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Review

Pseudomonas aeruginosa essentials: an update on investigation of essential genes

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Pseudomonas aeruginosa is the leading cause of nosocomial infections, particularly in immunocompromised, cancer, burn and cystic fibrosis patients. Development of novel antimicrobials against *P. aeruginosa* is therefore of the highest importance. Although the first reports on *P. aeruginosa* essential genes date back to the early 2000s, a number of more sensitive genomic approaches have been used recently to better define essential genes in this organism. These analyses highlight the evolution of the definition of an 'essential' gene from the traditional to the context-dependent. Essential genes, particularly those indispensable under the clinically relevant conditions, are considered to be promising targets of novel antibiotics against *P. aeruginosa*. This review provides an update on the investigation of *P. aeruginosa* essential genes. Special focus is on recently identified *P. aeruginosa* essential genes and their exploitation for the development of antimicrobials.

Introduction

Pseudomonas is an extremely versatile Gram-negative bacterium capable of thriving in a broad spectrum of environments (Juhas, 2015b). *Pseudomonas aeruginosa* is an opportunistic human pathogen that is a major causative agent of hard-to-eradicate nosocomial infections. Immunocompromised, cancer, burn, cystic fibrosis and intensive care unit patients with mechanical ventilation are amongst those with the highest risk of being infected by *P. aeruginosa* (Cramer *et al.*, 2012). A number of factors, such as quorum sensing, biofilm formation, horizontal gene transfer and intrinsic resistance to antibiotics, contribute to the pathogenicity of *P. aeruginosa* (Juhas, 2015b, c; Juhas *et al.*, 2004, 2005a, b, 2007a, b, 2008, 2009, 2013; Lister *et al.*, 2009; Sigurdsson *et al.*, 2012; Wiens *et al.*, 2014). Peer-reviewed and continually updated annotations for *P. aeruginosa* strain PAO1, in addition to the comparative analyses of other related *Pseudomonas* species, are stored in the Pseudomonas Genome database (Winsor *et al.*, 2011) and PseudoCyC (Romero & Karp, 2003).

Essential genes are required for the growth and survival of an organism. A number of experimental and computational approaches led to the identification of various sets of essential genes (Boutros & Ahringer, 2008; Christen *et al.*, 2011; de Berardinis *et al.*, 2008; French *et al.*, 2008; Gallagher *et al.*, 2011; Juhas *et al.*, 2012b; Langridge *et al.*, 2009; Lee *et al.*, 2015; McCutcheon & Moran, 2010; Moule *et al.*, 2014; Moya *et al.*, 2009; Rusmini *et al.*, 2014; Sigurdsson *et al.*, 2012; Skurnik *et al.*, 2013; Turner *et al.*, 2015). Essential genes are often divided into two categories, i.e. 'core' and 'accessory' essential genes. 'Core' essential genes are considered to be those genes that are universally indispensable for all living organisms. Therefore, it has been proposed that 'core' essential genes could be exploited as the basic building blocks of the tightly controlled minimal cell factories. 'Accessory' essential genes are required for the survival of individual species and cell types or under specific growth conditions. This makes bacterial 'accessory' essential genes promising targets for the development of novel antimicrobials (Juhas, 2015a; Juhas *et al.*, 2011, 2012a). Whilst there is the assumption that 'core' essential genes could be exploited as antimicrobial targets due to providing a broad host range, there is a real danger that antimicrobials inhibiting the 'core' essential gene would also target the essential human homologue (Juhas *et al.*, 2012a). Significant progress has been achieved in the investigation of essential genes in a number of bacterial species, including the Gram-positive bacterium *Bacillus subtilis* and the Gram-negative bacterium *Escherichia coli* (Hirokawa *et al.*, 2013; Pósfai *et al.*, 2006; Tanaka *et al.*, 2013). It has been shown that the outcome of essential gene identification studies is determined by a number of factors, such as environmental conditions, methodology used and species analysed (e.g. *B. subtilis* and *E. coli* K-12 share only ~50 % of their essential genes) (Juhas *et al.*, 2014).

