UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biologiche e Molecolari

XXVI Ciclo

Identification and characterization of new essential functions in the opportunistic pathogen *Pseudomonas aeruginosa*

Ruggero Rusmini

PhD Thesis

Scientific tutor: Prof. Giovanni Bertoni

Academic year: 2012-2013

SSD: BIO/18; BIO/19

Thesis performed at Department of Biosciences

Contents

Abstract

Pseudomonas aeruginosa is a highly adaptable bacterium that thrives in a broad range of ecological niches. In addition, it can infect hosts as diverse as plants, nematodes, and mammals. In humans, it is an important opportunistic pathogen in compromised individuals, such as patients with cystic fibrosis, severe burns, or impaired immunity. *P. aeruginosa* is difficult to control because of its ability to develop resistance, often multiple, to all current classes of clinical antibiotics. The discovery of novel essential genes or pathways that have not yet been targeted by clinical antibiotics can underlie the development of alternative effective antibacterials to overcome existing mechanisms of resistance.

The main focus of my PhD thesis was the discovery of novel essential genes of *P. aeruginosa* by shotgun antisense screening. Our antisense screenings identified 33 growth-impairing single-locus genomic inserts that allowed us to generate a list of 27 "*essential-for-growth*" genes: among them 15 were "*novel*" essential genes with no homologs reported to have an essential role in other bacterial species. Interestingly, the essential genes in our panel were suggested to take part in a broader range of cellular functions than those currently targeted by extant antibiotics. Our study also identified 43 growth-impairing inserts carrying multiple loci targeting 105 genes. Taken together, our results show the feasibility of antisense technology in *P. aeruginosa* for identifying novel essential genes and we expect that our methodology could be well suited for antisense-mediated searches of essential genes in other Gram-negative bacterial species. These results were presented in publication 1 (PART II).

Then, we focused on two *loci*, PA2873 and P0580, found in our antisense screenings in PAO1.

The PA2873 gene product was annotated as a hypothetical membrane protein endowed with a periplasmic region harboring a structural domain belonging to the transglutaminase-like superfamily, a group of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases. We show that the periplasmic portion of the PA2873 protein, which we named TgpA, does possess transglutaminase activity *in vitro*. This is the first report of transglutaminase activity in *P. aeruginosa*. In addition, we have provided strong evidence that TgpA plays a critical role in the viability of *P. aeruginosa*. The results on TgpA were presented in publication 2 (PART II).

The PA0580 *locus* encodes the protein Gcp belonging to the Kae1/Qri7/YgjD family. Combining several genetic approaches, we showed that Gcp is essential for *P. aeruginosa*. In addition, we focused on the product of PA3685 *locus*, the YeaZ protein, a putative partner of

Gcp. We obtained evidence that YeaZ also plays a critical role in *P. aeruginosa* viability. A manuscript describing the results on Gcp is in preparation and presented in PART III.

PART I

1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a motile, non-fermenting, Gram-negative organism belonging to the Pseudomonadaceae family that has the ability to thrive in a broad range of ecological niches. It is found in diverse habitats, including soil, water, plants, and animals (Rahme et al., 2000), and can infect multiple hosts (Mahajan-Miklos et al., 2000).

P. aeruginosa is both capable of a wide variety of acute, severe, invasive diseases and also - evading immune defenses - persistent human infections (Kerr and Snelling, 2009). Serious *P. aeruginosa* infections are often nosocomial and associated with compromised host defenses such as severe burns, urinary tract infections, AIDS (Manfredi et al., 2000), lung cancer, chronic obstructive pulmonary disease, and cystic fibrosis (CF)(Valderrey et al., 2010).

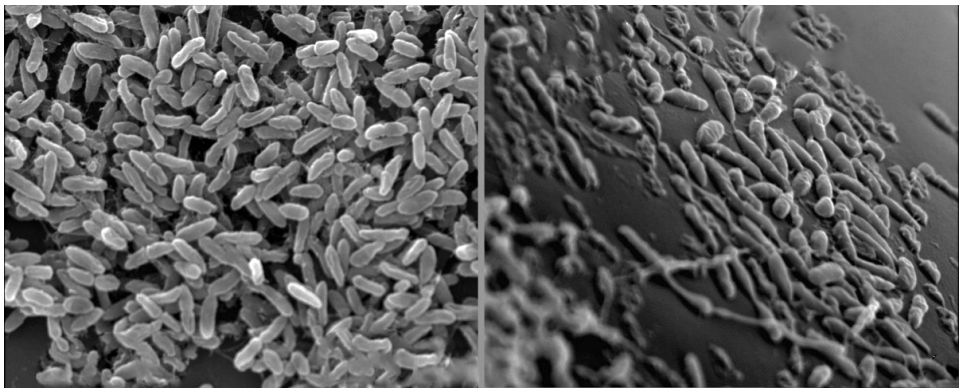


Figure 1. Scanning electron microscopy images of *P. aeruginosa* isolates attaching to glass surfaces.

The extreme versatility and adaptability encoded in its genome are the key features that make *P. aeruginosa* a very challenging opportunistic pathogen. As of January 2014, 48 strains of *P. aeruginosa* from both clinical and environmental sources have been fully or partly sequenced according to *Pseudomonas* Genome Database (PGD). Compared with most other pathogenic bacteria, *P. aeruginosa* has a relatively large genome, ranging from 6.22 to 6.91 Mb (Silby et al., 2011) arranged as a combination of conserved regions spaced by regions of genomic plasticity that contain genes unique to each strain.

P. aeruginosa is famously metabolically versatile and has been isolated from numerous nutrient-poor settings to the extent that it is able to survive even in distilled water.

Despite its optimum temperature for growth being 37°C, it is able to tolerate temperatures as high as 50°C and is capable of growing under both aerobic as well as anaerobic conditions, using preferentially nitrate as a terminal electron acceptor (Schreiber et al., 2007).

Even more striking than its metabolic diversity is the huge number of regulatory genes encoded by *P. aeruginosa* (Stover et al., 2000): nearly 10% of its genome, about 520 genes, is predicted to encode regulatory proteins. Bacteria that can adapt in diversified environments have a larger proportion of their genomes dedicated to regulatory genes than ones that are specialized to survive in a specific environment (Goodman and Lory, 2004). In *P. aeruginosa* many of these regulatory genes belong to the two-component class systems, which allow the bacterium to rapidly adapt to an environmental change (Alm et al., 2006).

Nevertheless, over a decade after *P. aeruginosa* PAO1 strain was sequenced (Stover et al., 2000), it was possible to assign a functional class to only 57% of its open reading frames (ORFs): out of these, 12% have functions experimentally demonstrated in *P. aeruginosa*; 8% is highly similar to a gene experimentally demonstrated in another organism; and 37% are homology-based bioinformatics predictions. Thus 43% of predicted ORFs contained genes for which no function could be assigned or predicted.

A large portion of its genome is involved in the expression of an impressive arsenal of virulence factors (Feinbaum et al., 2012) which explains the wide range of infections caused by the bacterium. A summary of virulence factors is illustrated in Figure 2 and Chapter 2.

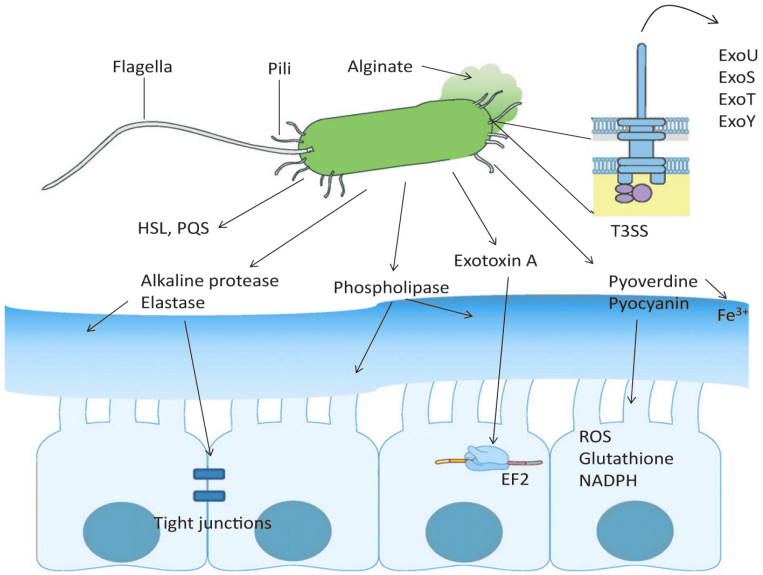


Figure 2. Summary of virulence factors produced by *P. aeruginosa.* Flagella and Type IV pili are the main adhesins, along with LPS, which are also highly inflammatory. Once contact with the host has occurred, the T3SS can be activated and starts to inject cytotoxins directly into the cells. Several virulence factors are secreted and proteases expressed. Pyocyanin can interfere with host cell electron transport pathways and redox cycling. Pyoverdine captures Fe^{3+} to allow for a competitive edge in an environment in which free iron is scarce. Adapted from Gellatly & Hancock, 2013.

2. Virulence factors of *P. aeruginosa*

Virulence factors include pili and flagella, which play a critical role in motility and adhesion to the epithelium as well as elastase, alkaline protease, the endotoxin lipopolysaccharide (LPS), and four effector proteins already identified: exotoxin A, exoenzyme S (ExoS), exoenzyme U (ExoU), exoenzyme T (ExoT), exoenzyme Y (ExoY).

All these factors together are thought to be crucial for maximum virulence of *P. aeruginosa*. However, based on several studies in diverse animal models, the relative contribution of any given factor may vary with the type of infection (Mahajan-Miklos et al., 2000).

2.1 Lipopolysaccharide

Lipopolysaccharide is a complex glycolipid that constitutes the main component of the outer membrane of Gram-negative bacteria and plays a crucial role in antigenicity, inflammatory response, exclusion of external molecules, and in mediating interactions with antibiotics (King et al., 2009). The molecule produced by *P. aeruginosa* is a typical Gram-negative bacterial LPS consisting of three different regions: the membrane-anchored lipid A, the polysaccharide core region, and a highly variable O-specific polysaccharide (O-antigen or O-polysaccharide).

While the structure of the inner core is rather preset (two D- *manno* -2-keto- octulosonic acid residues and two L- *glycero* -D- *manno* -heptose residues), significant variability can be detected in both the Lipid A moiety and in the O-antigen (Figure 3).

The lipid A domain is diglucosamine biphosphate backbone with *O*- and *N*-linked primary and secondary fatty acids that anchors the LPS in the bacterial outer membrane. The main structural differences are observed in the number, the position, the nature of the linked acyl groups, and the modification or type of substituent of the phosphate groups (Lam et al., 2011). Many lipid A modifications are regulated and can be induced as a response to an environmental change or during CF infection (Ernst et al., 2007; Cigana et al., 2009): among them, it is worth mentioning the altered bacterium's susceptibility to polymyxins and cationic antimicrobial peptides (Gutu et al., 2013) as well as changes in its inflammatory properties (Ernst et al., 2003).

Moreover, 85% of outer core molecules are uncapped (they also lack the terminal core rhamnose molecule) while 15% is capped by a variable number of repeated saccharide (O-antigen) units of 3–5 sugars with defined linkage.

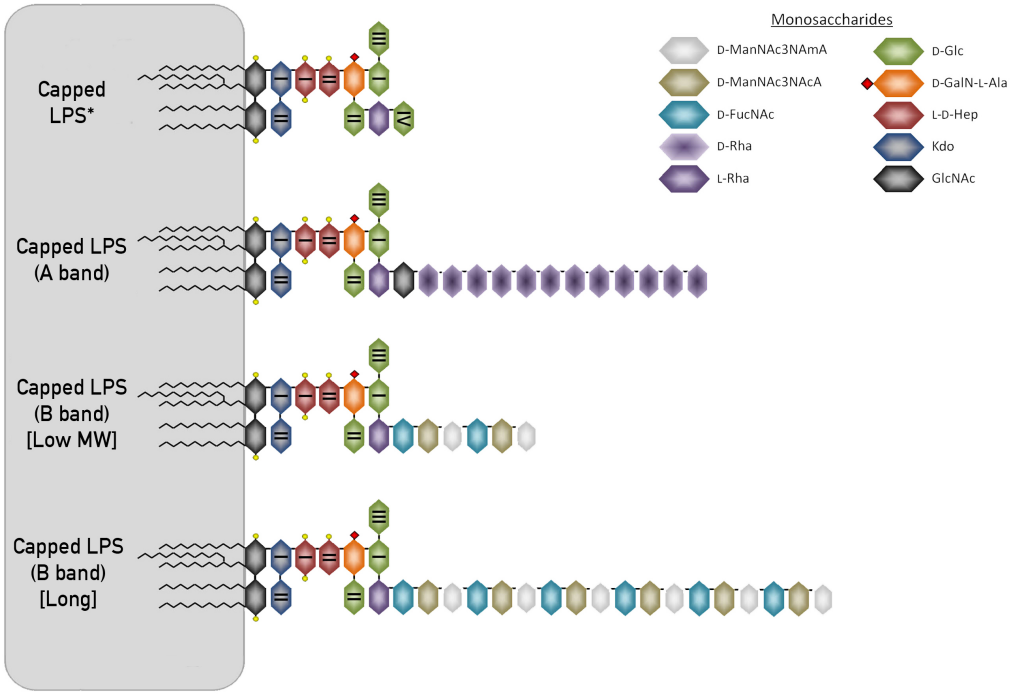


Figure 3. Representations of the heterogeneity of the LPS glycoforms present on the surface of *P. aeruginosa*. From Lam et al. 2011.

Two different types of O-antigen have been characterized: A-band LPS is a homopolymer of D-rhamnose which elicits a weak antibody response; B-band LPS is a heteropolymer with three to five distinct sugars in its repeat units with a strong antibody response and is the chemical basis for serotyping (Knirel et al., 2006). Some isolates, lacking the O-antigen, have a "rough" colony morphology compared to the smooth isolates producing it, while others, identified as "semi-rough", substitute the lipid A and core with only one O-saccharide unit (Knirel et al., 2001). The O-antigen is a major target for protective immunity as evidenced by numerous animal studies, and LPS relative amounts can change in a given strain dependant on the growth conditions affecting its pathogenicity (Ernst et al., 1999).

Significantly, many reports indicate that over time chronic *P. aeruginosa* isolates change the type of LPS expressed, from the more antigenic B-band to the A-band: most likely this switch is driven by selective pressure to evade host adaptive immune responses (King et al., 2009). A number of LPS vaccines have been investigated for use especially in CF patients and, although they have reached phase II and III clinical trials, these have not been successful (Jones et al., 1979, 1980; Langford and Hiller, 1984; Cryz et al., 1997; Erridge et al., 2002). Despite these extensive efforts over more than 30 years, realization of clinically applicable LPS-based vaccines remains elusive, providing little immunity and not showing protection against infection with *P. aeruginosa* (Pier, 2003; Sharma et al., 2011).

2.2 Flagella and Type IV pili

P. aeruginosa typically possesses a single unsheathed polar flagellum and several much shorter Type IV pili also localized at a cell pole. Through a rotating corkscrew motion, the flagellum contributes to the swimming motility in an aqueous environment and, on semi-solid media, provides swarming motility. Its movement is typically described as straight-line swimming interspersed with tumbles, in which flagella rotation is transiently reversed and motility is arrested in order to reorient itself (Köhler et al., 2000; Yeung et al., 2009).

However, its contribution in virulence goes beyond mere motility, since several different flagellar proteins have been reported to play critical roles in attachment, invasion, biofilm formation, and in the mediation of inflammatory responses (Toutain et al., 2007; Parker and Prince, 2013). Non-flagellated mutants are a very representative portion of the isolates from chronic infections in CF patients: loss of these appendages is considered to be an event involved in the evasion of the host immune system (Wolfgang et al., 2004), as described before for the switch in LPS nature. In fact, it has been demonstrated that flagellin - the structural protein forming the major portion of flagellar filaments - mediates the inflammatory response interacting specifically with a leucine-rich region of host Toll-like receptor 5 (Verma et al., 2005) and causing production and release of proinflammatory cytokines that recruit neutrophils to the infected region (Hayashi et al., 2001; Smith et al., 2003).

In an era of increasing drug resistance, immunotherapy is a desirable treatment against *P. aeruginosa* infections: flagellar vaccines have been investigated in pre-clinical studies in mouse models. However, limited protection was observed with a monovalent vaccine and future development of a bivalent vaccine has been terminated (Döring and Pier, 2008; Campodónico et al., 2010).

Regarding the Type IV pili, they are, without doubt, the most important adhesins of *P. aeruginosa* with a fundamental role in phenomena such as tissue tropism, initiation of biofilm formation (Barken et al., 2008), and non-opsonic phagocytosis (Heale et al., 2001). From the cell pole, they extend and retract like hooks to pull the cell along solid surfaces in a process called “twitching motility” (Kipnis et al., 2006) and can also lead to aggregation, causing the bacteria to form microcolonies on target tissues (Sriramulu et al., 2005). Like flagella, pili are targets of antipseudomonal therapy, including immunization; however, these attempts are frustrated by the extreme antigenic variability of pili across different strains (Giltner et al., 2012).

2.3 Type III Secretion

P. aeruginosa has a variety of secretion systems, of which at least four most probably play a role in virulence (Type I, II, III, and VI): in particularl, Type III (T3SS) is evolutionarily connected with the flagellar system, although it is not clear which developed first (Saier, 2004). The T3SS is a complex macromolecular machinery, encoded by 36 genes on five operons, spanning the inner bacterial membrane, the periplasmic space, the peptidoglycan layer, the outer bacterial membrane, the extracellular space, and, finally reaching the host cell membrane (Figure 4).

This secretion apparatus is able to deliver effector proteins from the bacterium directly into the host cell through a pore formed in the membrane. Despite extensive characterization by a number of laboratories, so far only four effector toxins have been identified - ExoY, ExoS, ExoT, ExoU - a number that is much lower compared to other bacterial pathogens, such as *Salmonella* (≈13) or *Shigella* (≈ 25) (Hauser, 2009). While all *P. aeruginosa* strains encode the T3SS, most strains do not carry a complete set of effector-encoding genes (Feltman et al., 2001): nearly all express one of the two major exotoxins *exoU* or *exoS*, but very rarely both (Shaver and Hauser, 2004).

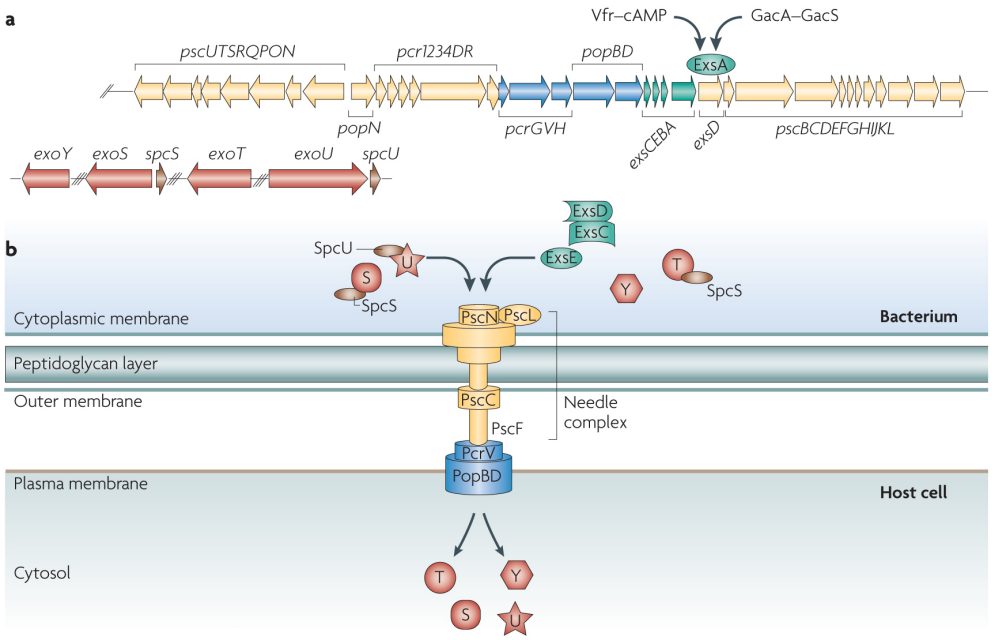


Figure 4. *P. aeruginosa* Type III secretion. **a.** Thirty-six genes, encoded in five clustered operons, are involved in the T3SS. **b.** T3SS can be functionally divided into five components: the needle complex, the translocation apparatus, the regulatory proteins, the effector proteins, and the chaperones. These five parts work together to inject effector proteins into host cells in a highly regulated manner. From Hauser 2009.

ExoS and ExoT are bifunctional cytotoxins, including both *N*-terminal GTPase-activating protein and *C*-terminal ADP ribosyltransferase activities. These molecules display a number of adverse effects on the host cell, including cell death, actin cytoskeletal disruption, inhibition of DNA synthesis, vesicular trafficking, and endocytosis (Pederson and Barbieri, 1998; Barbieri et al., 2001; Würtele et al., 2001).

Perhaps the most crucial activity is the one connected with irreversible disruption of the cytoskeletal structure: indeed, a substantial reduction in cell-cell adherence facilitates *P. aeruginosa* penetration through epithelial barriers and the spread of infection (Shaver and Hauser, 2004). ExoU is a potent - 100 times more than ExoS - phospholipase capable of causing rapid cell death in eukaryotic cells due to loss of plasma membrane integrity consistent with necrosis (Finck-Barbançon et al., 1997; Sato and Frank, 2004). ExoY is an adenylate cyclase that, when injected into mammalian cells, results in an elevation of intracellular cAMP concentration leading to differential expression of multiple genes and eventually disruption of the actin cytoskeleton (Cowell et al., 2005) and increased endothelial permeability (Sayner et al., 2004).

2.4 Proteases

Several proteases are synthesized and secreted by *P. aeruginosa*. These enzymes cooperate to dismantle host tissue, playing a critical role in both acute lung infections (Ingmer and Brøndsted, 2009) and in ocular infections (Hobden JA., 2002).

Alkaline protease (AprA) is a type 1 secreted zinc protease that degrades several components of the host immune system and host fibronectin (Hong and Ghebrehiwet, 1992). In addition, it assists *P. aeruginosa* to screw-on the immune response by degrading free flagellin and so preventing Toll-Like receptor 5 activation (Bardoel et al., 2011).

LasA and LasB are two elastases that are regulated by the lasI quorum-sensing system and secreted via Type II secretion apparatus (de Kievit and Iglewski, 2000). LasA, also known as staphylolysin, is a 20 kDa serine protease able to cleave the pentaglycine bonds in the peptidoglycan of *Staphylococcus aureus* (Vessillier et al., 2001). It possesses only a small fraction of the elastolytic abilities of LasB and rather is thought to enhance its partner's proteolytic activity (Matsumoto, 2004). On the contrary, it has been observed that LasB is capable of degrading a number of connective tissue proteins, including elastin, fibrin, and collagen (Hamdaoui et al., 1987; Miyajima et al., 2001).

Although their role in chronic infection is still not well understood, they clearly play a fundamental role in interference with host defense mechanisms (Alhede et al., 2014).

2.5 Quorum Sensing

Quorum-sensing (QS) represents a form of cell-cell communication based on small diffusible molecules called autoinducers - since one of their effects is to enhance their own synthesis - that allow bacteria to coordinate gene expression in a cell population density dependent manner (Miller and Bassler, 2001; Bassler, 2002).

The number of autoinducer molecules in the medium is proportional to the concentration of bacteria so, when a critical number of bacteria (i.e. "quorum") is reached, the concentration of autoinducers triggers the activation of specified downstream genes which affects the entire bacterial population (Vasil, 2003).

This cell-density response has evolved as a means to provide advantages to a group of cells, such as improving access to environmental niches or enhancing their defense capabilities against other microorganisms or eukaryotic host-defense mechanisms (Ng and Bassler, 2009). In Gram-negative bacteria, the most common signal molecule used is an acyl homoserine lactone (AHL) molecule, which was first described in the marine bioluminescent bacterium *Vibrio fischeri* (Ruby, 1996).

In *P. aeruginosa*, QS consists of two separate but interrelated systems, *las* and *rhl* (Gambello and Iglewski, 1991; Ochsner et al., 1994) that regulate - via three autoinducers - a wide range of cellular processes, such as biofilm formation, swarming motility, and a broad array of virulence factors (Venturi, 2006).

Two of these autoinducers are AHL molecules: the Las system is comprised of the transcriptional regulator LasR and its cognate AHL signal 3-oxo-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL), while the Rhl system is constituted of RhlR together with its partner *N*-butyryl-L-homoserine lactone (C_4-HSL).

Importantly, the Las and Rhl systems are not interchangeable, meaning that the C_4-HSL molecule cannot activate LasR and also the opposite, namely that 3-oxo-C_{12}-HSL cannot activate RhlR (Pearson et al., 1995, 1997).

P. aeruginosa produces a third signaling molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone, named *Pseudomonas* Quinolone Signal (PQS), which requires the presence of biosurfactant to increase its solubility and hence its bioactivity as a signal molecule (Calfee et al., 2005).

This molecule is synthesized by a complex multistep process involving two operons (*pqsABCDE* and *phnAB*), and three genes apart, *pqsR*, *pqsH*, and *pqsL* (Deep et al., 2011; Heeb et al., 2011).

These systems are intertwined in a hierarchical manner, auto-regulated and intimately connected forming a complicated regulatory cascade (Schuster and Greenberg, 2006; Williams and Cámara, 2009).

Novel QS systems are continually being discovered so that the challenge is not just to elucidate the chemical identity of the QS signal molecules but to understand why certain bacteria prefer one particular chemical class over another and how different systems cooperate in gene regulation. An extremely simplified view of the matter requires that the Las system is at the top of the hierarchy regulating both Rhl and the production of quinolones at either transcriptional or posttranslational levels.

Different independent studies have shown that as many as 10% of genes and more than 20% of the expressed bacterial proteome are regulated by QS systems (Schuster et al., 2003; Wagner et al., 2003; Deep et al., 2011), in particular those associated with the virulence itself (Table 1).

Factor	References
AHLs	(Seed et al., 1995)
Alkaline protease	(Gambello et al., 1993; Latifi et al., 1995)
Catalase	(Hassett et al., 1999)
Elastase	(Toder et al., 1994)
Exotoxin A	(Gambello et al., 1993)
Hydrogen cyanide	(Pessi and Haas, 2000)
Lectins	(Winzer et al., 2000)
Pyocyanin	(Brint and Ohman, 1995; Latifi et al., 1995)
Rhamnolipid	(Ochsner and Reiser, 1995; Pearson et al., 1997)
Secretion proteins	(Chapon-Hervé et al., 1997)
Superoxide dismutase	(Hassett et al., 1999)

Table 1. Known quorum-sensing-regulated virulence factors in *P. aeruginosa*.

2.6 Biofilm

Biofilms are complex organized structures of bacteria attached to one another and to a surface (Kolter and Greenberg, 2006), and their formation is intricately linked to QS (Bjarnsholt et al., 2010).

These highly organized structures are characterized by extracellular polymeric substances (EPS), composed of polysaccharides, nucleic acids, lipids, and proteins (O'Toole et al., 2000). EPS, the major component of biofilm volume (about 50 to 90%), is designed in order to maintain its architecture and functions as glue, holding the biofilm community together and protecting it from the environmental forces counterproductive to breaking it down (Stoodley et al., 2002). In addition, by forming such a multicellular aggregate, cells are deeply protected from penetration of toxic chemicals (*e.g.*, antibiotics, host defense molecules) and can also escape, or at least significantly slow down, the process of phagocytosis by mammalian host (Hall-Stoodley and Stoodley, 2009).

Furthermore, it has been reported that bacteria within the biofilm differ substantially with respect to their planktonic form, mainly regarding their transcriptional profile: indeed, more than 25% of the PAO1 genome was differentially expressed in the biofilm's condition (Waite et al., 2005, 2006). The transition of *P. aeruginosa* from the motile to the aggregate state in biofilms is a complex multistep process (Figure 5) that involves a multitude of physiological changes (O'Toole et al., 2000). The first phase is an initial reversible adhesion to the surface, which is mostly controlled by physiochemical properties such as Van der Waals interactions, electrical charge, and hydrophobicity (Jucker et al., 1996). In the presence of opportune conditions, adhesion, mediated by Type IV pili, flagella, and the more recently discovered Cup fimbria (Mikkelsen et al., 2011), become irreversible and cells start to duplicate forming a monolayer called microcolony. After this irreversible attachment, microcolonies multiply and, producing the EPS matrix, a mature bioflim could develop. Three different polysaccharidesalginate, Pel, and Psl - are synthesized by *P. aeruginosa* EPS, with various relevance and relative percentages according to different strains, environmental conditions, and the age of the biofilm (Harmsen et al., 2010).

Alginate is an acetylated polysaccharide composed of non-repetitive monomers of β-1,4-linked L-guluronic and D-mannuronic acids (Govan and Deretic, 1996) overproduced by mucoid strains often isolated from the lungs of patients with CF (Høiby et al., 2010). Its predicted role is to protect the bacterial community from the host response. In addition,

alginate-overproducing strain have been identified as more resistant to the antibiotic tobramycin (Hentzer et al., 2001): this anionic polysaccharide acts as a physical barrier for antibiotics because, if the alginate matrix is disrupted by an alginate lyase treatment, the strain becomes significantly more sensitive to the aminoglycosides (Hatch and Schiller, 1998). The glucose-rich Pel polysaccharide, encoded by the *pel* cluster, is produced by most strains, while the mannose-rich Psl polysaccharide is not fully encoded in all strains (López et al., 2010). These two polysaccharides mainly mediate cell-to-surface and cell-to-cell interactions, which are essential for *P. aeruginosa* biofilm formation and maintenance (Ma et al., 2006, 2009).

