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Use of genomics to select antibacterial targets

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ABSTRACT

The problem of antibiotic resistance has eroded the usefulness of our arsenal of effective antibiotics. There is a need for new strategies to discover and develop new, effective drugs. The advent of the microbial genomics era has provided a wealth of information on a variety of microorganisms. This has allowed the identification and/or validation of a number of gene products that could serve as targets for the discovery of novel antibacterial agents. New genetic techniques and approaches have arisen in an attempt to exploit this newly available genomic data. Both random and targeted gene disruption efforts have proven effective in this process. Many of these methods would have been difficult to accomplish without DNA sequence and bioinformatics analyses. Several targets have been selected to further characterize and screen for inhibitors and one has yielded two clinical candidates.

Antibiotic resistance has increased over the past two decades to involve virtually every human pathogen and every class of antimicrobials in clinical use today [1–3]. Contributing to the dilemma, only two new classes of anitibiotics have been introduced over the past 30 years: the oxazolidinones, represented by linezolid, and the lipopeptide daptomycin [4]. Most early antibiotics were discovered empirically, usually through a whole-cell screening campaign. The relatively few existing classes of antibiotics currently in use target only a small subset of essential bacterial targets. Clearly, a new approach to the discovery of new antibacterials is required and innovative strategies will be necessary to identify novel, effective agents. The question remains as to the number of additional essential targets that might yield effective inhibitors with the potential to be developed into new drugs. Over the past decade, a number of pharmaceutical and biotechnology companies have initiated target-based efforts to discover new inhibitory chemotypes and the ability to use this approach has been facilitated by the availability of genomic information.

The genomics era began in 1995 with the publication of the complete chromosomal sequence of *Haemophilus influenzae*, the first such sequence from a free-living organism [5]. The flood of genomic information over the following decade has had a great impact on microbial research [6–9] as the number of reported complete chromosomal sequences now numbers into the hundreds and continues to grow. Along with new species, different strains within species are also being added to this ever-expanding database allowing for comparative analyses. To view these microbial genomic sequences, see http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi or http://www.tigr.org/tdb. In regard to drug discovery, these databases provide both opportunities and questions. It was widely assumed that this information would identify a large number of new targets that would rapidly lead to the discovery and advancement of novel drugs. In retrospect, this was a naïve assumption considering the difficulties of advancing a compound from any therapeutic area into human clinical use. It also became apparent that selection of a target to pursue in the quest for a new inhibitor is not always an obvious choice as there are no guarantees that all essential bacterial targets are ''druggable''. A general listing of desirable properties for an ideal bacterial target are listed in Table 1. However, even if a target possesses all of these properties

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Table 1 – Desirable properties of a good antibacterial target

Target property	Why desirable	Alternative
Essential	Inhibition leads to bacterial stasis or death	Inhibition of virulence may also be effective
Present in multiple bacterial species	Potential for broad-spectrum inhibitor of bacterial growth	A narrower spectrum may also be desired
Selectivity	Greater selectivity for bacterial target may result in less toxicity in humans	Effective drugs are in use against targets with significant homology to human equivalents
Bactericidal	Killing bacteria is optimal	There are several effective bacteriostatic drugs on the market
In vitro functional assay	Enzymatic assay could aid drug discovery	There are alternative methods to discover inhibitors

there is no guarantee that inhibitors can be discovered that can be developed into useful drugs.

There are two sets of targets that have been identified over the past decade of the microbial genomics era. The first group of targets is one whose genes are essential for in vitro growth (i.e., growth on bacterial culture media). These are identified by some of the methods discussed below. The assumption is that inhibition of these targets would lead to cellular growth arrest or death of the pathogen. All currently used antibiotics in the clinic will inhibit or kill bacteria grown in vitro. This also allows for traditional susceptibility testing in the microbiology laboratory to identify which antibiotics are appropriate for use in treatment of the infection. A second group of targets include those that are only expressed in vivo or are essential for growth and survival in the infected host. Inhibition of these targets may or may not inhibit the bacteria in vitro but would presumably inhibit the progression of the infection and/or affect survival of the infectious organism in the host. These targets would be considered to be essential or attenuating in vivo and are identified by different means than in vitro essential targets.