Although the first reports on *P. aeruginosa* essential genes date back to the early 2000s (Jacobs *et al.*, 2003; Liberati *et al.*, 2006), a number of more sensitive genomic approaches have been used recently to better define essential genes in this organism (Gallagher *et al.*, 2011; Lee *et al.*, 2015; Rusmini *et al.*, 2014; Sigurdsson *et al.*, 2012; Skurnik *et al.*, 2013). This review provides an update on the investigation of *P. aeruginosa* essential genes with particular emphasis on recently identified essential genes and their exploitation for the development of antimicrobials.

Abbreviation: RND, resistance-nodulation-division.

P. aeruginosa essential genes: from the traditional to the context-dependent view

The identification of *P. aeruginosa* essential genes highlights the evolution of the definition of an 'essential' gene. Traditionally, genes were regarded as generally essential or non-essential, with very little information available about the impact of the strain's genetic background and the growth conditions. To identify essential genes, cells were routinely grown in a rich medium, such as LB. Moreover, the results of the essential gene identification study in one organism were often directly applied to another. It is only recently that the importance of the growth conditions and the analysed strain's genetic background for the outcome of the essential gene identification have started to be fully appreciated.

The first genome-wide identification of genes indispensable for the growth of *P. aeruginosa* was performed by Jacobs *et al.* (2003) (Fig. 1). The investigation of >30 000 mutants generated in that study revealed that 12 % (645 genes) of all ORFs of *P. aeruginosa* strain PAO1 lacked transposon insertions. Based on a comparison with the previously identified set of *E. coli* essential genes, it was hypothesized that ~300–400 genes are generally indispensable in *P. aeruginosa* (Jacobs *et al.*, 2003). The subsequent genome-wide analysis employed the mariner transposon *MAR2xT7* to identify essential genes in *P. aeruginosa* strain PA14 (Liberati *et al.*, 2006) (Fig. 1). The investigation of 20 530 unique insertion sites mutants generated in that study (5459 of which were non-redundant) showed that 1454 genes of *P. aeruginosa* strain PA14 lacked insertions (Liberati *et al.*, 2006). Based on the combination of the transposon mutant libraries of *P. aeruginosa* strains PA14 and PAO1, it has been hypothesized that the general *P. aeruginosa* essential gene set consists of 335 genes (Liberati *et al.*, 2006). Recently, the essential gene set of *P. aeruginosa* strain PA14 has been refined employing high-throughput sequencing of transposon libraries (Tn-seq) (Skurnik *et al.*, 2013) (Fig. 1). Investigation of >300 000 mariner transposon insertion mutants generated in that analysis led to the identification of 636 essential genes of *P. aeruginosa* strain PA14. In total, 198 genes of this gene set were found to be essential in *P. aeruginosa* strains PAO1 and PA14 in the previous transposon mutagenesis analyses. Interestingly, 210 putative essential genes of *P. aeruginosa* strain PA14 were completely missing from the genome of *P. aeruginosa* strain PAO1 (Skurnik *et al.*, 2013). These studies show clearly that the *P. aeruginosa* essential gene sets differ significantly between individual strains.

Growth conditions are also important for the outcome of essential gene identification studies (Juhas *et al.*, 2011; Umland *et al.*, 2012). In all the above analyses *P. aeruginosa* was grown in LB medium (Jacobs *et al.*, 2003; Liberati *et al.*, 2006; Skurnik *et al.*, 2013). To differentiate between general and growth-condition-specific *P. aeruginosa* essential genes, *P. aeruginosa* strain MPAO1 was recently grown on six different media, including a medium made from a cystic fibrosis patient's sputum (Lee *et al.*, 2015). The modified Tn-seq approach based on the generation and amplification of single-strand circles with transposon junction sequences (Tn-seq circle method) employed in that study achieved a very high genome coverage. The investigation of the ~1 000 000 transposon insertions mutants generated led to the identification of 551 essential genes (Lee *et al.*, 2015). A comparison with the above studies (Jacobs *et al.*, 2003; Lee *et al.*, 2015; Liberati *et al.*, 2006; Skurnik *et al.*, 2013) revealed that 141 genes were essential in all four genome-wide analyses. The largest proportion of the highly confirmed *P. aeruginosa* essential gene set plays a role in translation, post-translational modification and degradation. Interestingly, this is followed by hypotheticals with unknown function (Fig. 2). Large groups of highly confirmed *P. aeruginosa* essential genes are also involved in cell wall biogenesis, energy metabolism, biosynthesis of amino acids and cofactors, and DNA replication and modification (Fig. 2). However, this highly confirmed gene set is too small to represent all *P. aeruginosa* essential functions. Furthermore, it lacks some genes known to be essential, such as *dnaB* required for DNA replication and *rpoC*