As the microcolony develops, the EPS forms and encloses the biofilm in a mushroom-shaped structure that is crossed with specifically designed channels allowing the exchange of waste products and nutrients to the deep rooted cells (Ryder et al., 2007; Kaplan, 2010).

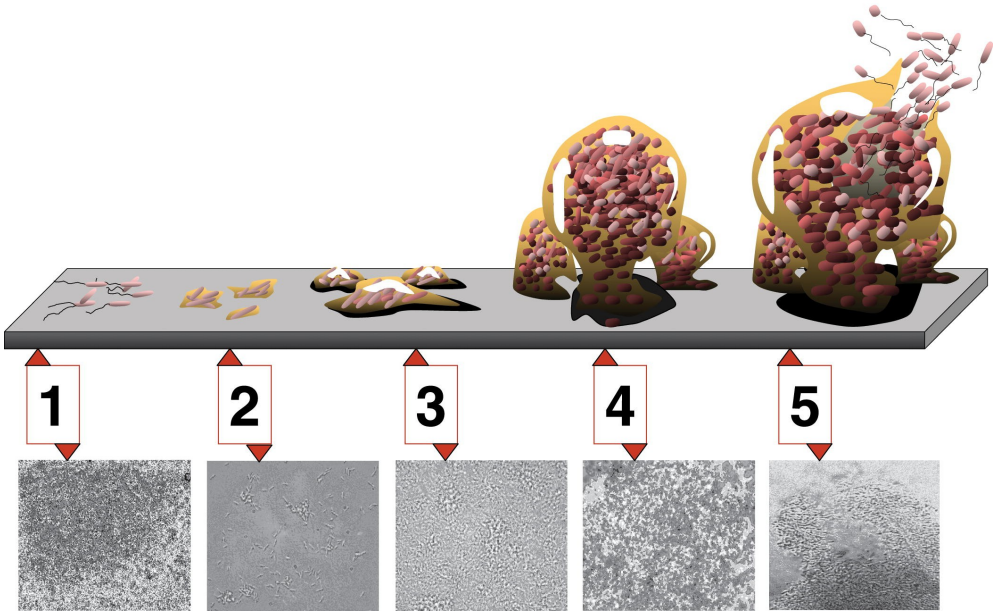


Figure 5. Schematic representation of biofilm maturation model. Stage 1, initial attachment; stage 2, irreversible attachment; stage 3, maturation I; stage 4, maturation II; stage 5, dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. From Monroe 2007.

In the last step of its life cycle, the biofilm undergoes a structural fragmentation with the release and dispersal of cells through the environment, where they are able to adhere to another surface, renewing the cycle. Although this process can be influenced by mechanical events, biofilm detachment and desegregation is a cell-driven process carried out in response to different environmental signals, such as nutrient reduction (Gjermansen et al., 2010) or the accumulation of oxygen and nitrogen reactive species (Barraud et al., 2006; Nijland et al., 2010).

3. Mechanisms of resistance of *P. aeruginosa*

All known mechanisms of antibiotic resistance can be displayed by this bacterium (intrinsic, acquired, and adaptive), sometimes all within the same isolate (Table 2).

Mechanism	Resistance class	Example(s)
Efflux pumps	Intrinsic	MexAB–OprM, MexCD–OprJ, MexEF–OprN, MexXY–OprM (cephalosporins, carbapenems, aminoglycosides, quinolones, ureidopenicillins)
Outer membrane impermeability	Intrinsic	OprF, OprD, OprB (carbapenems, aminoglycosides, quinolones)
β-lactamases	Intrinsic	AmpC (penicillins)
Targeted mutation	Acquired	DNA gyrase, DNA topoisomerase (quinolones)
		MexZ (quinolones, cefapimes, aminoglycosides)
Horizontal transfer	Acquired	Metallo-β-lactamases, ESBLs (penicillins, cephalosporins, carbapenems)
Membrane changes	Adaptive	Lipid A modification (aminoglycosides, polymyxins)
		AmpC upregulation (penicillins)

ESBL, extended spectrum β-lactamase.

Table 2. Example of resistance mechanisms in *P. aeruginosa*. From Gellatly & Hancock, 2013.

P. aeruginosa exhibits intrinsic resistance to almost all of the available antibiotics (Bonomo and Szabo, 2006; Lister et al., 2009). One of the major factors contributing to this intrinsic resistance is the low permeability of its outer membrane that plays a critical role in defining small molecules rate of uptake, and so antibiotics (Hancock, 1998).

Low outer membrane permeability works in collaboration with multidrug efflux whereby the slow uptake of antibiotics is countered by rapid efflux. The *P. aeruginosa* genome contains a large number of drug efflux systems belonging to Resistance-Nodulation-Division (RND) and major facilitator superfamily (MFS) types (Livermore, 2001).

These two intrinsic elements work in concert with chromosomally encoded AmpC β-lactamases to degrade many β-lactam antibiotics. β-lactamases are hydrolyzing enzymes that are able to cleave the lactam ring, leading to inactivation of the antibiotic (Livermore, 1995).

All these mechanisms act synergistically, defining the really high intrinsic resistance exhibited by *P. aeruginosa* to reduce active intracellular levels of many antibiotics (Nikaido, 2001). In addition, the most recent evidence supports the idea of a larger resistome with more than 100 genes that, when mutated, lead to altered susceptibility to several antibiotics (Schurek et al., 2008).

On the another hand, the acquired resistance could be the consequence of the genetic transfer and expression of a resistance cassette or it may be the result of mutations in targets or in the genes, including regulators, which stabilize or enhance intrinsic resistance mechanisms. (Breidenstein et al., 2011). DNA elements, such as plasmids, transposons, integrons,

prophages, and resistance islands, can harbor antibiotic resistance genes and can be obtained by conjugation, transformation, or transduction.

This can increase antibiotic resistance and, if plasmids contain multiple resistance cassettes, even multidrug resistance. These acquired elements can also reinforce the intrinsic resistance of *P. aeruginosa*: overexpression of a second β-lactamase can increase resistance to certain antibiotics or widen the range of β-lactams that can be resisted (Bagge et al., 2002).

A second form of acquired resistance is due to a mutation event in a regulatory gene that causes de-regulation of a preexistent resistance mechanism like those leading to overexpression of efflux pumps, reduced uptake of antibiotics, hyperproduction of β-lactamases, and altered antibiotic targets. For example, a point mutation in the suppressor *mexZ* leads to the overexpression of the MexXY efflux pump (Matsuo et al., 2004); or a mutation in DNA gyrase reduces the binding affinity for fluoroquinolones leading to resistance (Schweizer, 2003); or an overexpression of β-lactamases occurs upon regulatory mutations especially in *ampC* repressor gene *ampD* (Langaee et al., 1998).

Finally, significant differences in *in vitro* susceptibility determinations and time-kill studies for *P. aeruginosa* isolates and treatment results in CF patients have been reported and can be assigned to a phenomenon called “adaptive resistance” (Gilleland et al., 1989; Barclay et al., 1996). Adaptive resistance has been observed *in vitro* and *in vivo* for *P. aeruginosa* and other Gram-negative and it is defined as reduced antimicrobial killing in bacterial cultures pre-exposed to a certain agent (Karlowsky, Zelenitsky, & Zhanel, 1997). It is a form of inducible resistance that does not necessarily involve mutations and its best characterized mechanisms overlay with acquired ones, and may indeed foreshadow the development of mutational resistance. In *P. aeruginosa* a well-known mechanism causes resistance to cationic antimicrobial peptides: under specific conditions (limiting Mg^{2+}, exposure to peptides, and polymyxins), a large number of sensor kinases upregulate the expression of the *arnBCADTEF-udg* operon, which drives the addition of amino-arabinose to lipid A (McPhee et al., 2003, 2006). This modification, lowering the negative charge of lipopolysaccharide, reduces the interactions of these cationic peptides with the outer membrane in a transient manner since susceptibility is restored when inducing conditions are removed.

4. *P. aeruginosa* infections

Hospital-acquired (nosocomial) infections can be defined as those occurring within 48 hours of hospital admission, 3 days of discharge, or 30 days of an operation. They affect 1 in 10 patients admitted to hospital and annually result in more than 5000 deaths (Inweregbu, 2005). *P. aeruginosa* infections are typically of the late onset category, often presenting more than 5 days after admission.

As stated in the 2009–2010 report by the National Healthcare Safety Network (NHSN), *P. aeruginosa* is the 5th most common healthcare associated pathogen causing 12% of catheter-associated urinary tract infections, 4% of central line–associated bloodstream infections, 17% of ventilator-associated pneumonia (VAP) and 6% of surgical site infections (Sievert et al., 2012).

From these numbers it is easy to understand how *P. aeruginosa* is the second most common pathogen causing respiratory infections in hospitalized patients and also amongst the most lethal, since reports suggest up to 70–80% mortality when confined to the lungs (Hunter, 2006).

Infections of the respiratory tract are often grouped into two types: acute or chronic. Acute hospital infections are generally the effect of direct trauma, such as damage to the epithelium due to intubation or smoke inhalation. On the other hand, chronic ones affect primarily patients with weak or non-effective immune response, such as in the elderly or individuals with CF.

4.1 Acute lung infections

P. aeruginosa rarely infects the human lung without an underlying defect in immunity or mechanical barrier (Williams et al., 2010).

As mentioned before, patients with VAP often suffer from a breached epithelium induced by the insertion of the endotracheal tube, which, as a plastic surface, may serve as a cover and reservoir for *P. aeruginosa* growth as biofilms (Schaber et al., 2007).

These structures are extremely difficult to eradicate and, as biofilm-associated bacteria, *P. aeruginosa* exhibit even more resistance to all classes of antibiotics and disinfectants: for this reason, antibiotic treatments undertaken before the biofilm has developed are the most effective (Brooun et al., 2000).

Acute lung infections also occur in all those nosocomial categories who are not able to develop an adequate immune response, such as elderly people, patients with neutropenia due to cancer chemotherapy, or immunocompromised people due to organ transplant (Williams et al., 2010). Nosocomial infections are also of high incidence because immune deficient patients are frequently hospitalized and therefore exposed to *Pseudomonas* reservoirs in the healthcare setting.

4.2 Chronic lung infections and cystic fibrosis

P. aeruginosa, if not eradicated during the acute infection phase, can adapt to the lung environment, resulting in a chronic infection. Currently there is no universally accepted definition of chronic *P. aeruginosa* infection (Pressler et al., 2011): those most used are based on microbiological results from sputum cultures.

Nevertheless, chronic infection can be described as an infection that persists in spite of therapy, and in spite of the host's immune and inflammatory response. The most well-known cases of chronic pseudomonal lung infections are those borne by CF patients.

CF is an autosomal, recessive, multi-organ disorder affecting 1:2,500 in the Caucasian population (Ratjen and Döring, 2003). It is caused by the presence of one of more than 1,500 possible mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene; these mutations lead to the malfunction or loss-of-function of CFTR, a cAMP-regulated chloride ion channel, resulting in defective chloride ion transport across epithelial cell surfaces (Boucher, 2004).

These abnormalities lead to a thick and dehydrated mucus secretion that impairs mucociliary clearance of bacterial pathogens (Collins, 1992), which can therefore more easily colonize the airways and cause an initial acute infection and vigorous inflammatory response, including polymorphonuclear leukocytes and antibodies (Høiby et al., 2005). The hyperactive inflammatory response causes the progressive destruction of lung function and ultimately death.

Improvements in clinical care have resulted in a dramatic increase in the life expectancy of CF patients over the last 40 years, if one considers that, in 1974, the international median age at death was 8 years while nowadays, in several European countries, it is between 30 and 40 years (Cystic Fibrosis Foundation Patient Registry 2012 Annual Data Report).

The microbial flora of the airways of CF patients emblematize a really complex and heterogeneous ecosystem in which multispecies communities coexist (Harrison, 2007). Most

studies of CF pathogens have focused on four major bacterial species: *P. aeruginosa*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Burkholderia cepacia* complex species group. The prevalence of these species in the airways does not remain unchanged over time; on the contrary, it undergoes significant modifications, as illustrated in Figure 6.

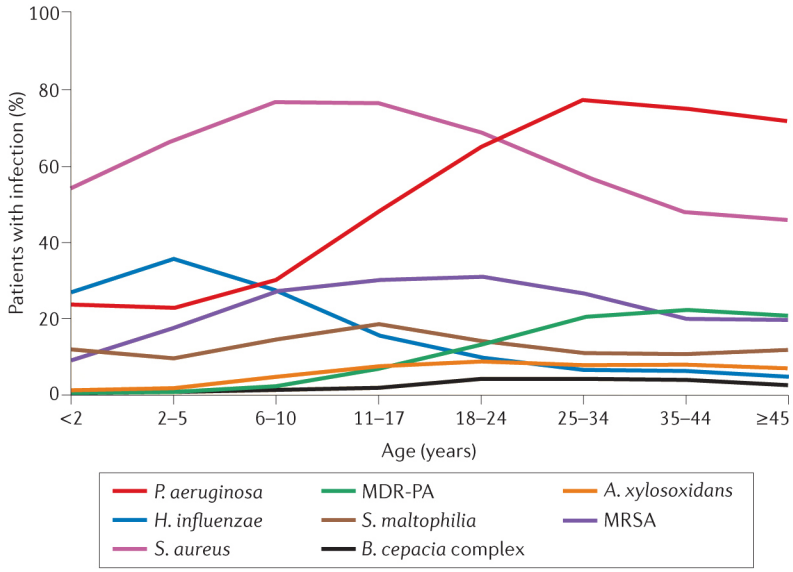


Figure 6. Progression of *P. aeruginosa* infections in patients with CF**.** Prevalence of several common human respiratory pathogens in patients with cystic fibrosis as a function of age. *P. aeruginosa* is the most frequently found pathogen in adults.

P. aeruginosa airways colonization frequently occurs after initial establishment by *S. aureus*; it then replaces the other bacterium becoming the predominant one in the CF lung. By the age of 25, approximately 70% of patients with CF are infected in the respiratory tract; however, the specific mode of colonization is still unknown. Generally the initial colonizing strains are unique environmental isolates (Burns et al., 2001), although epidemic isolates are also known (Salunkhe et al., 2005). Chronic airway infection with *P. aeruginosa* is usually preceded by a period of recurrent, intermittent colonization of the airway, as schematized in Figure 7.

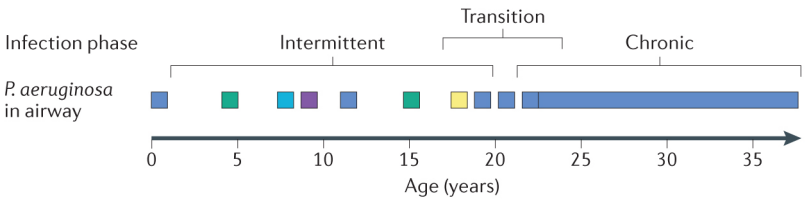


Figure 7. Schematic representation of the progression of *P. aeruginosa* infection. Different colors represent phylogenetically independent *P. aeruginosa* clones. From Folkesson et al., 2012.

At later times CF patients are reinfected with different *P. aeruginosa* strains, but about 25% of cases are re-colonizations with the same genotype (Munck et al., 2001).

This intermittent colonization phase should last for several years, but will inevitably transform into a chronic infection characterized by a high bacterial load that ultimately triggers a prolonged inflammatory response. This protracted and higher degree of inflammation causes the variety of symptoms (*e.g.*, cough, sputum production, damage to the epithelial layer, loss of lung function, and breathing problems) accompanying CF chronic infections and leads to respiratory distress and eventual failure and death (Lee et al., 2003).

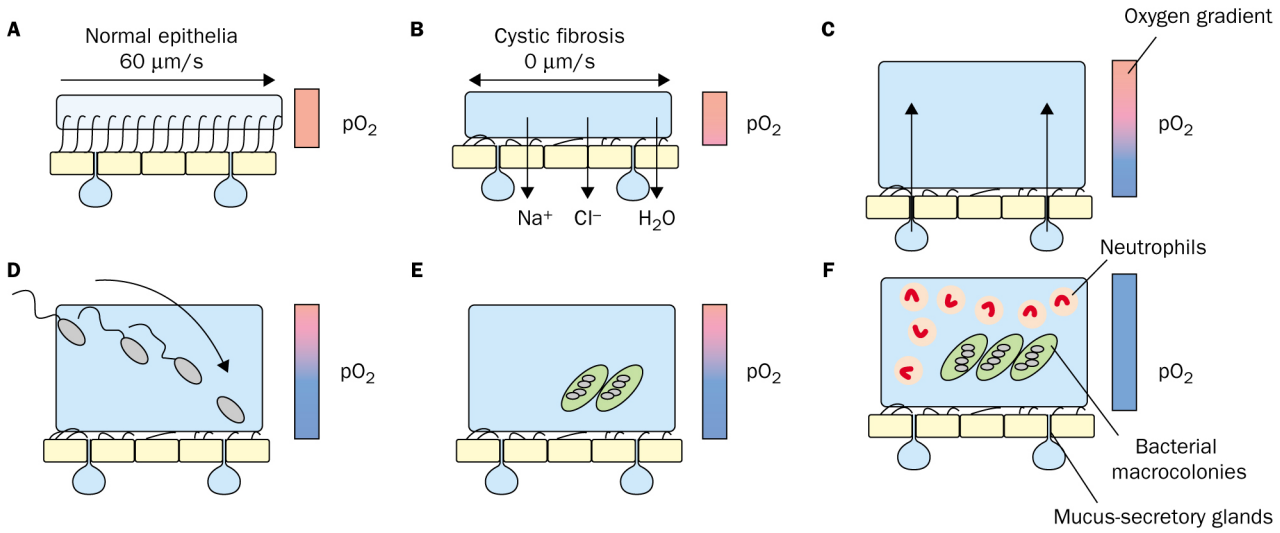


Figure 8. Blue circles=mucus layer; yellow squares=airway epithelial cells. For oxygen gradient: red=no gradient; blue=steep, hypoxic gradient. Because of blocked chloride secretion, excessive sodium absorption and water absorption, normal mucociliary clearance **(A)** is defective in cystic fibrosis **(B)**. Mucus secretion leads to plug formation **(C)**. Steep hypoxic gradients (blue bar) are sensed by penetrating bacteria **(D)** leading to increased alginate **(E)** and macrocolony formation **(F)**. Neutrophil functions are impaired by anoxic conditions and macrocolonies **(F)**. From Ratjen & Döring, 2003.

During the process of infection, *P. aeruginosa* undergoes a number of adaptations to highly stressful conditions leading to its characteristic persistence and antibiotic resistance (Figure 8). One of the most striking features of *P. aeruginosa* adaptation to the airways of CF patients is the conversion to mucoid phenotype due to overexpression of extracellular polysaccharide alginate (Govan and Deretic, 1996). Indeed, while the environmental isolates involved in the initial colonization usually present a non-mucoid phenotype, as the bacterium penetrates the thickened mucus of the airways, travelling down the oxygen gradient, it increases its expression of alginate and switches to a mucoid phenotype coincidently with the establishment of chronic infection (Starner, 2005).

The mucoid form of *P. aeruginosa* is associated with 90% of *P. aeruginosa* CF infections compared to only 2% of *P. aeruginosa* non-CF infections (Pritt et al., 2007).

Alginate production seems to be part of the general envelope stress response of several bacterial species, protecting them from adverse environmental stresses. During CF lung colonization, this extracellular polysaccharide preserves *P. aeruginosa* from the consequences of inflammation by inhibiting part of the innate immune response activation and decreasing phagocytosis by neutrophils and macrophages (Meshulam et al., 1984; Leid et al., 2005).

In general, as the process of infection progresses, a fundamental transition in the gene expression profile occurs in *P. aeruginosa* strain. This switch is mainly governed by the *algU* regulon, which is activated under stress conditions and leads to a coordinated downregulation of central metabolism, motility, and virulence (Aspedon et al., 2006), and a simultaneous upregulation of genes affecting membrane permeability, efflux and, as mentioned before, alginate production (Jones et al., 2010)(Figure 9).

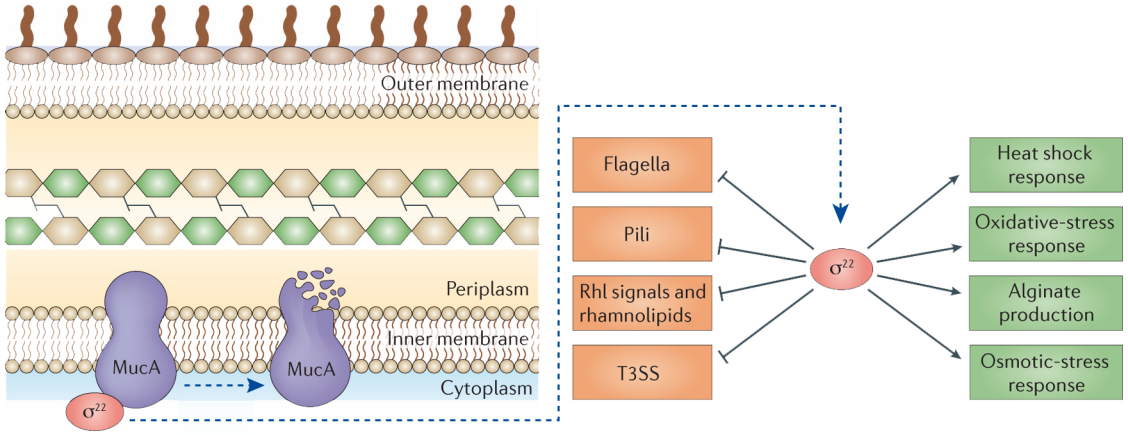


Figure 9. The function of the RNA polymerase σ-factor σ^{22} (encoded by *algU*) and pathway upregulated and downregulated in response. Adapted from Folkesson et al. 2012.

Not surprisingly, similar divergences in gene expression are observed when comparing early-colonizing CF-associated strains with chronical ones (Yang et al., 2011).

It is therefore evident that chronic colonization and infection with *P. aeruginosa* is an inevitable reality for the majority of adults with CF and, unfortunately, resistance to all clinically relevant classes of antibiotics is routinely exhibited by *P. aeruginosa* isolates (Lister et al., 2009).

5. *P. aeruginosa* as a Superbug

The simultaneous occurrence of multiple resistance mechanisms in the clinical isolates of *P. aeruginosa* has resulted in strains that are multidrug resistant (MDR). MDR bacterium is defined as non-susceptible to one or more antimicrobials in three or more antimicrobial classes, while strains that are non-susceptible to all antimicrobials are classified as extreme drug-resistant strains (Kallen and Srinivasan, 2010). This utmost resistance, together with demonstrated high morbidity and mortality infections, has led *P. aeruginosa* to be considered one of the most challenging superbugs (Boyle et al., 2012).

As stated by the NHSN, focused on nosocomial infections in intensive care units, not only were resistance rates increasing, so was the incidence of occurrence in most infection types (Gaynes and Edwards, 2005).

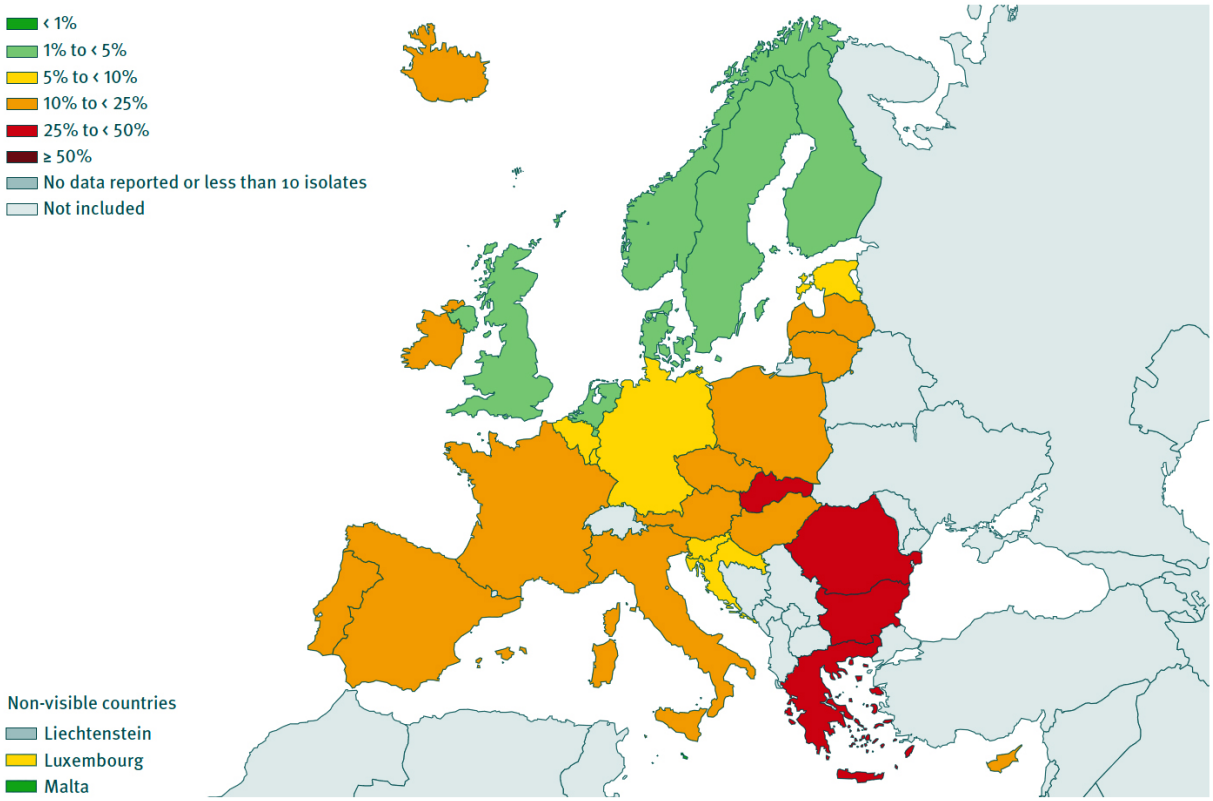


Figure 10. Percentage (%) of invasive isolates with combined resistance (resistance to three or more antimicrobial classes among piperacillin (±tazobactam), ceftazidime, fluoroquinolones, aminoglycosidesand carbapenems). From Antimicrobial Resistance Surveillance in Europe of 2012.

According to Antimicrobial Resistance Surveillance in Europe of 2012 (http://www.ecdc.europa.eu) high percentages of resistance in *P. aeruginosa* isolates were reported, with almost all the countries showing percentages above 10% for all antimicrobial

groups under surveillance. In particular, 37.7% of the isolates were resistant to one or more of the five considered antimicrobial classes, while 16.3% were resistant to at least three antimicrobial groups and 6% of the isolates were resistant to all five antimicrobial classes (piperacillin, ceftazidime, fluoroquinolones, aminoglycosides, and carbapenems). Trends for the period 2009–2012 were calculated for 23 countries and significant increases were observed for four countries (Austria, Ireland, Portugal, and Sweden) and slight increases for another 12 (Figure 10).

Nowadays, colistin, and polymyxin B are increasingly used as the last-line therapeutic options for treatment of infections caused by MDR *P. aeruginosa* (Li et al., 2006), and, while resistance rates remain low, mortality of 80% has been observed for infections caused by colistin resistant Gram-negative bacilli (Beno et al., 2006).

It is easy to observe how these kinds of numbers associated with extreme difficulties in the treatment of such infections, and high rates of morbidity and mortality, have made *Pseudomonas* one of the most dangerous and challenging hospital pathogens.

However, despite this alarming scenario, since the new millennium only three new classes of antibiotics have been introduced to the market for human use (Figure 11).

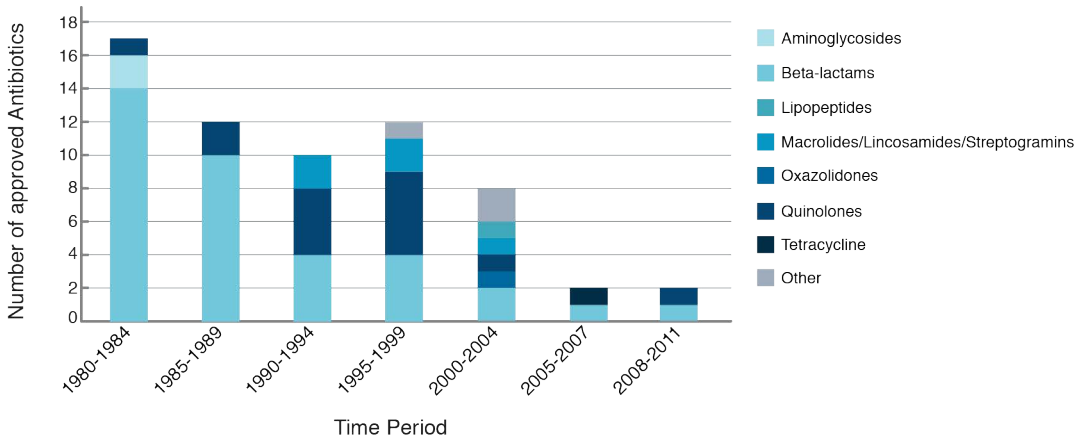


Figure 11. Number and functional classes of approved antibiotics during the last 30 years.

