Current knowledge and future directions in developing strategies to combat *Pseudomonas aeruginosa* infection

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Abstract

In the face of growing antimicrobial resistance, there is an urgent need for the development of effective strategies to target *Pseudomonas aeruginosa*. This metabolically versatile bacterium can cause a wide range of severe opportunistic infections in patients with serious underlying medical conditions, such as those with burns, surgical wounds or people with cystic fibrosis. Many of the key adaptations which arise in this organism during infection are centered on core metabolism and virulence factor synthesis. Interfering with these processes may provide a new strategy to combat infection, in combination with conventional antibiotics. This review will provide an overview of the most recent work which has advanced our understanding of *P. aeruginosa* infection. Strategies which exploit this recent knowledge to combat infection will be highlighted, alongside potential alternative therapeutic options and their limitations.

Introduction:

Pseudomonas aeruginosa (Pa) is a prominent Gram-negative bacterial pathogen which can survive in a variety of challenging environments. Recent evidence suggests that Pa may be closely associated with human activity as it is rarely detected in pristine environments. Pa is a major cause of illness and mortality in humans. Infections exist across a spectrum which ranges from the acute to lifelong, persistent manifestations in patients with immunosuppressive or chronic conditions, such as cystic fibrosis (CF), burns, wounds and cancer [1,2]. Physicians and microbiologists have witnessed the increasing resistance of Pa to conventional therapies which are commonly use in the clinic. Drug design strategies for this organism have frequently hit a wall as compounds with high efficacy against laboratory strains frequently fail against clinical isolates. Dwindling profits and antimicrobial efficacy challenges have led to numerous pharmaceutical companies abandoning antibacterial drug discovery [3].

The remarkable edge that Pa has over other human pathogens is difficult to pinpoint. However, it is thought to be mediated through a combination of the following overlapping characteristics: metabolic and phenotypic flexibility, prolific virulence factor production, persistence and a high intrinsic mutation rate [1]. Precisely how Pa coordinates the above systems at the site of human infection remains elusive. Comprehending how this organism accomplishes this feat is central to developing strategies to combat human infection. By furthering our understanding of the biology of Pa infection, we are more likely to expedite the development of new location-specific strategies to combat infection and increase the efficacy of existing therapies.

This review will provide an overview of the most recent developments which have advanced our understanding of Pa infection. Strategies which exploit this recent knowledge to combat infection will be highlighted, alongside current challenges and bottlenecks to future major advances.

Current knowledge of Pa infection – latest tools of the trade

What's going on *in-vivo*?

Advancements in omics and computing technologies over the last decade have led to an enormous leap in our understanding of acute and chronic Pa infection. High throughput genome sequencing allows for the direct inspection of the individual genomes for thousands of Pa isolates from diverse infection sites. This has facilitated the tracking of single strains over the course of an infection whilst also uncovering spatial and temporal heterogeneity, population structure, coexisting lineages, common SNPs, polymorphisms, and core genes. These recent advancements have also facilitated the examination of Pa megaplasmids and pangenomes [4–9].

Sequencing advances have also allowed us to examine the transcriptional signature of Pa*,* along with other key pathogens, at the site of infection [10–12]. Developments in proteomics instrumentation and bacterial enrichment methods are also allowing us to examine at a subset of the Pa proteome from the infection site [13]. Most of the genomic and *in-vivo*/*ex-vivo* transcriptome investigations from human samples to date are from CF patients chronically infected with Pa. This bias towards respiratory samples compared with other infection sites is largely due to the relative availability of 1) fresh sputum (which can be collected non-invasively from CF patients) and 2) freshly explanted diseased lungs (which are removed prior to transplantation and dissected). Moving away from this trend, Cornforth et al. examined the Pa transcriptome from chronic wound and burn wound infection samples alongside Pa from CF sputum samples, providing a diverse sample set from patients treated with a variety of therapeutic regimens [10]. The above studies have allowed us to glean some core insights into Pa infection:

- Pa infection is a spatially dynamic process with a convergent set of mutations evident over time in the population. Systems previously considered to be extremely important for infection are frequently mutated.

- Despite lung containing genetically distinct populations, a common gene expression program is evident for chronic CF infection across multiple independent studies. This includes the downregulation of central metabolism, motility and amino acid biosynthesis. Systems to tolerate redox stress and micronutrient starvation are also activated. This suggests the existence of convergent phenotypes exist which serve to maximise Pa survival during infection.
- The Pa transcriptome during human infection is distinct from *in-vitro* transcriptomes, despite a multitude of intrinsic differences such as infection site or therapeutic regimen.

Using the above technologies, we bear witness to temporal Pa evolution and adaptation, uncovering genetic mutations accumulated over the course of human infection. We also get a snapshot of the final output of this dynamic process; the transcriptional/proteomic signature of infection. Thankfully, the above *in-vivo* data can be examined in the light of a vast repertoire of *in-vitro* functional genomics studies which have attempted to recapitulate the infection environment, and animal models of Pa infection. The combination of this knowledge with detailed mechanistic studies affords us the opportunity to cast the spotlight on effective new strategies with which to combat infection.

Despite this extensive literature, we still struggle to fully interpret the above *in-vivo* omic data. This is because we have an elementary understanding of how several key pathways and systems operate in Pa. In fact, the core operations of several key Pa systems have been extrapolated from other bacteria, such as chemotaxis, central carbon metabolism, the electron transport chain (ETC), and redox tolerance. However, recent mechanistic studies are filling this knowledge gap [14–19]. The *in-vivo* transcriptomic studies have also highlighted several uncharacterised genes which appear to be expressed at a high level during the infection process. How do these uncharacterised nodes fit into the global picture of Pa infection? Our lack of an answer to this question alone demonstrates that we still have a lot to learn from *in-vitro* mechanistic studies in order to fully comprehend Pa behaviour during infection.

Supercharged Pa functional genomics – Transposon insertion sequencing

Advances in sequencing technologies have been combined with traditional transposon mutagenesis by several research groups in a technique referred to as transposon sequencing (Tn-seq) (related strategies are known as TraDIS, INSeq, RB-TnSeq and HITS). In this strategy, pooled populations of transposon mutants covering the entire genome are cultured in a selected environment. Sequencing tools are then used to pinpoint genomic regions which are important or essential for bacterial fitness [20]. When applied to Pa, this technique has facilitated the rapid identification of genes which are important for fitness in infection-relevant environments, single nutrient sources, energy limitation, antibiotic resistance, competing microorganisms, and motility [21–29]. This technique has also been combined with murine models of burn infection and chronic wound infection to determine the requirements of Pa in those environments [23,26].

A comprehensive Tn-seq study (nine Pa strains grown on five different media) has highlighted the extensive genomic plasticity of Pa. For example, the type IV pilus-associated adhesin gene, *pilY1*, did not tolerate insertions in the lung isolate BWH013 when grown on LB medium whilst readily tolerating insertions in the other eight strains [24]. These recent *in-vitro* studies have provided excellent contextualisation of the human infection omics data. We now know which genes are important for fitness during growth in *in-vivo*-like environments, which genes are expressed *in-vivo* and which genes are frequently mutated during infection. This is important as gene expression changes don't necessarily correlate with fitness during infection [23]. Using this information, we can put together a clear list of validated targets and strategies to disrupt Pa infection.

New strategies to combat Pa infection

All roads lead to metabolism: constraining the metabolic flexibility of Pa

Many of the important changes in Pa which occur during infection rather than growth in standard laboratory conditions are centered on core metabolism. This includes the processes

129 required for the acquisition of nutrients and for electron acceptors to fuel metabolism (Figure
130 1 A-C). For this reason, the recent *in-vivo* transcriptomic/proteomic data has reignited interest
131 in these processes. Although the metabolic versatility of Pa has been appreciated and studied
132 for decades, recent work continues to transform our understanding of these systems and
133 emphasize their importance during infection [30,31].

