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NEWS AND VIEWS

Evaluating the suitability of essential genes as targets for antibiotic screening assays using proteomics

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Regarded as one of the most fundamental discoveries in modern medicine, antibiotics have revolutionized the treatment of bacterial disease and provided the prerequisite for the development of the modern pharmaceutical industry.

Due to the extensive use of antibiotics, bacteria have evolved by developing an array of resistance mechanisms (Fischbach and Walsh, 2009; Van Hoek et al., 2011). Pathogens displaying resistance include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *enterococci* (VRE) and multidrug-resistant (XDR) *Mycobacterium tuberculosis* (Alekshun and Levy, 2007). With a decreasing antibiotic arsenal, there is an urgent need to discover and develop new antibiotics that can negate bacterial resistance. A central requirement of discovering new antibiotics is the possession of a novel mode of action, often determined by the type of cellular component targeted. The question is how we identify and assess the suitability of bacterial targets for the development of screening assays.

Modern antibiotic discovery has been facilitated by advances in genomics and bioinformatics, which have enabled the comparison of bacterial genomes. This in turn has enabled the identification of broadly conserved genes deemed essential for bacterial cell viability and therefore potential antibiotic targets (Fleischmann et al., 1995; McDevitt and Rosenberg, 2001). The ability to sequence and compare bacterial genomes also initiated the concept of a minimal bacterial genome following the study to examine the gene complement of *Mycoplasma genitalium*, a parasitic bacterium possessing the smallest genome (580 kb) of an independently replicating cell (Mushegian and Koonin, 1996). Further studies revealed that 265–350 of the 517 gene complements of *M. genitalium* were deemed essential for cell viability, including 111 coding sequences of unknown function. With the identification of essential genes, researchers have acquired a list of potential cellular targets that could be exploited for antibiotic discovery (Hutchison et al., 1999). Due to their indispensable role, essential genes provide ideal targets for antibiotics since they possess a high degree of conservation among bacteria (Jordan et al, 2002). A large proportion of these genes encode enzymes which have historically provided the most effective antibiotic targets due to their high affinity for ligand interaction (Gao and Zhang, 2011).

It was originally assumed that essential genes contributed equally to maintaining cell growth; however this was subsequently addressed by evaluating the degree of requirement for cell viability (stringency) of 4 suspected *E. coli* essential gene targets, *fabI*, *murA*, *acpP* and *ftsZ* using inducible antisense RNA expression to achieve a titration of mRNA transcripts and bacterial growth rate (Goh et al., 2009). Stringency was evaluated by measuring the relationship between a decrease in mRNA and reduction in bacterial growth rate to develop a Minimum Transcript Level (MTL50), the amount of mRNA necessary to maintain 50% cell viability. The results demonstrated a hierarchy of stringency with *acpP* displaying the most stringently required gene, followed by *ftsZ*, *fabI* and *murA*. Determining gene stringency using mRNA transcripts however may not provide the most accurate representation of actual target protein due to the complex regulatory nature of the transcriptome.

The expression of proteins is highly dependent on mRNA translation efficiency. This can be influenced by levels of secondary and tertiary structures within the transcript, which can dictate ribosome binding thus translation initiation. Cellular levels of mRNA are further complicated by the rate of mRNA turnover and post-translation modification (Maier et al., 2009). Consequently mRNA transcription is not coordinated in a proportional manner with translation resulting in a lack of correlation between mRNA and protein abundances (Laurent et al., 2010). Furthermore, proteins typically undergo

post-translational modification which cannot be inferred from RNA or genomic sequences; therefore mRNA levels are not necessarily indicative of active proteins. The measurement of mRNA must therefore be supplemented by direct measurement of the cell proteome if stringency of essential genes is to be determined conclusively. Advances in liquid chromatography tandem mass spectroscopy (LC-MS/MS) coupled with novel isotope quantification strategies have enabled sensitive and accurate quantification of targeted proteins from complex matrices, such as whole cell lysates.

LC-MS/MS typically involves the tryptic digestion of a protein sample into peptides which is separated by liquid chromatography. Specific precursor ions are subject to collision activated dissociation (CAD) and are directed to a mass analyzer producing a mass spectrum of fragmented peptides that can be used to determine amino acid sequence and consequently protein identification.