6. The crucial role of antibiotics

To fight bacterial infections, in the past 90 years, various classes of antimicrobial drugs have been developed following two parallel and independent lines of discovery. One approach has been the identification of small-molecule natural products with an observed antibacterial activity. Among these, the most famous and well-known examples are penicillins and cephalosporins, the glycopeptides like vancomycin, and the aminoglycosides (Clardy et al., 2009).

The other approach is represented by the discovery that "man-made" molecules had antibiotic activity: this is the case of the aromatic sulfa scaffolds originally from the chemical dyes industry or the fluoroquinolones (Blondeau, 2004).

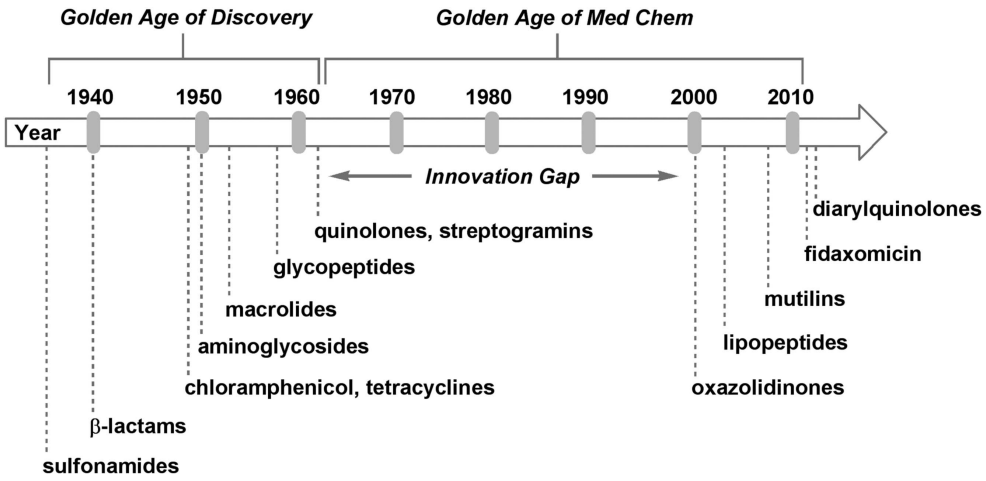


Figure 12. Timeline showing the "Golden Age" of antibiotics and of antibiotic medicinal chemistry. No new structural classes of antibiotics were introduced between 1962 and 2000. From Walsh & Wencewicz, 2013.

The so called "Golden Age" of discovery of natural antibiotics was actually a short two decades (Figure 12), between 1940 and 1960, when one-half of the drugs commonly used today were discovered. From the 1960s to the 1970s, the rise of antibiotic resistance, especially in the hospital environment, led to a frantic search for new active compounds, but unfortunately screening programs mostly rediscovered existing antibiotics (Lewis, 2013).

So pharmaceutical companies developed new antibiotics by improving the existing ones through chemical modifications, tailoring on the periphery of major antibiotic classes, while leaving the core intact (Bérdy, 2005). This approach obviously involved less risk as the target,

selectivity, and toxicity of the starting antibiotic backbone had already been defined (Fernandes, 2006).

Antibiotic	*Year deployed*	*Clinical resistance observed*[a]
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	Late 1960s
Nalidixic acid	1962	1962
Fluoroquinolones	1980s	1980s
Linezolid[b]	1999	1999
Daptomycin[b]	2003	2003
Retapamulin[b,c,d]	2007	2007
Fidaxomicin	2011	2011
Bedaquiline[b,e]	2013	?

[a]This table was modified from a previous report by Palumbi.[38]
[b]Represents a first-in-class drug for human use.
[c]Approved for topical use only.
[d]Resistance to retapamulin was observed in clinical isolates of *S. aureus* without previous exposure to pleuromutilins, but no case of resistance development during retapamulin therapy was found in the literature.[43]
[e]Approved only for use in combination therapy for treatment of MDR TB.

Table 3. Evolution of resistance to clinical antibiotics. Adapted from Walsh & Wencewicz, 2013.

Despite the short-term effectiveness of this strategy, resistances against new versions of old-fashioned antibiotics quickly arise (Table 3).

Simultaneously, the lack of success from natural compound screening led companies to redirect their efforts to small-molecule chemical libraries, obtaining, also in this case, a very poor return of new antibacterial agents.

Thus, the development of brand new molecules with antibiotic effects must be a priority in microbiological research.

6.1 Antibiotics in current use target a small number of cellular functions

Historically, the treatment of human bacterial infections by antibiotics targets a surprisingly small number of vital cellular functions: cell wall, DNA, RNA, folate and protein biosynthesis (Lewis, 2013)(Figure 13).

As pictured in Figure 13, β-lactams and vancomcyin-type glycopeptides are among the classes of antibiotics that interfere with specific steps in homeostatic cell wall biosynthesis. Their successful treatments result in changes to cell shape and size, induce cellular stress responses, and culminate in cell lysis (Tomasz, 1979; Barclay et al., 1996).

A second category comprises the blockade of DNA replication, most notably targeting DNA-topoisomerase complexes by the synthetic quinolone class of antimicrobials, including the clinically relevant fluoroquinolone. These molecules interfere with the maintenance of chromosomal topology by hitting DNA gyrase and topoisomerase IV, trapping these enzymes at the DNA cleavage stage and preventing strand rejoining (Drlica et al., 2008).

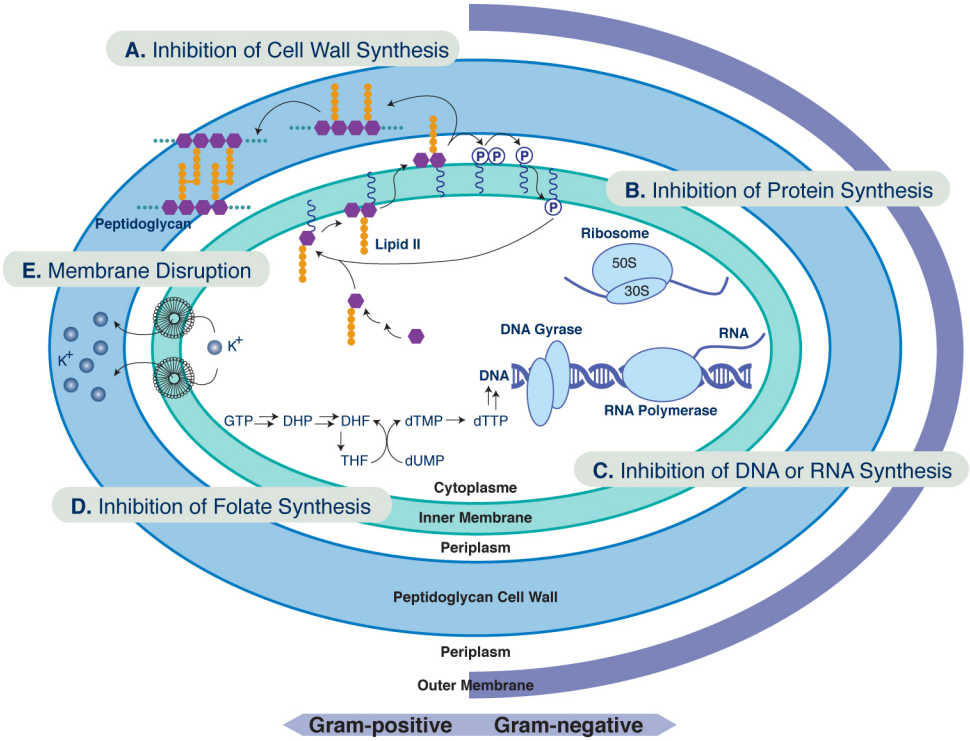


Figure 13. The five major clinically validated antibacterial targets/pathways. Adapted from Walsh & Wencewicz, 2013.

The inhibition of RNA synthesis is due to the action of the rifamycin class of semi-synthetic bactericidal antibiotics. These drugs inhibit DNA-dependent transcription by stable binding, with high affinity, to the subunit encoded by the *rpoB* gene and blocking RNA polymerase enzyme (Floss and Yu, 2005).

Regarding protein synthesis is one of the targets more explored by antibiotics which can inhibit, although with different degrees of specificity, virtually every step of the translation. In fact, there are more than 50 molecules targeting the small and large ribosomal subunits and the interface between them (Wilson, 2009).

The last category of antimicrobials targets the folate biosynthetic pathway, responsible for the *de novo* synthesis of thymidine and other key cellular components. Folate analogs and sulfa derivate drugs, mostly used in combination, block two sequential steps in that biosynthetic pathway (Lange et al., 2007).

7. Looking for novel essential genes as antimicrobial targets

Thus, there is absolutely no doubt about the utmost need to develop new antibacterial agents to combat the growing problem of antibiotic-resistant bacteria: targeting of novel essential pathways will likely play an important role in the discovery of these new molecules (Gerdes et al., 2002). In fact, a number of crucial cellular pathways, such as secretion, cell division, and many other metabolic functions, remains uncharacterized and untargeted up to today (Kohanski et al., 2010). Obviously, the most accurate knowledge possible of related biological processes in bacterial pathogens eases the choice of the target, thus the essential function expressed, which is one of the key moments in the long and expensive process of drug development.

In recent years, high-throughput automated genomic DNA sequencing, together with improvement in bioinformatic analysis, has provided plenty of information to help in the search for new targets to the extent that, in many cases, entire biochemical pathways can be reconstructed and compared (Zhang and Lin, 2009; Deng et al., 2011). Considering the matter from a different angle, the need to identify novel antibacterial targets has been one of the major drivers in the development of techniques designed to determine gene essentiality, recently concerning mainly the bioinformatic area (Duffield et al., 2010; Cooper and Duffield, 2011; Cheng et al., 2013; Wei et al., 2013). Although the genome sequences of approximately 2700 bacteria have already been released in public databases (Pagani et al., 2012; Huang et al., 2014), the function of a large fraction of these bacterial genes is still unknown, as mentioned in Chapter 1, as regards, for example, *P. aeruginosa*. Furthermore, although important, many processes and pathways are not ubiquitous and, in most cases, are only shared by a subset of bacterial strains that are not well characterized (Jordan et al., 2002). It is therefore evident that understanding and modeling the complexity of a living organism require global elucidation of gene function as well as the identification of the essential genes (de Berardinis et al., 2008). An essential gene is defined as one whose loss is lethal under a certain environmental condition (Xu et al., 2011) but, regarding laboratory conditions where this essentiality is assessed, it typically refers to the ability to grow on solid media and form a colony in different experimental requirements (Fang et al., 2005). Finally, the identification of essential genes plays a primary role not only in the research of potential targets for antimicrobial drug development but also in unraveling the minimal gene set for living organisms (Koonin, 2003; Gil et al., 2004; Glass et al., 2006) and deciphering bacterial relationships during evolution (Liao et al., 2006; Koonin, 2009).

7.1 Transposon mutagenesis based screenings

One powerful procedure for the detection of gene essentiality is based on transposon mutagenesis (TM). Transposons are discrete pieces of DNA that can move - transpose - from one location in a genome to another: hundreds of transposons have been identified, allowing a very large number of possible genetic tools to become available (Muñoz-López and García-Pérez, 2010). The simplest transposon is pictured in Figure 14 as a segment of DNA flanked by sequences recognized by a protein, transposase, which enables its movement.

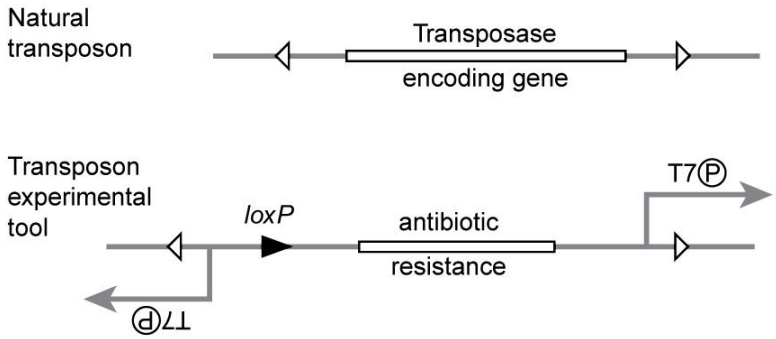


Figure 14. Transposon structure. The transposon ends are defined by two short inverted repeat sequences (open triangles). By supplying the transposase exogenously, the transposon can be simplified as an experimental tool. In this case, the terminal transposase recognition sequences bracket DNA that contains the desired sequences. For example, the transposon can be constructed to contain an appropriate antibiotic resistance gene, outward-facing T7 promoters, and a *lox*P site.

There are two different mechanisms by which transposition occurs: the first is cut-and-paste transposition (*e.g.*, *Tn5* and *Tn10*) (Reznikoff, 2008), in which the transposon is precisely excised from its original location in the donor DNA and is then inserted into a target sequence. The second is replicative transposition (*e.g.*, *Tn3*, and *Mu*) by one or both enzymes of transposase and resolvase, leaving one copy on the target DNA or two copies on both donor and target DNA respectively (Ton-Hoang et al., 2010).

Although the recognition sequence for some transposons is not clear or has not yet been fully determined, in general transposon-based gene integration does not require homologous sequences for transfer to the chromosome, and the genomic position to which a transposon can move depends only on the sequence recognized and cleaved by the enzyme (Choi and Kim, 2009). TM results in disruption of the region of the genome where the transposon has inserted. Failure to isolate such a knockout mutation in a particular ORF is taken as presumptive evidence that the targeted gene is essential in the tested growth conditions.

With TM there are two opposite approaches for identifying essential genes of the bacterial chromosome (Figure 15):

- the so called “negative” approach, which determines many ORFs as non-essential and assumes that non-inserted genome stretches are essential;
- the “positive” approach, which identifies genes that are essential by generating growth-conditional mutants and showing that they have a lethal phenotype.

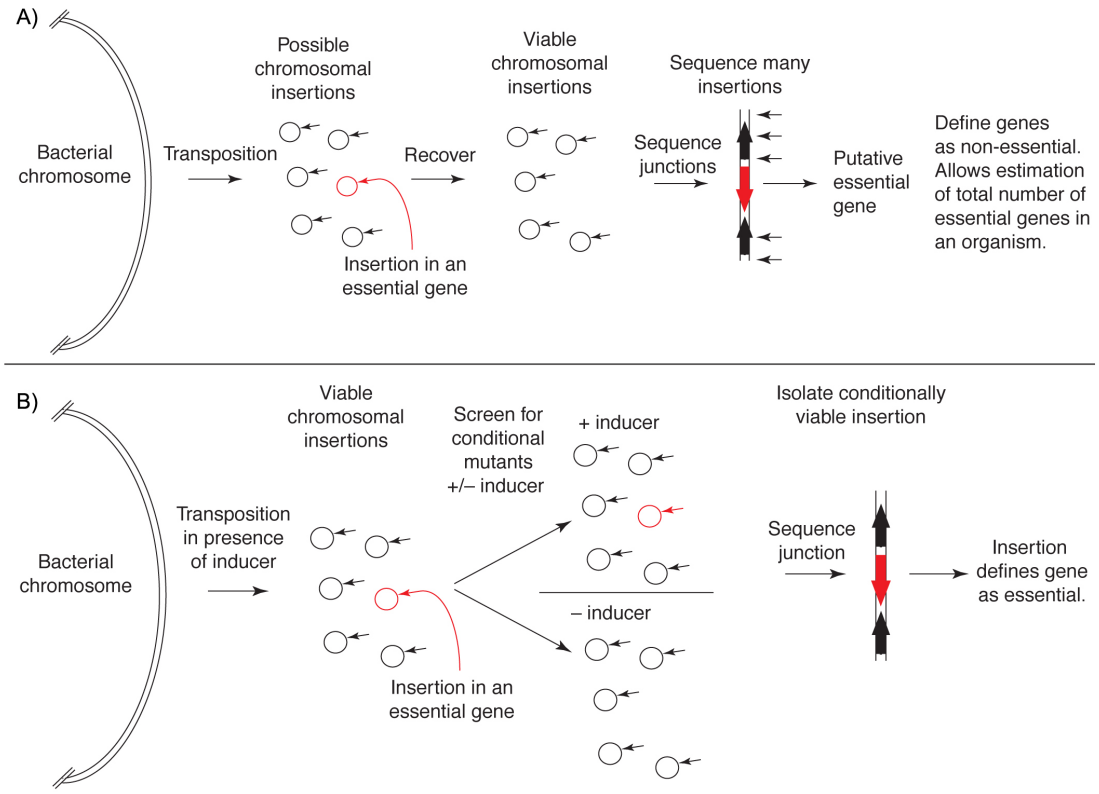


Figure 15. **A)** Negative approach to identify essential bacterial genes: the whole bacterial chromosome is the target for transposon mutagenesis generating a huge number of viable insertions subsequently analyzed and sequenced. This method requires many insertions to be sequenced before statistically significant conclusions can be drawn. **B)** Positive approach: transposon contains an outward-facing inducible promoter at one edge. Strains generated by such an insertion are dependent on the inducer for viability. The insertional junction is sequenced, allowing the identification of the downstream essential gene. Adapted from Judson & Mekalanos, 2000.

However, such large-scale systematic analysis of gene essentiality cannot be accomplished without unbiased and accurate annotations generating both false positives and negatives (Deng et al., 2013). Indeed, even if saturation is reached, it is unavoidable that some genes, particularly the shorter ones, may circumvent the insertion and be wrongly annotated as essential (Gerdes et al., 2003; Gallagher et al., 2007). On the other hand, false negative errors in which essential genes are determined as non-essential may take place if the insertion occurs at gene ends and does not fully inactivate the function (Akerley et al., 2002; Glass et al., 2006).

Type of approach	Name	Description	Advantages	Disadvantages
Negative	All negative approaches	Identify essential genes by defining non-essential regions and assuming what cannot be disrupted is essential.	Can define sites within an otherwise essential ORF that are permissive for insertions.	Does not identify essential genes. Further strain construction and testing of candidate ORFs is required to confirm essentiality of putative essential genes. Intermediate phenotypes especially require further analysis: 5′ or 3′ insertions in an ORF may or may not mean that the gene is essential. Operon structure can pose problems for analysis.
	PCR-mapping approaches	Mapping non-essential regions by *in vitro* transposition and PCR on a short, defined segment of DNA.	Analysis can be performed on a small or large scale: specific genome regions (5–10 kb) are analysed individually, this makes saturation easy (in a large-scale analysis, gives a defined endpoint). Small target sequence for both transposons [(TA dinucleotide for *mariner* (this occurs, on average 1 in 16 nucleotides), TGTT for Ty-1 (1 in 256)]. No sequencing required.	Large-scale analysis is resource intensive, requires many oligonucleotide primers. Restricted to naturally competent organisms.
	Global transposon mutagenesis	Analysis of a large number of random chromosomal insertions to define regions that cannot be hit.	Does not require a naturally competent organism.	Cannot define essential chromosomal regions or ORFs as essential unless saturation is approached, therefore can only be used as a method to estimate the number of essential genes. Need to approach saturation before any conclusions can be drawn. Resource intensive.
Positive	All positive approaches	Identify essential genes by substitution of an essential gene's natural promoter with an inducible one, generating a conditional mutation.	Analysis can be performed on a small or large scale. Every gene identified is a gene of interest; genes that are not strictly essential can be easily tested for essentiality under other growth conditions. The conditional strain can be used for further biochemical analysis. Essential ORFs with non-essential 5′ regions that are permissive for transposon insertions will still generate a conditional phenotype.	Insertions upstream of every essential gene might not be possible. Saturating mutagenesis of a genome is laborious to achieve. Expression levels of the inducible promoter will not be broad enough to identify every essential gene (basal expression of the promoter might be too high or maximal expression might not be enough). For operons, the essential gene might not be immediately downstream of the insertion; further analysis is required and operons with coupled translation might not be possible to hit.
	TnAraOut	A transposon containing an outward-facing arabinose-inducible promoter.	The arabinose promoter has a large induction ratio; small target sequence (TA dinucleotide); broad host range transposon.	
	Tn*5*tac1	A transposon containing an outward-facing IPTG-inducible promoter.		There is a higher level of basal expression and a smaller induction ratio for the tac promoter as compared with the arabinose P_{BAD} promoter.
	mini-Tn*10* transposons	Divergent transcription from the *tetA* and *tetR* genes in the presence of tetracycline results in tetracycline-inducible conditional phenotypes.		Larger target sequence for Tn*10* increases the difficulty of obtaining insertions upstream of essential genes.

Table 4. Advantages and disadvantages of transposon-based approaches to identify essential bacterial genes. Adapted from Judson & Mekalanos, 2000.

Some inconveniences of the "negative" TM approach were overcome by using the "positive" one described above. However, conditional TM can also be affected by systematic biases, deriving, for example, from transposon tools endowed with outward-facing promoters that are not strictly regulated in non-inducing conditions, resulting in a basal level of promoter expression (Table 4). Different approaches have been used for the transposon mutagenesis of *P. aeruginosa* (Jacobs et al., 2003; Lewenza et al., 2005). In the former, a library of 30,100 unique transposon insertions was generated using Tn*5* IS*50*L; in the latter, an insertion library of 34,000 mutants was developed using a *mariner*-based transposon. Both of these libraries represent multiple insertions into non-essential genes.

7.2 Single-gene knockout mutant collection

The availability of complete genome sequences has permitted the construction of large-scale collections of mutants and a comprehensive understanding of organisms at the molecular level. Indeed, an alternative strategy to TM has been represented by directed approaches such as the complete gene deletion method already effectively used for *Saccharomyces cerevisiae* (Giaever et al., 2002), *Acinetobacter baylyi* (de Berardinis et al., 2008), *Bacillus subtilis* (Kobayashi et al., 2003), *E. coli* (Gerdes et al., 2003; Baba et al., 2006), and *Streptococcus sanguinis* (Xu et al., 2011).

A single-gene deleted mutant collection should provide a fundamental tool for "reverse genetics" approaches, permitting analysis of the consequences of the complete loss of gene function, in contrast to forward genetics approaches, in which mutant phenotypes are associated with the corresponding genes.

The approach used for systematic gene deletion is based on homology directed recombination with linear fragments generated by PCR containing a resistance cassette, in most cases kanamycin, flanked, in some cases, by FLP recognition target (FRT) site and at least 50 bp homologous to adjacent chromosomal sequences.

To avoid polar effect on the downstream gene, the targeting PCR products were designed to create, after excision of the resistance cassette with the FLP recombinase, in-frame deletions of the 2nd through the 7th codon from the C-terminus, leaving the ORF start codon and translational signal intact. Regarding overlapping genes by more than 9 nt, fortunately they are only a small portion of the genome (*e.g.*, *E. coli*) and so a small number of primers were specifically re-designed to avoid altering two genes simultaneously (Baba et al., 2008).

Furthermore, the antibiotic cassette used lacked a promoter to prevent potential dysregulation of downstream genes, which could affect subsequent mutant library results.

As previously mentioned, the availability of a large number of sequenced and properly annotated genomes (Brent, 2005) allows highly accurate primers to be designed. The length of homologous sequences to the adjacent upstream or downstream flanking regions varies depending on the species studied: in *E.coli* 50 bp are sufficient (Baba et al., 2006) while in *A. baylyi* (de Berardinis et al., 2008) at least 300 bp are needed; on the other hand, in *S. sanguinis* long flanking sequences (approximately 1 kb) are required to ensure an efficient homologous recombination (Xu et al., 2011).

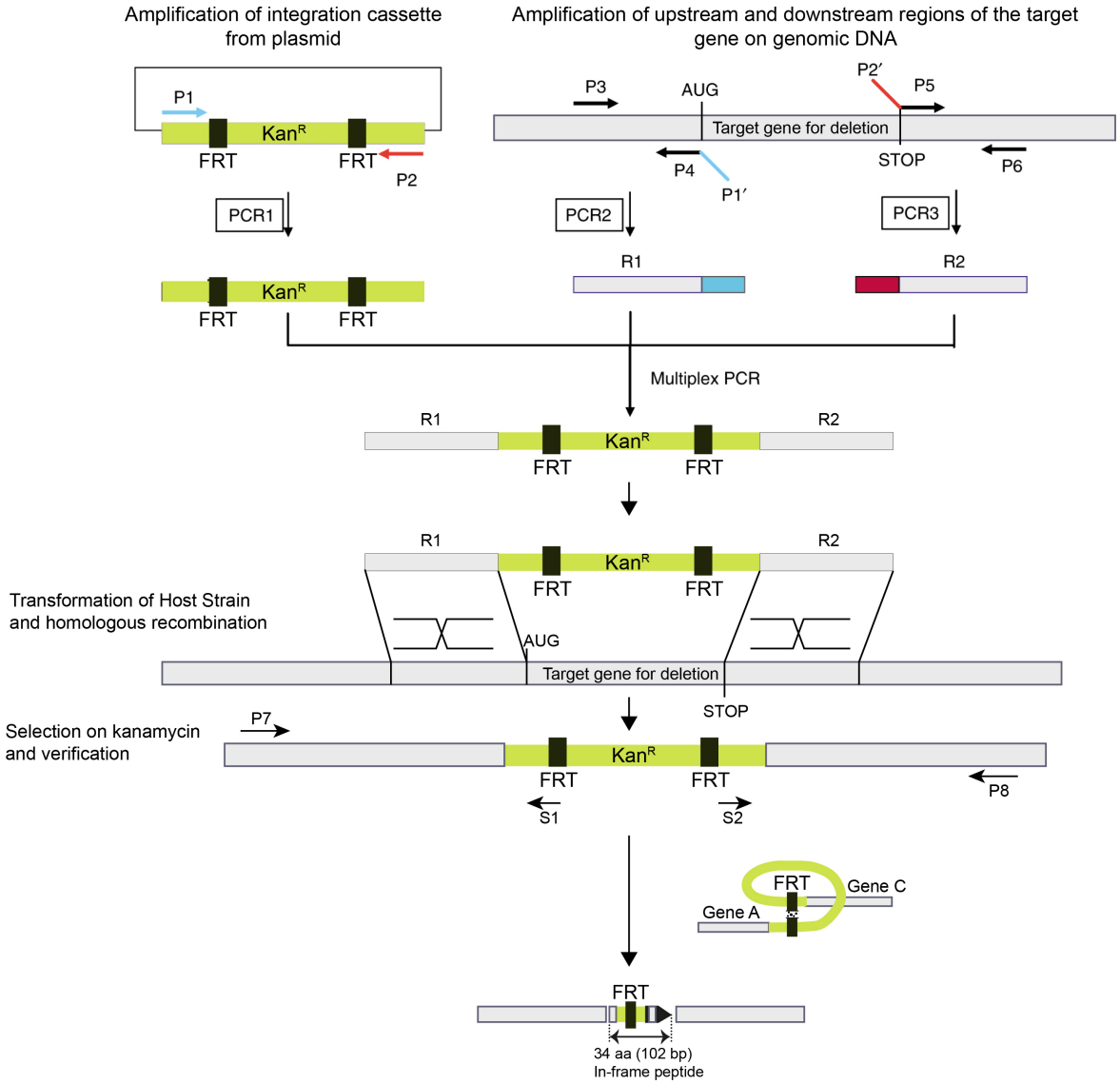


Figure 16 Schematic representation of the single-gene deletion mutants approach. Primers P1 to P6 are used for integration cassette construction while P7, P8, S1, and S2 for verifications. The *kan*^{R} integration cassette is obtained by PCR amplification using P1 and P2 primers on plasmid template. The flanking regions, specific for the target gene, are amplified on wild-type DNA template by P3/P4 primers (R1 region) and P5/P6 primers (R2 region). The primers P7 and P8 are used for external PCR verification of the correct replacement of the targeted gene by the integrative cassette. The primers S1 and S2 located within the kanR cassette are used to sequence junctions of the cassette on the P7/P8 PCR product. Structures created after excision of the resistance gene are verified by PCR with neighboring gene-specific primers and by direct DNA sequencing of the region encompassed as described by Datsenko and Wanner, 2000.

The PCR products, obtained after three different step reactions, were re-amplified to generate the auspicated linear fragments. These were usually digested with proper restriction enzyme and ethanol-precipitated to purify them from contaminating the template plasmid DNA (used for resistance cassette amplification) and excess primers. The resulting products were directly transformed into host cells and then clones were selected on minimal medium agar plates supplemented with kanamycin.

This is clearly one of the limiting steps of the technology: in fact, if on the one hand some species are naturally competent and allow an optimal transformation frequency, such as *E. coli* (Sinha and Redfield, 2012) or *A. baylyi* ADP1 (Metzgar et al., 2004), others are much more recalcitrant to the acquisition of genetic material (*e.g.*, *S. sanguinis*) and *ad hoc* experiments (Paik et al., 2005) are required to optimize transformation efficiency and avoid the false identification of genes as essential. Unfortunately, many other bacterial species, especially Gram-positive ones such as *Staphylococcus aureus* (Veiga and Pinho, 2009; Corvaglia et al., 2010), and *Bacillus subtilis* (Stephenson and Jarrett, 1991; Yang et al., 2010), are not naturally competent, and this extremely low transformation efficiency is an insurmountable limitation and the main reason why this technique is not applicable. Moreover, although this technology has been successfully applied for the creation of single mutants in "difficult strains" - *P. aeruginosa* (Lesic and Rahme, 2008) or *Salmonella enterica* (Husseiny and Hensel, 2005) - it is very difficult to envisage its application on a large scale for the creation of a library of mutants since, as stated by the authors of the technique (Baba et al., 2006), the most critical step is the preparation of highly electrocompetent cells (>10^9 transformants per 1 μg of plasmid DNA) with standards hardly reachable for the previously mentioned strains.

