General and condition-specific essential functions of *Pseudomonas aeruginosa*

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The essential functions of a bacterial pathogen reflect the most basic processes required for its viability and growth, and represent potential therapeutic targets. Most screens for essential genes have assayed a single condition—growth in a rich undefined medium—and thus have not distinguished genes that are generally essential from those that are specific to this particular condition. To help define these classes for *Pseudomonas aeruginosa*, we identified genes required for growth on six different media, including a medium made from cystic fibrosis patient sputum. The analysis used the Tn-seq circle method to achieve high genome coverage and analyzed more than 1,000,000 unique insertion positions (an average of one insertion every 6.0 bp). We identified 352 general and 199 condition-specific essential genes. A subset of assignments was verified in individual strains with regulated expression alleles. The profile of essential genes revealed that, compared with *Escherichia coli*, *P. aeruginosa* is highly vulnerable to mutations disrupting central carbon-energy metabolism and reactive oxygen defenses. These vulnerabilities may arise from the stripped-down architecture of the organism's carbohydrate utilization pathways and its reliance on respiration for energy generation. The essential function profile thus provides fundamental insights into *P. aeruginosa* physiology as well as identifying candidate targets for new antibacterial agents.

Tn-seq | ESKAPE | cystic fibrosis | antibiotic target | sputum

There is a pressing need for new drugs to treat bacterial infections, particularly those caused by pathogens frequently resistant to multiple antibiotics (1, 2). One of these organisms, the Gram-negative bacterium *Pseudomonas aeruginosa*, has emerged as a central threat in healthcare settings and can cause a variety of infections, including: acute pneumonia, bacteremia and wound, urinary tract, intra-abdominal, and chronic airway infections (3). Mortality rates for some of these infections can exceed 60%. Accordingly, *P. aeruginosa* is one of six pathogens for which new antibacterial agents are most desperately needed (1).

One approach to identify new drugs active against a pathogen like *P. aeruginosa* is to target processes required for its growth and survival (4). Such processes depend on the organism's essential gene set. Both general and growth condition-specific essential genes can be distinguished. General essential genes are required under virtually all growth conditions, whereas condition-specific essential genes are required under a subset of conditions. General essential functions can be further subdivided into those that are required in all organisms for fundamental processes like DNA replication, and those more limited in phylogenetic distribution, which are more likely to be important for the particular lifestyle of an organism. General essential functions represent potential drug targets, although essentiality is only one of several features that characterize good targets (5–7).

Essential genes have been identified at genome scale using several approaches (8). Targeted deletion mutagenesis was used to define a group of about 300 *Escherichia coli* genes essential for growth on nutrient medium (9). This group has been refined

and validated, and serves as a high quality reference set (10). An alternative method, sequencing of saturation-level pools of transposon mutants ("Tn-seq"), has become popular for essential gene screening because of its technical ease (11–15). However, most Tn-seq studies have included only limited confirmation of findings using individual mutants (8).

Three previous studies have identified *P. aeruginosa* essential genes. Two projects creating ordered transposon mutant libraries helped define such genes by exclusion, and the results were combined to specify candidate essential genes conserved in the strains analyzed (16, 17). More recent work identified genes essential for growth of strain PA14 at high genome coverage using Tn-seq. (18). All three studies examined bacteria grown only on Luria-Bertani (LB) nutrient agar and did not include follow-up verifications.

In the study reported here, we defined *P. aeruginosa* genes essential for growth under multiple conditions. The analysis used Tn-seq and achieved greater genome coverage than reached previously for any species. The present effort identified 352 general essential genes and 199 conditionally essential genes. These results provide insights into central elements of *P. aeruginosa* physiology and reveal vulnerabilities that could potentially be exploited therapeutically.

Results and Discussion

Identifying Essential Genes. We sought to identify both general and growth medium-specific essential genes of *P. aeruginosa*. To do this, we generated 13 fully independent, saturation-level transposon mutant pools on six different media and characterized them using Tn-seq. (15). To achieve high insertion density, we used a transposon with low insertion specificity derived from Tn5, and to limit false assignments caused by polarity, the transposon carried an outward facing promoter designed to express downstream genes (16, 19). Essential genes were defined as those with few or no insertions (*SI Materials and Methods*).

We analyzed three media in depth: LB nutrient agar, MOPS-pyruvate agar, and media made from cystic fibrosis sputa. We chose

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Significance

There is an urgent need for new antibiotics active against resistant bacterial pathogens like *Pseudomonas aeruginosa*. Target-directed drug development provides a potential path to such drugs, and essential gene products represent potential targets. Accordingly, the work reported here defines a highly verified set of such functions for *P. aeruginosa* required for growth under a variety of different conditions.

LB because it is a standard rich medium promoting rapid growth, and because it enables a direct comparison with the previous essentiality studies. We expected LB to be the most permissive medium, but this turned out not to be true. MOPS-pyruvate was used as a minimal medium to provide maximum nutritional contrast with LB. Sputum medium was used because it corresponds to the bacterial growth medium in vivo in cystic fibrosis infections, in which bacteria reach high cell densities. The makeup of mutant pools selected on each medium was defined using the Tn-seq circle method (15), with 49,000–253,000 unique insertion sites identified per pool (Table 1). Technical and biological replicate assays gave reproducible results (Fig. S1). Unique insertions at 1,043,276 sites were detected in the analysis, a genome average of one unique insertion per 6.0 bp.