Bioinformatic analyses of genomic data can greatly assist the target selection process by identifying genes that are conserved across numerous bacterial species. An assumption is that at least a subset of these genes would include those essential in multiple organisms and, therefore, candidates for further characterization. An example of one such bioinformatic study was described in a Concordance analysis of five microbial genomes [10]. Sequences with significant homology to a yeast chromosome were excluded. The final result was the selection of 89 sequences in common with all five species. Although such in silico analyses provide valuable information to the scientist, "wet biology" is still necessary to validate targets for further work.

1. Determination of gene essentiality

Gene essentiality can be considered from two approaches, both of which are aided greatly by the availability of DNA sequence information. One is by random mutagenesis where the target gene is not yet identified prior to analyses of the experimental data. An alternative tactic is targeted mutagenesis where potential targets are identified prior to experimental analyses and confirmed after study of the data. Both approaches are discussed below and are listed in Table 2. A summary of some methods that have been successfully applied over the recent past to whole bacterial genomes can be found in Table 3.

However, it was initially difficult to extend any analyses beyond *Escherichia coli* or *Salmonella typhimurium* due to the relative lack of required genetic manipulation in most other bacterial species. The majority of the original bacterial genetic techniques were developed in *E. coli*. Many of these methods work poorly or not at all in other organisms, particularly gram-positive bacteria with the exception of *Bacillus subtilis*. The arrival of the genomics era has spurred more attempts to genetically manipulate many other bacteria with some success [29]. The use of new shuttle vectors, inducible expression systems, and electroporation has led to an expansion of genomic analyses in other gram-negative and gram-positive bacteria beyond *E. coli* and *B. subtilis*.

A common feature found in prokaryotic genomes that must be kept under consideration is that of operons [30]. Many bacterial genes are located in transcriptionally linked units under the control of a common promoter. This can complicate the analysis of essentiality through gene disruption since genes downstream of an essential gene disruption cannot be assigned a designation unless individually disrupted themselves (Fig. 1). Genomic sequence data can assist this process by identifying potential operons throughout the chromosome

Table 2 – Examples of methods for determination of gene essentiality in bacteria

Method	Bacterial species	Reference
Random mutagenesis		
Plasmid insertion	*S. pneumoniae*	[11]
Conditional lethals	*E. coli*, *S. typhimurium*	[12,13]
Transposon	*E. coli*, *H. pylori*, *M. genitalium*	[14–16]
Shotgun antisense	*S. aureus*	[17]
Cassette mutagenesis	*H. influenzae*	[18]
Targeted gene disruption		
Plasmid insertion	*E. coli*, *S. pneumoniae*	[19,20]
Allelic exchange	*H. pylori*	[21]
Crossover PCR	*E. coli*	[22]
Targeted conditional lethals	*E. coli*, *S. aureus*	[12,23]
In vivo virulence		
Signature-tagged mutagenesis	*S. typhimurium*	[24]
In vivo expression technology	*S. typhimurium*	[25]
Differential fluorescence induction	*S. typhimurium*	[26]

Table 3 – Potential essential genes identified through gene disruptions of bacterial genomes

Organism	No. of genes	No. of potential essential genes	Method(s) used	Reference
Bacillus subtilis	4101	271	Plasmid insertions, conditional lethals	[27]
H. pylori	1590	344	Transposons	[15]
S. pneumoniae	2043	113	Plasmid insertions	[20]
S. aureus	2588	658	Antisense	[17]
E. coli	4291	620	Transposons	[14]
M. genitalium	484	265–350	Transposons	[16]
H. influenzae	1709	256	Transposons	[28]

as well as providing information to perform subsequent genetic experiments.