The LC-MS/MS process can be adapted to perform shotgun, directed or targeted detection and quantification (Domon and Aebersold, 2010). The shotgun approach is aimed at quantifying changes in global proteins by recording a mass spectrum at a particular time (survey scan) which is used in quantification. At the same time, a precursor ion is selected using heuristics based on signal intensity and undergoes fragmentation to generate a mass spectrum (product ion scanning). In the directed approach, peptide ions relating to the protein under investigation are detected in an initial survey scan and preselected to form an inclusion list used in a second LC-MS/MS analysis for the peptide fragments of interest. The targeted approach in contrast uses selective reaction monitoring (SRM) and requires prior identification of a target peptide including the retention time, m/z of precursor ion and measurement parameters.

Quantification in LC-MS/MS is achieved using an array of stable isotope labeling strategies. For absolute quantification, homologs of tryptic peptides are labeled with a heavy isotope (typically C13, 15N) and spiked at a known concentration into an experimental sample prior to LC-MS/MS to provide an internal standard, a method referred to as absolute quantification or AQUA (Gerber et al., 2003). Since the concentration of internal standard is known, it is directly related to the total area under extracted ion chromatograms, which can be compared to the extracted ion chromatograms for unlabelled experimental samples. Quantification of the experimental sample is determined by extrapolation from a standard curve (Kline and Sussman, 2010). Selection of a peptide for use as an internal standard is critical and requires it to be unique to the protein under investigation. The peptide chosen must undergo complete proteolysis, be of intermediate length (6–13 residues) to avoid complicated spectra, and not contain reactive residues that are prone to oxidation (methionine, tryptophan), reduction (cysteine) or deamidation (asparagine) (Mayya et al., 2006). Other variations include Protein Standard Absolute Quantification (PSAQ) (Brun et al., 2007), which uses full length labeled proteins synthesized *in vitro* and therefore overcomes limitations associated with AQUA, primarily the inability to account for variation in the efficiency of the proteolysis step. Recent developments of LC-MS/MS based protein quantitation include Absolute Protein Expression measurements (APEX), which can be performed without using heavy isotope labeling. Measurement of protein abundance is based on the theoretical proportionality between the fraction of expected peptides (upon tryptic digestion) for a protein of interest and observed peptides in the interpreted mass spectra. Both should be equal, however due to variation in ionization and other experimental factors; the observed peptides may vary. Application of a correction factor to account for the probability of observing expected peptides restores proportionality and enables protein concentration to be estimated (Lu et al., 2007).

In addition to measuring gene stringency, protein quantification has applications in identifying and validating essential genes as antibiotic targets through examination of the bacterial proteome. The proteome represents the definitive complement of expressed proteins of a cell when grown under a set of defined and stringently maintained growth conditions. The proteome reflects the ability to adapt to stimuli through quantitative expression of specific subsets of proteins unique to the stimulus encountered. An adaptive response can therefore generate a unique proteomic signature, which can be used to distinguish between stimuli, such as exposure to different antibiotics (Brötz-Oesterhelt et al., 2005). Quantifying the proteomic signature and comparing groups of expressed marker proteins to a control enables elucidation of the antibiotic mode of action (Bandow et al., 2003).

Quantifying proteins to determine gene stringency provides a means of selecting viable candidate targets for the development of target specific antibiotic screening assays. Current screening approaches have adopted the use of antisense expression for sensitizing cells through target depletion as a means of evaluating whole cell antibacterial activity of natural products (Singh et al., 2007). Conversely, a recent study utilized an alternate method by depleting target protein using an inducible protein degradation system to assess effects on cell growth (Wei et al., 2011). Irrespective of the method employed, an assessment of target stringency would enable priority targets to be selected. The ability to measure stringency enables a degree of assay standardization to be introduced, for example determining levels of antisense induction required to deplete target protein to 50% can be subsequently used as standard of comparison against other essential gene targets.

The emerging threat of pan-resistant bacteria has made the discovery of novel antibiotics imperative if humanity is to avoid increases in morbidity and mortality rates associated with untreatable bacterial disease. Current studies have provided insight into the subtleties of gene essentiality while advances in LC-MS/MS have provided a means of proteomic

quantification to fully validate the concept of gene stringency. The value in obtaining accurate data at the proteomic level can aid the assessment of the suitability of essential genes as targets in whole cell antibacterial assays for the discovery of novel antibiotics.

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