Two representative transposon insertion patterns help illustrate how essential genes were identified (Fig. 1). Fig. 1*A* shows an operon with two genes (20). The downstream gene on the right (PA0404) lacks insertions except at the very 5′ end and is assumed to be essential. The upstream gene on the left (PA0405) has a high density of insertions and is considered nonessential. Note that the orientation of the insertions in PA0405 is highly biased (bars above the line) such that the transposon outward-facing promoter should promote expression of PA0404. The forward-oriented insertions in PA0405 apparently circumvent polarity on PA0404 expression through the activity of the promoter. Fig. 1*B* shows an operon with three essential genes (*dnaA*, *dnaN*, and *gyrB*) and one nonessential gene (*recF*). In this case, insertions in *recF* were not polar on *gyrB*, regardless of orientation. This result suggests that a previously unrecognized secondary promoter exists for the *gyrB* gene. Even long operons of essential genes tolerated insertions in the appropriate orientation between genes (Fig. S2), indicating that polar effects were not a significant source of false essential assignments in this study.

Note that although the four essential genes shown in Fig. 1 were nearly entirely lacking insertions, they were not totally devoid of them. We assume that the exceptional insertions were not inactivating: for example, because of insertion in one copy of a transiently duplicated gene or to the presence of a suppressor mutation in the target cell (21).

Histograms of the number of insertion sequencing reads per annotated protein-coding gene identified well-separated groups corresponding to genes without, and with, insertions for each Tn-seq run (Fig. S3). Candidate essential genes for a given run were defined as those falling significantly below the main peak on the right (*SI Materials and Methods*). Genes falling in this region for more than half of the independent mutant pools prepared per condition were considered to be confirmed essentials.

To include additional genes with essential domains only, or essential genes that were absent from the genome annotation (20), we searched independently of the annotation for genome regions lacking insertions (*SI Materials and Methods* and Dataset S1) (13). We found 22 protein-coding genes with essential domains and one gene that had not previously been recognized in the annotation (PA0728.1) (see below). In addition, the analysis identified 18 candidate essential regions that overlap possible small regulatory RNA coding sequences (22) (Dataset S1).

General and Condition-Specific Essential Genes. For the three primary growth conditions analyzed, we identified consensus sets of confirmed essential genes (Dataset S1). We found that 352 genes were required on all three media, and refer to these as "general" essential genes (Fig. 2). An additional 199 genes were essential under a subset of the conditions and are called "condition-specific" essentials. Depending on the medium, 11–23% of essential genes were condition-specific.

The distribution of the general essential genes in functional categories was typical of that found in previous studies (8), with "translation, post-translational modification, and degradation" being most enriched (Table S1). Among the LB-specific essentials, there was an unexpected enrichment of genes in the "cell wall/LPS/capsule" category, which traced primarily to genes needed for LPS O-antigen synthesis (see below). Genes of unknown function were common in both general and condition-specific classes of essentials.

Nearly all of the general and condition-specific essential genes (99% and 90%, respectively) belong to the *P. aeruginosa* core genome (23) (Dataset S1). Two of the exceptional general essential genes absent from the core genome (*imm2* and PA0728.1) encode functions that block the expression or action of toxic proteins that are encoded by the corresponding strains (discussed below).

Comparison with previous studies. Three previous reports identified candidate essential genes for *P. aeruginosa* growth on LB medium (16–18). Comparison with our general essentials identifies 141 genes found in all four studies (Dataset S1). This overlap group represents a highly confirmed set of genes, but appears to be a severe underestimate based on its small number (8), and because it lacks many genes generally assumed to be essential, like the replication gene *dnaB*, the RNA polymerase gene *rpoC*, and the secretion apparatus gene *secE*.

Table 1. Tn-seq assays

Mutant pool	Tn-seq run	Colonies pooled	Mapped reads	Unique insertion sites	Essential coding genes
LB-1	1	139,100	2,303,025	94114	468
	2	139,100	13,436,777	88202	485
	3	139,100	8,997,565	63546	561
LB-2	1	106,400	14,904,269	81677	429
	2	106,400	18,640,542	81550	495
LB-3	1	172,800	24,322,071	109951	467
Minimal-1	1	240,650	28,214,377	193993	421
Minimal-2	1	312,000	13,427,239	205614	406
Minimal-3	1	285,800	5,160,085	252948	450
Sputum-1	1	76,400	5,661,970	79723	446
Sputum-2	1	144,000	7,630,737	181477	378
Sputum-3	1	46,800	14,522,500	48635	564
Sputum-4	1	96,600	2,992,340	91843	469
0.1X LB	1	180,800	8,736,912	121357	423
Human Serum	1	155,400	754,804	127933	488
BHI	1	216,000	4,439,772	174631	483
Total		2,172,750		1,043,276*	