In addition, this approach requires the phage λ Red recombinase expression in the bacterial strain for an efficient recombination between the targeted gene and a linear PCR product (Datsenko and Wanner, 2000). The Red system includes three genes: γ, β, and *exo*, whose products are called Gam, Bet, and Exo respectively. Gam binds to the bacterial RecBCD exonuclease V and inhibits its activities so that the other two components can promote recombination in concert: Exo processively digests the 5'-ended strand of a dsDNA while Bet binds to ssDNA and promotes strand annealing (Poteete, 2001; Sharan et al., 2009).

Expression of the λ Red recombinase under the control of an inducible promoter is required from low copy helper plasmids, such as pKD46 or pKD20, which are temperature-sensitive and thus can easily be eliminated after recombination event.

Thus, mutants of the targeted gene are isolated as antibiotic-resistant colonies: typically, for each gene deletion experiment, at least two Kan^R colonies were picked and each re-isolated twice. The mutants that could not be obtained in the high-throughput process were reattempted individually (up to five times), and, after this last failure, assessed as essential. Mutants were checked for correct structure and size by different PCR amplifications as schematized in Figure 16.

7.3 Antisense RNA approaches for essential genes detection

The genome-wide identification of "essential-for-growth" genes can be performed also via antisense RNA technology. Almost 20 years ago antisense RNA technology found its application in eukaryotic systems to inhibit gene expression (Davies et al., 1996). Since that time, in mammalian systems, RNA interference (RNAi) has become a powerful methodology for studying loss-of-function phenotypes, as well as being a proved crucial technique in interpreting gene function on a genome-wide scale (Fellmann and Lowe, 2014).

Furthermore, even in microorganism systems, RNAi has been used effectively to identify essential genes and virulence factors as well as to study gene functions of *Staphylococcus aureus* (Ji et al., 2001; Forsyth et al., 2002; Yin and Ji, 2002), mycobacteria (Parish and Stoker, 1997), *Streptococcus mutans* (Wang and Kuramitsu, 2005), and *Candida albicans* (De Backer et al., 2001).

The mechanisms of natural and artificially antisense RNAs are heterogeneous and are generally described as relating to mRNA destabilization (Storz, 2002): key factors for a competent and enduring RNAi, which is unfortunately very difficult to predict, are the possibility and accessibility to participate in RNA-RNA duplexes (Rana, 2007).

Furthermore, it has been observed that not all the different antisense RNAs that target a particular gene, attempting to saturate it, are able to generate the same structure and therefore inhibition (Ji et al., 2001; Forsyth et al., 2002). This differing efficacy of various antisense RNAs for a given gene can obscure the functional difference between a marginally effective antisense targeting an essential gene and, for example, a highly efficacious antisense targeting a gene required merely for rapid growth. For this reason it is easy to understand that, searching for new essential gene targets, a genome-wide antisense fragmentation approach is preferable with respect to a specific antisense RNA design directed against hypothetical essential genes.

This approach, already used on the bacterial pathogen *Staphylococcus aureus* (Forsyth et al., 2002), is based on the construction and screening of Shotgun Antisense Libraries (SALs) as schematized in Figure 17.

The cloning strategy employed should comprehensively represent the genome of interest in small random overlapping fragments. For this purpose, restriction digests, although easiest to obtain, are proven to produce poor randomization (Forsyth and Wang, 2008).

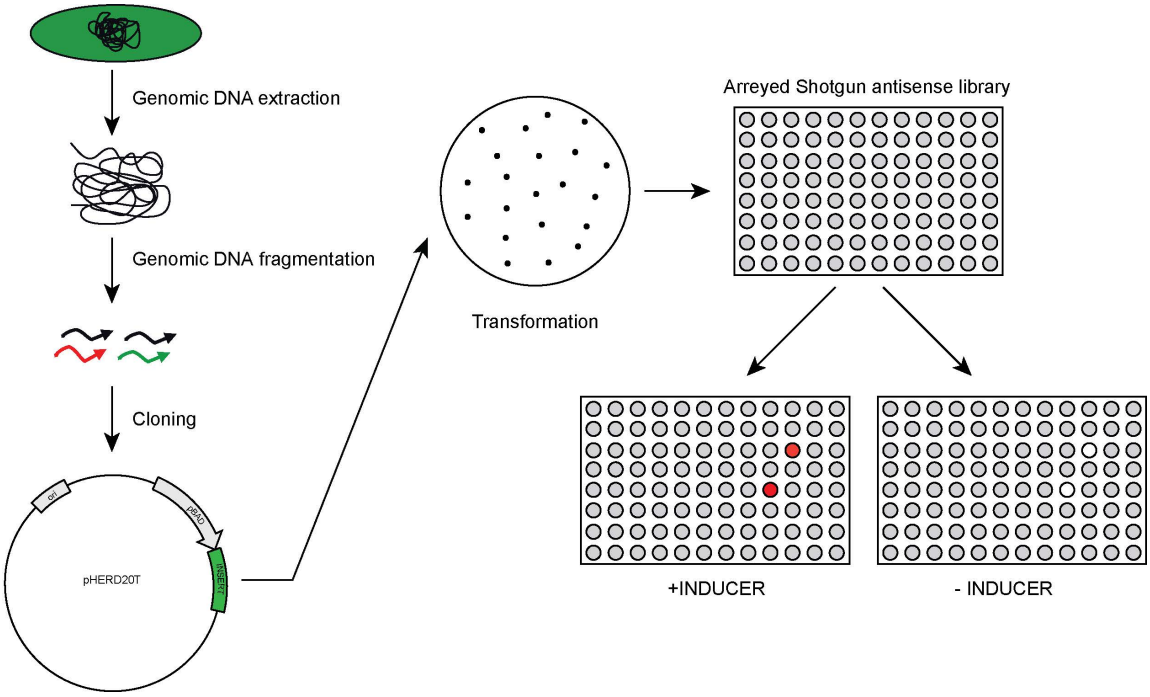


Figure 17. Schematic representation of construction and screening of a shotgun antisense library (SAL).

Alternatively, shearing genomic DNA using physical force (*e.g.*, with a nebulizer or sonicator) seems to allow a more complete coverage when one balances the size of the fragments with the number of clones in the library.

The resulting fragments are then size-selected on a gel (choosing ones spanning between 200–800 bp), bluntended using enzymes (*e.g.*, Klenow, T4 DNA polymerase), and cloned into an appropriate vector under the control of an inducible promoter.

The goal of the SAL approach is to identify those clones that produce an antisense RNA to an essential gene: to this end, fragments are screened to identify those whose expression, in the presence of promoter induction, impairs growth. Generally, only a really small percentage, approximately 1% of shotgun clones (Forsyth et al., 2002; Wang and Kuramitsu, 2005; Meng et al., 2012), will be sensitive to induction, of which about 70% will be antisense ones.

The genes targeted and insert orientation of clones are identified by DNA sequencing of the growth-impairing fragments previously amplified by polymerase chain reaction (PCR) with a set of vector-specific primers. Bioinformatics analyses are performed to determine the original genomic locations of the sensitive clone inserts and insert orientations.

Typically, two distinct phenotypic classes were identified after selection criteria (Yin and Ji, 2002; Rusmini et al., 2014): one class exhibits a lethal (total no-growth) phenotype after antisense induction; the second displays an impaired phenotype, mainly connected with slow or reduced growth, but does eventually give rise to definitive small colonies.

For the latter defective isolates, additional studies (*e.g.*, knockout via allele replacement) would be needed to definitely confirm the phenotypes observed.

As relates to the applicability and versatility, this technique originally only showed limited success in Gram-negative bacteria (Wagner and Flärdh, 2002): until 2012 no published reports were proposed describing the application of the SAL approach for comprehensive genome-wide essential gene characterization (Meng et al., 2012) due to an assumed inefficiency in conditional silencing mediated by antisense RNAs in Gram-negative bacteria.

In particular, although precise reasons have not yet been formulated, the involvement of different RNA chaperone requirements for *trans*-acting antisense RNAs was hypothesized (Waters and Storz, 2009). Furthermore, a lower abundance of the expressed antisense could be explained by a reduced stability of plasmid encoding probably due to the presence of RNase E in these bacteria while lacking in Gram-positive ones (Xu et al., 2010).

However, antisense inhibition technology could also show some complications. For instance, antisense destabilization of a polycistronic transcript could result in polar effects: in this unfortunate event, an antisense RNA complementary to a non-essential gene destabilizes an essential gene which is co-transcribed in the same mRNA (Wiedenheft et al., 2012).

A further possible limitation lies in the fact that, according to the scheme for SAL construction established in *S. aureus* (Ji et al., 2001; Forsyth et al., 2002), fragments generated *in vitro* are directly introduced into the original host, and selected in permissive conditions *(e.g.*, with the promoter vector in an off state) to allow the clones carrying inserts targeting essential genes to survive. However, basal vector promoter activity could be sufficient to elicit silencing effects against genes transcribed at low levels introducing a bias in the subsequent conditional screening and so favoring the identification of highly transcribed essential genes (*e.g.*, tRNAs, tRNA synthetases, ribosomal proteins, translation factors, components of the transcription machinery) as reported in a recent antisense screening of *E. coli* (Meng et al., 2012). To avoid missing the identification of low expressed essential genes owing to out-of-context use of the P_{BAD} promoter (Qiu et al., 2008), as described in PART II, we included some modifications to the original strategy. In detail, *P. aeruginosa* SALs were firstly generated in *E. coli*, and then arrayed and challenged by conjugative transfer into *P. aeruginosa*.

Moreover, this strategy assures a larger sized shotgun library because of the higher transformation efficiency of *E. coli* compared to *P. aeruginosa* and broadened the functional class variety of the identified essential genes in respect to previous reports.

References

Akerley, B.J., Rubin, E.J., Novick, V.L., Amaya, K., Judson, N., and Mekalanos, J.J. (2002) A genome-scale analysis for identification of genes required for growth or survival of Haemophilus influenzae. *Proc. Natl. Acad. Sci. U. S. A.* **99**: 966–971.

Alhede, M., Bjarnsholt, T., Givskov, M., and Alhede, M. (2014) Pseudomonas aeruginosa biofilms. Mechanisms of immune evasion. *Adv. Appl. Microbiol.* **86**: 1–40.

Alm, E., Huang, K., and Arkin, A. (2006) The evolution of two-component systems in bacteria reveals different strategies for niche adaptation. *PLoS Comput. Biol.* **2**: 1329–1342.

Aspedon, A., Palmer, K., and Whiteley, M. (2006) Microarray analysis of the osmotic stress response in Pseudomonas aeruginosa. *J Bacteriol* **188**: 2721–2725.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., et al. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**: 2006.0008.

Baba, T., Huan, H.-C., Datsenko, K., Wanner, B.L., and Mori, H. (2008) The applications of systematic in-frame, single-gene knockout mutant collection of Escherichia coli K-12. *Methods Mol. Biol.* **416**: 183–194.

De Backer, M.D., Nelissen, B., Logghe, M., Viaene, J., Loonen, I., Vandoninck, S., et al. (2001) An antisense-based functional genomics approach for identification of genes critical for growth of Candida albicans. *Nat. Biotechnol.* **19**: 235–241.

Bagge, N., Ciofu, O., Hentzer, M., Campbell, J.I.A., Givskov, M., and Høiby, N. (2002) Constitutive high expression of chromosomal beta-lactamase in Pseudomonas aeruginosa caused by a new insertion sequence (IS1669) located in ampD. *Antimicrob. Agents Chemother.* **46**: 3406–3411.

Barbieri, A.M., Sha, Q., Bette-Bobillo, P., Stahl, P.D., and Vidal, M. (2001) ADP-ribosylation of Rab5 by ExoS of Pseudomonas aeruginosa affects endocytosis. *Infect. Immun.* **69**: 5329–5334.

Barclay, M.L., Begg, E.J., Chambers, S.T., Thornley, P.E., Pattemore, P.K., and Grimwood, K. (1996) Adaptive resistance to tobramycin in Pseudomonas aeruginosa lung infection in cystic fibrosis. *J. Antimicrob. Chemother.* **37**: 1155–1164.

Bardoel, B.W., van der Ent, S., Pel, M.J.C., Tommassen, J., Pieterse, C.M.J., van Kessel, K.P.M., and van Strijp, J.A.G. (2011) Pseudomonas evades immune recognition of flagellin in both mammals and plants. *PLoS Pathog.* **7**:

Barken, K.B., Pamp, S.J., Yang, L., Gjermansen, M., Bertrand, J.J., Klausen, M., et al. (2008) Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in Pseudomonas aeruginosa biofilms. *Environ. Microbiol.* **10**: 2331–2343.

Barraud, N., Hassett, D.J., Hwang, S.-H., Rice, S.A., Kjelleberg, S., and Webb, J.S. (2006) Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa. *J. Bacteriol.* **188**: 7344–7353.

Bassler, B.L. (2002) Small talk: Cell-to-cell communication in bacteria. *Cell* **109**: 421–424.

Beno, P., Krcmery, V., and Demitrovicova, A. (2006) Bacteraemia in cancer patients caused by colistin-resistant Gram-negative bacilli after previous exposure to ciprofloxacin and/or colistin. *Clin. Microbiol. Infect.* **12**: 497–8.

De Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., et al. (2008) A complete collection of single-gene deletion mutants of Acinetobacter baylyi ADP1. *Mol. Syst. Biol.* **4**: 174.

Bérdy, J. (2005) Bioactive microbial metabolites. *J. Antibiot. (Tokyo).* **58**: 1–26.

Bjarnsholt, T., Tolker-Nielsen, T., Høiby, N., and Givskov, M. (2010) Interference of Pseudomonas aeruginosa signalling and biofilm formation for infection control. *Expert Rev. Mol. Med.* **12**: e11.

Blondeau, J.M. (2004) Fluoroquinolones: Mechanism of action, classification, and development of resistance. *Surv. Ophthalmol.* **49**:

Bonomo, R.A. and Szabo, D. (2006) Mechanisms of multidrug resistance in Acinetobacter species and Pseudomonas aeruginosa. *Clin. Infect. Dis.* **43 Suppl 2**: S49–S56.

Boucher, R.C. (2004) Relationship of airway epithelial ion transport to chronic bronchitis. *Proc. Am. Thorac. Soc.* **1**: 66–70.

Boyle, B., Fernandez, L., Laroche, J., Kukavica-Ibrulj, I., Mendes, C.M.F., Hancock, R.W., and Levesque, R.C. (2012) Complete Genome Sequences of Three Pseudomonas aeruginosa Isolates with Phenotypes of Polymyxin B Adaptation and Inducible Resistance. *J. Bacteriol.* **194**: 529–530.

Breidenstein, E.B.M., de la Fuente-Núñez, C., and Hancock, R.E.W. (2011) Pseudomonas aeruginosa: All roads lead to resistance. *Trends Microbiol.* **19**: 419–426.

Brent, M.R. (2005) Genome annotation past, present, and future: how to define an ORF at each locus. *Genome Res.* **15**: 1777–1786.

Brint, J.M. and Ohman, D.E. (1995) Synthesis of multiple exoproducts in Pseudomonas aeruginosa is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J. Bacteriol.* **177**: 7155–7163.

Brooun, A., Liu, S., and Lewis, K. (2000) A dose-response study of antibiotic resistance in Pseudomonas aeruginosa biofilms. *Antimicrob. Agents Chemother.* **44**: 640–646.

Burns, J.L., Gibson, R.L., McNamara, S., Yim, D., Emerson, J., Rosenfeld, M., et al. (2001) Longitudinal assessment of Pseudomonas aeruginosa in young children with cystic fibrosis. *J. Infect. Dis.* **183**: 444–452.

Calfee, M.W., Shelton, J.G., McCubrey, J.A., and Pesci, E.C. (2005) Solubility and bioactivity of the Pseudomonas quinolone signal are increased by a Pseudomonas aeruginosa-produced surfactant. *Infect. Immun.* **73**: 878–882.

Campodónico, V.L., Llosa, N.J., Grout, M., Döring, G., Maira-Litrán, T., and Pier, G.B. (2010) Evaluation of flagella and flagellin of Pseudomonas aeruginosa as vaccines. *Infect. Immun.* **78**: 746–755.

Chapon-Hervé, V., Akrim, M., Latifi, A., Williams, P., Lazdunski, A., and Bally, M. (1997) Regulation of the xcp secretion pathway by multiple quorum-sensing modulons in Pseudomonas aeruginosa. *Mol. Microbiol.* **24**: 1169–1178.

Cheng, J., Wu, W., Zhang, Y., Li, X., Jiang, X., Wei, G., and Tao, S. (2013) A new computational strategy for predicting essential genes. *BMC Genomics* **14**: 910.

Choi, K.-H. and Kim, K.-J. (2009) Applications of transposon-based gene delivery system in bacteria. *J. Microbiol. Biotechnol.* **19**: 217–228.

Cigana, C., Curcuru, L., Leone, M.R., Ierano, T., Lore, N.I., Bianconi, I., et al. (2009) Pseudomonas aeruginosa exploits lipid a and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. *PLoS One* **4**:

Clardy, J., Fischbach, M.A., and Currie, C.R. (2009) The natural history of antibiotics. *Curr. Biol.* **19**:

Collins, F.S. (1992) Cystic fibrosis: molecular biology and therapeutic implications. *Science* **256**: 774–779.

Cooper, I. and Duffield, M. (2011) The in silico prediction of bacterial essential genes. *Sci. Against Microb. Pathog.* 551–559.

Corvaglia, A.R., François, P., Hernandez, D., Perron, K., Linder, P., and Schrenzel, J. (2010) A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical Staphylococcus aureus strains. *Proc. Natl. Acad. Sci. U. S. A.* **107**: 11954–11958.

Cowell, B.A., Evans, D.J., and Fleiszig, S.M.J. (2005) Actin cytoskeleton disruption by ExoY and its effects on Pseudomonas aeruginosa invasion. *FEMS Microbiol. Lett.* **250**: 71–76.

Cryz, S.J., Lang, A., Rüdeberg, A., Wedgwood, J., Que, J.U., Fürer, E., and Schaad, U. (1997) Immunization of cystic fibrosis patients with a Pseudomonas aeruginosa O-polysaccharide-toxin A conjugate vaccine.

Datsenko, K. a and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**: 6640–5.

Davies, L., Farrar, N.A., Satre, M., Dottin, R.P., and Gross, J.D. (1996) Vacuolar H(+)-ATPase and weak base action in Dictyostelium. *Mol. Microbiol.* **22**: 119–126.

Deep, A., Chaudhary, U., and Gupta, V. (2011) Quorum sensing and Bacterial Pathogenicity: From Molecules to Disease. *J. Lab. Physicians* **3**: 4–11.

Deng, J., Deng, L., Su, S., Zhang, M., Lin, X., Wei, L., et al. (2011) Investigating the predictability of essential genes across distantly related organisms using an integrative approach. *Nucleic Acids Res.* **39**: 795–807.

Deng, J., Su, S., Lin, X., Hassett, D.J., and Lu, L.J. (2013) A statistical framework for improving genomic annotations of prokaryotic essential genes. *PLoS One* **8**: e58178.

Döring, G. and Pier, G.B. (2008) Vaccines and immunotherapy against Pseudomonas aeruginosa. *Vaccine* **26**: 1011–1024.

Drlica, K., Malik, M., Kerns, R.J., and Zhao, X. (2008) Quinolone-mediated bacterial death. *Antimicrob. Agents Chemother.* **52**: 385–92.

Duffield, M., Cooper, I., McAlister, E., Bayliss, M., Ford, D., and Oyston, P. (2010) Predicting conserved essential genes in bacteria: in silico identification of putative drug targets. *Mol. Biosyst.* **6**: 2482–2489.

Ernst, R.K., Hajjar, A.M., Tsai, J.H., Moskowitz, S.M., Wilson, C.B., and Miller, S.I. (2003) Pseudomonas aeruginosa lipid A diversity and its recognition by Toll-like receptor 4. *J. Endotoxin Res.* **9**: 395–400.

Ernst, R.K., Moskowitz, S.M., Emerson, J.C., Kraig, G.M., Adams, K.N., Harvey, M.D., et al. (2007) Unique lipid a modifications in Pseudomonas aeruginosa isolated from the airways of patients with cystic fibrosis. *J. Infect. Dis.* **196**: 1088–1092.

Ernst, R.K., Yi, E.C., Guo, L., Lim, K.B., Burns, J.L., Hackett, M., and Miller, S.I. (1999) Specific lipopolysaccharide found in cystic fibrosis airway Pseudomonas aeruginosa. *Science* **286**: 1561–1565.

Erridge, C., Stewart, J., Bennett-Guerrero, E., McIntosh, T.J., and Poxton, I.R. (2002) The biological activity of a liposomal complete core lipopolysaccharide vaccine. *J. Endotoxin Res.* **8**: 39–46.

Fang, G., Rocha, E., and Danchin, A. (2005) How essential are nonessential genes? *Mol. Biol. Evol.* **22**: 2147–2156.

Feinbaum, R.L., Urbach, J.M., Liberati, N.T., Djonovic, S., Adonizio, A., Carvunis, A.R., and Ausubel, F.M. (2012) Genome-wide identification of Pseudomonas aeruginosa virulence-related genes using a Caenorhabditis elegans infection model. *PLoS Pathog.* **8**: 11.

Fellmann, C. and Lowe, S.W. (2014) Stable RNA interference rules for silencing. *Nat. Cell Biol.* **16**: 10–8.

Feltman, H., Schulert, G., Khan, S., Jain, M., Peterson, L., and Hauser, a R. (2001) Prevalence of type III secretion genes in clinical and environmental isolates of Pseudomonas aeruginosa. *Microbiology* **147**: 2659–69.

Fernandes, P. (2006) Antibacterial discovery and development--the failure of success? *Nat. Biotechnol.* **24**: 1497–1503.

Finck-Barbançon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J.P., Fleiszig, S.M., et al. (1997) ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury. *Mol. Microbiol.* **25**: 547–557.

Floss, H.G. and Yu, T.-W. (2005) Rifamycin-mode of action, resistance, and biosynthesis. *Chem. Rev.* **105**: 621–32.

Forsyth, A. and Wang, L. (2008) Techniques for the isolation and use of conditionally expressed antisense RNA to achieve essential gene knockdowns in Staphylococcus aureus. *Methods Mol. Biol.* **416**: 307–321.

Forsyth, R.A., Haselbeck, R.J., Ohlsen, K.L., Yamamoto, R.T., Xu, H., Trawick, J.D., et al. (2002) A genome-wide strategy for the identification of essential genes in Staphylococcus aureus. *Mol. Microbiol.* **43**: 1387–1400.

Gallagher, L.A., Ramage, E., Jacobs, M.A., Kaul, R., Brittnacher, M., and Manoil, C. (2007) A comprehensive transposon mutant library of Francisella novicida, a bioweapon surrogate. *Proc. Natl. Acad. Sci. U. S. A.* **104**: 1009–1014.

Gambello, M.J. and Iglewski, B.H. (1991) Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression. *J. Bacteriol.* **173**: 3000–3009.

Gambello, M.J., Kaye, S., and Iglewski, B.H. (1993) LasR of Pseudomonas aeruginosa is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. *Infect. Immun.* **61**: 1180–1184.

Gaynes, R. and Edwards, J.R. (2005) Overview of nosocomial infections caused by gram-negative bacilli. *Clin. Infect. Dis.* **41**: 848–854.

Gellatly, S.L. and Hancock, R.E.W. (2013) Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathog. Dis.* **67**: 159–73.

Gerdes, S.Y., Scholle, M.D., Campbell, J.W., Balázsi, G., Ravasz, E., Daugherty, M.D., et al. (2003) Experimental determination and system level analysis of essential genes in Escherichia coli MG1655. *J. Bacteriol.* **185**: 5673–5684.

Gerdes, S.Y., Scholle, M.D., D'Souza, M., Bernal, A., Baev, M. V, Farrell, M., et al. (2002) From genetic footprinting to antimicrobial drug targets: examples in cofactor biosynthetic pathways. *J. Bacteriol.* **184**: 4555–4572.

Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., Véronneau, S., et al. (2002) Functional profiling of the Saccharomyces cerevisiae genome. *Nature* **418**: 387–391.

Gil, R., Silva, F.J., Peretó, J., and Moya, A. (2004) Determination of the core of a minimal bacterial gene set. *Microbiol. Mol. Biol. Rev.* **68**: 518–537.

Gilleland, L.B., Gilleland, H.E., Gibson, J.A., and Champlin, F.R. (1989) Adaptive resistance to aminoglycoside antibiotics in Pseudomonas aeruginosa. *J. Med. Microbiol.* **29**: 41–50.

Giltner, C.L., Nguyen, Y., and Burrows, L.L. (2012) Type IV pilin proteins: versatile molecular modules. *Microbiol. Mol. Biol. Rev.* **76**: 740–72.

Gjermansen, M., Nilsson, M., Yang, L., and Tolker-Nielsen, T. (2010) Characterization of starvation-induced dispersion in Pseudomonas putida biofilms: genetic elements and molecular mechanisms. *Mol. Microbiol.* **75**: 815–826.

Glass, J.I., Assad-Garcia, N., Alperovich, N., Yooseph, S., Lewis, M.R., Maruf, M., et al. (2006) Essential genes of a minimal bacterium. *Proc. Natl. Acad. Sci. U. S. A.* **103**: 425–430.

Goodman, A.L. and Lory, S. (2004) Analysis of regulatory networks in Pseudomonas aeruginosa by genomewide transcriptional profiling. *Curr. Opin. Microbiol.* **7**: 39–44.

Govan, J.R. and Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiol. Rev.* **60**: 539–574.

Gutu, A.D., Sgambati, N., Strasbourger, P., Brannon, M.K., Jacobs, M.A., Haugen, E., et al. (2013) Polymyxin resistance of Pseudomonas aeruginosa phoQ mutants is dependent on additional two-component regulatory systems. *Antimicrob. Agents Chemother.* **57**: 2204–15.

Hall-Stoodley, L. and Stoodley, P. (2009) Evolving concepts in biofilm infections. *Cell. Microbiol.* **11**: 1034–1043.

Hamdaoui, A., Wund-Bisseret, F., and Bieth, J.G. (1987) Fast solubilization of human lung elastin by Pseudomonas aeruginosa elastase. *Am. Rev. Respir. Dis.* **135**: 860–863.

Hancock, R.E. (1998) Resistance mechanisms in Pseudomonas aeruginosa and other nonfermentative gram-negative bacteria. *Clin. Infect. Dis.* **27 Suppl 1**: S93–S99.

Harmsen, M., Yang, L., Pamp, S.J., and Tolker-Nielsen, T. (2010) An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal. *FEMS Immunol. Med. Microbiol.* **59**: 253–268.

Harrison, F. (2007) Microbial ecology of the cystic fibrosis lung. *Microbiology* **153**: 917–23.

Hassett, D.J., Ma, J.F., Elkins, J.G., McDermott, T.R., Ochsner, U.A., West, S.E., et al. (1999) Quorum sensing in Pseudomonas aeruginosa controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol. Microbiol.* **34**: 1082–1093.

Hatch, R.A. and Schiller, N.L. (1998) Alginate lyase promotes diffusion of aminoglycosides through the extracellular polysaccharide of mucoid Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **42**: 974–977.

Hauser, A.R. (2009) The type III secretion system of Pseudomonas aeruginosa: infection by injection. *Nat. Rev. Microbiol.* **7**: 654–665.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., et al. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**: 1099–1103.

Heale, J.P., Pollard, A.J., Stokes, R.W., Simpson, D., Tsang, A., Massing, B., and Speert, D.P. (2001) Two distinct receptors mediate nonopsonic phagocytosis of different strains of Pseudomonas aeruginosa. *J. Infect. Dis.* **183**: 1214–1220.

Heeb, S., Fletcher, M.P., Chhabra, S.R., Diggle, S.P., Williams, P., and Cámara, M. (2011) Quinolones: from antibiotics to autoinducers. *FEMS Microbiol. Rev.* **35**: 247–274.

Hentzer, M., Teitzel, G.M., Balzer, G.J., Heydorn, A., Molin, S., Givskov, M., and Parsek, M.R. (2001) Alginate overproduction affects Pseudomonas aeruginosa biofilm structure and function. *J. Bacteriol.* **183**: 5395–5401.

Hobden JA. (2002) Pseudomonas aeruginosa proteases and corneal virulence. *DNA Cell Biol.* **21**: 391–6.

Høiby, N., Ciofu, O., and Bjarnsholt, T. (2010) Pseudomonas aeruginosa biofilms in cystic fibrosis. *Future Microbiol.* **5**: 1663–74.

Høiby, N., Frederiksen, B., and Pressler, T. (2005) Eradication of early Pseudomonas aeruginosa infection. *J. Cyst. Fibros.* **4**: 49–54.

Hong, Y. and Ghebrehiwet, B. (1992) Effect of Pseudomonas aeruginosa elastase and alkaline protease on serum complement and isolated components C1q and C3. In, *Clinical Immunology and Immunopathology*., pp. 133–138.

Huang, K., Brady, A., Mahurkar, A., White, O., Gevers, D., Huttenhower, C., and Segata, N. (2014) MetaRef: a pan-genomic database for comparative and community microbial genomics. *Nucleic Acids Res.* **42**: D617–24.

Hunter, J.D. (2006) Ventilator associated pneumonia. *Postgrad. Med. J.* **82**: 172–178.

Husseiny, M.I. and Hensel, M. (2005) Rapid method for the construction of Salmonella enterica Serovar Typhimurium vaccine carrier strains. *Infect. Immun.* **73**: 1598–1605.