134 Aerobic and anaerobic respiration have significant potential to be the cornerstone of future
135 antimicrobial therapies targeting this organism. Pa possesses an extensively branched
136 respiratory chain consisting of five terminal oxidases for aerobic respiration which it uses under
137 different growth conditions (Figure 1B) [32]. This setup allows Pa to proliferate in host
138 environments which contain variable levels of oxygen. The respiratory flexibility of Pa has
139 been found to be even more extensive than originally thought due to the recent discovery of
140 two 'orphan' terminal oxidase CcoN isosubunits - CcoN3 and CcoN4. These isosubunits are
141 produced in response to nitrite and cyanide respectively. Remarkably, by interchanging with
142 the integral cbb_3-type cytochrome *c* oxidase subunits (encoded by *ccoN1O1Q1P1* and
143 *ccoN2O2Q2P2*), at least 16 active cytochrome oxidase subunit isoforms can be produced by
144 Pa [33]. The CcoN4 subunit was subsequently shown to contribute to the reduction of
145 phenazines (redox-sensitive small molecule metabolites that support redox balancing in Pa)
146 and to virulence in a *Caenorhabditis elegans* model of infection [34]. The cbb3-type terminal
147 oxidases also contribute to ciprofloxacin tolerance when phenazines are produced but not in
148 a phenazine-null background, a key link between small molecule metabolites, primary
149 metabolism, and antibiotics [35]. Indeed, phenazine production has been shown to affect Pa
150 respiratory activity and terminal oxidase gene expression [36]. Furthermore, the challenge of
151 Pa with sublethal concentrations of diverse antibiotics was shown to promote the accumulation
152 of the phenazine pyocyanin (PYO), which in turn conferred general antibiotic tolerance. This
153 phenomenon may be linked to the modulation of terminal oxidase expression by PYO [36,37].
154 This underscores the potential of terminal oxidases (and more generally, aerobic respiration)
155 as a unique therapeutic target.

Microenvironments of the CF lung comprising of static mucus are thought to be hypoxic or anaerobic. These low oxygen environments are unlikely to sustain aerobic respiration [38,39]. However, CF sputum contains 73 to 792 μM nitrate, which Pa utilises for growth through the sequential eight-electron reduction of NO_3^- to N_2, a process known as denitrification (Figure 1A) [40,41]. Anaerobic survival is also facilitated by pyruvate fermentation and phenazine antibiotics [42,43]. The transition of Pa to this anaerobic lifestyle is followed by a series of other key physiological alterations. These include the increased formation of cellular aggregations to shield itself from the surrounding environment (biofilms), slow growth, and a drastic modification of the structure and function of its lipopolysaccharide outer membrane. These changes imbue Pa with an enhanced resistance to host immune defences and to a series of antibiotics commonly used to treat infection [39,44,45]. For this reason, the efficacy of several bactericidal antibiotics such as ciprofloxacin is enhanced by the stimulation of aerobic respiration. As a lack of O_2 increases the tolerance of Pa to antibiotics, the reoxygenation of O_2-depleted biofilms should increase susceptibility to antibiotics by reactivating aerobic respiration. This can be accomplished by means of hyperbaric oxygen treatment (HBOT) (100% O_2, 2.8 bar), which may be an effective adjuvant for the eradication of chronic Pa infections (Figure 2) [46,47].

Denitrification is a central process in Pa for another significant reason. A key toxic intermediate of this pathway, nitric oxide (NO), has been established as one of the key signals which lead to Pa biofilm dispersal [48,49]. These dispersed cells leave the biofilm to colonize new locations, contributing to acute virulence and chronic persistence [50,51]. Indeed, a nitrate reductase mutant (Δ*nirS*) which cannot generate metabolic NO was found to be incapable of dispersal in the presence of nitric oxide generators [52,53]. It has also been noted that dispersed cells, removed from the protection of biofilm growth, are substantially more sensitive to antimicrobial intervention [52]. NO-donors, such as NO-releasing alginates or the prodrug DEA-C3D (‘DiEthylAmin-Cephalosporin-3′-Diazeniumdiolate’), co-administered with antibiotics form a promising new adjunctive therapeutic strategy to combat infection [54,55].

Pa anaerobic metabolism can also be stimulated by either adding an electron acceptor that permits denitrification, or by adding arginine, which can be fermented by Pa. This may also present a potent antimicrobial strategy as both arginine and nitrate were shown to enhance the killing of Pa by ciprofloxacin and tobramycin under anaerobic, but not aerobic, growth conditions [56]. It may be possible to inhibit the ability of Pa to activate the denitrification machinery, blocking its transition into an anaerobic lifestyle. In addition, the handling of toxic endogenous NO is a carefully orchestrated process in Pa, which may provide a new antimicrobial opportunity (Figure 1A) [57]. Recent work has provided detailed structural and mechanistic insight into how these enzymes operate and may catalyse antimicrobial design targeting this pathway [58–60].

Central carbon metabolism during infection

Central carbon metabolism and associated nutrient assimilation pathways may also be a key target in the fight against infection (Figure 1C). Pa has a remarkable ability to consume available nutrients and to survive in a variety of challenging environments. These attributes may also play a key role in Pa outcompeting rival pathogens in a variety of infection sites [61,62]. Central carbon metabolic nodes and nutrient uptake systems are extensively modulated during growth in diverse infection sites (the metabolic requirements of Pa during CF infection have been reviewed in reference [31]).

In general, the expression of Pa central carbon metabolic pathways is lower in human infection (both soft-tissue wounds and CF lungs) in comparison with *in-vitro* growth. Processes such as ATP synthesis, coupled electron transport, amino acid biosynthesis, propionate metabolism, oxidative phosphorylation, and phospholipid metabolism were shown to be down regulated *in-vivo* compared to laboratory conditions. This reflects a concerted effort to reduce cellular activity and slow growth *in-vivo* [31,63]. Expression of the genes coding for most of the TCA cycle (including citrate synthase, aconitate synthase, both isocitrate dehydrogenases, and oxoglutarate dehydrogenase) were found to be repressed *in-vivo*. In contrast, isocitrate lyase (*aceA*) expression, which transforms isocitrate into glyoxylate, was increased in the human

CF samples, indicating a switch of carbon flux towards the glyoxylate shunt rather than to the decarboxylation steps of the TCA cycle (Figure 2). AceA expression was also significantly increased on the protein level for the CF samples in a separate study [13]. The glyoxylate cycle is also required for the degradation of purified mucins, the major macromolecular constituents of the mucus layer [27]. The reduced *in-vivo* expression of pyruvate dehydrogenase and pyruvate carboxylase which feed pyruvate into the TCA cycle suggests that Pa employs a gluconeogenic rather than a glycolytic configuration during human infection [10]. Expression of fumarate hydratase (*fumC1*), which catalyses the reversible conversion of fumarate to malate and modulates redox balance, was increased in both transcriptomic and proteomic profiles for the CF lung infection, but not the soft tissue wound infection. This observation is consistent with the presence of a series of redox stresses in the lung environment. Long-chain fatty acid catabolism, notably the genes *faoA* and *faoB,* were previously shown to be required in both murine surgical wound and burn wound infection models. These genes were also induced in the human soft tissue wound samples [10,11,23]. Sputum from patients with CF has been shown to contain millimolar concentrations of lactate. There is an extensive redundancy at this metabolic node as Pa is known to produce four enzymes annotated as lactate dehydrogenases. LdhA, which reduces pyruvate to d-lactate during anaerobic survival, LldE and LldD, which oxidize d-lactate and l-lactate, respectively, during aerobic growth and the newly annotated LldA, which performs redundant l-lactate oxidation during growth in aerobic cultures [14,64]. As expected, *lldA* was highly expressed in the CF samples. Interestingly, this gene also increased substantially in the wound samples [10,65].

