggravating hits of genes from the membrane biogenesis (M) class (Table 2). Nevertheless, although both drugs are derived from the same chemical backbone, the enriched categories do not greatly overlap. For amoxicillin the enriched categories include genes related to membrane biogenesis (M), coenzyme and lipid metabolism (H and I, respectively), amino acid transport and metabolism (E), and cell motility (N). For penicillin, however, translation (J) and nucleic acid transport and metabolism (F) were also enriched along with lipid metabolism genes (I) and membrane related genes (M). There is an overlap of only 42 genes between the two screens. Table 4 lists these genes and the COG classes to which they belong, of which 9 are involved in cell wall and membrane biogenesis, including mrcB, which is the penicillin binding protein 1B involved in the synthesis of cross-linked peptidoglycan from lipid intermediates. Lipopolysaccharide pathway-related genes and outer membrane proteins such as lolB and tolC were also among the overlapping hits along with some involved in transport and cell division. The lack of overlap between the amoxicillin and penicillin screens for hypersensitivity caused by mutations in genes from varying functional categories might suggest that there are different off target effects of these drugs.
<table>
<thead>
<tr>
<th>COG Class</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall and membrane biogenesis (M)</td>
<td>mrcB, glmS, glum, lolB, murl, kdsA, lpxB, toIC, ycfN</td>
</tr>
<tr>
<td>Cell Division and Chromosome partitioning (D)</td>
<td>ftsA, ftsE, ftsX, ftsK</td>
</tr>
<tr>
<td>Lipid metabolism (I)</td>
<td>plsC, ispU, ispG, acpP</td>
</tr>
<tr>
<td>Transcription (K)</td>
<td>lldR, ascG, cysB</td>
</tr>
<tr>
<td>Signal transduction mechanisms (T)</td>
<td>rcsD, dksA, pphB</td>
</tr>
<tr>
<td>Unknown or poorly characterized (R, S)</td>
<td>shiA, yzcX, yqjB, ycfM, ybgC, yhhW, yafJ, creA, yhbC</td>
</tr>
<tr>
<td>Energy production and conversion (C)</td>
<td>puuC, narl</td>
</tr>
<tr>
<td>Translation (J) and replication (L)</td>
<td>prfA, ycaJ</td>
</tr>
<tr>
<td>Carbohydrate (G), Nucleotide (F), Inorganic ion (P) transport</td>
<td>yeiQ, adk, pstS, zraP</td>
</tr>
<tr>
<td>and cellular transport (U)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5 - Overlapping aggravating hits between amoxicillin and penicillin screens
Phosphomycin, which binds to the \textit{murA} gene product (Eschenburg, Priestman et al. 2005; Eschenburg, Priestman et al. 2005), was unusual in that there was not significant enrichment for genes in the peptidoglycan biosynthesis pathway. As illustrated in Figure 13, the most enriched categories were O (chaperones, post translational modification and protein turnover) and C (energy production and conversion). The cell wall and membrane category (M) was not enriched. MurA, the product of an essential gene, catalyses the first commitment step in the peptidoglycan biosynthesis pathway. If MurA activity is compromised, then deletion of other genes in the pathway may have no additional effect on cell growth. When peptidoglycan biosynthesis is compromised, however, many of the chaperones become essential. It would appear, therefore, that a highly chaperone-dependent process is impaired when peptidoglycan synthesis is compromised. This maybe energy production, which occurs in association with the cell membrane. Resistance to phosphomycin occurs because of changes in the glycerophosphate transporter, the transporter through which the drug enters the cell.
Figure 13- Distribution of COG Categories for aggravating hits from the phosphomycin screen. For description of COGS please refer to Table 1 on page 16. Expected is the number of hits expected based on the population of annotated members of that particular category. Observed is what is seen in my data set.
3.3 DNA replication and repair pathway inhibitors