Ingmer, H. and Brøndsted, L. (2009) Proteases in bacterial pathogenesis. *Res. Microbiol.* **160**: 704–710.

Inweregbu, K. (2005) Nosocomial infections. *Contin. Educ. Anaesthesia, Crit. Care Pain* **5**: 14–17.

Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., et al. (2003) Comprehensive transposon mutant library of Pseudomonas aeruginosa. *Proc. Natl. Acad. Sci. U. S. A.* **100**: 14339–14344.

Ji, Y., Zhang, B., Van, S.F., Horn, Warren, P., Woodnutt, G., et al. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science* **293**: 2266–2269.

Jones, A.K., Fulcher, N.B., Balzer, G.J., Urbanowski, M.L., Pritchett, C.L., Schurr, M.J., et al. (2010) Activation of the Pseudomonas aeruginosa AlgU regulon through mucA mutation inhibits cyclic AMP/Vfr signaling. *J. Bacteriol.* **192**: 5709–5717.

Jones, R.J., Roe, E.A., and Gupta, J.L. (1980) Controlled trial of Pseudomonas immunoglobulin and vaccine in burn patients.

Jones, R.J., Roe, E.A., and Gupta, J.L. (1979) Controlled trials of a polyvalent pseudomonas vaccine in burns.

Jordan, I.K., Rogozin, I.B., Wolf, Y.I., and Koonin, E. V (2002) Essential genes are more evolutionarily conserved than are nonessential genes in bacteria. *Genome Res.* **12**: 962–968.

Jucker, B.A., Harms, H., and Zehnder, A.J. (1996) Adhesion of the positively charged bacterium Stenotrophomonas (Xanthomonas) maltophilia 70401 to glass and Teflon. *J. Bacteriol.* **178**: 5472–5479.

Kallen, A.J. and Srinivasan, A. (2010) Current epidemiology of multidrug-resistant gram-negative bacilli in the United States. *Infect. Control Hosp. Epidemiol.* **31 Suppl 1**: S51–S54.

Kaplan, J.B. (2010) Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J. Dent. Res.* **89**: 205–218.

Karlowsky, J.A., Zelenitsky, S.A., and Zhanel, G.G. (1997) Aminoglycoside adaptive resistance. *Pharmacotherapy* **17**: 549–55.

Kerr, K.G. and Snelling, A.M. (2009) Pseudomonas aeruginosa: a formidable and ever-present adversary. *J. Hosp. Infect.* **73**: 338–344.

De Kievit, T.R. and Iglewski, B.H. (2000) Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.* **68**: 4839–4849.

King, J.D., Kocíncová, D., Westman, E.L., and Lam, J.S. (2009) Review: Lipopolysaccharide biosynthesis in Pseudomonas aeruginosa. *Innate Immun.* **15**: 261–312.

Kipnis, E., Sawa, T., and Wiener-Kronish, J. (2006) Targeting mechanisms of Pseudomonas aeruginosa pathogenesis. *Med. Mal. Infect.* **36**: 78–91.

Knirel, Y.A., Bystrova, O. V, Kocharova, N.A., Zähringer, U., and Pier, G.B. (2006) Conserved and variable structural features in the lipopolysaccharide of Pseudomonas aeruginosa. *J. Endotoxin Res.* **12**: 324–336.

Knirel, Y.A., Bystrova, O. V, Shashkov, A.S., Lindner, B., Kocharova, N.A., Senchenkova, S.N., et al. (2001) Structural analysis of the lipopolysaccharide core of a rough, cystic fibrosis isolate of Pseudomonas aeruginosa. *Eur. J. Biochem.* **268**: 4708–4719.

Kobayashi, K., Ehrlich, S., Albertini, A., Amati, G., Andersen, K., and Arnaud, M. (2003) Essential Bacillus subtilis genes. *Proc. …* **100**: 4678–83.

Kohanski, M.A., Dwyer, D.J., and Collins, J.J. (2010) How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol.* **8**: 423–435.

Köhler, T., Curty, L.K., Barja, F., van Delden, C., and Pechère, J.C. (2000) Swarming of Pseudomonas aeruginosa is dependent on cell-to-cell signaling and requires flagella and pili. *J. Bacteriol.* **182**: 5990–5996.

Kolter, R. and Greenberg, E.P. (2006) Microbial sciences: the superficial life of microbes. *Nature* **441**: 300–302.

Koonin, E. V (2003) Comparative genomics, minimal gene-sets and the last universal common ancestor. *Nat. Rev. Microbiol.* **1**: 127–136.

Koonin, E. V (2009) Darwinian evolution in the light of genomics. *Nucleic Acids Res.* **37**: 1011–1034.

Lam, J.S., Taylor, V.L., Islam, S.T., Hao, Y., and Kocíncová, D. (2011) Genetic and Functional Diversity of Pseudomonas aeruginosa Lipopolysaccharide. *Front. Microbiol.* **2**: 118.

Langaee, T.Y., Dargis, M., and Huletsky, A. (1998) An ampD gene in Pseudomonas aeruginosa encodes a negative regulator of AmpC beta-lactamase expression. *Antimicrob. Agents Chemother.* **42**: 3296–3300.

Lange, R.P., Locher, H.H., Wyss, P.C., and Then, R.L. (2007) The targets of currently used antibacterial agents: lessons for drug discovery. *Curr. Pharm. Des.* **13**: 3140–54.

Langford, D.T. and Hiller, J. (1984) Prospective, controlled study of a polyvalent pseudomonas vaccine in cystic fibrosis--three year results.

Latifi, A., Winson, M.K., Foglino, M., Bycroft, B.W., Stewart, G.S., Lazdunski, A., and Williams, P. (1995) Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in Pseudomonas aeruginosa PAO1. *Mol. Microbiol.* **17**: 333–343.

Lee, T.W.R., Brownlee, K.G., Conway, S.P., Denton, M., and Littlewood, J.M. (2003) Evaluation of a new definition for chronic Pseudomonas aeruginosa infection in cystic fibrosis patients. *J. Cyst. Fibros.* **2**: 29–34.

Leid, J.G., Willson, C.J., Shirtliff, M.E., Hassett, D.J., Parsek, M.R., and Jeffers, A.K. (2005) The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-gamma-mediated macrophage killing. *J. Immunol.* **175**: 7512–7518.

Lesic, B. and Rahme, L.G. (2008) Use of the lambda Red recombinase system to rapidly generate mutants in Pseudomonas aeruginosa. *BMC Mol. Biol.* **9**: 20.

Lewenza, S., Falsafi, R.K., Winsor, G., Gooderham, W.J., McPhee, J.B., Brinkman, F.S.L., and Hancock, R.E.W. (2005) Construction of a mini-Tn5-luxCDABE mutant library in

Pseudomonas aeruginosa PAO1: a tool for identifying differentially regulated genes. *Genome Res.* **15**: 583–589.

Lewis, K. (2013) Platforms for antibiotic discovery. *Nat. Rev. Drug Discov.* **12**: 371–87.

Li, J., Nation, R.L., Turnidge, J.D., Milne, R.W., Coulthard, K., Rayner, C.R., and Paterson, D.L. (2006) Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect. Dis.* **6**: 589–601.

Liao, B.-Y., Scott, N.M., and Zhang, J. (2006) Impacts of gene essentiality, expression pattern, and gene compactness on the evolutionary rate of mammalian proteins. *Mol. Biol. Evol.* **23**: 2072–2080.

Lister, P.D., Wolter, D.J., and Hanson, N.D. (2009) Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin. Microbiol. Rev.* **22**: 582–610.

Livermore, D.M. (1995) beta-Lactamases in laboratory and clinical resistance. *Clin. Microbiol. Rev.* **8**: 557–84.

Livermore, D.M. (2001) Of Pseudomonas, porins, pumps and carbapenems. *J. Antimicrob. Chemother.* **47**: 247–250.

López, D., Vlamakis, H., and Kolter, R. (2010) Biofilms. *Cold Spring Harb. Perspect. Biol.* **2**: a000398.

Ma, L., Conover, M., Lu, H., Parsek, M.R., Bayles, K., and Wozniak, D.J. (2009) Assembly and development of the Pseudomonas aeruginosa biofilm matrix. *PLoS Pathog.* **5**:

Ma, L., Jackson, K.D., Landry, R.M., Parsek, M.R., and Wozniak, D.J. (2006) Analysis of Pseudomonas aeruginosa conditional psl variants reveals roles for the psl polysaccharide in adhesion and maintaining biofilm structure postattachment. *J. Bacteriol.* **188**: 8213–8221.

Mahajan-Miklos, S., Rahme, L.G., and Ausubel, F.M. (2000) Elucidating the molecular mechanisms of bacterial virulence using non-mammalian hosts. *Mol. Microbiol.* **37**: 981–8.

Manfredi, R., Nanetti, A., Ferri, M., and Chiodo, F. (2000) Pseudomonas spp. complications in patients with HIV disease: an eight-year clinical and microbiological survey. *Eur. J. Epidemiol.* **16**: 111–118.

Matsumoto, K. (2004) Role of bacterial proteases in pseudomonal and serratial keratitis. *Biol. Chem.* **385**: 1007–1016.

Matsuo, Y., Eda, S., Gotoh, N., Yoshihara, E., and Nakae, T. (2004) MexZ-mediated regulation of mexXY multidrug efflux pump expression in Pseudomonas aeruginosa by binding on the mexZ-mexX intergenic DNA. *FEMS Microbiol. Lett.* **238**: 23–28.

McPhee, J.B., Bains, M., Winsor, G., Lewenza, S., Kwasnicka, A., Brazas, M.D., et al. (2006) Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg2+-induced gene regulation in Pseudomonas aeruginosa. *J. Bacteriol.* **188**: 3995–4006.

McPhee, J.B., Lewenza, S., and Hancock, R.E.W. (2003) Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in Pseudomonas aeruginosa. *Mol. Microbiol.* **50**: 205–217.

Meng, J., Kanzaki, G., Meas, D., Lam, C.K., Crummer, H., Tain, J., and Xu, H.H. (2012) A genome-wide inducible phenotypic screen identifies antisense RNA constructs silencing Escherichia coli essential genes. *FEMS Microbiol. Lett.* **329**: 45–53.

Meshulam, T., Obedeanu, N., Merzbach, D., and Sobel, J.D. (1984) Phagocytosis of mucoid and nonmucoid strains of Pseudomonas aeruginosa. *Clin. Immunol. Immunopathol.* **32**: 151–165.

Metzgar, D., Bacher, J.M., Pezo, V., Reader, J., Döring, V., Schimmel, P., et al. (2004) Acinetobacter sp. ADP1: an ideal model organism for genetic analysis and genome engineering. *Nucleic Acids Res.* **32**: 5780–5790.

Mikkelsen, H., Sivaneson, M., and Filloux, A. (2011) Key two-component regulatory systems that control biofilm formation in Pseudomonas aeruginosa. *Environ. Microbiol.* **13**: 1666–1681.

Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**: 165–199.

Miyajima, S., Akaike, T., Matsumoto, K., Okamoto, T., Yoshitake, J., Hayashida, K., et al. (2001) Matrix metalloproteinases induction by pseudomonal virulence factors and inflammatory cytokines in vitro. *Microb. Pathog.* **31**: 271–281.

Munck, A., Bonacorsi, S., Mariani-Kurkdjian, P., Lebourgeois, M., Gérardin, M., Brahimi, N., et al. (2001) Genotypic characterization of Pseudomonas aeruginosa strains recovered from patients with cystic fibrosis after initial and subsequent colonization. *Pediatr. Pulmonol.* **32**: 288–292.

Muñoz-López, M. and García-Pérez, J.L. (2010) DNA transposons: nature and applications in genomics. *Curr. Genomics* **11**: 115–128.

Ng, W.-L. and Bassler, B.L. (2009) Bacterial quorum-sensing network architectures. *Annu. Rev. Genet.* **43**: 197–222.

Nijland, R., Hall, M.J., and Grant Burgess, J. (2010) Dispersal of biofilms by secreted, matrix degrading, Bacterial DNase. *PLoS One* **5**:

Nikaido, H. (2001) Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Semin. Cell Dev. Biol.* **12**: 215–223.

O'Toole, G., Kaplan, H.B., and Kolter, R. (2000) Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **54**: 49–79.

Ochsner, U.A., Koch, A.K., Fiechter, A., and Reiser, J. (1994) Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa. *J. Bacteriol.* **176**: 2044–54.

Ochsner, U.A. and Reiser, J. (1995) Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa. *Proc. Natl. Acad. Sci. U. S. A.* **92**: 6424–6428.

Pagani, I., Liolios, K., Jansson, J., Chen, I.-M.A., Smirnova, T., Nosrat, B., et al. (2012) The Genomes OnLine Database (GOLD) v.4: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Res.* **40**: D571–9.

Paik, S., Senty, L., Das, S., Noe, J.C., Munro, C.L., and Kitten, T. (2005) Identification of virulence determinants for endocarditis in Streptococcus sanguinis by signature-tagged mutagenesis. *Infect. Immun.* **73**: 6064–6074.

Parish, T. and Stoker, N.G. (1997) Development and use of a conditional antisense mutagenesis system in mycobacteria. *FEMS Microbiol. Lett.* **154**: 151–157.

Parker, D. and Prince, A. (2013) Epithelial Uptake of Flagella Initiates Proinflammatory Signaling. *PLoS One* **8**:

Pearson, J.P., Passador, L., Iglewski, B.H., and Greenberg, E.P. (1995) A second N-acylhomoserine lactone signal produced by Pseudomonas aeruginosa. *Proc. Natl. Acad. Sci. U. S. A.* **92**: 1490–1494.

Pearson, J.P., Pesci, E.C., and Iglewski, B.H. (1997) Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol.* **179**: 5756–5767.

Pederson, K.J. and Barbieri, J.T. (1998) Intracellular expression of the ADP-ribosyltransferase domain of Pseudomonas exoenzyme S is cytotoxic to eukaryotic cells. *Mol. Microbiol.* **30**: 751–759.

Pessi, G. and Haas, D. (2000) Transcriptional control of the hydrogen cyanide biosynthetic genes hcnABC by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in Pseudomonas aeruginosa. *J. Bacteriol.* **182**: 6940–6949.

Pier, G.B. (2003) Promises and pitfalls of Pseudomonas aeruginosa lipopolysaccharide as a vaccine antigen. *Carbohydr. Res.* **338**: 2549–2556.

Poteete, A.R. (2001) What makes the bacteriophage λ Red system useful for genetic engineering: Molecular mechanism and biological function. *FEMS Microbiol. Lett.* **201**: 9–14.

Pressler, T., Bohmova, C., Conway, S., Dumcius, S., Hjelte, L., Høiby, N., et al. (2011) Chronic Pseudomonas aeruginosa infection definition: EuroCareCF Working Group report. *J. Cyst. Fibros.* **10 Suppl 2**: S75–8.

Pritt, B., O'Brien, L., and Winn, W. (2007) Mucoid Pseudomonas in cystic fibrosis. *Am. J. Clin. Pathol.* **128**: 32–34.

Qiu, D., Damron, F.H., Mima, T., Schweizer, H.P., and Yu, H.D. (2008) PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in Pseudomonas and Burkholderia spp. and other bacteria. *Appl. Environ. Microbiol.* **74**: 7422–7426.

Rahme, L.G., Ausubel, F.M., Cao, H., Drenkard, E., Goumnerov, B.C., Lau, G.W., et al. (2000) Plants and animals share functionally common bacterial virulence factors. *Proc. Natl. Acad. Sci. U. S. A.* **97**: 8815–8821.

Rana, T.M. (2007) Illuminating the silence: understanding the structure and function of small RNAs. *Nat. Rev. Mol. Cell Biol.* **8**: 23–36.

Ratjen, F. and Döring, G. (2003) Cystic fibrosis. In, *Lancet.*, pp. 681–689.

Reznikoff, W.S. (2008) Transposon Tn5. *Annu. Rev. Genet.* **42**: 269–286.

Ruby, E.G. (1996) Lessons from a cooperative, bacterial-animal association: the Vibrio fischeri-Euprymna scolopes light organ symbiosis. *Annu. Rev. Microbiol.* **50**: 591–624.

Rusmini, R., Vecchietti, D., Macchi, R., Vidal-Aroca, F., and Bertoni, G. (2014) A shotgun antisense approach to the identification of novel essential genes in Pseudomonas aeruginosa. *BMC Microbiol.* **14**: 24.

Ryder, C., Byrd, M., and Wozniak, D.J. (2007) Role of polysaccharides in Pseudomonas aeruginosa biofilm development. *Curr. Opin. Microbiol.* **10**: 644–648.

Saier, M.H. (2004) Evolution of bacterial type III protein secretion systems. *Trends Microbiol.* **12**: 113–115.

Salunkhe, P., Smart, C.H.M., Morgan, J.A.W., Panagea, S., Walshaw, M.J., Hart, C.A., et al. (2005) A cystic fibrosis epidemic strain of Pseudomonas aeruginosa displays enhanced virulence and antimicrobial resistance. *J. Bacteriol.* **187**: 4908–4920.

Sato, H. and Frank, D.W. (2004) ExoU is a potent intracellular phospholipase. *Mol. Microbiol.* **53**: 1279–1290.

Sayner, S.L., Frank, D.W., King, J., Chen, H., VandeWaa, J., and Stevens, T. (2004) Paradoxical cAMP-induced lung endothelial hyperpermeability revealed by Pseudomonas aeruginosa ExoY. *Circ. Res.* **95**: 196–203.

Schaber, J.A., Triffo, W.J., Suh, S.J., Oliver, J.W., Hastert, M.C., Griswold, J.A., et al. (2007) Pseudomonas aeruginosa forms biofilms in acute infection independent of cell-to-cell signaling. *Infect. Immun.* **75**: 3715–3721.

Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M., and Jahn, D. (2007) The anaerobic regulatory network required for Pseudomonas aeruginosa nitrate respiration. *J. Bacteriol.* **189**: 4310–4314.

Schurek, K.N., Marr, A.K., Taylor, P.K., Wiegand, I., Semenec, L., Khaira, B.K., and Hancock, R.E.W. (2008) Novel genetic determinants of low-level aminoglycoside resistance in Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **52**: 4213–4219.

Schuster, M. and Greenberg, E.P. (2006) A network of networks: Quorum-sensing gene regulation in Pseudomonas aeruginosa. *Int. J. Med. Microbiol.* **296**: 73–81.

Schuster, M., Lostroh, C.P., Ogi, T., and Greenberg, E.P. (2003) Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* **185**: 2066–2079.

Schweizer, H.P. (2003) Efflux as a mechanism of resistance to antimicrobials in Pseudomonas aeruginosa and related bacteria: unanswered questions. *Genet. Mol. Res.* **2**: 48–62.

Seed, P.C., Passador, L., and Iglewski, B.H. (1995) Activation of the Pseudomonas aeruginosa lasI gene by LasR and the Pseudomonas autoinducer PAI: an autoinduction regulatory hierarchy. *J. Bacteriol.* **177**: 654–659.

Sharan, S.K., Thomason, L.C., Kuznetsov, S.G., and Court, D.L. (2009) Recombineering: a homologous recombination-based method of genetic engineering. *Nat. Protoc.* **4**: 206–223.

Sharma, A., Krause, A., and Worgall, S. (2011) Recent developments for Pseudomonas vaccines. *Hum. Vaccin.* **7**: 999–1011.

Shaver, C.M. and Hauser, A.R. (2004) Relative contributions of Pseudomonas aeruginosa ExoU, ExoS, and ExoT to virulence in the lung. *Infect. Immun.* **72**: 6969–6977.

Sievert, D.M., Ricks, P., Edwards, J.R., Schneider, A., Patel, J., Srinivasan, A., et al. (2012) Antimicrobial-Resistant Pathogens Associated with Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010.

Silby, M.W., Winstanley, C., Godfrey, S.A.C., Levy, S.B., and Jackson, R.W. (2011) Pseudomonas genomes: diverse and adaptable. *FEMS Microbiol. Rev.* **35**: 652–80.

Sinha, S. and Redfield, R.J. (2012) Natural DNA uptake by Escherichia coli. *PLoS One* **7**:

Smith, K.D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M.A., Barrett, S.L.R., et al. (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat. Immunol.* **4**: 1247–1253.

Sriramulu, D.D., Lünsdorf, H., Lam, J.S., and Römling, U. (2005) Microcolony formation: a novel biofilm model of Pseudomonas aeruginosa for the cystic fibrosis lung. *J. Med. Microbiol.* **54**: 667–676.

Starner, T.D. (2005) Pathogenesis of Early Lung Disease in Cystic Fibrosis: A Window of Opportunity To Eradicate Bacteria. *Ann. Intern. Med.* **143**: 816.

Stephenson, M. and Jarrett, P. (1991) Transformation of Bacillus subtilis by electroporation. *Biotechnol. Tech.* **5**: 9–12.

Stoodley, P., Sauer, K., Davies, D.G., and Costerton, J.W. (2002) Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* **56**: 187–209.

Storz, G. (2002) An expanding universe of noncoding RNAs. *Science* **296**: 1260–1263.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., et al. (2000) Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature* **406**: 959–964.

Toder, D.S., Ferrell, S.J., Nezezon, J.L., Rust, L., and Iglewski, B.H. (1994) lasA and lasB genes of Pseudomonas aeruginosa: analysis of transcription and gene product activity. *Infect. Immun.* **62**: 1320–1327.

Tomasz, A. (1979) The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. *Annu. Rev. Microbiol.* **33**: 113–37.

Ton-Hoang, B., Pasternak, C., Siguier, P., Guynet, C., Hickman, A.B., Dyda, F., et al. (2010) Single-stranded DNA transposition is coupled to host replication. *Cell* **142**: 398–408.

Toutain, C.M., Caizza, N.C., Zegans, M.E., and O'Toole, G.A. (2007) Roles for flagellar stators in biofilm formation by Pseudomonas aeruginosa. *Res. Microbiol.* **158**: 471–477.

Valderrey, A.D., Pozuelo, M.J., Jiménez, P.A., Maciá, M.D., Oliver, A., and Rotger, R. (2010) Chronic colonization by Pseudomonas aeruginosa of patients with obstructive lung diseases: Cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease. *Diagn. Microbiol. Infect. Dis.* **68**: 20–27.

Vasil, M.L. (2003) DNA microarrays in analysis of quorum sensing: strengths and limitations. *J. Bacteriol.* **185**: 2061–5.

Veiga, H. and Pinho, M.G. (2009) Inactivation of the SauI type I restriction-modification system is not sufficient to generate Staphylococcus aureus strains capable of efficiently accepting foreign DNA. *Appl. Environ. Microbiol.* **75**: 3034–3038.

Venturi, V. (2006) Regulation of quorum sensing in Pseudomonas. *FEMS Microbiol. Rev.* **30**: 274–291.

Verma, A., Arora, S.K., Kuravi, S.K., and Ramphal, R. (2005) Roles of specific amino acids in the N terminus of Pseudomonas aeruginosa flagellin and of flagellin glycosylation in the innate immune response. *Infect. Immun.* **73**: 8237–8246.

Vessillier, S., Delolme, F., Bernillon, J., Saulnier, J., and Wallach, J. (2001) Hydrolysis of glycine-containing elastin pentapeptides by LasA, a metalloelastase from Pseudomonas aeruginosa. *Eur. J. Biochem.* **268**: 1049–1057.

Wagner, E.G. and Flärdh, K. (2002) Antisense RNAs everywhere? *Trends Genet.* **18**: 223–226.

Wagner, V.E., Bushnell, D., Passador, L., Brooks, A.I., and Iglewski, B.H. (2003) Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* **185**: 2080–2095.

Waite, R.D., Paccanaro, A., Papakonstantinopoulou, A., Hurst, J.M., Saqi, M., Littler, E., and Curtis, M.A. (2006) Clustering of Pseudomonas aeruginosa transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. *BMC Genomics* **7**: 162.

Waite, R.D., Papakonstantinopoulou, A., Littler, E., and Curtis, M.A. (2005) Transcriptome analysis of Pseudomonas aeruginosa growth: comparison of gene expression in planktonic cultures and developing and mature biofilms. *J. Bacteriol.* **187**: 6571–6576.

Wang, B. and Kuramitsu, H.K. (2005) Inducible antisense RNA expression in the characterization of gene functions in Streptococcus mutans. *Infect. Immun.* **73**: 3568–3576.

Waters, L.S. and Storz, G. (2009) Regulatory RNAs in Bacteria. *Cell* **136**: 615–628.

Wei, W., Ning, L.W., Ye, Y.N., and Guo, F.B. (2013) Geptop: A Gene Essentiality Prediction Tool for Sequenced Bacterial Genomes Based on Orthology and Phylogeny. *PLoS One* **8**:

Wiedenheft, B., Sternberg, S.H., and Doudna, J.A. (2012) RNA-guided genetic silencing systems in bacteria and archaea. *Nature* **482**: 331–338.

Williams, B.J., Dehnbostel, J., and Blackwell, T.S. (2010) Pseudomonas aeruginosa: host defence in lung diseases. *Respirology* **15**: 1037–1056.

Williams, P. and Cámara, M. (2009) Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. *Curr. Opin. Microbiol.* **12**: 182–191.

Wilson, D.N. (2009) The A-Z of bacterial translation inhibitors. *Crit. Rev. Biochem. Mol. Biol.* **44**: 393–433.

Winzer, K., Falconer, C., Garber, N.C., Diggle, S.P., Camara, M., and Williams, P. (2000) The Pseudomonas aeruginosa lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS. *J. Bacteriol.* **182**: 6401–6411.

Wolfgang, M.C., Jyot, J., Goodman, A.L., Ramphal, R., and Lory, S. (2004) Pseudomonas aeruginosa regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 6664–6668.

Würtele, M., Renault, L., Barbieri, J.T., Wittinghofer, A., and Wolf, E. (2001) Structure of the ExoS GTPase activating domain. *FEBS Lett.* **491**: 26–29.

Xu, H.H., Trawick, J.D., Haselbeck, R.J., Forsyth, R.A., Yamamoto, R.T., Archer, R., et al. (2010) Staphylococcus aureus TargetArray: comprehensive differential essential gene expression as a mechanistic tool to profile antibacterials. *Antimicrob. Agents Chemother.* **54**: 3659–3670.

Xu, P., Ge, X., Chen, L., Wang, X., Dou, Y., Xu, J.Z., et al. (2011) Genome-wide essential gene identification in Streptococcus sanguinis. *Sci. Rep.* **1**:

Yang, L., Jelsbak, L., Marvig, R.L., Damkiær, S., Workman, C.T., Rau, M.H., et al. (2011) Evolutionary dynamics of bacteria in a human host environment. *Proc. Natl. Acad. Sci. U. S. A.* **108**: 7481–7486.

Yang, M.-M., Zhang, W.-W., Bai, X.-T., Li, H.-X., and Cen, P.-L. (2010) Electroporation is a feasible method to introduce circularized or linearized DNA into B. subtilis chromosome. *Mol. Biol. Rep.* **37**: 2207–2213.

Yeung, A.T.Y., Torfs, E.C.W., Jamshidi, F., Bains, M., Wiegand, I., Hancock, R.E.W., and Overhage, J. (2009) Swarming of Pseudomonas aeruginosa is controlled by a broad spectrum of transcriptional regulators, including MetR. *J. Bacteriol.* **191**: 5592–5602.

Yin, D. and Ji, Y. (2002) Genomic analysis using conditional phenotypes generated by antisense RNA. *Curr. Opin. Microbiol.* **5**: 330–333.

Zhang, R. and Lin, Y. (2009) DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes. *Nucleic Acids Res.* **37**: D455–D458.

PART II

Contents

Research article 1:

Milani, A., Vecchietti, D., **Rusmini, R**., and Bertoni, G. (2012) TgpA, a Protein with a Eukaryotic-Like Transglutaminase Domain, Plays a Critical Role in the Viability of *Pseudomonas aeruginosa*. *PLoS One* **7**: e50323.

Research article 2:

Rusmini, R., Vecchietti, D., Macchi, R., Vidal-Aroca, F., and Bertoni, G. (2014) A shotgun antisense approach to the identification of novel essential genes in *Pseudomonas aeruginosa*. *BMC Microbiol*. **14**: 24.

BMC Microbiology

RESEARCH ARTICLE Open Access

A shotgun antisense approach to the identification of novel essential genes in *Pseudomonas aeruginosa*

Ruggero Rusmini[1†], Davide Vecchietti[1†], Raffaella Macchi[1], Faustino Vidal-Aroca[1,2] and Giovanni Bertoni[1*]

Abstract

Background: Antibiotics in current use target a surprisingly small number of cellular functions: cell wall, DNA, RNA, and protein biosynthesis. Targeting of novel essential pathways is expected to play an important role in the discovery of new antibacterial agents against bacterial pathogens, such as *Pseudomonas aeruginosa*, that are difficult to control because of their ability to develop resistance, often multiple, to all current classes of clinical antibiotics.