The Tn-seq results for 16 experiments corresponding to 13 independent mutant pools are summarized. The essential coding genes for each run were combined to identify consensus essentials for each media type.
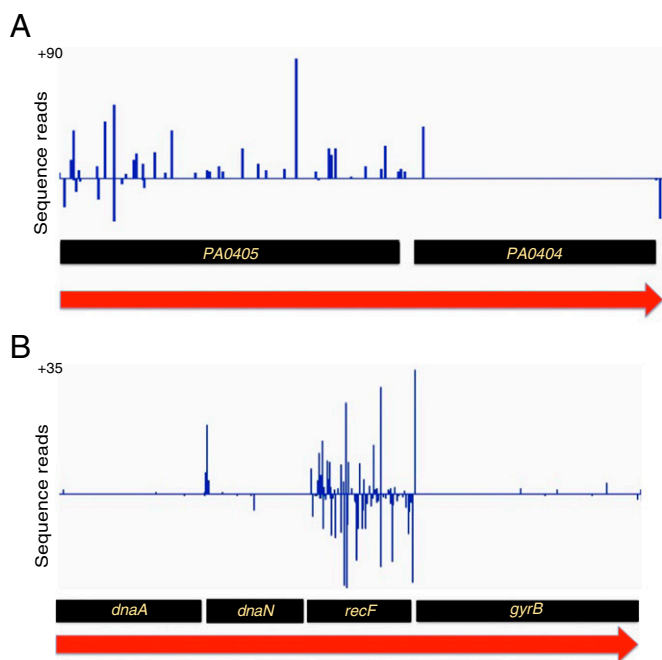*Corresponds to one insertion every 6.0 bp.

Fig. 1. Transposon insertions in two regions of the genome. The distributions of insertions in two predicted operons are shown. The height of each bar shows the Tn-seq read number at the corresponding position, with the placement above or below the line corresponding to the transposon orientation. (A) PA0405-PA0404 operon; (B) dnaA-dnaN-recF-gyrB operon.

We also compared the *P. aeruginosa* essential genes to the highly verified *E. coli* set (10). There are 270 *P. aeruginosa* orthologs of the 304 *E. coli* genes essential for growth on nutrient agar (24). Nearly all (87%) of the *P. aeruginosa* orthologs were essential for growth on LB (Table 2). This group corresponds to about half (48%) of all of the *P. aeruginosa* genes essential on LB. We assume that the remainder of the genes in the set consists of *P. aeruginosa*-specific essentials, unrecognized homologs, and any false assignments that may have been included. The general essential set of *P. aeruginosa* genes also provided good coverage of the orthologs of the *E. coli* essentials (85%), with fewer additional genes (35% of the total). Two previous lists of *P. aeruginosa* essential genes provided somewhat lower coverage with higher fractions of additional genes. The results imply that orthologs of most *E. coli* essential genes are also essential in *P. aeruginosa*, and that such genes are nearly all generally essential.
Additional media. Genes classified as general essentials based on the three primary media should be required for growth on most or all additional media. As a test of this, we carried out Tn-seq analyses of individual mutant pools generated on three additional media: another high nutrient medium (brain-heart infusion, BHI), a dilute nutrient medium with supplements (0.1 × LB), and medium made from human serum (*SI Materials and Methods*). We found that 97–100% of the general essential genes were also required on these media (Dataset S1), strongly supporting their assignments.
Mutants with regulated expression of individual genes. A potential limitation of Tn-seq screening for essential genes is that because mutants grown in competition are analyzed, slow-growing as well as nongrowing mutants may be lost from the population and their genes counted as essential (15). As a test of the extent to which this is true, we constructed strains with regulated versions of a sample of general essential genes. The strains were constructed by chromosomal integration of plasmids carrying inducible promoters with different expression ranges (*E. coli araBAD* and *trp-lac*) (*SI Materials and Methods*). Of 46 operons examined (corresponding to 62 essential genes), 43 led to inducer-

stimulated growth and 25 showed nearly complete inducer-dependence (Table S2). We assume that some of the strains may show only partial inducer-dependence because of residual expression in the absence of inducer. The results indicate that most of the general essential genes are required for optimal growth of individual colonies, and that at least half are strongly required.
Phylogenetically widespread essential functions. The 551 essential genes we identified underlie diverse biological processes. About two-thirds of the *P. aeruginosa* general essential genes correspond to genes essential in *E. coli* and most other bacteria (8). These genes underlie the most fundamental cellular processes: DNA replication, transcription and translation, protein folding and export, RNA degradation, peptidoglycan synthesis, and cofactor, nucleotide, and lipid synthesis (Table S1). The functions represent potential broad host range antibiotic targets.
Less-widespread functions. A number of essential genes of *P. aeruginosa* are not essential in *E. coli*. Two processes in which such genes are conspicuous are central carbon-energy metabolism and protection from reactive oxygen damage.
Central carbon-energy metabolism. Sixteen steps of central carbon metabolism in *P. aeruginosa* are essential under multiple growth conditions, with eight essential under all of the conditions examined (Table S3). The essential functions are found in glycolysis/gluconeogenesis (Embden–Meyerhof pathway, EMP), the pentose phosphate shunt (pentose phosphate pathway, PPP) and the tricarboxylic acid cycle (TCA) (Fig. 3). Only four of the genes in these pathways are essential in *E. coli* for growth on LB. The difference between the two species reflects their remarkably different central carbon metabolisms.