1.1. *Random mutagenesis*

Some of the earliest experimental determinations of essential genes were accomplished through the random generation of conditional lethal mutants that were incapable of growth under non-permissive growth conditions [13,31]. These were often selected after treatment of bacterial cultures with chemical mutagens such as nitrososguanidine (NTG). The most common conditional lethal mutants displayed a temperature-sensitive (ts) phenotype where growth was halted or greatly diminished at elevated or reduced temperatures. These were most often isolated in *E. coli* strains that grow optimally at 35–37 °C. Many ts mutants were capable of growth at the permissive temperature of 30 °C but were not viable at a non-permissive temperature of 42 °C. It is likely that single point mutations can result in thermolabile proteins that are still capable of supporting growth at the permissive temperature. The isolation and characterization of ts mutants led to early assignments of essential genes. However, even at the permissive temperature, protein function is probably not optimal and, therefore, may be more susceptible to inhibition. Taking advantage of this assumption, screening assays were developed against selected targets in effort to identify new inhibitors [32].

Another means of identification of potential essential genes through random mutagenesis is through the use of transposons [33]. These mobile genetic elements can randomly insert themselves into a bacterial chromosome often causing the disruption of the gene located at the site of this insertion [34]. Transposons can carry antibiotic resistance markers that can be used to select for insertion events. DNA sequence elucidation of flanking sequence followed by a search of genomic databases can precisely map the location of the transposon on the chromosome. If the insertion event is in an essential gene, the result is often an inviable cell. Therefore, transposon insertions actually identify non-essential genes that can then be subtracted from the genetic complement. Such a global transposon mutagenesis approach was used in *Mycoplasma genitalium* which contains the smallest number of genes, 517, in an independently replicating organism [15]. After more than 2200 insertion events were analyzed, it was determined that 265–350 of the 484 protein-encoding genes were essential for the growth of this bacterium. More recently, a similar analysis was performed on *Helicobacter pylori* to identify essential genes [14]. Microarray tracking was used to map the positions of over 5000 transposon mutants. The results showed that 344 genes had no insertions and most were presumed to be essential. Interestingly, only 11% of these genes overlapped the essential gene sets of all other bacteria tested while 55% were essential in some organisms but not others.

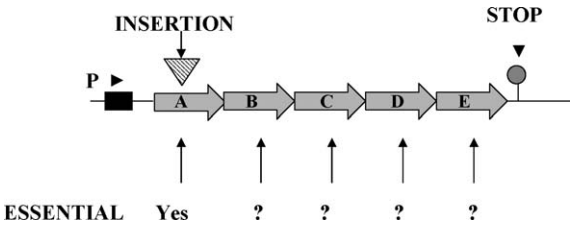

Fig. 1 – Drawing of a bacterial genomic operon. Box with arrow above indicates a common promoter for the operon. Triangle indicates a DNA insertion event. Circle on top of line indicates a transcriptional stop. An insertion in essential gene A can obscure the essentiality analyses of downstream genes.

Another method to localize transposon insertions is "genetic footprinting" using in vitro *mariner* mutagenesis [35]. This technique, designated genomic analysis and mapping by in vitro transposition (GAMBIT), generates a pool of in vitro transposon insertion mutants. Primers are generated from both the transposon and from genes of interest to generate PCR products that are then run on a gel. Since insertions into essential genes will not be represented, gaps of band sizes for that gene will appear and the gene can be assigned as essential. This represents a systematic but indirect method of identifying essential genes since only non-essential genes can acquire transposon insertions. This technique was first applies to naturally transformable organisms such as *H. influenzae* and *Streptococcus pneumoniae* [35] and later extended to other bacteria such as *Pseudomonas aeruginosa* [36], *H. pylori* [37], and *E. coli* [13].

Cassette mutagenesis offers another means of accomplishing random mutagenesis on a bacterial genome. Restriction enzyme-digested chromosomal DNA fragments are circularized, re-cut with a second restriction endonuclease, and an antibiotic resistance marker is inserted. After re-circularization, digestion with the original restriction enzyme linearizes the DNA for transformation into the recipient organism. Selection for the marker antibiotic resistance phenotype identifies clones that have integrated the fragment into the chromosome and retained viability. As with transposon mutagenesis, this technique identifies genes non-essential

to viability on in vitro growth media. It does not directly identify essential genes but does allow for the removal of non-essential genes from the genetic complement of the organism. Cassette mutagenesis has been successfully applied to the naturally transformable *H. influenzae* [17].