Reactivating cellular respiration

Antibiotic efficacy is intimately linked to bacterial cellular respiration (Figure 2). It has been established that a reduced susceptibility to bactericidal antibiotics with different modes of action is coupled with a reduced flux through the TCA cycle, by downregulating TCA cycle genes and/or by reducing the levels of metabolites feeding into the TCA cycle. These

metabolic adaptations lead to a reduced proton motive force (PMF), limiting the influx of antibiotics into the cell. In addition, these metabolic adaptations are frequently shown to reduce intracellular levels of ROS, which likely contributes to increased survival (Figure 2B) [66]. Meylan et al. demonstrated that supplementation with metabolites from the lower part of the TCA cycle (fumarate, succinate) and metabolites from lower glycolysis (pyruvate) sensitized Pa cells to tobramycin. Conversely, upper TCA metabolites, such as citrate, had little effect. In contrast, glyoxylate supplementation was shown to protect against tobramycin, conceivably by inhibiting α-ketoglutarate dehydrogenase and redirecting isocitrate into the glyoxylate shunt. It was finally shown that fumarate enhances the activity of the ETC by boosting the expression of the TCA cycle and ETC genes, leading to increased respiration, increased PMF, and increased tobramycin uptake [67,68]. Mammals may use a similar approach to potentiate antimicrobial efficacy. Lung epithelial cells cultured in a three-dimensional cell-culture model were shown to secrete endogenous metabolites (succinate and glutamate), which in turn potentiated the bactericidal activity of aminoglycosides against Pa. Biochemical and phenotypic assays indicated that the epithelial cell conditioned medium stimulated the PMF of Pa, resulting in increased intracellular pH [69]. This suggests that the stimulation Pa metabolism and respiration by carbon flux-altering metabolites could be a promising future strategy.

The TCA cycle is an understudied target in the development of novel therapeutics to combat Pa. It may be possible to target specific enzymes in central metabolism to boost aerobic respiration and antibiotic efficacy. The triosephosphate isomerase TpiA reversibly converts glyceraldehyde 3-phosphate to dihydroxyacetone phosphate, a key step connecting glucose metabolism with glycerol and phospholipid metabolism (Figure 1C). Deletion of *tpiA* from Pa enhances central carbon metabolism, respiration, and oxidative phosphorylation, which in-turn increases the PMF and promotes the uptake of aminoglycoside antibiotics [70]. Natural or synthetic inhibitors of the TCA cycle may also have significant potential. The natural product promysalin, originally isolated from the rhizosphere, exhibits narrow-spectrum

antibacterial activity against Pa. Using affinity-based protein profiling, succinate dehydrogenase (Sdh) was identified as the biological target of the natural product. This was further validated through the selection and sequencing of a resistant mutant [71]. However, based on the strategy of boosting cellular respiration outlined above (Figure 2),

it is possible that promysalin may drive Pa into a low metabolic state if applied incorrectly, reducing antibiotic efficacy. Promysalin and other inhibitors which specifically target essential metabolic enzymes in Pa will be invaluable for testing the feasibility of the carbon flux re-routing strategies described above.

An additional layer of complexity in targeting Pa metabolism is its high intrinsic mutation rate, leading to rapid phenotypic reorganisation, persistence, and the loss-of-function of numerous traits which are dispensable in the chronic infection site. These mutations, leading to slow growth and increased biofilm formation, are a significant issue and dramatically reduce the efficacy of current antimicrobial strategies [4]. Is it possible to 'reactivate' these slow growing, adapted Pa isolates using the strategies outlined to boost conventional antimicrobial treatments? Future work will be necessary to elucidate this.

Finding the off switch for infection: targeting Pa virulence factors

Pa infects and disseminates within hosts using a multitude of virulence factors, including the type III and IV secretion systems, cyanide, pyocyanin, and proteases. Although virulence factors are host and site specific, several of these virulence factors appear to be crucial for human infection. It is also likely that important Pa virulence factors remain uncharacterised. For example, Filamentous bacteriophage (Pf phage) has only recently been appreciated as a potential therapeutic target for the treatment of chronic Pa infections. CF patients who tested positive for Pf phage were more likely to have chronic infection and had greater declines in pulmonary function during exacerbation. Pf phage also promoted antibiotic resistance and interacted with the mammalian immune system [72–74]. For this reason, targeting the

289 synthesis of virulence factors by an “antivirulence strategy” represents a promising alternative
290 to antibiotic therapy.

291 **Communication is key – quorum sensing**

292 The cell-cell communication system known as quorum sensing (QS) regulates the expression
293 of several key virulence factors in Pa. QS occurs when a critical cell density (or quorum) is
294 reached as relayed through the production of small molecules known as autoinducers. These
295 autoinducers are released into the extracellular matrix and are generally recognized by the
296 same species triggering a collective response such as biofilm formation or virulence (Figure
297 3). The QS autoinducer molecules used by several Gram-negative bacteria, including Pa, are
298 acylated homoserine lactones (AHLs). As QS activates a wealth of pathogenic weapons,
299 unsurprisingly, QS mutants have reduced virulence in several animal models. Pyocyanin, the
300 terminal signalling molecule in the QS network, is one of the most prominent virulence factors
301 released by Pa during acute and chronic infections [75,76]. Although this phenazine was noted
302 earlier as an electron carrier important for respiration, this redox-active blue pigment also has
303 cytotoxic and immunomodulating properties. Moreover, it has been demonstrated that
304 pyocyanin promotes the development of a pulmonary pathophysiology in mice which may
305 have parallels to the lung of infected cystic fibrosis patients [77]

306 As summarised in Figure 3, the core QS network of Pa is extensive and exhibits crosstalk at
307 multiple levels. LasR, the master QS signal receptor, was initially hailed as the main anti-
308 virulence target for Pa and became a key target in small-molecule inhibitor screens.
309 Subsequent work showed that many patients harbour *lasR* mutants. The reason for this is still
310 unclear, but it may involve a combination of “QS cheating” and increased microoxic fitness of
311 this mutant [78,79]. Although initially discouraging, further investigation showed that most
312 LasR mutant isolates have rewired their communication circuitry to place the lesser studied
313 Rhl system at the top of the QS hierarchy (Figure 3). Precisely how this rewiring occurs, and
314 its consequences for infection, are under active investigation [80–82].

For the reasons outlined above, a RhlR inhibitor or an inhibitor that simultaneously targets both RhlR and LasR might be an appropriate therapy for Pa infection. Such a compound has been synthesized: meta-bromo-thiolactone (mBTL), an analog of the native RhlR autoinducer BHL (N-butanoyl-L-homoserine lactone). mBTL represses expression of the genes encoding pyocyanin, prevents biofilm formation, and protects human lung epithelial cells from killing by Pa (Figure 3). Both the LasR and RhlR quorum-sensing receptors are inhibited by mBTL; however, it appears that RhlR, not LasR, is the relevant *in-vivo* target. [81]. Pushing these designs forward, the molecular nature of RhlR activation is also being examined through the design of hybrid AHL analogs with structures which merge features of reported lead RhlR ligands and head groups with improved hydrolytic stabilities [83]. In addition to blocking virulence, targeting RhlR may lead to metabolic suicide of Pa in the low oxygen lung environment. It was shown that bacteria lacking RhlR succumb via metabolic NO intoxication due to dysregulatory increases in nitrate reductase (NAR) and nitrite reductase (NIR) activities. This imbalance is thought to result in the generation of intracellular NO faster than the protective nitric oxide reductase (NOR) enzyme can detoxify this potent intermediate (Figure 1B). Thus, the Rhl QS circuit and critical components of anaerobic respiration could be targets for the killing of Pa anaerobic biofilm in CF lung disease [39].