E. coli metabolizes glucose and other sugars by three pathways: glycolysis (EMP), the PPP, and the Entner–Doudoroff pathway (EDP) (25). In *P. aeruginosa*, glycolysis and the upper (oxidative) branch of the PPP are not available because of the absence of key enzymes (phosphofructokinase and phosphogluconate dehydrogenase) (26, 27). In addition, *P. aeruginosa* has a highly active glucose oxidation pathway that limits direct glucose phosphorylation by glucokinase under aerobic growth conditions. As a result of these differences, sugars are metabolized in *P. aeruginosa* primarily by the EDP (26, 27). Accordingly, essential intermediates of the EMP and PPP, such as fructose-6-phosphate and ribose-5-phosphate, must be made by gluconeogenesis and the lower (nonoxidative) branch of the PPP. Thus, in *P. aeruginosa*, in contrast to *E. coli*, there are only single—rather than double—routes of biosynthesis available for such intermediates. The constraints introduced by this architecture apparently make upper EMP and PPP pathway functions, such as fructose bisphosphate phosphatase and transketolase, which are dispensable for *E. coli*, essential in *P. aeruginosa*.
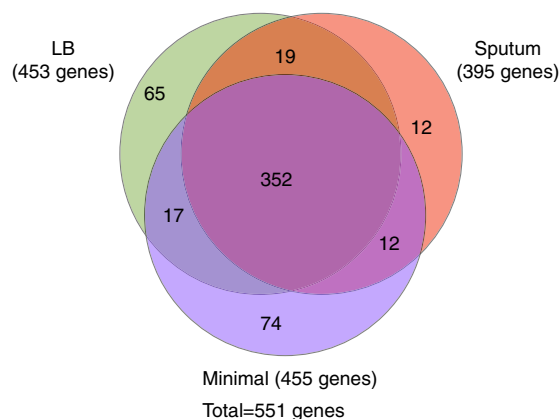

Fig. 2. Overlap among genes essential under different growth conditions. The distribution of 551 genes found to be essential under at least one of the three growth conditions examined in depth is shown.

Table 2. Comparison of *P. aeruginosa* and *E. coli* essential genes

Category	Essential genes (present study)		Essential genes (previous studies)	
	LB	General	LB (17)	LB (18)
Strain	PAO1	PAO1	PA14/ PAO1*	PA14
Candidate essential genes	453	352	335	634[†]
Coverage of essential *E.coli* orthologs	0.87 (236/270)	0.85 (229/270)	0.52 (139/269)	0.58 (156/269)
Additional PA genes (fraction of total)	217 (0.48)	123 (0.35)	196 (0.59)	478 (0.75)

P. aeruginosa orthologs of *E. coli* essential genes (9) (including four *dap* genes and *rpoH*) were identified for strain PAO1 (270 orthologs) and PA14 (269 orthologs) (19).
*Consensus of two studies (16, 17).
[†]Omits one duplicate in published list.

P. aeruginosa relies mainly on respiration for energy, with very limited fermentation capability (28). This reliance helps explain the essentiality of most steps of the TCA, because the TCA is the primary source of reducing equivalents for electron transport. The finding that not all TCA steps are essential may be rationalized by known metabolic redundancies (Fig. 3). In addition, the essentiality of aconitase (AcnB) on all primary media, but citrate synthase only on MOPS-pyruvate, seems paradoxical but may be because of secondary activity of 2-methylcitrate synthase (29).

Its orientation to respiration for ATP production probably also explains why, unlike *E. coli*, the *P. aeruginosa* proton ATPase genes (PA5554–PA5560) are general essentials. *E. coli* ATPase mutants are viable because they can grow on substrates supporting fermentative ATP production, bypassing the need for oxidative phosphorylation (30). Cytochrome oxidase genes are not essential for aerobic growth of *P. aeruginosa,* presumably because of the multiplicity of such functions (31). Remarkably, however, three general essential genes (PA2951– PA2953) were identified whose (mitochondrial) homologs function in respiration to transfer electrons from dehydrogenases to the ubiquinone pool (32, 33). In addition, a putative cytochrome c1 (PA4429) is essential for growth on LB.

Reactive oxygen defense. Several *P. aeruginosa* functions that protect cells from superoxide and hydrogen peroxide are essential. One of two cytoplasmic superoxide dismutases (SodB) is a general essential under our conditions, consistent with the slow-growth phenotype of mutants characterized previously (34). Other essential functions may protect cells from reactive oxygen species (ROS) through repair of damaged Fe-S clusters (PA0759, YfgZ), replenishing NADPH pools depleted by superoxide (ferredoxin-NADPH reductase, Fpr) and controlling free iron levels in the cytoplasm (ferric uptake regulator, Fur) (35–37). *E. coli* mutations in the corresponding genes increase sensitivity to ROS, but are not lethal under unstressed growth conditions (although inactivation of both cytoplasmic superoxide dismutases is lethal) (38).

Two factors that may contribute to the high sensitivity of *P. aeruginosa* to loss of ROS defenses are the organism's production of redox active phenazines and the essentiality of iron-containing proteins sensitive to inactivation by ROS. Phenazines are toxic to organisms ranging from other bacteria to metazoans, and it may be that an important role of superoxide defenses is to protect *P. aeruginosa* from its own poison. Fe-S–dependent dehydratases and mononuclear iron enzymes are key targets of superoxide and hydrogen peroxide (38). Several such enzymes of central carbon metabolism are essential in *P. aeruginosa*: aconitase (AcnB, PA1787) and fumarase (PA4333) of the TCA, and ribulose-5-phosphate 3-epimerase of the PPP (39). An additional target of reactive oxygen is the Isc Fe-S cluster synthesis system (40), which is essential in *P. aeruginosa*. The corresponding system in *E. coli* is not essential because it has a backup system (Suf); inactivation of both systems is lethal (41). Other targets of reactive oxygen that are essential, such as peptide deformylase (PA0019) and Fur (PA4764), presumably also contribute to the *P. aeruginosa* dependence on reactive oxygen defense functions.

Other Essential Processes.

Lipopolysaccharide synthesis. As in *E. coli* and other Gram-negative bacteria, genes required for production of lipid A and KDO (3-deoy-D-manno-octulosonic acid) are essential in *P. aeruginosa*. However, inner-core heptose and outer-core galactosamine are also generally essential in *P. aeruginosa* (42).