A shotgun antisense RNA approach has also been described for random target gene identification [16,38]. Antisense RNA binds to mRNA inhibiting subsequent translation and protein production. If the gene product is essential for growth, this can lead to cell death. A chromosomal fragment library is created in shuttle plasmids downstream of a regulatable promoter and amplified in *E. coli* to increase DNA quantities. The pooled library then is introduced into the strain of interest. Clones are screened in the presence and absence of inducer for detectable phenotypes. DNA sequence analyses then can determine the identity and orientation of the gene fragment and those in the correct antisense orientation are selected for further study. This method identified 658 candidate essential genes in *Staphylococcus aureus* with more than 150 of these found to be conserved in additional bacterial species [16]. Another antisense study yielded similar results on *S. aureus* [38].

1.2. *Targeted gene disruption*

The availability of genomic DNA sequence data has allowed for a more direct way to identify essential bacterial genes through targeted gene disruptions. There are several methods for creating such disruptions but the resulting mutants would be expected to be viable only in genes non-essential for growth. Experiments should be repeated multiple times and include positive and negative controls before a gene should be designated as essential. Examples of bacterial targeted disruptions are discussed below.

Plasmid insertion mutagenesis is probably the most rapid, direct means of generating gene disruptions (Fig. 2A). A short (100–500 base pair) internal sequence from the target gene is cloned into a suicide vector, incapable of replication in the host bacterial cell, and containing a selectable antibiotic resistance marker. When this recombinant plasmid is introduced into the host cell, a single crossover recombination event occurs between the internal gene fragment and the homologous sequence on the chromosome. The result is an insertion of the plasmid and disruption of the target gene. Although a gene duplication event occurs, two incomplete copies of the gene are created in the chromosome because an internal fragment was used. If antibiotic selective pressure is maintained, the mutation is a stable event. In the absence of the antibiotic, there is the possibility of plasmid excision and restoration of a wild-type phenotype.

An example of plasmid insertion mutagenesis was described in an attempt to identify essential genes in *S. pneumoniae* [19]. After identification of potential gene targets through bioinformatic analysis [10], internal gene fragments were cloned into a suicide plasmid vector and transformed into the host strain. Insertion-duplication events were selected by resistance to chloramphenicol conferred by the resistance gene carried by the plasmid. All such transformants were considered as indicative of non-essential genes and catalogued while events that resulted in no antibiotic-resistant colonies after multiple attempts were assumed to involve essential genes. This was done in a relatively high-throughput manner and resulted in the designation of 113 essential genes out of the total of 347 candidates tested.