Other prospects also exist to target the Pa QS system. The LysR-type transcriptional regulator protein PqsR (or MvfR) is a key component of the alkyl-quinolone (AQ)-dependent QS system in Pa (Figure 3). Consequently, PqsR could potentially block virulence in this organism [84,85]. In contrast to RhlR inhibitors, the design of and development of PqsR inhibitors has been aided by high-resolution structural data of this key QS regulator [85,86]. This has facilitated the guided optimisation of PqsR inhibitors, such as M64, through target-driven approaches [87]. Though a surprising strategy, the introduction of flexible linkers rather than rigid motifs led to a boost in PqsR inhibitor activity and anti-virulence potency. These recent advances have led to the discovery and optimization of 2-amino-pyridines as promising new PqsR inhibitors [88]. An additional component of the quinolone system, the thioesterase PqsE, is

also a vital component of the overall Pa QS system, making this enzyme an attractive target. Several groups are working on inhibitors of this QS node [89–91]. However, it has been noted that the thioesterase activity of PqsE itself does not appear to be responsible for the QS signalling properties of this enzyme [92]. Promising recent leads include as a series of 2-(phenylcarbamoyl)benzoic acid inhibitors that noncompetitively inhibit PqsE. However, future optimisation will be necessary to obtain *in-vivo* PqsE inhibitory activity [93].

Challenges with targeting quorum sensing

Like with all strategies, drawbacks exist when attempting to exploit the QS system in Pa. In recent years, QS research has demonstrated that these systems are intricately complex and have a high degree of plasticity. Our understanding of QS crosstalk in Pa is constantly evolving, with on-going revisions on which nodes are central and the best intervention strategies to target this system [90,94–98]. It is possible that QS inhibition may not be the best approach to curb Pa virulence; the small-molecule activation of RhlR can reduce pyocyanin production, possibly through a RhlR-mediated suppression of PQS signalling [99]. In addition, partial agonism of RhlR can reduce virulence in a *C. elegans* infection model [81]. These non-native ligands may have the potential to upregulate QS to promote the premature dispersal of biofilms, rendering Pa more susceptible to conventional antibiotic therapies. Finally, although QS inhibitors have significant potential to block infection at numerous sites, knowing which infections to target with a QS inhibitor is extremely important. For example, QS is an important factor in controlling Pa virulence in burn wounds, but not in chronic wounds [100]. Therefore, QS inhibitors targeting Pa in chronic wound infections may ultimately prove ineffective. This indicates that further basic research will be necessary to understand the importance of QS in specific infection sites in order to maximise their efficacy.

The Type III Secretion System injectisome

Pa and many other gram-negative bacteria utilize a complex type III secretion system (T3SS) to inject effector proteins into host cells. This highly regulated apparatus is comprised of over

20 proteins, which assemble a complex syringe-like machinery spanning from the inner bacterial membrane, through the extracellular space, and into the host cell membrane. There are four distinct exoenzymes that Pa injects into the target host cell via the T3SS: ExoT, ExoU, ExoS, and ExoY [23,26,101]. The T3SS appears to be a central determinant in human infection by Pa. In support of this, genes which encode components of the T3SS were significantly upregulated in an *ex-vivo* explanted CF lung transcriptome study when compared to *in-vitro* growth [12]. Additionally, the injection of ExoS or ExoU into phagocytes is critical for pathogenesis in a murine acute pneumonia model [102]. On top of this, exoenzyme specific differences appear to be prognostic in infection outcomes. In comparison to strains expressing ExoS, ExoU-expressing Pa has been associated with more severe outcomes in keratitis, acute pneumonia, and intensive care unit acquired pneumonia [103,104]

Given its contribution to clinical severity, therapeutic targeting of T3SS or its effectors may attenuate the morbidity and mortality of acute Pa infections. Several avenues to block T3SS are currently being explored [105]. These include MEDI3902, a bispecific human monoclonal antibody that binds to both the PcrV protein involved in host cell cytotoxicity and Psl exopolysaccharide involved in Pa colonization and tissue adherence. This antibody is currently undergoing a phase 2 proof-of-concept study (NCT02696902) for the prevention of nosocomial pneumonia caused by Pa in high-risk patients [106]**.** It may also be possible to inhibit assembly of the T3SS needle by disrupting interactions between the needle subunits, thereby blocking exoenzyme release. Ngo et al. identified compounds inhibiting the protein–protein interaction between PscE and PscG, the two cognate chaperones of the needle subunit PscF of the T3SS. Two promising candidates from these small molecule library screens specifically inhibited the T3SS and reduced the *ex-vivo* cytotoxicity of bacteria and their virulence in a *Galleria mellonella* infection model [107].

As with many virulence factors, targeting T3SS may only be a viable strategy at specific Pa infection stages or sites [23,26]. T3SS-negative bacteria have been isolated from non-CF patients with acute infections. Likewise, although initial Pa infections in patients with CF are

associated with T3SS-positive strains, T3SS-negative strains are isolated with increasing frequency from chronically infected CF patients over time. This may be explained by cheating strategies employed by Pa to exploit the production of public goods. Indeed, a new taxonomic group has recently emerged from analysis of the Pa pangenome, characterized by major alterations in their virulence factor repertoire; including the absence of T3SS effectors and the associated secretion and regulatory machinery. This suggests that some Pa strains could be inherently resistant to this anti-virulence strategy [9]. It is likely that other virulence factors, such as quorum sensing, play a larger role in these cases [108,109]

Pa surface colonization and aggregation

Host cell membranes and cell surfaces are the first line of defence against bacterial invasion. Pa has evolved several surface colonisation strategies in order to attack host cells and secure nutrients or to develop biofilms to shield itself from the surrounding environment. Biofilm formation by Pa is hypothesized to follow a developmental programme involving three discrete steps: surface attachment, microcolony formation and then maturation into an antibiotic-resistant population encased in an extracellular polymeric matrix.

In the initial stages of surface attachment, Pa uses fiber-like appendages called type IV pili (TFP) to sense contact with surfaces. This activates a signaling cascade, resulting in the expression of a plethora of genes associated with pathogenicity and twitching motility. TFP also mediate virulence through the coordinated and mutually dependent action with T3SS to facilitate exoenzyme injection into host cells. TFP and twitching were both required for pathogenicity in a murine model of corneal infection, specifically these features were required for Pa to traverse human corneal epithelial multilayers, and efficiently exit invaded epithelial cells [110–113]. Pa must respond in a rapid and orchestrated manner to ensure successful colonisation. Recent work is providing mechanistic insight into this fine balance of virulence, proliferation, and dissemination behaviours. Upon surface contact, the concentration of the second messenger c-di-GMP increases within seconds. This leads to surface adherence and virulence induction by stimulating pili assembly through activation of

the c-di-GMP receptor FimW. These surface-attached bacteria then divide asymmetrically to generate a piliated subpopulation committed to microcolony formation (striker) and a flagellated, motile offspring that explores the surface (spreader). Cell differentiation is driven by a cyclic nucleotide phosphodiesterase which asymmetrically positions to the flagellated pole, maintaining low c-di-GMP levels in the motile offspring. This cellular asymmetry program was shown to boost enhance spread and tissue damage [114]. In addition, Armbruster et al. found that the Wsp chemosensory surface sensing system generates heterogeneity in surface sensing, resulting in the two physiologically distinct subpopulations of cells [115]. It has also been shown that surface-associated Pa induces LasR QS targets more intensely in response to autoinducer signalling molecules in comparison to planktonic cultures. TFP retraction motors and the minor pilins are thought to play a role in this process. Thus, bacteria use pili not only to attach and move, but also to sense their environment and regulate surface-associated cellular processes. The coupling of physical surface responses and chemical QS responses in this manner may enable Pa to hyper-activate community behaviours when they are more beneficial [116].