LPS outer-core rhamnose and O-antigen were essential on LB nutrient agar. Previous studies have found that loss of O-antigen can reduce PAO1 growth because it leads to sensitivity to a potent self-produced bacteriocin (pyocin R2) (43). To test whether this mechanism explained our finding, we constructed three mutants deleted of O-antigen biosynthetic genes in pyocin-producing and nonproducing strains. In the pyocin[+] genetic background, all of the mutations severely inhibited growth on LB agar, confirming
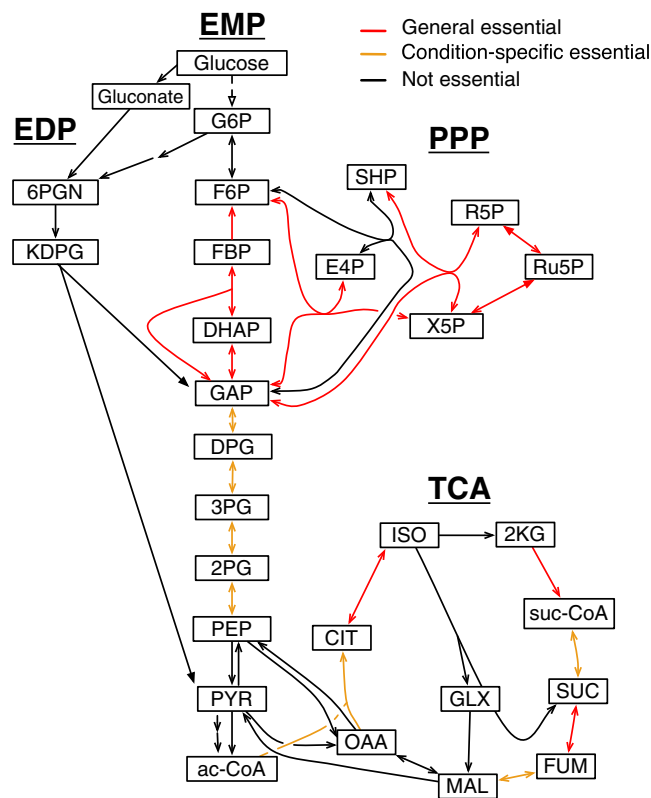

Fig. 3. Essential functions of central carbon metabolism. The essentialities of different steps of the EDP, glycolysis (EMP), PPP, and TCA are indicated. Some biochemical steps may be essential but not detected because of genetic redundancy: pyruvate kinase (*pyrA, pyrK*), malate oxidoreductase (*mqoA, mqoB*, PA1252), pyruvate carboxylase (PA1400, PA5435-PA5436), and isocitrate dehydrogenase (*icd, idh*).

the Tn-seq findings (Fig. S4). However, in a strain background deleted of pyocin R2 genes, the O-antigen mutations had little effect on growth. Thus, it appears that O-antigen was indeed essential in our screens because it protects cells from the pyocin. Because pyocin R2 orthologs are found in many *P. aeruginosa* strains, this mechanism of outer core and O-antigen essentiality may also be widespread (44).

Antitoxin and pyocin repression genes. Our analysis identified a general essential gene absent from the PAO1 genome annotation that appears to encode the antitoxin member of a ParDE-like toxin-antitoxin pair. The new gene (PA0728.1) is situated in the phage Pf1 region and presumably contributes to maintenance of the phage genome. Two additional essential genes (PA0125 and PA4674) also encode putative antitoxins of RelBE- and HigBA-like toxin-antitoxin pairs (45), and two others inhibit pyocin expression (*prtR*) or activity (*imm2*). In the absence of any of these inhibitory functions, toxin or pyocin activity presumably kills producer cells (43, 46, 47).

Asparagine-tRNA^Asn formation. The GatABC complex generates Asn-tRNA^{Asn} by transamidation of Asp-tRNA^{Asn}, an essential reaction that compensates for the absence of an asparginyl-tRNA synthetase in *P. aeruginosa* (48). The process is not essential in mammals (49) and represents a potential drug target.

Medium-specific essentials. A number of processes were essential on a subset of the media examined and help identify differences in growth physiology on them.

Growth on nutrient media (LB and BHI). Mutants lost from nutrient media may be sensitive to components of the media. For example, both a sodium-proton antiporter (PA1054-1059) and a membrane protease (FtsH), essential only on LB and BHI, are probably necessary for bacteria to cope with the NaCl in the media (50, 51). Glycerol-3-phosphate dehydrogenase (GlpD) may be essential because toxic glycerol-3-phosphate made from glycerol in the nutrient media accumulates in its absence (52).

Other genes selectively essential on nutrient media may be needed for rapid growth and division. For example, the Rep and PriA helicases may be essential during rapid growth to resolve replication-transcription conflicts (53). A 16S rRNA methylase (RsmH) that acts at the decoding center may be needed for translational fidelity at high growth rates (54), and a highly conserved GTPase (ObgE) may be essential to modulate the stringent response to a level commensurate with rapid growth (55).

Growth on minimal medium (MOPS-pyruvate). Nearly all of the genes specifically required for growth on MOPS-pyruvate medium are annotated as amino acid, nucleotide, and cofactor biosynthetic functions, and our findings help confirm these assignments. An additional function strongly essential on minimal medium was RpoN, an RNA polymerase σ-factor involved in nitrogen assimilation and other processes (56).