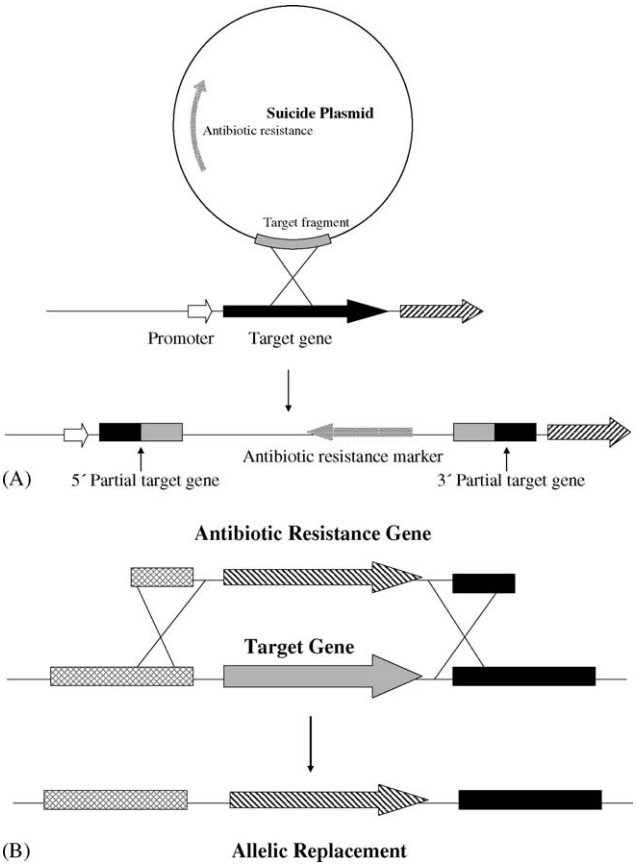

Fig. 2 – (A) Plasmid insertion mutagenesis. An internal fragment of the target gene is cloned onto a plasmid containing a selectable marker. The suicide plasmid vector (incapable of replication in the host cell) is introduced into the bacterium under selection for the antibiotic resistance marker. Bacterial colonies result from homologous recombination of the entire plasmid into the chromosomal gene followed by a single-crossover, insertion-duplication event for the target gene where two incomplete and inactive gene copies are generated. If the target gene is essential, this will be a lethal event and cells will not be viable. (B) Allelic replacement. An antibiotic resistance gene is cloned between flanking sequences of the target gene resulting in replacement of the target with the resistance marker. When introduced into the bacterial cell under antibiotic selection, a double-crossover event results in a replacement of the chromosomal gene copy with the resistance gene. If the target gene is essential, this will be a lethal event cells will not be viable.

Another method of targeted gene disruption is allelic exchange ([20]; Fig. 2B). DNA sequences flanking each end of the gene of interest are placed on each end of an antibiotic resistance gene and the resulting construct is then cloned into a suicide vector and introduced into the desired host cells by transformation or electroporation. Selection for allelic exchange, where the antibiotic resistance marker replaces

the target gene in the chromosome via a double crossover event, occurs in the presence of the antibiotic in the growth medium. The outcome is a stable event since the target gene is now no longer present on the chromosome and cannot be regenerated as in the plasmid insertion method described above. Allelic exchange can also be done using overlapping PCRs to create the target gene cassette as a DNA fragment versus cloned into a plasmid. However, the host cell must be capable of internalizing and recombining DNA fragments into its genome, thus, allelic exchange can be difficult to successfully accomplish in some bacteria. Also, the frequency of double crossover events can be low with the possibility of illegitimate recombination that can result in integrations in unexpected locations. This can lead to misinterpretations of experimental results. The use of counter-selectable markers on the plasmid vector can help eliminate single crossover or illegitimate recombinations [39]. Expression of counter-selectable markers leads to the death of the host bacterial cell under defined growth conditions.

Crossover PCR can create in-frame, tagged deletions in bacterial chromosomal DNA. This has been successfully performed in *E. coli* using a temperature-sensitive plasmid vector carrying markers for both positive and negative selection of chromosomal integration and excision events [21]. This technique can be especially useful in the analyses of operons since precise deletions occur without disruption of adjacent genes thus eliminating the potential complication of polar mutations. The temperature-sensitive plasmid is transformed into the target strain with selection for integrative events at the non-permissive temperature. The integrates then are resolved by growth at 30 °C and selection using the counter-selectable marker. Colonies are screened for loss of the original antibiotic-resistance phenotype and target gene deletion verified by PCR. Of course, a successful target deletion will result in a viable colony only for non-essential genes.

Another method of targeted gene disruption that has been recently described in *E. coli* uses targeted conditional lethal amber mutations (UAG stop codons) created in essential genes [22]. Using DNA sequence information and PCR, linear DNA fragments are created containing an amber mutation in the gene of interest. The fragments are electroporated into the bacterial cells where they integrate by a double crossover event in the chromosome. This is only possible in the presence of an inducible amber suppressor also introduced into the cells. The result is replacement of the wild-type gene with a copy containing an amber mutation that can be successfully expressed under permissive conditions.

1.3. *Examples of bacterial genomic-validated targets*

The use of the above techniques for target analysis has resulted in a number of bacterial targets validated for essentiality. Some of these have been found to be widespread in numerous bacteria while others appear unique to a species. Some have assigned functions while others remain unidentified. The known targets include proteins involved in numerous cellular processes including: DNA replication, transcription, translation/protein synthesis, cell wall, and cell division among others [27]. One target, peptide deformylase, was validated with the assistance of genomic information [40]. Subsequently, several inhibitors of peptide deformylase have been identified with two compounds advancing into human clinical trials [41]. Other targets under intensive study include tRNA synthetases, DNA gyrase subunit GyrB, and the fatty acid biosynthetic pathway especially FabI. Enzymes in cofactor biosynthetic pathways also appear promising [42]. The expectation is that inhibitors for several additional targets will also advance into the drug development process in the near future.