DNA replication and repair processes are very well conserved among bacterial species and are ideal targets for broad spectrum antibiotics. We screened two fluoroquinolones, ciprofloxacin and perfl Roxacin, whose targets are DNA gyrase and DNA topoisomerase subunit A, along with trimethoprim and nalidixic acid, which target dihydrofolate reductase and gyrA, respectively (Crumplin and Smith 1975; Maxwell 1999; Maxwell and Howells 1999; Imming, Sinning et al. 2006). The COG categories enriched in these screen are summarized in Table 4. As expected, the hits from these screens included genes in the same pathways as the drug targets. For example, in the trimethoprim screen, the top aggravating hits were genes related to the folic acid biosynthesis pathway, including the hypomorph of the drug target, folA, along with genes involved in the SOS response and chromosome segregation. There was significant enrichment for the COG category, coenzyme metabolism, which includes the drug target pathway, as well as replication and repair (category L). As shown in Figure 14, surprisingly, the COG categories translation (J) and membrane biogenesis (M) also showed enrichment after treatment with trimethoprim.

Nalidixic acid, which targets DNA gyrase, showed an enrichment profile similar to that of trimethoprim. The most enriched categories included replication and repair genes (L) along with membrane biogenesis (M) and translation (J) genes as shown in Figure 15. Interestingly, the COG category for nucleotide metabolism (F) was also enriched, suggesting that a combination of nalidixic acid plus inhibition of precursor pathways for DNA synthesis results in synthetic sickness.
Figure 14- Distribution of COG categories for aggravating hits from the trimethoprim screens. For description of COGS please refer to Table 1 on page 16. Expected is the number of hits expected based on the population of annotated members of that particular category. Observed is what is seen in my data set.
Figure 15-Distribution of COG categories for aggravating hits from the nalidixic acid screens. For description of COGS please refer to Table 1 on page 16. Expected is the number of hits expected based on the population of annotated members of that particular category. Observed is what is seen in my data set.
For the structurally similar drugs, ciprofloxacin and perfloxacin, which target DNA gyrase and DNA topoisomerase, the COG enrichment profiles were different. For ciprofloxacin the most enriched categories involved cell wall and membrane biogenesis (M) and nucleotide metabolism and transport (F) (Figure 16). Mutants in some replication and repair genes did cause hypersensitivity to the drug, but the functional group as a whole was not significantly enriched. For perfloxacin, however, translation (J), carbohydrate metabolism (G) and inorganic ion transport and metabolism (P), were enriched, whereas replication and repair (L) was de-enriched (Figure 17). As was the case for the structurally related drugs, penicillin and amoxicillin, these observations suggest that the profiles for ciprofloxacin and perfloxacin may be greatly influenced by off-target drug effects. Indeed, it is conceivable that some or many of the advantages derived from replacing one antibiotic with a structurally related one may derive from enhancements of off-target effects.
Figure 16 - Distribution of COG categories for aggravating hits from the ciprofloxacin screen. For description of COGS please refer to Table 1 on page 16. Expected is the number of hits expected based on the population of annotated members of that particular category. Observed is what is seen in my data set.
Figure 17 - Distribution of COG categories for aggravating hits from the perfloxacin. For description of COGS please refer to Table 1 on page 16. Expected is the number of hits expected based on the population of annotated members of that particular category. Observed is what is seen in my data set.
3.4 Inhibitors of protein Synthesis

The COG categories enriched in screens with protein synthesis inhibitors, such as aminoglycosides, tetracycline derivatives and macrolides, are summarized in Table 3. Among the aminoglycosides that I tested were amikacin, gentamicin, streptomycin, hygromycin B and tobramycin, which target the 30S ribosome and the 16S rRNA (Gill and Amyes 2004). However, our strain collection did not include hypomorphs of the ribosomal protein genes. These genes are difficult to tag, probably because the ribosomal proteins are small and close to each other and the rRNAs are within the ribosome. In spite of this, the aggravating hits were still enriched for other genes involved in translation (category J) in the screens with amikacin and tobramycin.

In general, there was substantial diversity in the COG categories that were enriched among the aggravating hits with protein synthesis inhibitors. This may not be surprising, because inhibiting protein synthesis has the potential of indirectly effecting virtually every other process in the cell. No single category was enriched for more than half of the 12 protein synthesis inhibitors that were screened, but among the more commonly enriched categories were M (cell wall and membrane biogenesis, 5 screens), J (translation, 4 screens), E (Amino acid transport and metabolism, 3 screens), and K (transcription, 3 screens).