Growth on sputum. Several functions associated with outer-membrane integrity and synthesis were specifically essential for sputum growth. These functions included the abundant outer-membrane OprI lipoprotein covalently attached to murein that helps stabilize the cell envelope (57) and the function attaching it to murein (PA2854). An ortholog of outer-membrane chaperone protein Skp (PA3647) was also specifically essential on sputum medium (58). The global translational regulator RsmA (59) was required for growth on sputum and LB, but not on minimal medium.

Conversely, most genes for amino acid and nucleotide biosynthesis were not essential on sputum. The result fits well with studies showing that cystic fibrosis patient sputum is rich in free amino acids (60). Genes needed for unsaturated fatty acid biosynthesis (*fabA*, *fabB*, PA5174, and *fabV*/PA2950) were essential on minimal and LB but not sputum medium, suggesting that sputum contains unsaturated fatty acids. Unsaturated fatty acid synthesis has been proposed as an antimicrobial drug target, but this finding suggests that its inhibition in *P. aeruginosa* may not be effective for treating cystic fibrosis pulmonary infections (61).

Concluding Remarks. The principle goal of this work was to specify gene functions that are essential in different growth media, and

may thus have the greatest potential as antibiotic targets in diverse conditions. To identify such functions, we screened for genes required for *P. aeruginosa* growth using Tn-seq and then identified the subset required on all of the media. This group of genes is likely to represent the most fundamental processes needed for *P. aeruginosa* viability and growth.

The principle advantage of using Tn-seq to identify essential genes is that it makes it possible to carry out a genomic-level analysis in a single experiment. The analysis of multiple independent mutant pools grown under a given condition provides statistically robust essential gene assignments. In addition, the analysis is not necessarily tied to a genome annotation and can identify new genes, as was done here. Tn-seq also has two significant limitations. First, Tn-seq has a limited capacity to distinguish mutants growing very slowly from those not growing at all. Thus, confirmatory experiments with individual mutants are crucial to verify Tn-seq findings. Second, because Tn-seq assays the fitness of mutants in pools, mutants whose phenotypes are "complemented" by secreted factors from other bacteria may be missed.

The identification of essential genes provides an important starting point for further tests to distinguish the most promising functions for further development as targets. Examples of useful tests are examining whether partial loss of a function inhibits growth and whether a function is essential during infection (6, 7). Strains with conditional expression of different essential genes should allow such attributes to be systematically evaluated.

Materials and Methods

Strains and Plasmid. The *P. aeruginosa* parent strain was MPAO1 (16). The donor strain for transposon mutagenesis was *E. coli* SM10λ*pir*/pIT2. Plasmid pIT2 is a suicide plasmid that carries Tn5 derivative IS*lacZhah*-Tc (16).

Transposon Mutagenesis. Transposon mutagenesis was carried out by conjugation of *P. aeruginosa* with *E. coli* SM10λ*pir*/pIT2, followed by selection for *P. aeruginosa* derivatives carrying the transposon tetracycline-resistance determinant (16). Donor *E. coli* cells (SM10λ*pir*/pIT2) in midexponential growth (OD_{600} 0.8–1.0) were mixed with overnight cultures of MPAO1 grown without agitation at 42 °C (OD_{600} 1.1–1.4). Cultures were mixed 1:1 and pelleted, then resuspended in 10 mM $MgSO_4$, spotted onto 0.45-μm Millipore filters, placed on LB agar, and incubated for 90 min at 37 °C. Mating mixtures were then suspended in either LB or MOPS-pyruvate medium, DMSO was added to 5% (vol/vol) final concentration and cell suspensions flash-frozen in a dry ice-ethanol bath.

Tn-seq. The makeup of transposon mutant pools was profiled using the Tn-seq circle method (15). DNA from mutant pools was isolated using DNeasy Blood & Tissue Kit, (Qiagen No. 69506), and 8–18 μg of DNA per pool was sheared to a target size of 300 bp (Covaris E210 focused-ultrasonicator, duty cycle 10%, intensity 5, 100 cycles per burst, duration of 250 s per tube), end-repaired (New England Biolabs Next End Repair Module), and dA-tailed (New England Biolabs Klenow), followed by Illumina adaptor attachment (New England Biolabs Quick Ligation Kit). DNA was purified following the end-repair, A-tailing and adaptor ligation steps using AMPure XP beads (Agencourt, No. A63881), with the beads carried through and recharged for the final two purifications by addition of PEG 8,000 + 2.5 M NaCl. Restriction enzyme digestion, size selection, circularization, and exonuclease treatment were carried out as described previously (15). The transposon–genome junctions were amplified from the circularized DNA fragments by real-time PCR (Bio-Rad Mini Opticon) in 50-μL reactions containing 3-μL template DNA, 25 μL 2× KAPA HiFi HotStart Readymix, 0.125 μL 100× SYBR Green dye (Life Technologies), and 300 nM each of primers T8_SLXA_PAIR_AmpF_1 and SLXA_PAIR_REV_AMP. Thermocycling was 95 °C for 3 min, then 33 cycles of 95 °C for 20 s, 66 °C for 15 s, and 72 °C for 30 s. Samples were removed at 50% maximum amplification and purified (QIAgen MinElute Kit), then sequenced on Illumina Genome Analyzer II or MiSeq platforms using sequencing primers T8_SEQ_G or T8_INDEX_1. Sequence data analysis (*SI Materials and Methods*) included gap size analysis (Fig. S5).