Bacterial flagellar motility is a fundamental mechanism exploited by host phagocytes to enable recognition and ingestion of bacteria. Correspondingly, loss of bacterial motility (which is consistently observed in clinical isolates from chronic Pa infections), enables bacteria to evade immune cells. Lorenz et al. have shown that surprisingly, flagellin appears to be an important factor also for survival of the Δ*lasR* mutant during chronic biofilm-associated infections [117,118]. During chronic infection in the CF lung Pa appears to persist in immotile aggregates. Surrounding immune cells (polymorphonuclear leukocytes) may constrain the growth of Pa through aerobic respiration, rewiring Pa towards anaerobic respiration [119–121]. The exact mechanisms producing aggregation remain unclear, but entropic forces generated by local polymers and macromolecules present at chronic infection sites (mucin, DNA, F-actin, etc.) may facilitate cell aggregation by a passive mechanism known as “depletion aggregation” [122].

Most Pa anti-virulence strategies require a detailed structural and mechanistic dissection of the target in question to develop an effective strategy for inhibition. An exciting way to inhibit Pa surface colonisation may be through a bottom-up approach via the combinatorial discovery of polymers resistant to bacterial attachment. Hook et al. screened hundreds of polymeric materials in a high-throughput microarray format to identify polymers that reduce the surface attachment of pathogenic bacteria including Pa [123]. This strategy has also led to the development of a predictive quantitative structure–activity relationship (QSAR) model to predict bacterial biofilm formation on a range of polymers which were not included in the initial library. Predictions were validated through the design of new coatings found to be resistant to biofilm formation [124]. These materials have the potential to revolutionise the design of biofilm resistant medical devices.

It has been established that the dispersal of biofilm microbes into their planktonic form increases their susceptibility to antimicrobials. While biofilm degrading and dispersing agents may represent attractive adjunctive therapies for biofilm-associated chronic infections, Fleming et al. have shown that a sudden dispersal of biofilm cells can be detrimental to the host. They found that large-scale, *in-vivo* dispersal of motile biofilm bacteria by glycoside hydrolases caused lethal septicaemia in a mouse wound model in the absence of antibiotic therapy. However, when administered cautiously, dispersal agents may increase the efficacy of current antimicrobial therapies [125].

Understanding and altering how Pa virulence programs are activated.

The concept of virulence factor inhibition as an antimicrobial strategy is typically pursued through the development of targeted ‘antivirulence’ drugs which block specific effectors or associated global regulators. It is often proposed that such treatments exert weaker selection for resistance than conventional antibiotics, based on the idea that these actions disarm rather than kill directly. However, the evolutionary robustness of antivirulence drugs is still debated [126,127]. An alternate antivirulence strategy may be to target how pathogenic ‘virulence programs’ are activated. By understanding how environmental cues elicit virulence activation

in specific infection sites, it may be possible to manipulate these innate reactions to change how Pa behaves *in-vivo*. Several examples of this approach exist in the literature, suggesting that the creation of microenvironmental conditions that suppress virulence is a feasible strategy.

Nutrient availability has long been established as a means to modulate bacterial behaviour. Phosphate (Pi) depletion develops rapidly following major surgery or organ injury and can be used to predict the development of lethal sepsis. Pa senses this reduction in phosphate as an environmental cue for enhanced virulence *in-vitro* and lethality *in-vivo*. Building on this discovery, intestinal phosphate supplementation may be a novel strategy to contain pathogens associated with lethal gut-derived sepsis. In fact, phosphate crosslinked nanoparticles that result in sustained delivery of Pi were effective compounds for suppressing virulence activation in Pa *in-vitro* [128–131].

Another key nutrient, L-arginine (L-Arg), was shown to be significantly depleted in burn wounds when compared to non-inured tissue. This is a result of elevated arginase production by myeloid-derived suppressor cells, which are recruited to the site of injury in response to inflammation. L-Arg is a potent chemoattractant for Pa, with low concentrations leading to increased motility. Indeed, topical administration of L-Arg resulted in reduced Pa motility and enhanced biofilm formation, which translated to decreased Pa spread and increased animal survival in infection models. This suggests that the development of an arginine-based topical treatment has the potential to reduce the spread of infection and aid patient recovery [132,133]. Further work is warranted in order to uncover additional nutrient cues at the site of infection which can modulate Pa virulence.

Host factors also have a clear influence on Pa behaviour during infection. QS is inhibited by physiological levels of serum albumin, which sequesters homoserine lactone quorum signals (3-oxo-C12-HSL), suppressing the ability of Pa to produce QS controlled virulence factors. As QS is an important factor controlling virulence in burn wounds, the local administration of exogenous albumin may attenuate Pa virulence [134]. The biological hydrogel mucus,

consisting of biopolymers called mucins, has also been recognised to play a role in host-microbe interactions. Mucin is also the primary polymer in CF sputum which impacts on the diffusion of QS signalling molecules [135,136]. When Pa is exposed to native mucus, this triggers downregulation of virulence genes that are involved in QS (*lasR*), siderophore biosynthesis (*pvdA*), and the T3SS (*pcrV*). Purified mucins also mediated biofilm dispersal by a mechanism dependent on an intact flagellum. Extraordinarily, glycans isolated from the mucin backbone can regulate these bacterial phenotypes, even at relatively low concentrations. Thus, it may be possible to use purified mucin glycans to lessen Pa virulence, rendering it less harmful to the host [137].

The modulation of Pa central metabolism dynamics may be another opportunity to curb virulence. Using fluorescence lifetime imaging microscopy (FLIM), which reports the activity of NADH in live cells, Perinbam et al. reported that a shift in central metabolism accompanies virulence activation in Pa. Rewiring surface-attached Pa central metabolism with citrate and pyruvate supplementation decreased the enzyme-bound NADH pool, reduced total NAD(H) production, and abolished Pa host-killing activity. In contrast, glucose and glycerol addition had little impact on host-killing activity. It was also possible to induce Pa virulence at an earlier time using the ETC oxidase inhibitor, antimycin A. This suggests that the glyoxylate cycle bypass (Figure 1C) may be involved in the virulence activation of surface-attached populations, as flux thorough this metabolic node may be inhibited by citrate or pyruvate [138,139].

Barriers to success: what knowledge limitations are holding us back?

Which Pa strain should I use?

Pa strain selection is an important factor when it comes to unravelling infection mechanisms. PAO1 and PA14 are arguably the most common laboratory-adapted Pa research strains which are used worldwide [140,141]. Although these two strains have undoubtedly catalysed our

528 understanding of Pa biology, ignoring their limitations may also be holding us back from future
529 advances.