Results: We aimed to identify novel essential genes in *P. aeruginosa* by shotgun antisense screening. This technique was developed in *Staphylococcus aureus* and, following a period of limited success in Gram-negative bacteria, has recently been used effectively in *Escherichia coli*. To also target low expressed essential genes, we included some variant steps that were expected to overcome the non-stringent regulation of the promoter carried by the expression vector used for the shotgun antisense libraries. Our antisense screenings identified 33 growth-impairing single-locus genomic inserts that allowed us to generate a list of 28 "essential-for-growth" genes: five were "classical" essential genes involved in DNA replication, transcription, translation, and cell division; seven were already reported as essential in other bacteria; and 16 were "novel" essential genes with no homologs reported to have an essential role in other bacterial species. Interestingly, the essential genes in our panel were suggested to take part in a broader range of cellular functions than those currently targeted by extant antibiotics, namely protein secretion, biosynthesis of cofactors, prosthetic groups and carriers, energy metabolism, central intermediary metabolism, transport of small molecules, translation, post-translational modification, non-ribosomal peptide synthesis, lipopolysaccharide synthesis/modification, and transcription regulation. This study also identified 43 growth-impairing inserts carrying multiple loci targeting 105 genes, of which 25 have homologs reported as essential in other bacteria. Finally, four multigenic growth-impairing inserts belonged to operons that have never been reported to play an essential role.

Conclusions: For the first time in *P. aeruginosa*, we applied regulated antisense RNA expression and showed the feasibility of this technology for the identification of novel essential genes.

Background

Pseudomonas aeruginosa is a highly adaptable bacterium that thrives in a broad range of ecological niches. In addition, it can infect hosts as diverse as plants, nematodes, and mammals. In humans, it is an important opportunistic pathogen in compromised individuals, such as patients with cystic fibrosis, severe burns, or impaired immunity [1,2]. *P. aeruginosa* is difficult to control because of its ability to develop resistance, often multiple, to all current classes of clinical antibiotics [3-5]. The discovery of novel essential genes or pathways that have not yet been targeted by clinical antibiotics can underlie the development of alternative effective antibacterials to overcome existing mechanisms of resistance. Whole-genome transposon-mutagenesis (TM) followed by identification of insertion sites is one of the most practical and frequently used experimental approaches to screen for

* Correspondence: giovanni.bertoni@unimi.it
†Equal contributors
[1]Department of Life Sciences, Università degli Studi di Milano, via Celoria 26, 20133 Milan, Italy
Full list of author information is available at the end of the article

essential bacterial genes [6-8]. Genome-wide surveys of essential genes in *P. aeruginosa* have been accomplished by saturating TM through a "negative" approach [9,10], specifically, by identifying non-essential genomic regions by transposon insertion and deducing that non-inserted genome stretches are essential. However, this approach can suffer from some systematic biases that generate both false positives and negatives [7]. For example, even if comprehensive insertion libraries are produced, it is inevitable that some genes, especially the shortest ones, could elude insertion and be spuriously annotated as essential, while transposon insertions that occur at gene ends and do not fully inactivate the function could lead to genes being incorrectly classified as non-essential. To filter errors resulting from these intrinsic biases in the "negative" TM approach, a statistical framework has recently been developed and tested in *P. aeuginosa* PAO1 and *Francisella tularensis novicida* [7] TM datasets. Some drawbacks of the "negative" TM approach were overcome by using growth-conditional TM, which allows identification of essential genes by transcriptionally fusing them to an outward-facing inducible promoter located at one end of the transposon [11]. However, conditional TM can also be affected by systematic biases, deriving, for example, from transposon tools endowed with outward-facing promoters that are not strictly regulated in non-inducing conditions, resulting in a basal level of promoter expression. In fact, promoter leakage under non-inducing conditions would not completely switch off the gene downstream of the insertion site, significantly increasing the false-negative identification rate. The TM tools applicable for use with *P. aeruginosa* [12] are based on elements used for tightly regulated gene expression in *E. coli*, and are expected to not be completely switched off in non-inducing conditions when used "out-of-context". For these reasons, we set out to screen novel essential genes of *P. aeruginosa* using a method other than TM. To this end, we selected shotgun antisense RNA identification of essential genes, a technique that was developed a decade ago in *Staphylococcus aureus* [13,14]. This technique originally only showed limited success in Gram-negative bacteria [15,16], but has recently been used effectively in *E. coli* [17]. In this approach, essential genes are identified after shotgun-cloned genomic fragments are conditionally expressed. The fragments are screened to identify those whose expression impairs growth [18]. The genes targeted by antisense RNA are identified by DNA sequencing of the growth-impairing fragments. This study shows for the first time the feasibility of the antisense technology in *P. aeruginosa* for identifying novel essential genes. Moreover, we included some modifications to the original strategy that could have broadened the functional class variety of the identified essential genes in respect to a recent report in *E. coli* [17].

Results

Ad hoc procedure to screen for essential *P. aeruginosa* genes by antisense RNA effects

According to the scheme for antisense-mediated identification of essential genes established in *S. aureus* [13,14], the shotgun genomic libraries generated *in vitro* are directly introduced into the original host by transformation, and selected in permissive conditions, i.e., with the promoter vector in an off state, to allow the clones carrying inserts targeting essential genes to survive. However, basal vector promoter activity could be sufficient to elicit silencing effects against genes transcribed at low levels. This effect may introduce a bias in the subsequent conditional screening, favoring the identification of highly transcribed essential genes (e.g., tRNAs, tRNA synthetases, ribosomal proteins, translation factors, components of the transcription machinery). Cells transformed using constructs targeting essential genes expressed at low levels will fail to form a colony in the permissive conditions. The high basal P_{trc} activity was suggested to cause the identification of a relevant proportion of highly expressed essential genes in a recent antisense screening of *E. coli* [17]. A not entirely negligible basal activity is frequent in the commonly used expression system tools, especially when they are used outside the source organism. This is the case in the P_{BAD} promoter-based systems, like those selected for this study, which have been used for tightly regulated gene expression in *E. coli*, and for efficient arabinose-induced overexpression in other hosts. However, outside of the *E. coli* regulatory context, for instance in *Burkholderia pseudomallei* [19] and *P. aeruginosa* (Bertoni *et al.*, unpublished), these systems can display, also in the presence of glucose, a basal level of activity. To avoid missing the identification of low expressed essential genes owing to out-of-context use of the P_{BAD} promoter, we set out to generate *P. aeruginosa* genomic shotgun libraries in *E. coli* first, and to then array and challenge them by conjugative transfer into *P. aeruginosa* (Figure 1). Moreover, this strategy assures a larger sized shotgun library because of the higher transformation efficiency of *E. coli* compared with *P. aeruginosa.* To test the robustness of this approach, we checked the false-positive rate due to failure of vector mating transfer and assessed that it was negligible.

Construction of arrayed shotgun genomic libraries of *P. aeruginosa*

Genomic DNA was purified from *P. aeruginosa* PAO1 and then mechanically sheared to generate DNA fragments in a size range spanning 200–800 bp (Additional file 1: Figure S1A). In pilot experiments,

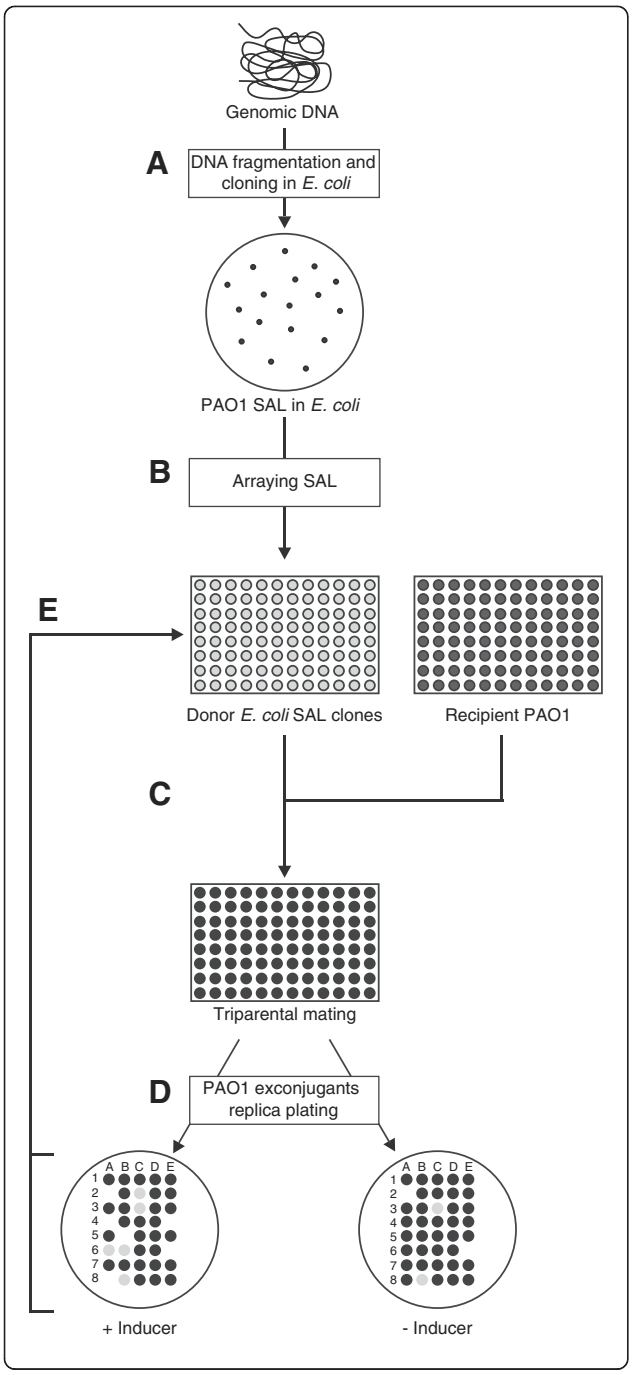


Figure 1 Construction and screening of PAO1 SALs. (A) Genomic DNA was isolated from *P. aeruginosa* PAO1 and nebulized to obtain sheared fragments of 200–800 bp. After treatment with exonuclease BAL-31 and Klenow polymerase, the genomic DNA fragments were cloned into the *E. coli* strain JM109, downstream of the arabinose-inducible promoter P_{BAD} of the pHERD20T vector. **(B)** *E. coli* transformants, representing the PAO1 shotgun antisense library (SAL), were arrayed in 96-well microplates and **(C)** mated with *P. aeruginosa* PAO1 in the presence of a helper strain (triparental mating). **(D)** SAL recipient PAO1 exconjugants were selected by spotting on PIA plates supplemented with Cb both in the absence and in the presence of the P_{BAD} inducer arabinose. Recipient PAO1 exconjugant spots were inspected for growth defects following 24 h of incubation at 37°C. **(E)** The identity of the genomic fragments eliciting growth defects (lethal effects, indicated by a lack of a spot: only with inducer, e.g. clones A4, A8, B5, and E4, and with and without an inducer, e.g. clones A2 and E6; growth impairment, indicated as gray spots: only with an inducer, e.g. clones C2, A6, and B6, and with and without an inducer, e.g. C3 and B8) was determined by sequencing the inserts in the corresponding clones of *E. coli* SAL.

following treatment with exonuclease BAL-31 and Klenow polymerase, the 200–800 bp DNA fragments were cloned into *E. coli* downstream of the arabinose-inducible promoter, P_{BAD}, of the broad host-range vector pVI533EH. Approximately 800 transformant clones were then arrayed in 96-well microplates. Analysis of cloning efficiency by PCR indicated that about 30% of transformant *E. coli* colonies carried a PAO1 genomic insert. To generate shotgun antisense libraries (SALs) with a lower background of clones carrying an empty vector, we selected the broad host-range vector pHERD-20 T, which facilitates the identification of clones carrying an insert based on blue/white screening. We obtained a 7:3 ratio between dark blue (absence of an insert) and white-light blue (potential presence of an insert) colonies, with 95% of white-light blue colonies carrying an insert with the expected average size (Additional file 1: Figure S1B). Thus, the probability of selecting a clone with an insert (Additional file 1: Figure S1C) increased from about 30% to 95% using pHERD-20 T. A pHERD-20 T-based SAL library was constructed by arraying approximately 10,000 white-light blue transformant clones in 96-well microplates.

Screenings of SALs for growth-impairing inserts

The genomic inserts of both pVI533EH- and pHERD-20 T-based SALs were screened for their ability to impair PAO1 growth, supposedly by antisense transcription effects, by mating transfer of SALs from *E. coli* to PAO1 (Figure 1C), and then replica plating of exconjugants on *Pseudomonas* Isolation Agar (PIA) supplemented with carbenicillin (Cb), both in the absence and presence of the P_{BAD} inducer arabinose (Figure 1D). Recipient PAO1 exconjugant spots were inspected for growth defects following 24 h of incubation at 37°C. Insert-induced

impairment ranged from growth defect to arrest, which could be displayed in some cases even in the absence of arabinose (Additional file 1: Figure S1C). This suggested that basal insert expression in PAO1, a regulatory context for P_{BAD} that is not as restrictive as *E. coli*, was sufficient to produce deleterious effects on growth. These screenings resulted in the identification of five and 71 growth-impairing inserts in the pVI533EH- and pHERD-20 T-based SALs, respectively. These 76 inserts, recovered in the corresponding *E. coli* donor clones (Figure 1E), were subjected to sequence analysis, and their features are listed in Additional file 2: Table S2.

Analysis of the growth-impairing inserts

Bioinformatic analysis of the DNA sequences obtained indicated that 33 of the 76 positive clones (44%) contained single intragenic fragments. Of these, 20 (26% of the positive clones) were in antisense orientation. As listed in Table 1, some of these fragments derived from conserved genes involved in DNA replication, transcription, and translation, such as *dnaG*, *rpoC*, *rpoB*, *infB*, and *rbfA*, which can be considered "classical" essential genes. Fragments derived from *rpoC*, *rpoB*, *infB*, and *rbfA* were antisense oriented. Two different fragments were derived from *dnaG,* one antisense and the other sense oriented. As previously suggested [13], it is likely that sense-oriented intragenic fragments can act as dominant-negative interfering sequences. Alternatively, we suggest that transcription noise in the vector backbone from the other side of P_{BAD} could produce sufficient amounts of insert antisense transcripts to silence the target essential gene. One insert targeted PA3820 (*secF*), which was previously shown to play an essential role in several bacterial species [20]. Six intragenic fragments derived from PA4669 (*ipk*), PA2951 (*etfA*), PA3687 (*ppc*), PA3758 (*nagA*), PA1183 (*dctA*), and PA1805 (*ppiD*), which are homologous to genes previously shown to be essential in a limited number of bacterial species [20].

The other inserts shown in Table 1 are derived from 16 genes with no homologs annotated as essential [20]. We recently validated the essential role of one of these hits, TgpA (PA2873), in *P. aeruginosa* by insertional and conditional mutagenesis [21]. In addition, the critical role of PA1554 (*ccoN1*) in the aerobic growth of *P. aeruginosa* PAO1 was reported previously [22].

The remaining positive clones contained fragments including multiple loci and targeted a total of 103 genes (Additional file 3: Table S3). Nineteen of these multigenic fragments included 25 genes with homologs described as essential in other bacterial species [20]. The rest of the multigenic fragments carried genes with no evidence of an essential role. Interestingly, four multigenic inserts included gene sequences belonging to a single operon (Table 2).

Discussion

The discovery of novel essential genes or pathways that have not yet been targeted by clinical antibiotics can underlie the development of alternative effective antibacterials to overcome the extant mechanisms of resistance. In *P. aeruginosa*, a genome-wide assessment of essential genes has been performed previously by constructing an ordered, nonredundant random transposon (Tn) insertion library [9,10,23]. An approach of this kind has proven invaluable in studying bacterial genomes and in detecting novel essential genes. However, there can be some degree of imprecision in tagging for essentiality owing to Tn insertions into possible permissive site(s) of essential genes. For example, "classical" essential genes involved in DNA replication, transcription, translation, and cell division (e.g. *polA*, *holE*, *holB*, *holC, dnaG*, *dnaJ*, *dnaK*, *rpoC*, *infC*, and *ftsYEX*) were Tn inserted in previous investigations (Additional file 4: Table S4) [9,10,23] and, for this reason, *P. aeruginosa* alleles were not included in the Database of Essential Genes (DEG) [20]. Some disadvantages of this kind of approach could be overcome by using growth-conditional mutagenesis.

To generate growth-conditional phenotypes, we decided to use the antisense-mediated strategy established previously in *S. aureus* [13,14]. This technique is not affected by some of the bias linked to transposon mutagenesis mentioned above. However, it can present limitations in the multi-step process of antisense libraries preparation such as the blunt-end cloning of mechanically sheared DNA fragments, library clones carrying multigenic inserts, the reintroduction efficiency of libraries into the original host. In addition, the efficiency of antisense inhibition, supposed to affect gene translatability and/or mRNA stability, can be gene-dependent and also differential for distinct DNA fragments belonging to the same gene.

We report here, for the first time, successful application of regulated antisense RNA technology to discover novel essential functions in *P. aeruginosa*. To also screen for low expressed essential genes, we added a preliminary shotgun library construction in *E. coli* to the previous strategy, followed by mating transfer to *P. aeruginosa*.

The subset of growth-impairing fragments that targeted single loci (Table 1) directly defined 28 "essential-for-growth" genes. Only five of these genes were "classical" essential genes involved in DNA replication, transcription, and translation. The remaining 23 genes are suggested to take part in disparate cellular functions, including protein secretion, biosynthesis of cofactors, prosthetic groups, and carriers, energy metabolism, central intermediary metabolism, transport of small molecules, translation, post-translational modification, non-ribosomal peptide synthesis, lipopolysaccharide synthesis/modification, and transcriptional regulation. Finally, some of the gene products described in Table 1 were annotated as "hypothetical"

Table 1 *Pseudomonas aeruginosa* PAO1 genes targeted by growth-impairing inserts including a single *locus*

Insert name[a]	Insert-included *locus*	Gene name and product annotation[b]	Function class[b]	Orthologous proteins in DEG[c]
S2F1	PA0577	*dnaG* - DNA primase (2)	DNA replication, recombination, modification and repair	Bs, Sa, Ec, Hi, Sp, Mt, Fn, Ab, Mp, Se, Cc, Ss
S3D3				
S11F7	PA4269	*rpoC* - DNA-directed RNA polymerase beta chain (2)	Transcription, RNA processing and degradation	Bs, Sa, Ec, Mg, Sp, Mt, St, Fn, Ab, Mp, Se, Ss, Pg
B1	PA4270	*rpoB* - DNA-directed RNA polymerase beta chain (2)	Transcription, RNA processing and degradation	Bs, Sa, Vc, Ec, Hi, Mg, Mt, St, Fn, Ab, Mp, Pa, Se, Cc, Ss, Pg, Bt
S6A10	PA4744	*infB* - translation initiation factor IF-2 (2)	Translation, post-translational modification, degradation	Bs, Sa, Vc, Ec, Mg, Mt, St, Fn Ab, Mp, Pa, Se, Cc, Pg, Bt
S2F6	PA4743	*rbfA* - ribosome-binding factor A (2)	Translation, post-translational modification, degradation	Sa, Vc, Mg, Hp, Fn, Ab, Se
S6E7	PA3820	*secF* - secretion protein SecF (2)	Protein secretion/export apparatus	Ec, Hi, St, Fn, Ab, Pa, Se
S5A10	PA1709[d]	*popD* - Translocator outer membrane protein (1)	Protein secretion/export apparatus	
S11B13	PA4669	*ipk* - isopentenyl monophosphate kinase(2)	Biosynthesis of cofactors, prosthetic groups and carriers	Vc, Se, Pa
G2	PA2951	*etfA* - electron transfer flavoprotein alpha-subunit (2)	Energy metabolism	Ab, Cc
H2				
S10F8	PA5186	probable iron-containing alcohol dehydrogenase (3)	Energy metabolism	
F1	PA1554	*ccoN1*\ *fixN* \ Cytochrome c oxidase, cbb3-type, CcoN subunit (1)	Energy metabolism	
S11G10	PA3687	*ppc* - phosphoenolpyruvate carboxylase (2)	Energy metabolism	Hi
S86C	PA3758	*nagA* - probable N-acetylglucosamine-6-phosphate deacetylase (3)	Central intermediary metabolism	Hi, Mt
E5	PA1183	*dctA* - C4-dicarboxylate transport protein (2)	Transport of small molecules	Ab
S11C9	PA3382	*phnE* - phosphonate transport protein PhnE (2)	Transport of small molecules	
S4E6	PA4903	*vanK* - probable major facilitator superfamily (MFS) transporter (3)	Transport of small molecules	
B3	PA5548	probable major facilitator superfamily (MFS) transporter (3)	Transport of small molecules	
S4H12				
A5	PA1590	*braB* - branched chain amino acid transporter (1)	Transport of small molecules	
S3D4	PA1805	*ppiD* - peptidyl-prolyl cis-trans isomerase D - Rotamase D (2)	Translation, post-translational modification, degradation	Cc, Bs
S9G5	PA2402	Probable non-ribosomal peptide synthetase (3)	Putative enzymes	
S5D4				
S5A4	PA5238	probable O-antigen acetylase (3)	Membrane proteins, Cell wall/LPS/capsule	
S5G6				
S4B10	PA3433	*ywbI* - probable transcriptional regulator (3)	Transcriptional regulators	
S5A1	PA2220	*oprR* - probable transcriptional regulator (3)	Transcriptional regulators	
M4G6	PA2873[e]	*tgpA* - transglutaminase protein A TgpA (1)	Adaptation, Protection, Membrane proteins	
S10A3	PA0307	hypothetical protein (4)	Hypothetical, unclassified, unknown	

Table 1 *Pseudomonas aeruginosa* PAO1 genes targeted by growth-impairing inserts including a single *locus* (Continued)

S841F	PA4926	conserved hypothetical protein (4)	Hypothetical, unclassified, unknown
S9A9	PA0262	conserved hypothetical protein (4)	Hypothetical, unclassified, unknown
F2	PA5264	hypothetical protein (4)	Hypothetical, unclassified, unknown

[a]Inserts with antisense orientation are in bold.
[b]Annotations according to the Pseudomonas Genome Database (www.pseudomonas.com) [27]. Numbers inside parenthesis indicate the classes of product name confidence. Class1: Function experimentally demonstrated in *P. aeruginosa*; Class 2: Function of highly similar gene experimentally demonstrated in another organism; Class 3: Function proposed based on presence of conserved amino acid motif, structural feature or limited sequence similarity to an experimentally studied gene. Class 4: Homologs of previously reported genes of unknown function, or no similarity to any previously reported sequences.
[c]DEG: Database of Essential Genes (DEG 7.0) (www.essentialgene.org) [20]. For each hit, the orthologue proteins, extracted from the Ortholuge DB [30], that were found in DEG are indicated with the abbreviation of the harboring bacterial species. Bacterial species: Ab (*Acinetobacter baylyi*), Bs (*Bacillus subtilis*), Bt (*Bacteroides thetaiotaomicron*), Cc (*Caulobacter crescentus*), Ec (*Escherichia coli*), Fn (*Francisella novicida*), Hi (*Haemophilus influenzae*), Hp (*Helicobacter pylori)*, Mt (*Mycobacterium tuberculosis*), Mp (*Mycoplasma pulmonis*), Mg (*Mycoplasma genitalium*), Pg (*Porphyromonas gingivalis*), Pa (*Pseudomonas aeruginosa*), Se (*Salmonella enterica*), St (*Salmonella typhimurium*), Sp (*Streptococcus pneumoniae*), Ss (*Streptococcus sanguinis*), Sa (*Staphylococcus aureus*), Vc (*Vibrio cholerae*).
[d]Previous reports [24-26] did not mention growth defects associated to deletion of *popD* gene.
[e]Hit not present in DEG whose essentiality was experimentally demonstrated in *P. aeruginosa* [21].

proteins. We suggest that these proteins may be involved in unexplored essential functions, either as stand-alone proteins or connected to classical housekeeping processes. This is the case for the inner membrane protein TgpA (PA2873; Table 1) [21], which was found in our antisense screenings and was previously reported as hypothetical, whose transglutaminase activity associated with the periplasmic domain might be either linked to cell wall metabolism or be involved in unknown key functions of protein maturation, secretion, and/or modification.

Only two of the 23 non-classical essential genes, PA4669 (*ipk*) and PA3820 (*secF*), were already indicated as essential in *P. aeruginosa* [9,20]. For the remaining 21 genes, no evidence for essentiality has been reported previously in *P. aeruginosa* [20]. We propose these genes as novel essential genes in *P. aeruginosa*. PA2951 (*etfA*), PA3687 (*ppc*), PA3758 (*nagA*), PA1183 (*dctA*), and PA1805 (*ppiD*) are homologous to genes previously shown to be essential in a limited number of bacterial species [20]. Interestingly, for the remaining 16 genes, no homologs have been reported as essential in other bacteria [20]. Among these, PA1709 (*popD*), coding for a subunit of the PopB/D translocon complex of the type III secretion-translocation system (TTSS), is implicated in effector translocation across the host plasma membrane.

Table 2 PAO1 growth-impairing inserts including loci belonging to a single operon

Insert name[a]	Operon *loci*[b]	Gene name and product annotation[c]	Function class[c]	Species containing orthologs in DEG[d]
E6	**PA1037**	*yicG* - conserved hypothetical protein (4)	Hypothetical, unclassified, unknown	
	PA1038	hypothetical protein (4)		
	PA1039	*ychJ* - hypotetical protein (4)		
	PA1040	hypothetical protein (4)		
S9B6a	**PA1089**	conserved hypothetical protein (4)	Hypothetical, unclassified, unknown	
	PA1090	conserved hypothetical protein (4)		
	PA1088	hypothetical protein (4)		
S9B6b	**PA0393**	*proC* - pyrroline-5-carboxylate reductase (1)	Amino acid biosynthesis and metabolism	*E. coli*, *M. tuberculosis*, *A. baylyi*
	PA0392	*yggT* - conserved hypothetical protein (4)	Hypothetical, unclassified, unknown	
	PA0394	*yggS* - conserved hypothetical protein (4)		
S2A4	**PA1001**[e]	*phnA* - anthranilate synthase component I (1)	Adaptation, protection; amino acid biosynthesis	
	PA1002[e]	*phnB* - anthranilate synthase component II (1)		

[a]Inserts with antisense orientation are in bold.
[b]Loci included in the insert are in bold.
[c]Annotations according to the Pseudomonas Genome Database (www.pseudomonas.com) [27]. Numbers inside parenthesis indicate the classes of product name confidence. Class1: Function experimentally demonstrated in *P. aeruginosa*; Class 2: Function of highly similar gene experimentally demonstrated in another organism; Class 3: Function proposed based on presence of conserved amino acid motif, structural feature or limited sequence similarity to an experimentally studied gene. Class 4: Homologs of previously reported genes of unknown function, or no similarity to any previously reported sequences.
[d]DEG: Database of Essential Genes (DEG 7.0) (www.essentialgene.org) [20].
[e]Previous reports [34,35] did not mention growth defects associated to deletion of *phnAB* genes.

Previous reports on *P. aeruginosa* PopD function [24-26] did not mention growth defects associated to deletion of *popD* gene. Therefore, the growth-impairing effects of S5A10 insert corresponding to PA1709 (Table 1) did not seem to match the PopD role characterized so far. These discrepancies could be due to differences in experimental conditions between our study and earlier works.

We evaluated the set of 21 novel candidate essential genes for degree of conservation in *Pseudomonas* species according to the computationally-based analysis of orthologs of the Pseudomonas Genome Database [27] (Additional file 5: Table S5). Interestingly, they are well-conserved in the sequenced *Pseudomonas* species, with the exceptions of PA5548 and PA1709 (*popD*) that are unique in *P. aeruginosa*. However, PA5548 and PA1709 (*popD*) orthologs can be found in other bacterial species. Remarkably, 17 of 21 novel essential candidates are conserved in all twelve sequenced *P. aeruginosa* genomes (Additional file 5: Table S5). Instead, PA2220 (*oprR*), PA5264, PA1709 (*popD*) and PA3687 (*ppc*) are present in 3, 8, 9 and 10 of the sequenced genomes, respectively. Essential genes that are not fully conserved in all strains of a bacterial species can occur infrequently. As an example, the *Escherichia coli* genes *ytfI, ypjF, ymfJ, ymfI* and *ymcD*, coding for hypothetical proteins, were reported as essential in the K12-MG1655 strain [28,29] and are conserved in only a limited number of the sequenced *E. coli* genomes [30].

Moreover, we compared the novel essential candidates with a panel of "classical" essential genes that were not included in the Database of Essential Genes (DEG) [20] because of the occurence of Tn insertions in previous screenings in *P. aeruginosa* [9,10,23]. The Tn insertion patterns of the novel essential candidates (i.e. number of insertions and insertion site(s)- terminal *vs* internal; Additional file 5: Table S5) were similar to those of "classical" essential genes (Additional file 4: Table S4).

This study also identified growth-impairing inserts carrying multiple genes. Because of their multigenic composition, the tagging of genes in these constructs for essentiality is not as direct as for single locus inserts (see above). However, among the multigenic inserts, we identified sequences corresponding to 25 genes with homologs already annotated as essential in other bacterial species [20] (Additional file 3: Table S3). Seven of these genes were indicated previously as essential in *P. aeruginosa* [9,20]. The 25 genes were annotated as involved in multiple cellular functions: lipid A biosynthesis (*lpxA, lpxB*; *lpxD, fabZ*) [31], amino acid biosynthesis and metabolism (*glyA3, proC, hom, lysC, ldh*), DNA replication and recombination (*dnaX, recB, recR*), transport of small molecules (*potD, mgtA, fadL, fepG, pstC),* biosynthesis of cofactors, prosthetic groups and carriers (*folD*), translation and post-translational modification (*tufB*), nucleotide biosynthesis (*purL*), protein secretion (*secE*), tRNA modification (*gcp*) [32], central intermediary metabolism (*glpK),* and energy metabolism (*fdx2*). Other genes present in the multigenic inserts might be essential, but their identification would require further analysis via subcloning and/or conditional mutagenesis.