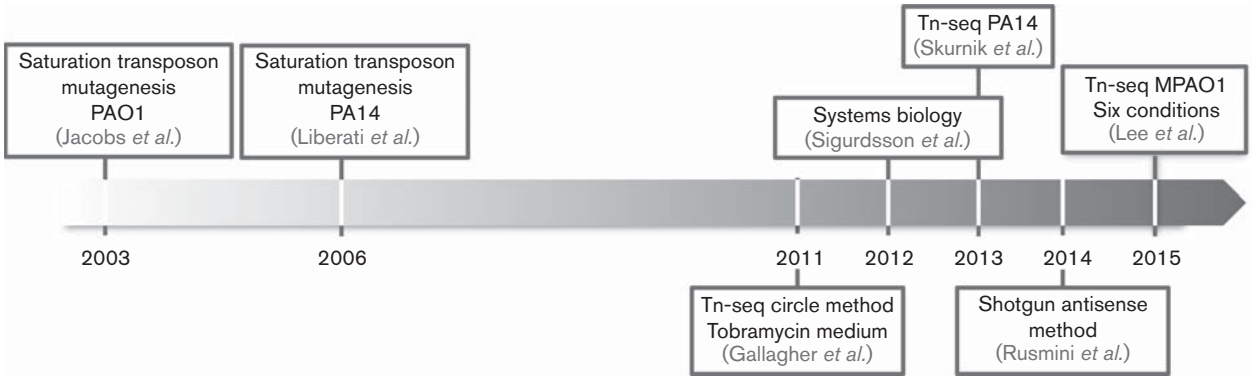

Fig. 1. Timeline of identification of key *P. aeruginosa* essential genes.