Nucleotide Accession Numbers. The Tn-seq sequence reads have been deposited in the Sequence Read Archive (SRA) (www.ncbi.nlm.nih.gov/sra) under accession number SRP052838.

1. Rice LB (2010) Progress and challenges in implementing the research on ESKAPE pathogens. *Infect Control Hosp Epidemiol* 31(Suppl 1):S7–S10.
2. Moir DT, Opperman TJ, Butler MM, Bowlin TL (2012) New classes of antibiotics. *Curr Opin Pharmacol* 12(5):535–544.
3. Page MG, Heim J (2009) Prospects for the next anti-*Pseudomonas* drug. *Curr Opin Pharmacol* 9(5):558–565.
4. Juhas M, Eberl L, Church GM (2012) Essential genes as antimicrobial targets and cornerstones of synthetic biology. *Trends Biotechnol* 30(11):601–607.
5. Gwynn MN, Portnoy A, Rittenhouse SF, Payne DJ (2010) Challenges of antibacterial discovery revisited. *Ann N Y Acad Sci* 1213:5–19.
6. Murima P, McKinney JD, Pethe K (2014) Targeting bacterial central metabolism for drug development. *Chem Biol* 21(11):1423–1432.
7. Projan SJ (2008) Whither antibacterial drug discovery? *Drug Discov Today* 13(7-8):279–280.
8. Luo H, Lin Y, Gao F, Zhang CT, Zhang R (2014) DEG 10, an update of the database of essential genes that includes both protein-coding genes and noncoding genomic elements. *Nucleic Acids Res* 42(Database issue):D574–D580.
9. Baba T, et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol* 2:2006.0008.
10. Zhou J, Rudd KE (2013) EcoGene 3.0. *Nucleic Acids Res* 41(Database issue):D613–D624.
11. Barquist L, Boinett CJ, Cain AK (2013) Approaches to querying bacterial genomes with transposon-insertion sequencing. *RNA Biol* 10(7):1161–1169.
12. van Opijnen T, Camilli A (2013) Transposon insertion sequencing: A new tool for systems-level analysis of microorganisms. *Nat Rev Microbiol* 11(7):435–442.
13. Christen B, et al. (2011) The essential genome of a bacterium. *Mol Syst Biol* 7:528.
14. Barquist L, et al. (2013) A comparison of dense transposon insertion libraries in the *Salmonella* serovars Typhi and Typhimurium. *Nucleic Acids Res* 41(8):4549–4564.
15. Gallagher LA, Shendure J, Manoil C (2011) Genome-scale identification of resistance functions in *Pseudomonas aeruginosa* using Tn-seq. *MBio* 2(1):e00315–e10.
16. Jacobs MA, et al. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 100(24):14339–14344.
17. Liberati NT, et al. (2006) An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* 103(8):2833–2838.
18. Skurnik D, et al. (2013) A comprehensive analysis of in vitro and in vivo genetic fitness of *Pseudomonas aeruginosa* using high-throughput sequencing of transposon libraries. *PLoS Pathog* 9(9):e1003582.
19. Oberhardt MA, Puchałka J, Fryer KE, Martins dos Santos VA, Papin JA (2008) Genome-scale metabolic network analysis of the opportunistic pathogen *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 190(8):2790–2803.
20. Winsor GL, et al. (2011) *Pseudomonas* Genome Database: Improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Res* 39(Database issue):D596–D600.
21. Reams AB, Kofoid E, Savageau M, Roth JR (2010) Duplication frequency in a population of *Salmonella enterica* rapidly approaches steady state with or without recombination. *Genetics* 184(4):1077–1094.
22. Gómez-Lozano M, Marvig RL, Molin S, Long KS (2012) Genome-wide identification of novel small RNAs in *Pseudomonas aeruginosa*. *Environ Microbiol* 14(8):2006–2016.
23. Ozer EA, Allen JP, Hauser AR (2014) Characterization of the core and accessory genomes of *Pseudomonas aeruginosa* using bioinformatic tools Spine and AGEnt. *BMC Genomics* 15:737.
24. Whiteside MD, Winsor GL, Laird MR, Brinkman FS (2013) OrthologeDB: A bacterial and archaeal orthology resource for improved comparative genomic analysis. *Nucleic Acids Res* 41(Database issue):D366–D376.
25. Fraenkel DG (1986) Mutants in glucose metabolism. *Annu Rev Biochem* 55:317–337.
26. Lessie TG, Phibbs PV, Jr (1984) Alternative pathways of carbohydrate utilization in pseudomonads. *Annu Rev Microbiol* 38:359–388.
27. Temple LM, Sage AE, Schweizer HP, Phibbs PV (1998) Carbohydrate catabolism in *Pseudomonas aeruginosa*. *Pseudomonas*, ed Montie T (Plenum, New York), pp 35–72.
28. Vander Wauven C, Piérard A, Kley-Raymann M, Haas D (1984) *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: Evidence for a four-gene cluster encoding the arginine deiminase pathway. *J Bacteriol* 160(3):928–934.
29. Gerike U, Hough DW, Russell NJ, Dyall-Smith ML, Danson MJ (1998) Citrate synthase and 2-methylcitrate synthase: Structural, functional and evolutionary relationships. *Microbiology* 144(Pt 4):929–935.
30. Futai M, Kanazawa H (1983) Structure and function of proton-translocating adenosine triphosphatase (F0F1): Biochemical and molecular biological approaches. *Microbiol Rev* 47(3):285–312.