Interestingly, four multigenic inserts contained genes belonging to a single operon (Table 2), a feature that suggests a functional association. One such gene, *proC*, codes for pyrroline-5-carboxylate reductase [33] and was reported as essential in *E. coli*, *Mycobacterium tuberculosis* and *Acinetobacter baylyi* [20]. Other gene products of these operons are annotated as hypothetical proteins. Therefore, we suggest that these operonic genes might be involved in novel essential pathways. Overall, they are well-conserved in the sequenced *Pseudomonas* species (Additional file 5: Table S5). Exceptions are PA1088-1089-1090 which appear restricted to few *Pseudomonas* species and not conserved in all sequenced *P. aeruginosa* strains. Finally, one operonic growth-impairing insert included PA1001-1002 (*phnAB*) implicated in the biosynthesis of pyocyanin. Previous reports on *P. aeruginosa* PAO1 *phnA* and PA14 *phnAB* function [34,35] did not mention growth defects associated to deletion of these genes. As in the case of PA1709 (*popD*), discrepancies between our results and previous works could be attributable to differences in experimental conditions.

Conclusions

Taken together, our results show the feasibility of antisense technology in *P. aeruginosa* for identifying novel essential genes. Because of its supposed inefficiency [16], this approach has been neglected in Gram-negative bacteria for several years, and was only recently recovered in *E. coli* [17]. By comparison with this previous work, the results presented here strongly suggest that our modification of the antisense strategy could broaden the class variety of the identified essential genes. We expect that our methodology could be well suited for antisense-mediated searches of essential genes in other Gram-negative bacterial species.

Methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Additional file 6: Table S1. Bacteria were grown at 37°C in Luria-Bertani (LB) broth, or in M9 minimal medium supplemented with 0.2% citrate (M9-citrate). Antibiotics were added at the following concentrations (μg/ml): Cb, 300; kanamycin; 50. Arabinose was added to a final concentration of 10 mM. In mating experiments, exconjugant *P. aeruginosa* PAO1 clones were selected on PIA (Difco) containing Cb.

Construction and screening of PAO1 shotgun antisense libraries

Genomic DNA was isolated from *P. aeruginosa* PAO1 using an illustra GenomicPrep Cells and Tissue DNA Isolation Kit (GE Healthcare). DNA was diluted in 10 mM TE buffer (pH 8.0) and nebulized to obtain sheared fragments spanning 200–800 bp (Additional file 1: Figure S1A). Following ethanol precipitation, fragmented DNA was treated with nuclease BAL-31 and Klenow (New England Biolabs) for 10 min at 30°C to obtain blunt ends. After enzyme inactivation with 1 mM EDTA, DNA was dialyzed against 20 mM Tris–HCl (pH 8.0). pVI533EH and pHERD20T were digested with *Sma*I (New England Biolabs) and dephosphorylated using shrimp alkaline phosphatase (Roche). Fragmented DNA was ligated to dephosphorylated vectors using T4 Ligase (Takara Bio) at 16°C overnight. Ligation mixtures were transformed into *E. coli* JM109 by electroporation, and transformants were selected on LB plates supplemented with Cb. The resulting transformant colonies composing the SAL were arrayed and cultured in 96-well microplates. Quality control by PCR of single colonies, using primers flanking the multi-cloning site (Additional file 1: Figure S1B), was performed to check the presence and the size of a genomic insert.

SALs were mobilized from *E. coli* to *P. aeruginosa* PAO1 by conjugative triparental mating. *E. coli* donor strains were grown overnight in 96-well microplates in LB broth supplemented with Cb. The recipient *P. aeruginosa* PAO1 and helper *E. coli* HB101/pRK2013 strains were grown overnight in flasks in LB broth. Thirty microliters each of helper, recipient, and donor strains were mixed in microplate wells. After mixing, microplates were centrifuged at 750 × g for 5 min and incubated for 3 h at 37°C. Cell pellets resulting from triparental mating were resuspended in 90 μl of LB, and 2 μl of each mating mixture were spotted on PIA plates supplemented with Cb, both in the absence and presence of 10 mM arabinose, to counter select *E. coli* donor and helper strains. Exconjugant cell spots were inspected for growth defects following 24–48 h of incubation at 37°C. The PAO1 growth-impairing inserts in pVI533EH/pHERD20T derivatives were sequenced following PCR amplification using oligo pVI533-F/pVI533-R and pHERD-F/pHERD-R, respectively (Additional file 6: Table S1). The resulting sequences were matched to the PAO1 genome at the *Pseudomonas* Genome Database [27].

Additional files

Additional file 1: Figure S1. Construction and screening for growth defects of *P. aeruginosa* shotgun antisense libraries. A. Agarose gel electrophoresis showing two fractions, F1 and F2 (lanes 2 and 3), of DNA fragments generated from *P. aeruginosa* PAO1 genomic DNA (lane 1). The DNA fragments from F1 and F2 were generated by nebulization at 2.5 and 5 bar pressure, respectively. B. Quality control for cloning: pHERD vector used for library preparation allows white/blue screening for positive inserts. White clones were checked by PCR for the presence of an insert using oligos annealing at both sides of the polylinker sequence. As an example, a check of a randomly selected pool of 25 white colonies is shown (M: molecular weight marker; E. empty vector). It is noteworthy that more than 90% of clones from F1 (23/25) carried an insert within the expected size range (200–800 bp; average size: 500 bp), and were used for shotgun cloning. C. SAL recipient PAO1 exconjugants were selected by spotting on PIA plates supplemented with Cb, both in the absence and in the presence of the P_{BAD} inducer arabinose. Recipient PAO1 exconjugant spots were inspected for growth defects following 24 h of incubation at 37°C. For example: red circle indicates growth impairment only with inducer; yellow circle indicates lethal effects only with inducer; green circle indicates lethal effects both in the presence and absence of the inducer. The identity of the genomic fragments eliciting growth was determined by sequencing the inserts in the corresponding clones of *E. coli* SAL.

Additional file 2: Table S2. Growth-impairing inserts resulting from PAO1 SAL screenings.

Additional file 3: Table S3. PAO1 growth-impairing inserts including multiple loci.

Additional file 4: Table S4. Additional information on a selection of PAO1 "classical" essential genes.

Additional file 5: Table S5. Additional information on novel *P. aeruginosa* candidate essential genes.

Additional file 6: Table S1. List of bacterial strains, plasmids, and oligonucleotides.

Authors' contributions

RR, DV, FV, and GB conceived and designed the experiments. RR, RM, and FV performed the experiments. RR, DV, and GB analyzed the data. DV and GB wrote the paper. All authors read and approved the final manuscript.

Acknowledgments

The authors are grateful to Andrea Milani and all members of the laboratory for their helpful discussions and technical support. This work was funded by the Italian Cystic Fibrosis Research Foundation (grant FFC#10/2004) and by the European Commission (grant NABATIVI, EU-FP7-HEALTH-2007-B contract number 223670).

Author details

[1]Department of Life Sciences, Università degli Studi di Milano, via Celoria 26, 20133 Milan, Italy. [2]Alcon Italia S.p.A., viale Richard 1/B, 20143 Milan, Italy.

Received: 2 July 2013 Accepted: 23 January 2014
Published: 5 February 2014

References

1. Pier GB, Ramphal R: **Pseudomonas aeruginosa**. In *Principles and Practice of Infectious Diseases*. Edited by Mandell GL, Bennett JE, Dolin R. Philadelphia, PA: Elsevier Churchill Livingstone; 2005:2587–2615.
2. Wagner VE, Filiatrault MJ, Picardo KF, Iglewski BH: ***Pseudomonas aeruginosa* virulence and pathogenesis issues**. In *Pseudomonas Genomics and Molecular Biology*. Edited by Cornelis P. Norfolk: Caister Academic Press; 2008:129–158.
3. Bonomo RA, Szabo D: **Mechanisms of multidrug resistance in Acinetobacter species and *Pseudomonas aeruginosa*.** *Clin Infect Dis* 2006, **43:**S49–S56.
4. Lister PD, Wolter DJ, Hanson ND: **Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms.** *Clin Microbiol Rev* 2009, **22:**582–610.
5. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, *et al*: **Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance.** *Clin Microbiol Infect* 2012, **18:**268–281.

6. Reznikoff WS, Winterberg KM: **Transposon-based strategies for the identification of essential bacterial genes.** *Methods Mol Biol* 2008, **416:**13–26.
7. Deng J, Su S, Lin X, Hassett DJ, Lu LJ: **A statistical framework for improving genomic annotations of prokaryotic essential genes.** *PLoS One* 2013, **8:**e58178.
8. Barquist L, Boinett CJ, Cain AK: **Approaches to querying bacterial genomes with transposon-insertion sequencing.** *RNA Biol* 2013, **10:**1–9.
9. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM: **An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants.** *Proc Natl Acad Sci U S A* 2006, **103:**2833–2838.
10. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, *et al*: **Comprehensive transposon mutant library of *Pseudomonas aeruginosa*.** *Proc Natl Acad Sci U S A* 2003, **100:**14339–14344.
11. Judson N, Mekalanos JJ: **TnAraOut, a transposon-based approach to identify and characterize essential bacterial genes.** *Nat Biotechnol* 2000, **18:**740–745.
12. de Lorenzo V, Timmis KN: **Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons.** *Methods Enzymol* 1994, **235:**386–405.
13. Ji Y, Zhang B, Van SF, Horn, Warren P, Woodnutt G, Burnham MK, Rosenberg M: **Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA.** *Science* 2001, **293:**2266–2269.
14. Forsyth RA, Haselbeck RJ, Ohlsen KL, Yamamoto RT, Xu H, Trawick JD, Wall D, Wang L, Brown-Driver V, Froelich JM, *et al*: **A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*.** *Mol Microbiol* 2002, **43:**1387–1400.
15. Engdahl HM, Lindell M, Wagner EGH: **Introduction of an RNA stability element at the 5 ′-end of an antisense RNA cassette increases the inhibition of target RNA translation.** *Antisense Nucleic Acid Drug Dev* 2001, **11:**29–40.
16. Wagner EGH, Flardh K: **Antisense RNAs everywhere?** *Trends Genet* 2002, **18:**223–226.
17. Meng J, Kanzaki G, Meas D, Lam CK, Crummer H, Tain J, Xu HH: **A genome-wide inducible phenotypic screen identifies antisense RNA constructs silencing *Escherichia coli* essential genes.** *FEMS Microbiol Lett* 2012, **329:**45–53.
18. Yin D, Ji Y: **Genomic analysis using conditional phenotypes generated by antisense RNA.** *Current Opin Microbiol* 2002, **5:**330–333.
19. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD: **PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia spp.* and other bacteria.** *Appl Environ Microbiol* 2008, **74:**7422–7426.
20. Zhang R, Lin Y: **DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes.** *Nucleic Acids Res* 2009, **37:**D455–D458.
21. Milani A, Vecchietti D, Rusmini R, Bertoni G: **TgpA, a protein with a eukaryotic-like transglutaminase domain, plays a critical role in the viability of *Pseudomonas aeruginosa*.** *PLoS One* 2012, **7:**e50323.
22. Comolli JC, Donohue TJ: **Differences in two *Pseudomonas aeruginosa* cbb3 cytochrome oxidases.** *Mol Microbiol* 2004, **51:**1193–1203.
23. Lewenza S, Falsafi RK, Winsor G, Gooderham WJ, McPhee JB, Brinkman FS, Hancock RE: **Construction of a mini-Tn5-luxCDABE mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes.** *Genome Res* 2005, **15:**583–589.
24. Goure J, Pastor A, Faudry E, Chabert J, Dessen A, Attree I: **The V antigen of *Pseudomonas aeruginosa* is required for assembly of the functional PopB/PopD translocation pore in host cell membranes.** *Infect Immun* 2004, **72:**4741–4750.
25. Tomalka AG, Stopford CM, Lee PC, Rietsch A: **A translocator-specific export signal establishes the translocator-effector secretion hierarchy that is important for type III secretion system function.** *Mol Microbiol* 2012, **86:**1464–1481.
26. Tomalka AG, Zmina SE, Stopford CM, Rietsch A: **Dimerization of the *Pseudomonas aeruginosa* translocator chaperone PcrH is required for stability, not function.** *J Bacteriol* 2013, **195:**4836–4843.
27. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS: **Pseudomonas genome database: improved comparative analysis and population genomics capability for Pseudomonas genomes.** *Nucleic Acids Res* 2011, **39:**D596–D600.
28. Gerdes SY, Scholle MD, Campbell JW, Balazsi G, Ravasz E, Daugherty MD, Somera AL, Kyrpides NC, Anderson I, Gelfand MS, *et al*: **Experimental determination and system level analysis of essential genes in Escherichia coli MG1655.** *J Bacteriol* 2003, **185:**5673–5684.
29. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H: **Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection.** *Mol Sys Biol* 2006, **2:**2006.0008.
30. Whiteside MD, Winsor GL, Laird MR, Brinkman FS: **OrthologueDB: a bacterial and archaeal orthology resource for improved comparative genomic analysis.** *Nucleic Acids Res* 2013, **41:**D366–D376.
31. King JD, Kocincova D, Westman EL, Lam JS: **Review: Lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*.** *Innate Immun* 2009, **15:**261–312.
32. Deutsch C, El YB, de C-LV, Iwata-Reuyl D: **Biosynthesis of threonylcarbamoyl adenosine (t6A), a universal tRNA nucleoside.** *J Biol Chem* 2012, **287:**13666–13673.
33. Savioz A, Jeenes DJ, Kocher HP, Haas D: **Comparison of proC and other housekeeping genes of *Pseudomonas aeruginosa* with their counterparts in *Escherichia coli*.** *Gene* 1990, **86:**107–111.
34. Essar DW, Eberly L, Hadero A, Crawford IP: **Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications.** *J Bacteriol* 1990, **172:**884–900.
35. Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM: **Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model.** *Cell* 1999, **96:**47–56.

doi:10.1186/1471-2180-14-24
Cite this article as: Rusmini *et al.*: A shotgun antisense approach to the identification of novel essential genes in *Pseudomonas aeruginosa*. *BMC Microbiology* 2014 **14**:24.

SUPPLEMENTARY FIGURES

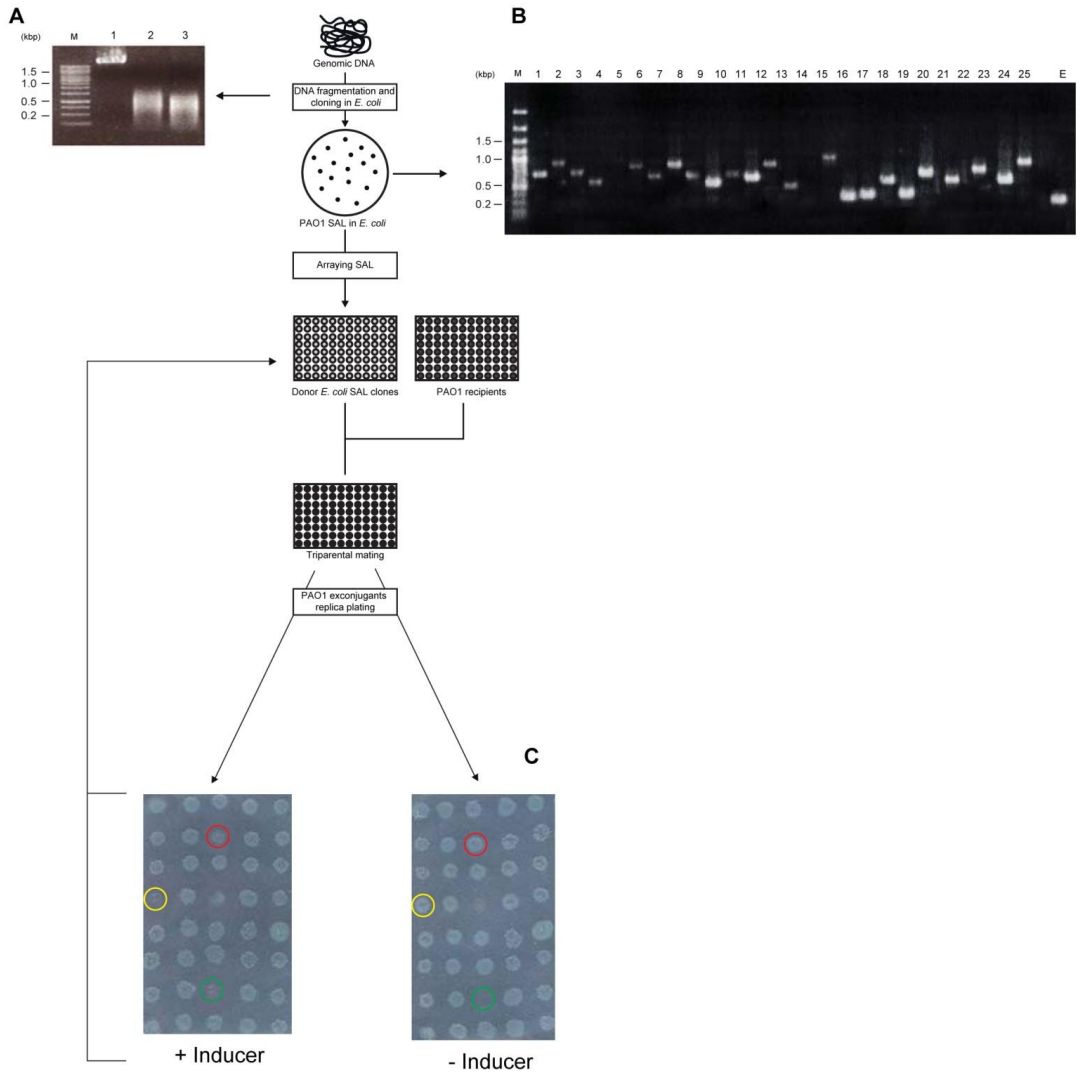


Figure S1.Construction and screening for growth defects of *P. aeruginosa* shotgun antisense libraries. A. Agarose gel electrophoresis showing two fractions, F1 and F2 (lanes 2 and 3), of DNA fragments generated from *P. aeruginosa* PAO1 genomic DNA (lane 1). The DNA fragments from F1 and F2 were generated by nebulization at 2.5 and 5 bar pressure, respectively. **B**. Quality control for cloning: pHERD vector used for library preparation allows white/blue screening for positive inserts. White clones were checked by PCR for the presence of an insert using oligos annealing at both sides of the polylinker sequence. As an example, a check of a randomly selected pool of 25 white colonies is shown (M: molecular weight marker; E. empty vector). It is noteworthy that more than 90% of clones from F1 (23/25) carried an insert within the expected size range (200–800 bp; average size: 500 bp), and were used for shotgun cloning. **C**. SAL recipient PAO1 exconjugants were selected by spotting on PIA plates supplemented with Cb, both in the absence and in the presence of the P_{BAD} inducer arabinose. Recipient PAO1 exconjugant spots were inspected for growth defects following 24 h of incubation at 37°C. For example: red circle indicates growth impairment only with inducer; yellow circle indicates lethal effects only with inducer; green circle indicates lethal effects both in the presence and absence of the inducer. The identity of the genomic fragments eliciting growth was determined by sequencing the inserts in the corresponding clones of *E. coli* SAL.

Table S1: List of bacterial strains, plasmids and oligonucleotides.

Strains,plasmids,oligos	Genotype or description	Reference
Pseudomonas aeruginosa		
PAO1		[1]
Escherichia coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44, relA1, Δ(lac-proAB)* F' *(traD36, proAB+ lacIqZ* ΔM15)	[2]
HB101 [RK2013]	*recA, thi, hsdR, hsdM, proA, leu, strA*	[3]
Plasmids		
pHERD20T	Ap^R, Cb^R broad-host-range *araC-*P_{BAD} expression vector; blue/white screening for recombinants	[4]
pVI533EH	Ap^R, Cb^R broad-host-range *araC-*P_{BAD}	[5]
Oligonucleotides		
pVI533-F	ATCACGGCAGAAAAGTCCAC	
pVI533-R	CTTCTCTCATCCGCCAAAAC	
E2Eco5'	CGCGAATTCTCAGACCGCCGGCAGCGAC	
E2Hind3'	CGCAAGCTTCTGGCAGCGTTGCGTCGA	
pHERD-F	ATCGCAACTCTCTACTGTTTCT	
pHERD-R	TGCAAGGCGATTAAGTTGGGT	

References

1. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406: 959-964.
2. Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. 33: 103-119.
3. Boyer HW, Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. 41: 459-472.
4. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD (2008) PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. 74: 7422-7426.
5. Sze CC, Shingler V (1999) The alarmone (p)ppGpp mediates physiological-responsive control at the sigma 54-dependent Po promoter. 31: 1217-1228.

Table S2. Growth-impairing inserts resulting from PAO1 SAL screenings.

Insert name[a]	Phenotype[b]		Start[c]	Stop[c]	Length (bp)	Orientation[d]	*Locus*[e]	Gene name and product annotation[f]
	+ ARA	- ARA						
M4G6	Lethal	Lethal	3226136	3226491	355	ANTISENSE	PA2873	tgpA - transglutaminase protein A TgpA (Class 1)
M7E2	Lethal	Defect	4443964	4443811	153	ANTISENSE	PA3964	hypothetical protein (Class 4)
			4443854	4443417	437	ANTISENSE	PA3963	yiiP - probable transporter (Class 3)
			596188	595707	481	SENSE	PA0536	hypothetical protein (Class 4)
M2D2	Defect	Normal	5121704	5121898	194		IR	
			5121899	5122199	300	ANTISENSE	PA4574	conserved hypothetical protein (Class 4)
			2651256	2650985	271	ANTISENSE	PA2394	pvdN (Class 1)
M7G4	Lethal	Defect	5950869	5951115	246		IR	
			5951115	5951612	497	ANTISENSE	PA5287	amtB - ammonium transporter AmtB (Class 2)
			4189311	4189547	236		IR	
			4189547	4189730	183	ANTISENSE	PA3736	hom - homoserine dehydrogenase (Class 1)
			3467036	3466960	76		IR	
			3466960	3466730	230	SENSE	PA3088	conserved hypothetical protein (Class 4)
M7H8	Defect	Normal	1953080	1952665	415	ANTISENSE	PA1800	tig - trigger factor (Class 2)
			1952665	1952433	232		IR	
			1952433	1952250	183	SENSE	PA1799	parR - two-component response regulator, ParR (Class 1)
A1	Lethal	Defect	5720756	5720945	189	SENSE	PA5081	hypothetical protein (Class 4)
			5720950	5721501	551	ANTISENSE	PA5082	probable binding protein component of ABC transporter (Class 3)
			5159950	5160615	665	SENSE	PA4601	morA - motility regulator (Class 1)
			5160616	5160737	121		IR	
			5160738	5160846	108	SENSE	PA4602	glyA3 - serine hydroxymethyltransferase (Class 2)
A5	Defect	Normal	1733430	1733642	212	SENSE	PA1590	braB - branched chain amino acid transporter (Class 1)
B1	Lethal	Defect	4778201	4778555	354	ANTISENSE	PA4270	rpoB - DNA-directed RNA polymerase beta chain (Class 2)
B3	Defect	Normal	6242757	6242573	184	ANTISENSE	PA5548	probable major facilitator superfamily (MFS) transporter (Class 3)
B5	Lethal	Defect	2124389	2124637	248	ANTISENSE	PA1941	hypothetical protein (Class 4)
			1946604	1946417	187	SENSE	PA1796	folD - 5,10-methylene-tetrahydrofolate dehydrogenase / cyclohydrolase (Class 2)
D1	Defect	Normal	6237168	6237657	489	ANTISENSE	PA5544	conserved hypothetical protein (Class 4)
			4044648	4044984	336	SENSE	PA3610	potD - polyamine transport protein PotD (Class 2)
			4044984	4045152	168		IR	
D2	Defect	Normal	1114549	1114297	252	ANTISENSE	PA1027	probable aldehyde dehydrogenase (Class 3)
			4267513	4267432	81	SENSE	PA3809	fdx2 - ferredoxin [2Fe-2S] (Class 2)
D5	Defect	Normal	2048624	2048570	54	SENSE	PA1878	hypothetical protein (Class 4)
			2048569	2048463	106		IR	
			2048462	2048258	204	ANTISENSE	PA1877	probable secretion protein (Class 3)
			6162859	6163204	345	SENSE	PA5473	conserved hypothetical protein (Class 4)
E2	Lethal	Defect	640687	640420	267	SENSE	PA0581	ygiH - conserved hypothetical protein (Class 4)
			640419	640342	77		IR	
			640341	640687	346	ANTISENSE	PA0580	gcp\ygjD O-sialoglycoprotein endopeptidase (Class 2)
E4	Defect	Normal	5138686	5138754	68	ANTISENSE	PA4588	gdhA - glutamate dehydrogenase (Class 2)

			5138755	5139049	294		IR	
			5139050	5139121	71	ANTISENSE	PA4589	fadL - probable outer membrane protein precursor (Class 3)
			3636174	3636093	81	SENSE	PA3249	probable transcriptional regulator (Class 3)
E4	Defect	Normal	5138686	5138754	68	ANTISENSE	PA4588	gdhA - glutamate dehydrogenase (Class 2)
			5138755	5139049	294		IR	
			5139050	5139121	71	ANTISENSE	PA4589	fadL - probable outer membrane protein precursor (Class 3)
			3636174	3636093	81	SENSE	PA3249	probable transcriptional regulator (Class 3)
E5	Lethal	Lethal	1285145	1284513	632	ANTISENSE	PA1183	dctA - C4-dicarboxylate transport protein (Class 2)
E6	Lethal	Defect	1125143	1125465	322	ANTISENSE	PA1037	yicG - conserved hypothetical protein (Class 4)
			1125466	1125547	81		IR	
			1125548	1125868	320	ANTISENSE	PA1038	hypothetical protein (Class 4)
F1	Defect	Normal	1692754	1692568	186	SENSE	PA1554	ccoN1 \ fixN \ Cytochrome c oxidase, cbb3-type, CcoN subunit (Class 1)
F2	Defect	Normal	5926859	5926602	257	SENSE	PA5264	hypothetical protein (Class 4)
G1	Defect	Normal	5197806	5197881	75		IR	
			5197881	5198071	190	SENSE	PA4630	hypothetical protein (Class 4)
			2007983	2007807	176	ANTISENSE	PA1847	yhgI - conserved hypothetical protein (Class 4)
H2	Lethal	Defect	3310951	3311112	161	ANTISENSE	PA2951	etfA - electron transfer flavoprotein alpha-subunit (Class 2)
G2	Lethal	Defect	3310792	3311282	490	ANTISENSE	PA2951	etfA - electron transfer flavoprotein alpha-subunit (Class 2)
G4	Defect	Normal	5862829	5862260	569	SENSE	PA5207	probable phosphate transporter (Class 3)
			3826157	3826019	138	ANTISENSE	PA3419	hypothetical protein (Class 4)
			3826019	3825774	245		IR	
			3825773	3825719	54	SENSE	PA3418	ldh - leucine dehydrogenase (Class 2)
S2A4	Lethal	Lethal	1087451	1085564	93	SENSE	PA1001	phnA - anthranilate synthase component I (Class 1)
			1085542	1085603	61	SENSE	PA1002	phnB - anthranilate synthase component II (Class 1)
S2F1	Lethal	Defect	637810	638141	331	ANTISENSE	PA0577	dnaG - DNA primase (Class 2)
S2F6	Defect	Normal	5327423	5327324	99	ANTISENSE	PA4743	rbfA - ribosome-binding factor A (Class 2)
S3C3	Defect	Normal	4784032	4784139	107	ANTISENSE	PA4276	secE \ prlG - secretion protein SecE (Class 2)
			4784140	4784183	43		IR	
			4784184	4784259	75	ANTISENSE	PA4276.1	tRNA-Trp (Class 2)
			4784260	4784315	55		IR	
			4784316	4784670	354	ANTISENSE	PA4277	tufB - elongation factor Tu (Class 2)
S3D3	Lethal	Defect	637230	636304	926	SENSE	PA0577	dnaG - DNA primase (Class 2)
S3D4	Lethal	Defect	1960531	1960182	349	ANTISENSE	PA1805	ppiD - peptidyl-prolyl cis-trans isomerase D (Class 2)
S4B7	Defect	Normal	4524644	4524114	530	SENSE	PA4041	hypothetical protein (Class 4)
			3131111	3131159	48	ANTISENSE	PA2772	hypothetical protein (Class 4)
			3131160	3131448	288		IR	
S4B10	Lethal	Defect	3841063	3841483	420	SENSE	PA3433	ywbI - probable transcriptional regulator (Class 3)
S4D10	Lethal	Defect	4082574	4082961	387	ANTISENSE	PA3644	lpxA - UDP-N-acetylglucosamine acyltransferase (Class 2)
			4082958	4083398	440	ANTISENSE	PA3645	fabZ - sefA - (3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase (Class 2)
			4083399	4083443	44		IR	
			4083444	4084133	689	ANTISENSE	PA3646	lpxD \ omsA \ firA - UDP-3-O-[3-hydroxylauroyl] glucosamine N-acyltransferase (Class 2)
S4E6	Defect	Lethal	5502636	5502339	297	SENSE	PA4903	vanK - probable major facilitator superfamily (MFS) transporter (Class 3)