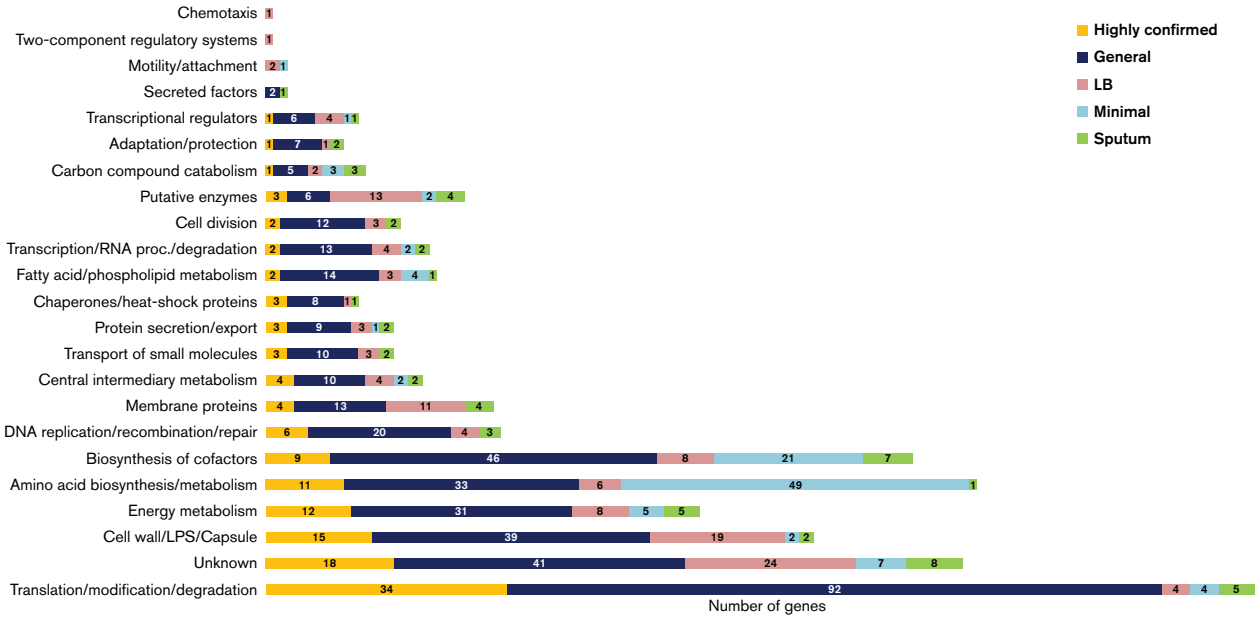

Fig. 2. Functional categories of *P. aeruginosa* essential genes: highly confirmed essential genes found to be indispensable in four genome-wide analyses; genes generally essential regardless of the growth condition; and condition-specific genes essential in specific environments (LB, minimal medium, cystic fibrosis sputum). Numbers represent number of essential genes in each category. The majority of *P. aeruginosa* essential genes play a role in fundamental cellular functions. Interestingly, a number of essential genes are hypotheticals with unknown function.

encoding RNA polymerase (Lee *et al.*, 2015). Out of 551 genes found essential by Lee *et al.* (2015), 352 genes were required for growth in all three primary growth conditions (LB medium, minimal medium, sputum) and are therefore considered to be generally essential in *P. aeruginosa*. The majority of the general *P. aeruginosa* essential genes play a role in fundamental cellular functions, such as DNA replication, transcription and translation, RNA metabolism, protein export, biosynthesis of cofactors and amino acids, and cell wall biogenesis (Juhas *et al.*, 2011; Lee *et al.*, 2015; Luo *et al.*, 2014). As in the highly confirmed essential gene set, many general essential genes are hypotheticals with unknown function (Fig. 2). Large portions of general essential genes are also required for energy metabolism, fatty acid and phospholipid metabolism and central intermediary metabolism, transport of small molecules, cell division and membrane biogenesis. A number of general essential genes are involved in central carbon metabolism and in protection against reactive oxygen species, such as superoxide and hydrogen peroxide. The former encompass genes encoding the components of the tricarboxylic cycle, the pentose phosphate pathway and glycolysis, whilst the latter include superoxide dismutase SodB, ferredoxin-NADPH reductase Fpr, ferric uptake regulator Fur, and YfgZ and PAO759 (Arai *et al.*, 2014; Imlay, 2013; Lee *et al.*, 2015). Other generally essential *P. aeruginosa* genes identified in the study are involved in the inhibition of pyocin expression (*imm2* and *ptR*), antitoxins (PAO125, PA4674 and PAO728.1), and asparagine-tRNA and LPS biosynthesis (Lee *et al.*, 2015; Penterman *et al.*, 2014a, b; Sheppard *et al.*, 2008). The remaining 199 genes were indispensable only in a subset of growth conditions analysed (Lee *et al.*, 2015) (Fig. 2). From the clinical point of view, genes required for growth in cystic fibrosis sputum are particularly interesting. A number of genes involved in the biogenesis and maintenance of the outer membrane, such as the outer membrane lipoprotein OprI and the outer membrane chaperone protein Skp, were shown to be essential for the growth of *P. aeruginosa* MPAO1 in cystic fibrosis sputum (Goemans *et al.*, 2014; Lee *et al.*, 2015; Wessel *et al.*, 2013). Other genes, such as *rsmA* encoding the global translational regulator, were shown to be essential for growth in cystic fibrosis sputum and LB medium, but not in the minimal (MOPS-pyruvate) medium (Lee *et al.*, 2015). Notably, genes involved in nucleotide and amino acid biosynthesis were not essential in cystic fibrosis sputum, thus confirming that cystic fibrosis sputum contains amino acids. Similarly, genes involved in the biosynthesis of unsaturated fatty acids were not required for growth in cystic fibrosis sputum, despite being indispensable in LB and minimal medium (Lee *et al.*, 2015). This study clearly demonstrated that the essentiality of *P. aeruginosa* genes is growth-condition-dependent.