31. Arai H, et al. (2014) Enzymatic characterization and in vivo function of five terminal oxidases in *Pseudomonas aeruginosa*. *J Bacteriol* 196(24):4206–4215.
32. Tsai MH, Saier MH, Jr (1995) Phylogenetic characterization of the ubiquitous electron transfer flavoprotein families ETF-alpha and ETF-beta. *Res Microbiol* 146(5):397–404.
33. Watmough NJ, Frerman FE (2010) The electron transfer flavoprotein: ubiquinone oxidoreductases. *Biochim Biophys Acta* 1797(12):1910–1916.
34. Hassett DJ, Schweizer HP, Ohman DE (1995) *Pseudomonas aeruginosa* sodA and sodB mutants defective in manganese- and iron-cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. *J Bacteriol* 177(22):6330–6337.
35. Waller JC, et al. (2012) Evidence that the folate-dependent proteins YgfZ and MnmEG have opposing effects on growth and on activity of the iron-sulfur enzyme MiaB. *J Bacteriol* 194(2):362–367.
36. Giró M, Carrillo N, Krapp AR (2006) Glucose-6-phosphate dehydrogenase and ferredoxin-NADP(H) reductase contribute to damage repair during the soxRS response of *Escherichia coli*. *Microbiology* 152(Pt 4):1119–1128.
37. Barton HA, Johnson Z, Cox CD, Vasil AI, Vasil ML (1996) Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. *Mol Microbiol* 21(5):1001–1017.
38. Imlay JA (2013) The molecular mechanisms and physiological consequences of oxidative stress: Lessons from a model bacterium. *Nat Rev Microbiol* 11(7):443–454.
39. Sobota JM, Imlay JA (2011) Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese. *Proc Natl Acad Sci USA* 108(13):5402–5407.
40. Nachin L, Loiseau L, Expert D, Barras F (2003) SufC: An unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. *EMBO J* 22(3):427–437.
41. Takahashi Y, Tokumoto U (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. *J Biol Chem* 277(32):28380–28383.
42. Lam JS, Taylor VL, Islam ST, Hao Y, Kocincova D (2011) Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. *Front Microbiol* 2:118.
43. Penterman J, et al. (2014) Rapid evolution of culture-impaired bacteria during adaptation to biofilm growth. *Cell Reports* 6(2):293–300.
44. Nakayama K, et al. (2000) The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. *Mol Microbiol* 38(2):213–231.
45. Pandey DP, Gerdes K (2005) Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res* 33(3):966–976.
46. Penterman J, Singh PK, Walker GC (2014) Biological cost of pyocin production during the SOS response in *Pseudomonas aeruginosa*. *J Bacteriol* 196(18):3351–3359.
47. Sano Y, Matsui H, Kobayashi M, Kageyama M (1993) Molecular structures and functions of pyocins S1 and S2 in *Pseudomonas aeruginosa*. *J Bacteriol* 175(10):2907–2916.
48. Akochy PM, Bernard D, Roy PH, Lapointe J (2004) Direct glutaminyl-tRNA biosynthesis and indirect asparaginyl-tRNA biosynthesis in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 186(3):767–776.
49. Sheppard K, et al. (2008) From one amino acid to another: tRNA-dependent amino acid biosynthesis. *Nucleic Acids Res* 36(6):1813–1825.
50. Kosono S, et al. (2005) Characterization of a multigene-encoded sodium/hydrogen antiporter (sha) from *Pseudomonas aeruginosa*: Its involvement in pathogenesis. *J Bacteriol* 187(15):5242–5248.
51. Hinz A, Lee S, Jacoby K, Manoil C (2011) Membrane proteases and aminoglycoside antibiotic resistance. *J Bacteriol* 193(18):4790–4797.
52. Cozzarelli NR, Koch JP, Hayashi S, Lin EC (1965) Growth stasis by accumulated L-alpha-glycerophosphate in *Escherichia coli*. *J Bacteriol* 90(5):1325–1329.
53. Merrikh H, Zhang Y, Grossman AD, Wang JD (2012) Replication-transcription conflicts in bacteria. *Nat Rev Microbiol* 10(7):449–458.
54. Kimura S, Suzuki T (2010) Fine-tuning of the ribosomal decoding center by conserved methyl-modifications in the *Escherichia coli* 16S rRNA. *Nucleic Acids Res* 38(4):1341–1352.
55. Raskin DM, Judson N, Mekalanos JJ (2007) Regulation of the stringent response is the essential function of the conserved bacterial G protein CgtA in *Vibrio cholerae*. *Proc Natl Acad Sci USA* 104(11):4636–4641.
56. Potvin E, Sanschagrin F, Levesque RC (2008) Sigma factors in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* 32(1):38–55.
57. Wessel AK, Liew J, Kwon T, Marcotte EM, Whiteley M (2013) Role of *Pseudomonas aeruginosa* peptidoglycan-associated outer membrane proteins in vesicle formation. *J Bacteriol* 195(2):213–219.
58. Goemans C, Denoncin K, Collet JF (2014) Folding mechanisms of periplasmic proteins. *Biochim Biophys Acta* 1843(8):1517–1528.
59. Brencic A, Lory S (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* 72(3):612–632.
60. Palmer KL, Aye LM, Whiteley M (2007) Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol* 189(22):8079–8087.
61. Schweizer HP, Choi KH (2012) Characterization of molecular mechanisms controlling fabAB transcription in *Pseudomonas aeruginosa*. *PLoS ONE* 7(10):e45646.