1.4. *In vivo essential genes*

Genomics can also assist in the identification of targets that are essential to the establishment and/or maintenance of an in vivo infection or in the expression of virulence factors. This is more challenging than identifying in vitro essential targets since these may or may not have effects on growth on medium in the laboratory. Three methods developed to seek genes important in pathogenesis are listed in Table 3. These are signature-tagged mutagenesis (STM), in vivo expression technology (IVET), and differential fluorescence induction (DFI). Details for each of these methods can be found in the listed references. Comparative genomics has also proven useful in the analysis of bacterial pathogenesis. For example, a recent comparison of the genomes of an early methicillin-resistant *S. aureus* (MRSA) and a later biofilm-producing MRSA strain revealed that many differences in pathogenicity could be attributed to genes contained in pathogenicity islands [43].

1.5. *Other uses of genomics for target selection*

There have been recent efforts using structural genomics to assist in the bacterial target selection process and drug discovery [44]. Genomic sequence data has been used to assist the process of determining a large number of protein structures by high-throughput strategies. Bacterial proteins are amenable to this approach since they are generally smaller, have less post-translational modification, and can often be produced in multiple milligram amounts with *E. coli* host strains and vectors. The resulting structural data of potential targets could help in prioritization. Analysis of enzyme active sites might identify binding pockets that appear to be more ''druggable'', i.e., having a better chance of discovering drug-like inhibitors. With structural information in hand, the in silico techniques such as virtual screening and de novo design can be employed to identify potential lead compounds. Structural knowledge of relevant target binding sites can also be used in the optimization of inhibitory chemotypes, providing the chemist with important information that can be used in the design of additional analogs.

Bacteriophage genomics has been used to identify potential antibacterial targets in *S. aureus* [45]. Bacteriophages have evolved unique mechanism to inhibit the growth of their bacterial hosts. Through DNA sequence analyses of 26 bacteriophages, 31 polypeptide families were found that inhibited growth upon expression in *S. aureus*. This information was used to discover the cellular targets for these peptides and later to develop screens to search for small molecule inhibitors of these essential bacterial targets.

Another area where genomics can assist in target selection is in vaccines [46]. Bioinformatic analyses can predict which proteins are likely to be located on the cell surface or are secreted proteins through the identification of signature motifs or homology to such proteins in other organisms. This so-called ''reverse vacccinology'' has led to some early success with *Neisseria meningiditis* and *S. pneumoniae* [47]. Genomic data can also indicate if the vaccine candidate is unique to the strain of origin, conserved across multiple seroytpes, or is found in other bacteria. One limitation is that, at present, antigenicity cannot be predicted nor can the expression of proteins in vivo although immunization with DNA may circumvent this issue [48,49]. Genomic DNA sequence data in combination with one of the gene disruption techniques described above can create mutants in potential candidates whose virulence can then be determined in vivo.

The microbial genomics era was ushered in with great promise and high expectations. Much progress has been made over the past 10 years in regard to approaches, methods, and data analyses. However, perhaps the most important goal has yet to be achieved: the delivery of a new drug to the clinic aided by the availability of microbial genomics. There are several possibilities for why more success has not been apparent over the past decade. These include the reduction or cessation of antibacterial drug discovery efforts at a number of large pharmaceutical companies due primarily to the perception of insufficient return on investment. Also, the length of time that is often required to move from identification of a valid target to a drug suitable for human clinical trials can be substantial. Without sufficient perseverance and commitment of resources by the parent company, a potential drug may never reach the market place. Finally, it is possible that many of the new targets identified through the use of genomic information may not be valid targets for drugs. Nature may have already shown us the majority of antibacterial targets through natural product inhibitors. Perhaps the next decade will reveal the true value of genomics in the antibacterial drug discovery process.

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