530 The microevolution of individually maintained sublines of PAO1 has resulted in significant
531 phenotypic variability and the altered production of key secreted factors (rhamnolipid,
532 pyoverdine, pyocyanin, PQS, exopolysaccharide). It is likely that small alterations exist within
533 PAO1 and PA14 strains held in laboratories across the world. These changes may potentially
534 affect the outcomes of *in-vitro* phenotypic analyses and *in-vivo* pathogenesis studies [140]. In
535 a recent transposon sequencing study using nine different Pa isolates (including five from
536 various human infection sites), the laboratory strain PA14 was an outlier in two growth
537 conditions, M9 minimal media and urine. In these two media, PA14 had more essential genes
538 involved in oxidative phosphorylation and the TCA cycle when compared to the other isolates.
539 This behaviour could be a consequence of PA14 microevolution to laboratory conditions,
540 highlighting the risks of extrapolating PA14 behaviour to the species in general [24]. There
541 also appears to be lineage specific differences in key Pa pathogenicity determinants.
542 Evaluation of the reversible attachment regime for PAO1 and PA14 suggest the existence of
543 two distinct surface colonization strategies. PAO1 lineages appear to commit quickly to a
544 surface when compared to PA14 lineages. These differences may be explained by
545 modifications of their corresponding Wsp-based and Pil-Chp-based surface-sensing systems
546 [142]. A simple solution to this strain issue is to increase the use of Pa clinical isolates for
547 infection-site specific analyses; however, these strains can be challenging to work with
548 because of inherent growth defects or issues with genetic tractability. As per the reversible
549 attachment study [142], a solution may lie in examining multiple laboratory strains
550 simultaneously when elucidating core mechanisms. This will also unravel strain specific
551 attributes and increase our knowledge of strain divergence.

552 **All models are wrong, but some are useful: recapitulating Pa infection**

553 A variety of laboratory models are available to study Pa infection, each with their own
554 advantages and disadvantages. As with strain choice, poor infection model choice can lead to

555 inconsistent or opposing studies, including virulence factor importance or antimicrobial
556 efficacy during infection. This in turn can lead to erroneous results in drug development or
557 synergy studies [143]. This issue is compounded by the fact that current preclinical animal
558 models do not satisfactorily predict the complexities of human respiratory disease, further
559 endangering the success of antimicrobial drug efficacy testing [144]. In addition, experimental
560 protocols when using infection models, such as inoculum CFU or immunosuppression
561 regimen, are not universally standardized or reported. The introduction of minimum
562 information guidelines, as recently introduced for *in-vitro* biofilm formation assays, may help
563 to alleviate this issue [145].

564 A stark example of changes in Pa phenotypes and behavior when grown in different model
565 systems is apparent in relation to glycoside hydrolase activity. These enzymes are known to
566 cause the dispersal of Pa from biofilms into a free-swimming state, increasing antimicrobial
567 efficacy. Several strains of Pa biofilms were grown *in-vivo* (in a mouse model of wound
568 infection) and then excised and treated *ex-vivo*. These same strains were also grown as an
569 *in-vitro* biofilm for comparison. Surprisingly, the biofilms that had the greatest dispersal
570 response when treated *ex-vivo* were those for which the *in-vitro* treatments had the least
571 effect. This highlights the possibility that effective approaches for biofilm eradication may
572 depend strongly on the growth environment, stressing the need for taking growth conditions
573 into account when devising antibiofilm strategies [146].

574 Recent work is applying machine learning approaches to optimise and evaluate the quality
575 of models to recapitulate Pa infection sites. This work uncovered that the *in-vitro* synthetic CF
576 sputum medium model and a CF airway epithelial cell model had the highest genome-wide
577 accuracy when attempting to recreate the Pa transcriptomic signature during CF lung infection.
578 Nevertheless, these models still underperformed on distinct categories, including porins and
579 polyamine biosynthesis for the synthetic sputum medium and protein synthesis for the
580 epithelial cell model. Interestingly, over 200 genes that were not mimicked in a Pa model strain
581 were largely captured by using a clinical isolate, illustrating the limitations of laboratory strains

582 [147]. This evidence-based method of selecting and improving laboratory models is a move in
583 the right direction. Paradigm shifts need to occur in assessing a variety of Pa infection sites,
584 particularly chronic wounds and burns [148]. Using metabolomic and transcriptomic data from
585 infection sites as a reference point, the challenge ahead will be to empirically develop
586 reproducible models to mimic these environments. This may be aided by the inclusion of
587 additional variables such as polymicrobial communities, therapeutics, and reproducible spatial
588 structure.

589 **Knowledge gaps in Pa infection biology**

590 While the above studies have proposed several new approaches to combat infection, there
591 are still significant gaps in our understanding of Pa infection biology. It's important to note that
592 although the *in-vivo* Pa omics data have provided a snapshot of a largely advanced stage of
593 infection, these data do not explain how Pa establishes itself during infection or how it
594 dominates in various infection sites, eliminating competing pathogens. Many testable models
595 exist to explain how these processes occur and they are likely to be the focus of further
596 extensive studies. There is a clear need for greater understanding of the mechanisms
597 underlying both the initiation of Pa infection and dispersal from established biofilms (for both
598 acute and chronic infections), as these processes appear to be at the intersection of virulence
599 and antimicrobial susceptibility. It is also worth noting that the rationale behind several key
600 metabolic and virulence rearrangements which are known to occur in Pa as it transitions from
601 an acute to a chronic infection is of significant interest. Additional factors such as spatial
602 structure and environmental heterogeneity are likely to drive the diversification of Pa within
603 the infection site. For this reason, it is likely that multiple evolutionary trajectories to
604 pathoadaptation may exist, complicating an empirical disentanglement of this phenomenon
605 [149].

606 Metabolic flexibility, interconnected nodes and rapid pathway reorganisation through mutation
607 are major impediments in targeting Pa. The last decade has resulted in unprecedented
608 developments in our understanding of the complexity and crosstalk within Pa QS systems,

609 virulence factor production and central metabolism, and systems level interactions across
610 these key nodes. We need to further refine our understanding of these central targetable
611 pathways on a systems level in order to screen for inhibitors *in-silico* [150]. This review has
612 highlighted a series of core metabolic components in Pa which appear to be crucial for
613 sustaining infection (Figure 1 – red boxes). If further drug discovery resources are directed
614 towards modulating these key systems, it is likely to yield effective antipseudomonal therapies.
615 However, in order to fully grasp Pa infection, there must be a directed effort towards strong
616 functional genomics approaches which will elucidate the precise role of uncharacterized genes
617 which are essential for Pa growth or virulence during infection. It is likely that *in-vitro* studies
618 which neglect the importance of polymicrobial interactions, *in-vivo* stresses or genetic
619 mutation are only allowing us to grasp a fraction of Pa physiology, masking the importance of
620 infection-relevant genetic networks. The clear importance of polymicrobial interactions on Pa
621 physiology during infection have been reviewed elsewhere [151,152].

622 What technologies will lead these advances? Machine learning will undoubtedly continue to
623 play a large role in future leaps in understanding Pa infection [10,153,154]. Developments in
624 structural biology are also providing new mechanistic insight into how Pa develops resistance
625 to current therapies. The cryo-electron microscopy (Cryo-EM) structures of the Pa ribosome
626 and a uL6 mutant ribosome from a CF isolate were recently elucidated. The uL6 deletion
627 causes resistance to members of the aminoglycoside family of antibiotics, ribosomal
628 instability, and a reduced growth rate. The structural data have offered explanations for these
629 phenotypic alterations and will act as a template for unravelling the consequences of ribosomal
630 variant strains obtained during infection [155]. Understanding drug resistance on a molecular
631 level may then enable rapid screening approaches for personalized therapies. Although
632 sequencing technologies will continue to play a large role in driving Pa biology forward, the
633 combined use of other technologies, such as microscopy, machine learning, mass
634 spectrometry, structural biology, and non-invasive metabolic sensors will be essential to
635 catalyse future research advances.