Other recent studies include the Tn-seq circle method-based identification of 117 genes essential for the intrinsic resistance of *P. aeruginosa* to the aminoglycoside

antimicrobial tobramycin (Gallagher *et al.*, 2011). Furthermore, gene deletions were used to identify putative drug targets amongst *P. aeruginosa* essential genes in the biofilm-like and planktonic setting (Sigurdsson *et al.*, 2012). The single gene deletions identified 26 potential drug targets required for growth under both conditions, whilst the double deletions identified 17 combinations of 21 different essential genes (Sigurdsson *et al.*, 2012). The shotgun antisense method (Ji *et al.*, 2001; Meng *et al.*, 2012) led to the identification of 28 *P. aeruginosa* essential genes, including 16 novel genes that do not have homologues amongst previously identified essential gene sets in any other bacterial species (Rusmini *et al.*, 2014). In addition to functions traditionally associated with essential genes, such as DNA replication, transcription and translation, the putative essential genes identified by the shotgun antisense method are implicated in energy metabolism, protein secretion, transport of small molecules, central intermediary metabolism and synthesis of LPSs (Rusmini *et al.*, 2014).

Essential genes as antimicrobial targets

Pathogenic bacteria, particularly those resistant to multiple antibiotics, are amongst the main threats to public health. As essential genes are vital for the survival of bacteria, they are considered to be good targets for the development of novel antimicrobials (Juhas *et al.*, 2012a). Examples of antimicrobials inhibiting essential cellular processes include rifamycins interfering with RNA synthesis (Chopra, 2007), quinolones inhibiting DNA gyrase (Marcusson *et al.*, 2009), tetracyclines, puromycins, benzoxaboroles and peptide–peptide nucleic acid conjugates targeting protein biosynthesis (Ghosal & Nielsen, 2012; Rock *et al.*, 2007), borinic esters inhibiting the cell cycle (Benkovic *et al.*, 2005), and penicillin and peptidomimetic antibiotics targeting the cell wall (Chung *et al.*, 2009; Werneburg *et al.*, 2012). The essential second messenger c-di-AMP, recently discovered in a number of Gram-positive pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *B. subtilis*, is also considered to be a potential target of novel antimicrobials (Corrigan & Gründling, 2013; Mehne *et al.*, 2013).

P. aeruginosa essential genes as antimicrobial targets

Although there is still the assumption that only 'core' essential genes encoding universal cellular functions are good antimicrobial targets due to providing a broad host range, this is not always the case. Antibiotics interfering with a 'core' essential gene could also inhibit the function of the essential homologue in human cells. As described above, recent analyses led to the identification of a number of general and condition-specific *P. aeruginosa* essential genes. A number of these, such as those implicated in asparagine-tRNA biosynthesis, are not essential in mammalian cells and are therefore candidate drug targets (Lee *et al.*, 2015). Recent genome-wide analyses have also revealed a number of essential genes that are indispensable in *P. aeruginosa*, but not in other bacteria, such as *E. coli*. These include genes involved in central carbon energy metabolism and protection from reactive oxygen species (Lee *et al.*, 2015). As the essentiality of these processes stems from the *P. aeruginosa* lifestyle (e.g. the reliance of *P. aeruginosa* on respiration for energy generation), they are considered to be good targets for *P. aeruginosa*-specific antibiotics. Furthermore, the 'accessory' essential genes required for *P. aeruginosa* growth under clinically relevant conditions, such as in cystic fibrosis sputum and in the presence of antibiotics, and those indispensable for pathogenicity appear to be promising targets. Recently described and validated examples of essential *P. aeruginosa* antimicrobial targets include tobramycin antibiotic resistance-conferring genes, resistance-nodulation-division (RND)-type efflux pumps and *lptD* (highlighted below).