3.5 Screening with anti-psychotics

Initially, we decided to screen many kinds of compounds. While doing this, we realized that most non-antibiotics were very hard to screen with a plate-based assay. For example a very high concentration of a compound was often required to see the effect; which make the plates cloudy. Since our analysis was based on imaging, this
became unfeasible. However, I was successful in performing screens with three structurally similar compounds, haloperidol, chlorpromazine and imipramine, used to treat mental health disorders. In humans, these drugs bind cell surface receptors. One previous study showed that chlorpromazine changes the membrane morphology of *E. coli* (Amaral 1991). When aggravating hits from these screens were analyzed, there was significant enrichment for COG category M (cell wall and membrane biogenesis) in all cases, especially a subset of the *rfa* genes involved in lipopolysaccharide biosynthesis (Figure 18).
Figure 18- Aggravating hits from screens with anti-psychotic drugs are enriched for COG category M (cell wall and membrane biogenesis), especially genes involved in lipopolysaccharide synthesis.
Chapter 4
Discussion

4.1 Patterns of COG category enrichment across antibiotic screens

When enrichment profiles for COG categories across all the screens were examined, certain patterns emerged. First, more than half the drugs (16/24), regardless of their class, showed an enrichment for mutants in the cell wall and membrane biogenesis category (M), as shown in Figure 19. Specifically, 17 mutants in the cell wall and membrane biogenesis category showed hypersensitivity with one or more of the drugs from the four drug classes that were examined. As might be expected, a few of these genes were drug efflux pumps and large membrane spanning proteins (e.g. tolC) but a surprisingly large proportion of the mutants involved genes required for lipopolysaccharide (LPS) synthesis (Figure 20). Some small scale studies showed previously that deletion of the LPS genes can make bacteria more vulnerable to certain antibiotics (Amaral 1991), but I have identified a set of core LPS genes whose deletions sensitize

*E. coli* to many different antibiotics at sub-minimal inhibitory concentrations. One possible explanation might be that deletion of these proteins can make the cells hyperpermeable. The products of these genes may be potential targets for combination antibiotic therapy.
Figure 19 – Heat map of COG categories enriched among the aggravating hits for all the drug screens. Note the enrichment across most drug screens of the categories: M (membrane) and J (Translation). For description of COGs please refer to table on page 16.
Figure 20 - Common aggravating cell wall and membrane hits across all the screens
It was also obvious that for nearly half the screens (11/24), there was significant enrichment for genes in the translation category (J), and this was true across all classes of antibiotics. Mutations in 39 genes caused hypersensitivity to one or more of the drugs in all classes, these genes including 16 of the 20 *E. coli* tRNA synthetases as well as many other translation factors (Figure 21). These genes are all essential and their hypomorphs appear to lose viability in the presence of many different kinds of antibiotics. It was recently found that reactive oxygen species (ROS) damage the proof-reading capacity of the tRNA synthetases (Ling and Soll) (Figure 22). It has been proposed that cell death in the presence of many classes of bacteriocidal drugs results from the production of ROS (Ling and Soll). These observations were made using lethal concentrations of the various drugs. In my studies, I found that hypomorphic alleles of translation proteins cause similar kinds of sensitivity to a variety antibiotics at sublethal concentrations, presumably for the same reasons.
Figure 21 - Translation related genes where mutations cause hypersensitivity across all categories of antibiotics.
Modified from Ling, J – PNAS 2010

Figure 22- Model of antibiotic-induced cell death via oxidative stress
Moreover, genes in the energy production and conversion categories (C) were highly enriched for alleviating interactions across most of the screens (17/24) and across almost all categories of drugs (tetracycline and doxycycline were two exceptions) (Figure 23). The enriched genes involved almost all subunits of NADH dehydrogenase I and many proteins in the TCA cycle. It was recently found that deletions of some of these genes reduce the free iron in the cell, thereby reducing the production of ROS (Girgis, Hottes et al. 2009).