Concluding remarks

Tremendous progress has been achieved in understanding Pa infection, however, there are still many open questions to be answered. The large-scale testing and validation of new antimicrobial therapies is ongoing, but it is likely that we're beholden to the therapies that we have now for the foreseeable future. For this reason, enhancing the efficacy of current antimicrobial therapies by the potentiation of cellular respiration (hyperbaric oxygen treatment, metabolic activators), or through biofilm dispersal strategies (NO generators, glycoside hydrolases) may be the easiest road forward. Virulence reduction strategies targeting QS, T3SS and Pa attachment hold promise as adjuvants and may offer a new path for narrow spectrum therapies against multidrug-resistant clinical isolates. Although strategies targeting core metabolism or virulence appear as separate entities in this review, the extensive crosstalk between these systems makes the specific targeting of any one system extremely difficult in this organism.

Carle Gessard's interest in Pa was piqued over 130 years ago by its "radiant blue/green secretions" (pyocyanin) and its ability to grow "equally well in saliva, perspiration, albumin, blister fluid and liquid from hydroceles" [156]. We now appreciate that combatting this pathogen will depend on exploiting this metabolic flexibility and its ability to synthesise these potent virulence factors. The above research directions have taken us closer to accomplishing this aim. It is clear that it will be a hard-won war, but each battle brings its own new and interesting challenges which make for rewarding and exciting research.

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Figure Legends

Figure 1: A schematic overview of the interconnected physiologic networks of Pa; Cellular respiration [A], denitrification [B] and carbon metabolism [C]. Avenues to target these systems (discussed in the main text) are highlighted with red boxes. **Cellular respiration:** Pa encodes a collection of aerobic terminal oxidases (Cco1, Cco2, Cyo, Cox and CIO) which catalyze electron transfer from the respiratory apparatus to oxygen (cellular respiration – represented by NADH dehydrogenase (NDH), succinate-Q-reductase complex (SDH), terminal oxidases and ATP synthase) The different terminal oxidases vary in their affinities, electron donor (ubiquinol or cytochrome *c*) and efficiencies. Pa phenazines (such as pyocyanin) can synergise with these terminal oxidases to confer antibiotic tolerance and aid in aerobic and anaerobic functionality. Therefore, the Pa terminal oxidases and associated reactions are a potential antimicrobial target [157]. Increased production of electron donors increases the proton motive force (PMF), driving metabolism and leading to increased levels of intracellular reactive oxygen species (ROS). Cell-damaging ROS can be harnessed to increase the efficacy of antibiotics targeting Pa [158]. **Denitrification:** The Pa denitrification pathway is activated in the presence of low oxygen. Here, nitrate (NO_3^-) is reduced to molecular nitrogen (N_2) in four steps, each catalyzed by a specific reductase, namely, nitrate reductase (NAR), nitrite reductase (NIR), NO reductase (NOR), and nitrous oxide reductase (N_2OR). Q, coenzyme Q or ubiquinone; QH_2, reduced form of ubiquinone [159]. Modulation key steps of this pathway by using targeted inhibitors and/or selective reductase overactivation/inhibition can lead to the uncontrolled accumulation of reactive nitrogen species (RNS), such as nitric oxide (NO) and nitrite ($NO2^-$). These toxic intermediates can potentiate the efficacy of therapeutic antibiotics. **Central carbon metabolism**: the Pa central metabolic network consists of six main metabolic blocks, identified with different colours: (i) the peripheral pathways, that encompass the oxidative transformation of glucose, acetate, lactate and glycerol (grey); (ii) the Embden-Meyerhoff-Parnas pathway, orange); (iii) the pentose

phosphate pathway (PPP, green); (iv) the Entner-Doudoroff pathway (EDP, purple); (v) the tricarboxylic acid cycle (blue) and glyoxylate cycle (red); and (vi) anaplerotic and gluconeogenic bioreactions (yellow) [15,16]. The first enzyme of the glyoxylate shunt (AceA) is highlighted. The triosephosphate isomerase TpiA is another validated metabolic target. The natural product promysalin targets succinate dehydrogenase (SDH), an essential reaction in Pa carbon metabolism. SDH is also a central ETC component (succinate-Q reductase complex).

Abbreviations; AcCoA, acetyl-coenzyme A; CIT, citrate; PYR, pyruvate, ICIT, isocitrate; AKG, alpha-ketoglutarate; FUM, fumarate; MAL, malate; OAA, oxaloacetate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; G3P, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; G6P, glucose 6-phosphate; DHAP, dihydroxyacetone phosphate; 6PG, 6-phosphogluconate; Ri5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; FMN, flavin mononucleotide; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; CO_2, carbon dioxide; QH_2, ubiquinol; UQ_8H_2, ubiquinol-8; Pi, inorganic phosphate; H^+, proton; $FADH_2$, flavin adenine dinucleotide; H_2O, water.

Figure 2: Stimulating metabolic flux drives cellular respiration, which in turn sensitises Pa to antimicrobial intervention. A; The production of electron donors (NADH, FADH) stimulates the proton motive force (PMF), which is essential for the uptake of many antibiotics (tobramycin uptake depicted). This ramp up of metabolism may also lead to increased levels of intracellular reactive oxygen species (ROS), which potentiate antibiotic efficacy. The uptake of tobramycin is an energy dependent process that relies on the electron transport chain (ETC) to maintain a sufficiently high PMF [158]. **B;** Central metabolism is largely downregulated during Pa infection, with increased expression of the glyoxylate shunt (red)d, skipping the decarboxylation steps of the TCA cycle. This reduced cellular respiration and growth

consequently lowers the uptake of PMF dependent antibiotics, such as the aminoglycosides, increasing tolerance. **C;** Reactivating Pa respiration in hypoxic Pa aggregates using hyperbaric oxygen (O_2) treatment (HBOT) or metabolic potentiators will then increase cellular respiration (full TCA cycle flux - green) and the PMF, increasing antibiotic efficacy . **B;** Central metabolism is largely downregulated during Pa infection, with increased expression of the glyoxylate shunt (red), skipping the decarboxylation steps of the TCA cycle. This reduced cellular respiration and growth consequently lowers the uptake of PMF dependent antibiotics, such as the aminoglycosides, increasing tolerance. **C;** Reactivating Pa respiration in hypoxic Pa aggregates using hyperbaric oxygen (O_2) treatment (HBOT) or metabolic potentiators will then increase cellular respiration (full TCA cycle flux - green) and the PMF, increasing antibiotic efficacy [160].

Figure 3: A schematic overview of the Pa quorum sensing network, their respective signalling molecules and validated inhibition sites. As the bacterial cell density increases, QS signalling molecules build-up until reaching a threshold concentration. This accumulation results in the transcription of genes under QS regulation, activating a battery of virulence factors. The **las system** consists of a transcriptional activator, LasR, and the acyl-homoserine lactone (AHL) synthase, LasI, which catalyses synthesis of the autoinducer N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL, also known OdDHL). The transcriptional regulator RsaL negatively regulates *lasI* synthesis. This signalling molecule binds to the transcriptional activator LasR, and the OdDHL-LasR complex then stimulates the expression of target genes. The **rhl system** consists of the LuxI type autoinducer synthetase, RhlI, which synthesises the autoinducer molecule *N*-butyryl-L-homoserine lactone (C4-HSL, also known BHL). This signalling molecule binds to the transcriptional activator RhlR, and the BHL-RhlR complex then stimulates the expression of target genes. The QS inhibitor compound meta-bromo-thiolactone (mBTL) partially inhibits both LasR and RhlR, blocking both the production of pyocyanin and biofilm formation. The **pqs system** (PQS - *Pseudomonas* quinolone signal) consists of PqsA-E, PhnAB, PqsH and a LysR family

regulator PqsR. Anthranilate synthesised by PhnAB is processed by the enzymes PqsA to PqsD to generate 2-heptyl-4-quinolone (HHQ). HHQ is then converted into PQS (2-heptyl-3-hydroxy-4-quinolone) by PqsH. The PQS:PqsR and HHQ:PqsR complexes stimulate the expression of target genes. A key target in this system, PqsE, is a thioesterase which cleaves 2-aminobenzoylacetyl-CoA, resulting in 2-aminobenzoylacetate, the precursor of HHQ. The benzamide-benzimidazole compound M64 inhibits PqsR, which in turn interferes with biofilm formation and potentiates antibiotic efficacy [76,81,87].