Recent Tn-seq circle method-based analysis led to identification of 117 genes essential for the growth of *P. aeruginosa* on the aminoglycoside antibiotic tobramycin (Gallagher *et al.*, 2011). In addition to the previously identified tobramycin resistance-conferring genes (Lee *et al.*, 2009), these include genes for a number of novel tobramycin targets, such as PAO392, PA1805 and PA4077. PAO392, PA1805 and PA4077 encode an unknown membrane protein implicated in osmotic stress tolerance, a peptidyl–prolyl *cis–trans* isomerase (*ppiD*) involved in folding of the membrane and exported proteins, and the transcriptional regulator of the envelope stress response, respectively (Gallagher *et al.*, 2011). Gallagher *et al.* (2011) showed that the Tn-seq-based approach combined with analysis of individual mutants is suitable for identification of antibiotic resistance-conferring genes.

The RND-type efflux pumps, such as AcrAB–TolC and MexAB–OprM, contribute to the intrinsic resistance of *P. aeruginosa* to antibiotics by pumping antimicrobials from the periplasm to the outside of the cell (Nikaido & Pagès, 2012; Opperman & Nguyen, 2015). A number of efflux pump inhibitors, including phenylalanyl arginyl β-naphthylamide, 1-(1-naphthylmethyl)-piperazine and D13-9001, have been described; however, none has yet been tested clinically (Lamers *et al.*, 2013; Nakashima *et al.*, 2013; Schuster *et al.*, 2014).

The recently developed peptidomimetic antimicrobials show activity in the nanomolar range against *P. aeruginosa* (Srinivas *et al.*, 2010). Genetic and biochemical analyses revealed that peptidomimetics do not lyse the bacterial membrane, but target the β-barrel outer-membrane protein LptD. In complex with the lipoprotein LptE, LptD transports LPS from the periplasm to the outer membrane (Fig. 3). The conditional mutant with the native *lptD* promoter replaced by the rhamnose-inducible promoter

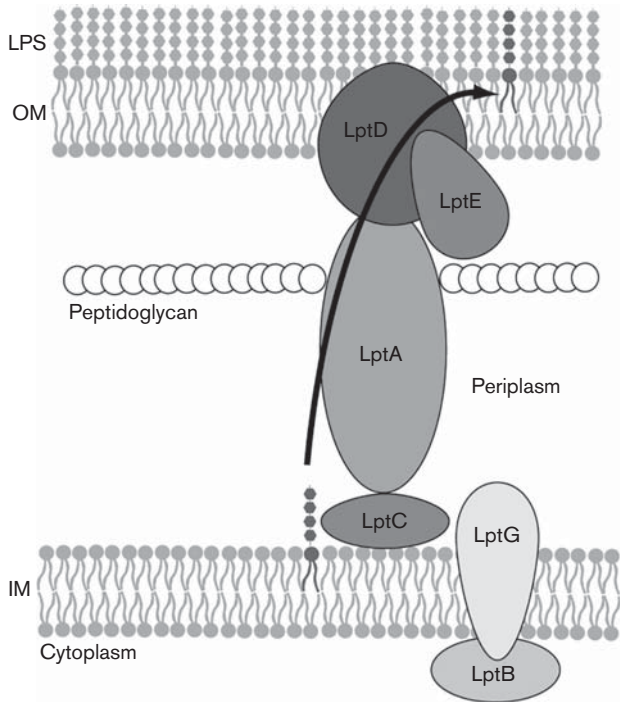

Fig. 3. Essential role of LptD in LPS transport in *P. aeruginosa*. LptD is essential for the transport of LPS from the periplasm to the outer membrane of *P. aeruginosa*. *lptD* is the target of the recently developed peptidomimetic antimicrobials. IM, inner membrane; OM, outer membrane.

P_{rhaB} enabled regulation of *lptD* expression by the level of rhamnose in the growth medium. This allowed direct comparison of the effects of LptD depletion and peptidomimetic treatment on *P. aeruginosa* (Werneburg *et al.*, 2012). The conditional *lptD* mutant grew only in medium with rhamnose, whilst complementation *in trans* restored the growth defect, thus showing that *lptD* is essential in *P. aeruginosa* (Werneburg *et al.*, 2012). Depleting LptD in the conditional mutant resulted in the accumulation of membrane-like material in the cell and blebbing of the outer membrane (Werneburg *et al.*, 2012). Furthermore, the *lptD* mutation led to modifications of the lipid A core of LPS. Similar effects were observed when *P. aeruginosa* was treated with peptidomimetics. This showed that peptidomimetics inhibit transport of LPS to the outer membrane of *P. aeruginosa* by targeting the essential *lptD*.