It is, therefore, possible that the generation of ROS following exposure to sub-minimal inhibitory concentrations of antibiotics causes cell sickness or death when the concentration of one of the tRNA synthetases targeted by ROS is lower in a strain with a hypomorphic allele. In situations where an antibiotic may not reach concentrations sufficient for bactericidal or bacteriostatic effects (e.g. in biofilms), simultaneous treatment with a chemical developed to target a translation factor (e.g. a tRNA synthetase) may enhance clinical effectiveness.
Figure 23 - Heat map of COG categories enriched among the alleviating hits across all the screens. There is a significant enrichment for COG class C (energy production and conversion) for all screens. Most aminoglycosides, cell wall and membrane inhibitors, and DNA replication and repair inhibitors show significant enrichment for genes in this category.
4.2 Mutants causing hypersensitivity across a large number of drugs

Below is a list of mutants that showed up as hits across screens from the antipsychototics drugs and all the antibiotic classes. These mutants are classified into a variety of different categories. Though some genes are clearly membrane related such as tolA and ompA are in varied categories such as replication and repair.

<table>
<thead>
<tr>
<th>gene</th>
<th>COG</th>
<th>description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ftsA</td>
<td>D</td>
<td>ATP-binding cell division protein involved in recruitment of FtsK to Z ring</td>
</tr>
<tr>
<td>acrA</td>
<td>M</td>
<td>multidrug efflux system</td>
</tr>
<tr>
<td>holA</td>
<td>L</td>
<td>DNA polymerase III, delta subunit</td>
</tr>
<tr>
<td>tolA</td>
<td>M</td>
<td>membrane anchored protein in TolA-TolQ-TolR complex</td>
</tr>
<tr>
<td>fabA</td>
<td>I</td>
<td>beta-hydroxydecanoyl thioester dehydrase</td>
</tr>
<tr>
<td>ompA</td>
<td>M</td>
<td>outer membrane protein A</td>
</tr>
<tr>
<td>hns</td>
<td>NU</td>
<td>global DNA-binding transcriptional dual regulator H-NS</td>
</tr>
<tr>
<td>ascG</td>
<td>K</td>
<td>DNA-binding transcriptional repressor</td>
</tr>
<tr>
<td>tolC</td>
<td>MU</td>
<td>transport channel</td>
</tr>
<tr>
<td>rfaE</td>
<td>M</td>
<td>fused heptose 7-phosphate kinase -1- heptose 1-phosphate adenylyltransferase</td>
</tr>
<tr>
<td>rfaP</td>
<td>-</td>
<td>kinase that phosphorylates core heptose of lipopolysaccharide</td>
</tr>
<tr>
<td>dnaA</td>
<td>L</td>
<td>chromosomal replication initiator protein DnaA, DNA-binding transcriptional dual regulator</td>
</tr>
</tbody>
</table>

Table 6 - Frequent Fliers (hits that appear in one or more drug screens across different categories of drugs)
4.3 Perspectives

The genome-wide drug hypersensitivity screens described here provide some insight into what is happening in the cell at low concentrations of the drugs. There are microniches in the body, along with biofilms, where the antibiotic concentrations never reach inhibitory levels. Consequently, genomic screens at sub-lethal concentrations of antibiotics are quite relevant. The drugs screens also provide a glimpse of the interconnectivity between the different functional categories of genes.

Antimicrobials existed for millions of years before their therapeutic powers were harnessed. For the last fifty years, biochemical approaches related to drug-target interactions and inhibiting the activities of “target” proteins have been the focus in antibiotic research. Pharmaceutical companies have designed large scale screening assays to screen thousands of compounds against essential proteins thought to be “good” targets. Over the decades, finding new drugs, which can ward off resistance also became important. Antibiotic resistance is partly a by-product of the extensive use of antibiotics in agriculture and clinical settings today. This increased rate of resistance has been attributed at least partially to overuse and improper use of antibiotics in clinical settings, reducing the shelf life of current antibiotics.