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Figure1

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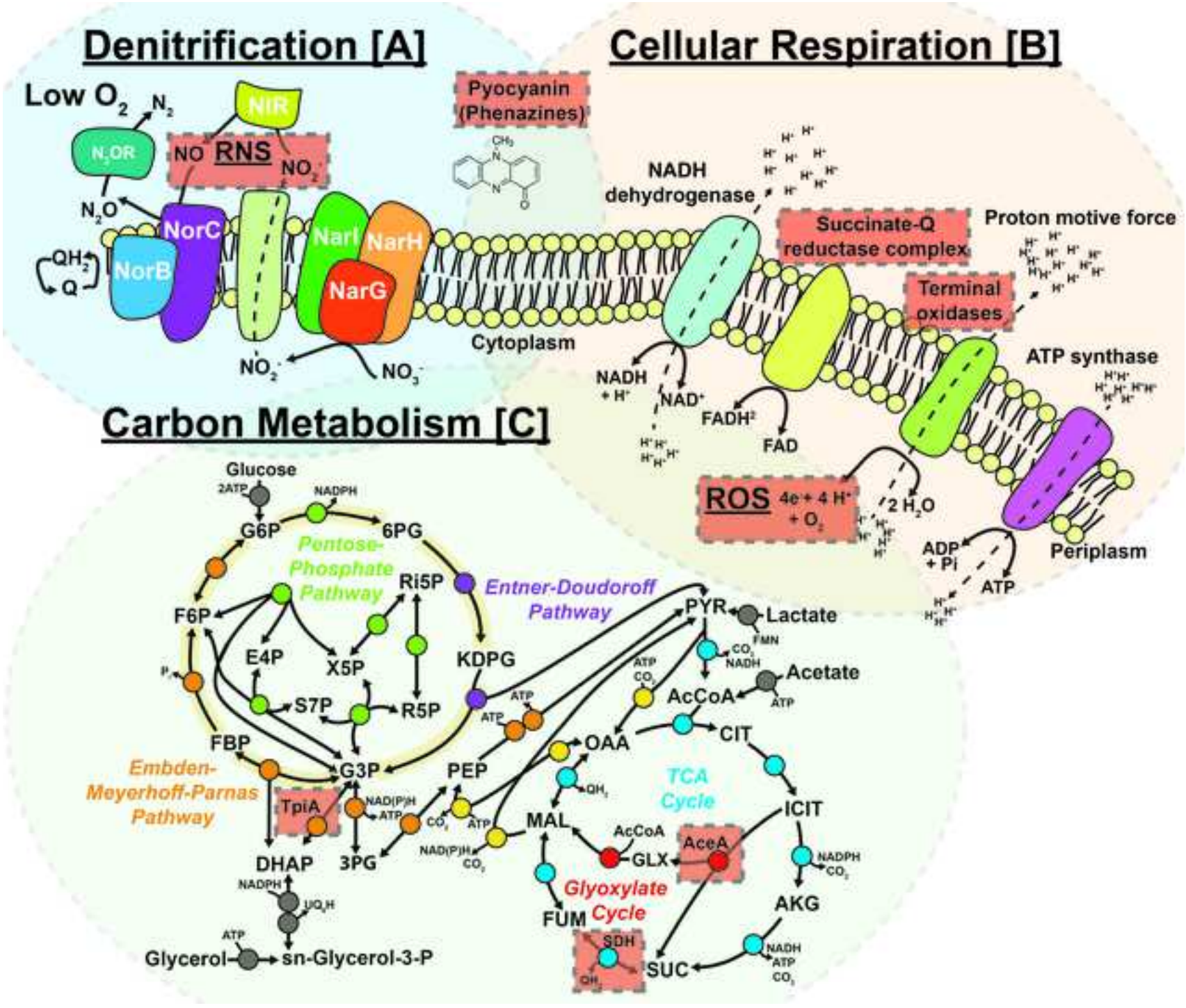

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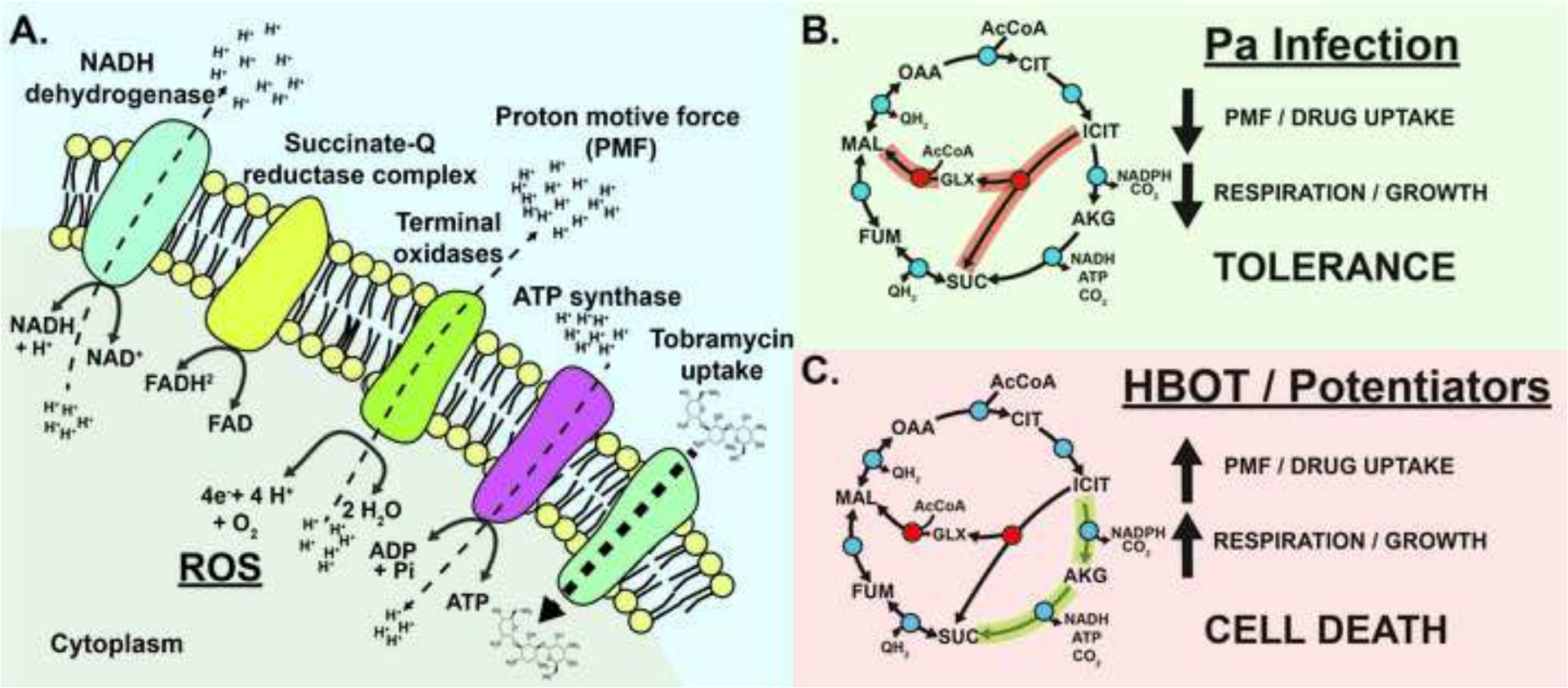

Figure3

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