Conclusions

P. aeruginosa is amongst the major causative agents of multi-resistant infections in both communal and hospital settings (Cramer *et al.*, 2012). Development of novel antimicrobials against *P. aeruginosa* is therefore of the highest importance. *P. aeruginosa* essential genes have recently been better defined by a number of studies employing more sensitive genomic approaches. These analyses highlight the role of growth conditions and strain on the outcome of essential gene identification studies and underline the importance of empirical validation of essential genes. Experimental methods used for identification of essential genes, such as saturation transposon mutagenesis, antisense RNA inhibition and gene deletions, have their limitations. Antisense RNA inhibition can only be used for a subset of genes with high inhibitory RNA expression, whilst deletions of individual genes are not suitable for multicopy genes and for genes whose essentiality is dependent on the function of other genes (Juhas *et al.*, 2011). Transposon mutagenesis, routinely used to identify essential genes, is not flawless for a number of reasons. (1) Transposon insertion is not entirely random and mutagenesis employing different transposons or the same transposon with different antibiotic resistance genes can lead to variable essential gene sets. (2) The affected gene is often considered essential even if the transposon insertion only slows down growth. (3) Polar effects can complicate the identification of essential genes as the growth defect might be the result of disruption of another gene of the same operon. (4) Mutants in essential genes can be recovered if the insertion is in a non-essential domain.

However, the impact of some of the above limitations on the outcome of gene identification studies can be minimized. The last caveat of transposon mutagenesis described above is more related to genome coverage than an inherent limitation of the transposon-based approach. High coverage transposon mutant studies, such as sequencing of saturation-level transposon mutants pools (Tn-seq) described above (Gallagher *et al.*, 2011; Lee *et al.*, 2015; Skurnik *et al.*, 2013), should identify essential domains. The recently developed Tn-seq circle method used to differentiate between general and growth-condition-specific *P. aeruginosa* essential genes achieved a very high genome coverage (transposon insertion every 6 bp). Although the Tn-seq circle method can be used with virtually any transposon, this study utilized a transposon with low insertion specificity derived from Tn*5* to achieve high genome coverage (Lee *et al.*, 2015). Therefore, at present, the Tn-seq circle method using low insertion specificity transposons appears to be the most suitable transposon-based approach for essential gene identification. Polar effects could indeed influence the outcome of saturation transposon mutagenesis (and gene deletion) studies; however, the quality of the particular gene essentiality analysis can be assessed with the help of conditional mutants where the gene's native promoter is exchanged for a promoter whose expression is easily controlled by the inducer (Juhas *et al.*, 2012a, b). A good example is the construction of the rhamnose-inducible *P. aeruginosa* *lptD* mutant described above that confirmed the essential role of LptD in LPS transport (Werneburg *et al.*, 2012). A similar approach could be applied to define essential genes rigorously in bacteria less well characterized than *P. aeruginosa*. Furthermore, polar effects could be minimized

by using transposons harbouring an outward-facing promoter for the expression of the downstream genes (Lee *et al.*, 2015). In particular, those 'accessory' *P. aeruginosa* essential genes that do not have human homologues, that are required for the specific *P. aeruginosa* 'lifestyle' and for growth under clinically relevant conditions, such as in cystic fibrosis sputum and in the presence of antibiotics, and those encoding main virulence factors are considered to be promising antimicrobial targets. Examples of *P. aeruginosa* essential gene/antimicrobial systems include tobramycin targeting PAO392, PA1805 and PA4077, inhibitors of RND-type efflux pumps, and peptidomimetics targeting *lptD*, as highlighted in this review. Notably, a large proportion of *P. aeruginosa* essential genes are hypotheticals with unknown function (Romero & Karp, 2003; Winsor *et al.*, 2011) (Fig. 2). Further analyses of essential cellular functions, including the unknown *P. aeruginosa* essential genes, will lead to the development of novel antimicrobials against this hard-to-eradicate pathogen.

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