Since the full sequencing of many bacterial genomes, we now have new tools for bacterial research including microarrays, protein arrays, full genome over-expression arrays and gene deletion arrays. These new genomic tools have expanded the current number of targets for antibiotics from 30 to some 300 potential essential gene targets in *E. coli*. There are also over 3000 non-essential *E. coli* genes which can potentially also be exploited for antimicrobial research (Schaechter 2001; Riley, Abe et al. 2006).
Recent genomic studies have indicated that the molecular circuitry in bacteria is far more complex than was previously thought (Butland, Babu et al. 2008; Typas, Nichols et al. 2008). In particular, over 90% of *E. coli* genes are non-essential, and pathway redundancy is very extensive. In light of these recent findings, our approach of finding magic bullets which target single gene products to kill bacteria might not be the ideal way to develop new antibiotics.

Much like full genome epistasis studies among genes *E.coli*, chemical genomic methods can help identify pathways and elucidate molecular mechanisms. In this study I created chemical genomic profiles for 21 antibiotics and 3 anti-psychotic drugs in the hopes of identifying unifying themes in drug mechanisms of action. Recently, several other groups used the Keio collection for similar screens. For example, Miller’s group screened 7 antibiotics and defined a set of genes that showed hypersensitivity to particular antibiotics (Tamae, Liu et al. 2008). One key difference between Miller’s work and the work presented here is that the Miller group used a subjective method of scoring for sick or lethal phenotype, whereas in my study I used a object method of quantifying the colony sizes and performed further statistical analysis. Another such study done by Kohanski’s group used this type of screening combined with classical biochemical experiments to show that ROS-mediated cell death occurs after treatment with bactericidal antibiotics (Kohanski, Dwyer et al. 2008). Tavazoie`s group showed that deletions removing key components of aerobic respiration or iron sulffer cluster genes result in resistance to killing via antibiotic treatment (Girgis, Hottes et al. 2009).

Recently a larger compendium of chemical-genetic profile has been created by Carol Gross’ group. They screened over 300 different chemicals against the collection (Nichols, RJ et al.2011). Similar to my screen results, they were able to see a set of
mutants that showed hypersensitivity to a variety of drug classes. This larger study also provided some great insight into gene essentiality under various conditions.

An important difference in my study compared to some of the above mentioned was that the screens were done at lower than MIC concentrations. My results are pertinent to clinical settings, because there are niches in particular organs where that the levels of antibiotics never reach bacteriostatic or bactericidal concentrations. This is especially true for biofilms, which are also of special concern for public health. Therefore, it is important for us to examine genes that govern sensitivity to low levels of antibiotics. By creating sensitivity profiles based on low antibiotic concentrations, one identifies the cell’s intrinsic resistome (i.e. genes whose deletions hypersensitize the cell to antibiotics). Not surprisingly, deletions of efflux pumps and large membrane spanning proteins cause sensitivity to a large number of drugs, indicating the presence of generalized mechanisms of resistance towards the chemical backbones of commonly used antibiotics. More interestingly, a large number of mutants in the core lipopolysacharide synthesis pathway also showed sensitivity across many classes of drugs. There has been some evidence in small scale studies showing that deletion of the LPS genes can make bacteria more vulnerable to certain antibiotics (Tamaki and Matsuhashi 1973). I have identified a set of core LPS genes whose deletions sensitize *E. coli* to diverse antibiotics at sub-minimal inhibitory concentrations. The products of these LPS biosynthesis genes could serve as potential targets for drugs to be used in combination therapies. A similar argument could be made for targeting certain translation proteins (e.g. tRNA synthetases) with combination therapies.
There is a strong argument for performing such screens because hypersensitivity profiles such as these can help us identify the core set of genes that enable us to design combination therapies. Other extensions of this work could be: A) taking the short lists of hits from deletion screens and using over-expression data or proteomic approaches to identify targets of drugs; B) using the information from the screens regarding genes and pathways whose deletions show hypersensitivity to certain drugs to design cocktails of drugs used at sub-minimal inhibitory concentrations, reducing toxicity and possibly reducing rates of resistance: C) further characterizing some of the unknown genes that come up as hits in the screens; D) creating a compendium of chemical genetic profiles which can be combined with other genetic and proteomic data to elucidate molecular mechanism in bacterial cells; and E) extending such screens to other microbial communities such as soil and gut microbiota, which can give us insights into the spread of resistance in nature and also drug metabolism in the body.
References


Appendices (if any)

Note due to the large data size tables are attaches in electronic format
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