S4H12	Defect	Lethal	6241851	6242650	799	SENSE	PA5548	probable major facilitator superfamily (MFS) transporter (Class 3)
S5A1	Defect	Normal	2443160	2442692	468		IR	
			2442691	2442652	39	ANTISENSE	PA2220	oprR - probable transcriptional regulator (Class 3)
S5A4	Defect	Normal	5898131	5898063	68	SENSE	PA5238	probable O-antigen acetylase (Class 3)
S5A10	Lethal	Normal	1855763	1855737	26		IR	
			1855736	1855297	439	ANTISENSE	PA1709	popD \ pepD - Translocator outer membrane protein PopD precursor (Class 1)
S5D4	Defect	Normal	2677220	2676516	704	SENSE	PA2402	probable non-ribosomal peptide synthetase (Class 3)
S5D11	Lethal	Normal	3122034	3122265	231	ANTISENSE	PA2762	hypothetical protein (Class 4)
			3122266	3122297	31		IR	
			1671373	1672034	661	SENSE	PA1532	dnaX - DNA polymerase subunits gamma and tau (Class 2)
			1672035	1672079	44		IR	
			1672080	1672406	326	SENSE	PA1533	conserved hypothetical protein (Class 4)
			1672407	1672484	77		IR	
			1672485	1672830	345	SENSE	PA1534	recR - recombination protein RecR (Class 2)
S5G6	Lethal	Lethal	5898063	5898131	68	ANTISENSE	PA5238	probable O-antigen acetylase (Class 3)
S6E7	Lethal	Defect	4276723	4276362	361	SENSE	PA3820	secF - secretion protein SecF (Class 2)
S6A10	Defect	Normal	5327427	5327562	135	ANTISENSE	PA4744	infB - translation initiation factor IF-2 (Class 2)
S86C	Lethal	Defect	4208263	4208429	167	SENSE	PA3758	nagA - probable N-acetylglucosamine-6-phosphate deacetylase (Class 3)
S817E	Lethal	Defect	507855	507631	224	ANTISENSE	PA0451	conserved hypothetical protein (Class 4)
			507630	507570	60		IR	
			4081062	4081045	17	SENSE	PA3643	lpxB \ pgsB - lipid A-disaccharide synthase (Class 2)
			4081045	4080947	98	SENSE	PA3644	lpxA - UDP-N-acetylglucosamine acyltransferase (Class 2)
S828F	Lethal	Defect	5439259	5439758	499	SENSE	PA4844	probable chemotaxis transducer (Class 3)
			5439759	5439771	12		IR	
			1237546	1237480	66	ANTISENSE	PA1145	probable transcriptional regulator
			1237551	1238001	450	ANTISENSE	PA1146	probable iron-containing alcohol dehydrogenase (Class 3)
S841F	Lethal	Defect	5525057	5525437	382	ANTISENSE	PA4926	conserved hypothetical protein (Class 4)
S82G	Defect	Normal	3081915	3081418	498	ANTISENSE	PA2727	hypothetical protein (Class 4)
			5200935	5201381	446	SENSE	PA4633	probable chemotaxis transducer (Class 3)
S9A9	Defect	Normal	294329	294798	469	ANTISENSE	PA0262	conserved hypothetical protein (Class 4)
S9B6a	Lethal	Defect	1176726	1176982	256	SENSE	PA1089	conserved hypothetical protein (Class 4)
			1176958	1177036	78	SENSE	PA1090	conserved hypothetical protein (Class 4)
S9B6b	Lethal	Defect	434830	434894	64	ANTISENSE	PA0393	proC - pyrroline-5-carboxylate reductase (Class 1)
			434669	434819	150	ANTISENSE	PA0392	yggT - conserved hypothetical protein (Class 4)
S9C8	Lethal	Defect	234467	234112	355	ANTISENSE	PA0205	probable permease of ABC transporter (Class 3)
			153848	153837	11		IR	
			153836	153717	119	ANTISENSE	PA0135	hypothetical protein (Class 4)
S9C9	Defect	Normal	6037340	6036772	568	SENSE	PA5368	pstC - membrane protein component of ABC phosphate transporter (Class 1)
			2100707	2100289	418	ANTISENSE	PA1923	hypothetical protein (Class 4)
S9C59	Defect	Normal	154366	153837	529		IR	
			153836	153717	119	ANTISENSE	PA0135	hypothetical protein (Class 4)
			4569651	4569624	27		IR	
			4569623	4569283	340	SENSE	PA4086	cupB1 - probable fimbrial subunit CupB1 (Class 2)

S9E5	Defect	Normal	4145666	4144939	727	SENSE	PA3703	wspF - probable methylesterase (Class 3)
			4144938	4144832	106			
			4144831	4144743	88	SENSE	PA3702	wspR - WspR (Class 1)
S9G5	Defect	Normal	2674616	2674704	88	ANTISENSE	PA2402	probable non-ribosomal peptide synthetase (Class 3)
S10A3	Lethal	Normal	348093	348439	346		IR	
			348545	348440	105	ANTISENSE	PA0307	hypothetical protein (Class 4)
S10C8	Lethal	Normal	587898	587417	481	SENSE	PA0528	probable transcriptional regulator (Class 3)
			4014406	4014039	367	ANTISENSE	PA3582	glpK [Glycerokinase] - glycerol kinase (Class 1)
SC10D4	Lethal	Normal	2601262	2601490	228	ANTISENSE	PA2354	probable transcriptional regulator (Class 3)
			3453439	3452908	531	SENSE	PA3079	hypothetical protein (Class 4)
S10E3	Lethal	Normal	4657107	4657418	311	SENSE	PA4161	ferric enterobactin transport protein FepG (Class 2)
			4657429	4657617	188	ANTISENSE	PA4162	probable short-chain dehydrogenase (Class 3)
S10F8	Defect	Normal	5838470	5838167	303	SENSE	PA5186	probable iron-containing alcohol dehydrogenase (Class 3)
S11D9	Lethal	Normal	4615670	4616010	340	SENSE	PA4127	hpcG | hpaH - 2-oxo-hept-3-ene-1,7-dioate hydratase (Class 2)
			4215993	4216467	474	ANTISENSE	PA3763	purL - phosphoribosylformylglycinamidine synthase (Class 2)
			3118114	3118193	79	ANTISENSE	PA2757	hypothetical protein (Class 4)
			3118194	3118269	75		IR	
			2593153	2592957	196	ANTISENSE	PA2346	conserved hypothetical protein (Class 4)
S11E4	Lethal	Normal	1642573	1642998	425	ANTISENSE	PA1511	conserved hypothetical protein (Class 4)
			6093091	6093166	75		IR	
			6093167	6093317	150	ANTISENSE	PA5414	hypothetical protein (Class 4)
			2835461	2835306	155	SENSE	PA2518	xylX -toluate 1,2-dioxygenase alpha subunit-17 (Class 2)
S11F7	Lethal	Lethal	4775550	4775812	262	ANTISENSE	PA4269	rpoC-DNA-directed RNA polymerase beta* chain (Class 2)
S11F9a	Lethal	Normal	3453439	3452912	527	SENSE	PA3079	hypothetical protein (Class4)
			2601262	2601490	228	ANTISENSE	PA2354	probable transcriptional regulator (Class 3)
S11B4	Defect	Normal	3256405	3256611	206	ANTISENSE	PA2902	hypothetical protein (Class 4)
			3256612	3256680	68		IR	
			3256681	3256742	61	ANTISENSE	PA2903	precorrin-3 methylase CobJ (Class 2)
			3278160	3278060	100	ANTISENSE	PA2922	probable hydrolase (Class 3)
			5945438	5945933	495	SENSE	PA5281	yigB -probable hydrolase (Class 3)
			5945934	5945962	28		IR	
			5945963	5946045	82	ANTISENSE	PA5282	probable major facilitator superfamily (MFS) transporter (Class 3)
S11C6a	Lethal	Lethal	3453439	3452908	531	SENSE	PA3079	hypothetical protein (Class 4)
			2601262	2601490	228	ANTISENSE	PA2354	probable transcriptional regulator (Class 3)
S11C9	Lethal	Normal	3788559	3789144	585	ANTISENSE	PA3382	phosphonate transport protein PhnE-13 (Class 2)
S11B8	Lethal	Lethal	990798	990828	30	ANTISENSE	PA0904	lysC (ask,akaB) aspartate kinase alpha and beta chain (Class 2)
			990829	991012	183		IR	
			991013	991198	185	ANTISENSE	PA0905	rsmA (csrA) carbon storage regulator (Class 1)
			991199	991260	61		IR	
			991260	991350	90	ANTISENSE	PA0905.1	tRNA-Ser (Class 2)
			991351	991468	117		IR	
			991469	991477	8	ANTISENSE	PA0905.2	tRNA-Arg (Class 2)
			5417750	5417935	185	ANTISENSE	PA4825	mgtA -Mg(2+) transport ATPase, P-type 2 (Class 2)

			2717298	2717430	132	ANTISENSE	PA2424	pvdL (Class 1)
S11C13	Lethal	Lethal	6125830	6126057	227	ANTISENSE	PA5441	hypothetical protein(Class 4)
			4347794	4347698	96	ANTISENSE	PA3880	conserved hypothetical protein (Class 4)
S11B13	Lethal	Lethal	5238083	5237858	225	ANTISENSE	PA4669	ipk isopentenyl monophosphate kinase ychB (Class 2)
S11C5	Defect	Defect	5276309	5276735	426	SENSE	PA4697	hypothetical protein (Class 4)
			5276736	5276842	106		IR	
			5276843	5276989	146	ANTISENSE	PA4698	hypothetical protein (Class 4)
S11G10	Lethal	Normal	4129492	4129146	346	SENSE	PA3687	ppc - phosphoenolpyruvate carboxylase (Class 2)
S11E5	Lethal	Normal	2825463	2824722	741	SENSE	PA2507	catA - catechol 1,2-dioxygenase (Class 2)
			1556884	1556766	118	ANTISENSE	PA1429	probable cation-transporting P-type ATPase (Class 3)
S11F13	Defect	Normal	1622665	1622929	264	ANTISENSE	PA1494	conserved hypothetical protein (Class 4)
			5196570	5196456	114	ANTISENSE	PA4628	lysP - lysine-specific permease (Class 2)
S11F14	Lethal	Normal	1931144	1931474	330	ANTISENSE	PA1783	nasA - nasT nitrate transporter (Class 2)
			6074820	6074559	261	ANTISENSE	PA5396	hypothetical protein (Class 4)
			4803013	4803126	113	ANTISENSE	PA4284	recB - rorA - exodeoxyribonuclease V beta chain (Class 2)
			1295287	1295255	32	SENSE	PA1194	probable amino acid permease (Class 3)
S11D5	Defect	Normal	534945	534676	269	ANTISENSE	PA0473	psfA - probable glutathione S-transferase (Class 3)
			535085	534946	139		IR	
			535399	535086	313	ANTISENSE	PA0474	hypothetical protein (Class 4)

[a] Inserts resulting from the first pilot SAL screening are in bold.
[b] SAL recipient PAO1 exconjugants phenotype in presence (+ ARA) and absence (- ARA) of the P_{BAD} inducer.
[c] End coordinates of the insert according to the Pseudomonas Genome Database (www.pseudomonas.com) [1].
[d] Insert orientation relatively to the P_{BAD} of the cloning vector.
[e,f] PA number and annotations according to the Pseudomonas Genome Database (www.pseudomonas.com) [1]. IR: intergenic region.

1. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS: **Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes.** *Nucleic acids research* 2011, **39:**D596-600.

Table S3. PAO1 growth-impairing inserts including multiple *loci*

Insert name[a]	Insert-included *loci*[b]	Gene name and product annotation[c]	Species containing orthologs in DEG[d]
S3C3	PA4276	*secE* \ *prlG* - secretion protein SecE (Class 2)	Bs, Vc, Ec, Hi, Mg, Mt, Fn, Ab, Se, Sa, Ec, Ss
	PA4276.1	*tRNA-Trp* Rho-independent transcription terminator (Class 2)	
	PA4277	*tufB* - elongation factor Tu (Class 2)	Ec
S4D10	**PA3644**	*lpxA* - UDP-N-acetylglucosamine acyltransferase (Class 2)	Ec, Hp, St, Fn, Pa, Se,Ec
	PA3645	*fabZ* - sefA - (3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase (Class 2)	Vc, Ec, Hi, Sp, Fn, Ab, Pa, Se, Sa, Ec, Cc, Ss
	PA3646	*lpxD* \ *omsA* \ *firA* - UDP-3-O-[3-hydroxylauroyl] glucosamine N-acyltransferase (Class 2)	Vc, Ec, Hi, Hp, Fn, Ab, Se, Cc, Pg
E2	PA0581	*ygiH* - conserved hypothetical protein (Class 4)	
	PA0580	*gcp**ygjD* O-sialoglycoprotein endopeptidase (Class 2)	Bs, Vc, Ec, Hi, Mg, Mt, Fn, Ab, Se, Sa, Ec, Ss
B5	PA1941	hypothetical protein (Class 4)	
	PA1796	*folD* - 5,10-methylene-tetrahydrofolate dehydrogenase (Class 2)	Bs, Ec, Mg, Sp, Mt, St, Fn, Ab, Mp, Pa, Se, Ec
A1	PA5081	hypothetical protein (Class 4)	
	PA5082	probable binding protein component of ABC transporter (Class 3)	
	PA4601	*morA* - motility regulator (Class 1)	
	PA4602	*glyA3* - serine hydroxymethyltransferase (Class 2)	Bs, Sa, Ec, Mg, Fn, Ab, Mp, Pa, Se, Ec
S817E	PA0451	conserved hypothetical protein (Class 4)	
	PA3643	*lpxB* \ *pgsB* - lipid A-disaccharide synthase (Class 2)	Vc, Ec, Fn, Ab, Pa, Se
	PA3644	*lpxA* - UDP-N-acetylglucosamine acyltransferase (Class 2)	Ec, Hp, St, Fn, Pa, Se,Ec
S5D11	PA2762	hypothetical protein (Class 4)	
	PA1532	*dnaX* - DNA polymerase subunits gamma and tau (Class 2)	Bs, Sa, Ec, Fn, Ab, Mp, Se, Ss
	PA1533	conserved hypothetical protein (Class 4)	
	PA1534	*recR* - recombination protein RecR (Class 2)	Pg
S9B6b[e]	PA0393	*proC* - pyrroline-5-carboxylate reductase (Class 1)	Ec, Mt, Ab,
	PA0392	*yggT* - conserved hypothetical protein (Class 4)	
D1	PA5544	conserved hypothetical protein (Class 4)	
	PA3610	*potD* - polyamine transport protein PotD (Class 2)	Ec, Hi, Mp
S11D9	PA4127	*hpcG* | hpaH - 2-oxo-hept-3-ene-1,7-dioate hydratase (Class 2)	
	PA3763	*purL* - phosphoribosylformylglycinamidine synthase (Class 2)	Hi, Mt, Ab
	PA2757	hypothetical protein (Class 4)	
	PA2346	conserved hypothetical protein (Class 3)	
S10C8	PA0528	probable transcriptional regulator (Class 3)	
	PA3582	*glpK* - glycerol kinase (Class 1)	Sa, Mg
M7G4	PA5287	*amtB* - ammonium transporter AmtB (Class 1)	
	PA3736	*hom* - homoserine dehydrogenase (Class 1)	Ab
	PA3088	conserved hypothetical protein (Class 3)	
S11B8	PA0904	*lysC* (*ask*,*akaB*) aspartate kinase alpha and beta chain (Class 2)	Hp, Ab
	PA0905	rsmA (csrA)carbon storage regulator (Class 1)	
	PA0905.1	tRNA-Ser	
	PA0905.2	tRNA-Arg	
	PA4825	*mgtA* -Mg(2+) transport ATPase, P-type 2 (Class 2)	Mp
	PA2424	*pvdL* (Class 1)	
E4	PA4588	*gdhA* - glutamate dehydrogenase (Class 2)	
	hPA4589	*fadL* - probable outer membrane protein precursor (Class 3)	St
	PA3249	probable transcriptional regulator (Class 3)	
G4	PA5207	probable phosphate transporter (Class 3)	

	PA3419	hypothetical protein (Class 4)	
	PA3418	*ldh* - leucine dehydrogenase (Class 2)	Hi
S9C9	PA5368	*pstC* - membrane protein component of ABC phosphate transporter (Class 1)	Ss
	PA1923	hypothetical protein (Class 4)	
S10E3	PA4161	ferric enterobactin transport protein FepG (Class 2)	Se
	PA4162	probable short-chain dehydrogenase (Class 3)	
D2	PA1027	probable aldehyde dehydrogenase (Class 3)-	
	PA3809	*fdx2* - ferredoxin [2Fe-2S] (Class 2)	Pa
S11F14	PA1783	*nasA* - nitrate transporter (Class 2)	
	PA5396	hypothetical protein (Class 4)	
	PA4284	*recB* - *rorA* - exodeoxyribonuclease V beta chain (Class 2)	Hi
	PA1194	probable amino acid permease (Class 3)	
E6[e]	PA1037	*yicG* - conserved hypothetical protein (Class 4)	
	PA1038	hypothetical protein (Class 4)	
S2A4[e]	PA1001	*phnA* - anthranilate synthase component I (Class 1)	
	PA1002	*phnB* - anthranilate synthase component II (Class 1)	
S9B6a[e]	PA1089	conserved hypothetical protein (Class 4)	
	PA1090	conserved hypothetical protein (Class 4)	
M7H8	PA1800	*tig* - trigger factor (Class 2)	
	PA1799	*parR* - two-component response regulator, ParR (Class 1)	
S9E5	PA3703	*wspF* - probable methylesterase (Class 3)	
	PA3702	*wspR* - WspR (Class 1)	
S11C5	PA4697	hypothetical protein (Class 4)	
	PA4698	hypothetical protein (Class 4)	
S11D5	PA0473	*psfA* - probable glutathione S-transferase (Class 3)	
	PA0474	hypothetical protein (Class 4)	
M7E2	PA3964	hypothetical protein (Class 4)	
	PA3963	*yiiP* - probable transporter (Class 3)	
	PA0536	hypothetical protein (Class 4)	
M2D2	PA4574	conserved hypothetical protein (Class 4)	
	PA2394	*pvdN* (Class 1)	
D5	PA1878	hypothetical protein (Class 4)	
	PA1877	probable secretion protein (Class 3)	
	PA5473	conserved hypothetical protein (Class 4)	
G1	PA4630	hypothetical protein (Class 4)	
	PA1847	*yhgI* - conserved hypothetical protein (Class 4)	
S4B7	PA4041	hypothetical protein (Class 4)	
	PA2772	hypothetical protein (Class 4)	
S828F	PA4844	probable chemotaxis transducer (Class 3)	
	PA1145	probable transcriptional regulator (Class 3)	
	PA1146	probable iron-containing alcohol dehydrogenase (Class 3)	
S82G	PA2727	hypothetical protein (Class 4)	
	PA4633	probable chemotaxis transducer (Class 3)	
S9C8	PA0205	probable permease of ABC transporter (Class 3)	
	PA0135	hypothetical protein (Class 4)	
S9C59	**PA0135**	hypothetical protein (Class 4)	
	PA4086	*cupB1* - probable fimbrial subunit CupB1 (Class 2)	
SC10D4	**PA2354**	probable transcriptional regulator (Class 3)	
	PA3079	hypothetical protein (Class 4)	

S11E4	PA1511	conserved hypothetical protein (Class 3)
	PA5414	hypothetical protein (Class 4)
	PA2518	*xylX* -toluate 1,2-dioxygenase alpha subunit (Class 2)
S11F9a	**PA3079**	hypothetical protein (Class4)
	PA2354	probable transcriptional regulator (Class 3)
S11B4	PA2902	hypothetical protein(Class4)
	PA2903	precorrin-3 methylase CobJ (Class 2)
	PA2922	probable hydrolase (Class 3)
	PA5281	*yigB* -probable hydrolase (Class 2)
	PA5282	probable major facilitator superfamily (MFS) transporter (Class 3)
S11C6a	**PA3079**	hypothetical protein (class 4)
	PA2354	probable transcriptional regulator (Class 3)
S11C13	PA5441	hypothetical protein(Class 4)
	PA3880	conserved hypothetical protein (Class 4)
S11E5	PA2507	*catA* - catechol 1,2-dioxygenase (Class 2)
	PA1429	probable cation-transporting P-type ATPase (Class 3)
S11F13	PA1494	conserved hypothetical protein (Class 3)
	PA4628	*lysP* - lysine-specific permease (Class 2)

[a] Non-chimeric inserts are in bold.
[b] *Loci* present in more than one insert are in bold.
[c] Annotations according to the Pseudomonas Genome Database (www.pseudomonas.com) [1].
[d] DEG: Database of Essential Genes (DEG 7.0) (www.essentialgene.org) [2]. Bacterial species: Ab (*Acinetobacter baylyi*), Bs (*Bacillus subtilis*), Bt (*Bacteroides thetaiotaomicron*), Cc (*Caulobacter crescentus*), Ec (*Escherichia coli*), Fn (*Francisella novicida*), Hf (*Haemophilus influenzae*), Hp (*Helicobacter pylori)*, Mt (*Mycobacterium tuberculosis*), Mp (*Mycoplasma pulmonis*), Mg (*Mycoplasma genitalium*), Pg (*Porphyromonas gingivalis*), Pa (*Pseudomonas aeruginosa*), Se (*Salmonella enterica*), St (*Salmonella typhimurium*), Sp (*Streptococcus pneumoniae*), Ss (*Streptococcus sanguinis*), Sa (S*taphylococcus aureus*), Vc (*Vibrio cholerae*).
[e] Inserts spanning adjacent ORFs belonging to an operon.

1. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS: **Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes.** *Nucleic acids research* 2011, **39:**D596-600.
2. Zhang R, Lin Y: **DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes.** *Nucleic acids research* 2009, **37:**D455-458.

Table S4. Additional information on a selection of *P. aeruginosa* "classical" essential genes.

Locus	**Gene name and product annotation[a]**	**Putative orthologs in *Pseudomonas* species[b]**	**Transposon insertion (N[c], IP[d])**	**Notes**
PA0577	*dnaG* DNA primase	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	1(TA), 1992/1995; 1(TL), 1990/1995	PA14 ortholog was Tn inserted in [1]
PA5493	*polA* DNA polymerase I	Pae(10/12),Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TA), 2153/2742 - 1628/2742; 3(TL), 1158/2742 - 2328/2742 - 2309/2742	PA14 ortholog was Tn inserted in [1]
PA3832	*holC* DNA polymerase III, chi subunit	Pae(12/12),Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy		PA14 ortholog was Tn inserted in [1]
PA2961	*holB* DNA polymerase III, delta subunit	Pae(12/12) Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TA), 971/987 - 963/987; 2(TL), 953/987	PA14 ortholog was Tn inserted in [1]
PA4760	*dnaJ* protein	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppr, Ppu , Pre, Pst, Psy		PA14 ortholog was Tn inserted in [1]
PA4761	*dnaK* molecular chaperone DnaK	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy		PA14 ortholog was Tn inserted in [1]
PA4269	*rpoC* RNA polymerase beta* chain	Pae(10/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy		PA14 ortholog was Tn inserted in [1]
PA2473	*infC* Translation initiation factor IF-3	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy		PA14 ortholog was Tn inserted in [1]
PA0373	*ftsY* cell division protein	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	1(TA), 1359/1368	PA14 ortholog was Tn inserted in [1]
PA0374	*ftsE* cell division protein	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TA), 196/672 - 268/672	PA14 ortholog was Tn inserted in [1]
PA0375	*ftsX* cell division protein	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	1(TA), 36/1008; 1(TL) 548/1008	PA14 ortholog was Tn inserted in [1]

[a] Annotations according to the Pseudomonas Genome Database (www.pseudomonas.com)[2].

[b] For each hit, the computationally-predicted putative orthologs that were found in the Pseudomonas Genome Database (www.pseudomonas.com) [2] are indicated with the abbreviation of the harboring bacterial species. For *Pseudomonas aeruginosa*, the number of strains harboring orthologs over the total of 12 sequenced strains considered for the analysis at the Pseudomonas Genome Database is indicated inside parenthesis. Species name abbreviations: Pae (*P. aeruginosa*), Pen (*P. entomophila*), Pme (*P. mendocina*), Pre (*P. resinovorans*), Pfl (*P. fluorescens*), Pbr (*P. brassicacearum*), Ppr (*P. protegens*), Pde (*P. denitrificans*), Ppu (*P. putida*), Pfu (*P. fulva*), Pst (*P. stutzeri*), Psy (*P. syringae*), Ppo (*P. poae*).

[c] **N**: number of transposon insertions in the PA Two Allele Transposon Library (TA) [3] and/or in the Tn5 lux Transposon Mutant Library (TL) [4].

[d] **IP**: transposon insertion site given as number of base pairs from the gene 5' / total gene length.

References

1. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM: **An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants.** *P Natl Acad Sci USA* 2006, **103:**2833-2838.
2. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS: **Pseudomonas Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes.** *Nucleic acids research* 2011, **39:**D596-600.
3. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, et al: **Comprehensive transposon mutant library of *Pseudomonas aeruginosa*.** *Proc Natl Acad Sci U S A* 2003, **100:**14339-14344.
4. Lewenza S, Falsafi RK, Winsor G, Gooderham WJ, McPhee JB, Brinkman FS, Hancock RE: **Construction of a mini-Tn5-luxCDABE mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes.** *Genome Res* 2005, **15:**583-589.

Table S5. Additional information on novel *P. aeruginosa* candidate essential genes.

Locus[a]	Gene name and product annotation[b]	Putative orthologous in *Pseudomonas* species[c]	Transposon insertion (N[d], IP[e])	Notes
PA2951	*etfA* - electron transfer flavoprotein alpha-subunit	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	0(TA) ; 0(TL)	PA2951 orthologs are reported as essential in *Acinetobacter baylyi*, *Caulobacter crescentus* [1].
PA3687	*ppc* - phosphoenolpyruvate carboxylase	Pae(10/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TA), 2357/2637 - 1408/2637	PA3687 orthologs are reported as essential in *Haemophilus influenzae* [1]
PA3758	*nagA* - probable N-acetylglucosamine-6-phosphate deacetylase	Pae(12/12), Pbr, Pen, Pfl, Ppo, Ppr	2(TA), 552/1092 - 556/1092	PA3785 orthologs are reported as essential in *Haemophilus influenzae*, *Mycobacterium tuberculosis* [1]
PA1183	*dctA* - C4-dicarboxylate transport protein	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TA), 286/1311 - 685/1311	PA1183 orthologs are reported as essential in *Acinetobacter baylyi* [1]
PA1805	*ppiD* - peptidyl-prolyl cis-trans isomerase D - Rotamase D	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	1(TA), 1429/1866	PA1805 orthologs are reported as essential in *Caulobacter crescentus*, *Bacillus subtilis* [1]
PA5186	probable iron-containing alcohol dehydrogenase	Pae(12/12), Pde, Pfu, Pme, Ppu, Pre, Pst	2(TA), 42/1164 - 797/1164; 1(TL), 524/1164	
PA1554	*ccoN1*\Cytochrome c oxidase, cbb3-type, CcoN subunit	Pae(12/12), Pbr, Pd, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy,	3(TA), 1188/1428 - 885/1428 - 419/1428	Shown to be critical for aerobic growth [2].
PA3382	*phnE* - phosphonate transport protein PhnE	Pae(12/12), Pde, Pfl, Pme, Ppu, Pst, Psy	3(TA), 661/795 - 450/795 - 298/795; 1(TL), 652/795	The phosphonate transporter function was reported as essential in *Mycoplasma pulmonis* [1].
PA4903	*vanK* - probable major facilitator superfamily transporter	Pae(12/12), Pde, Pfl, Pfu, Ppr, Ppu, Pre	1(TA), 713/1335	
PA5548	probable major facilitator superfamily transporter	Pae(12/12)	1(TA), 611/1206	
PA1709	*popD* - Translocator outer membrane protein	Pae(9/12)	1(TA), 871/888	
PA1590	*braB* - branched chain amino acid transporter	Pae(12/12), Pme, Pst	1(TA), 1080/1314	
PA2402	Probable non-ribosomal peptide synthetase	Pae(12/12), Pbr, Pde, Pen, Pfl, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TA), 10034/15450 - 462/15450	50% identity with the peptide synthase MbtE reported as essential in *Haemophilus influenzae* [1].
PA5238	probable O-antigen acetylase	Pae(12/12), Pbr, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TL), 915/1989 - 1698/1989	
PA3433	*ywbI* - probable transcriptional regulator	Pae(12/12), Pde, Pen, Pfl, Ppo, Ppr, Ppu, Pre, Psy	0(TA) ; 0(TL)	
PA2220	*oprR* - probable transcriptional regulator	Pae(3/12), Pbr, Pde, Pfl, Ppr, Ppu, Pst, Psy	1(TA), 58/921	
PA2873	*tgpA* - transglutaminase protein A TgpA	Pae(12/12), Pde, Pfl, Pme, Pre, Pst, Psy	1(TA), 1568/2007; 1(TL) 1042/2007	
PA0307	hypothetical protein	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst	2(TA), 451/612 - 408/612	
PA4926	conserved hypothetical protein	Pae(12/12), Pbr, Pfl, Pfu, Pme, Ppo, Ppr, Pre, Psy	1(TA), 29/936	
PA0262	conserved hypothetical protein	Pae(12/12), Ppu, Pre, Psy	9(TL), 1703/3060 - 1223/3060 - 1214/3060 - 1203/3060 - 396/3060 - 1242/3060 - 1244/3060 - 1483/3060 - 1219/3060	
PA5264	hypothetical protein	Pae(8/12), Pde, Pst, Psy	2(TA), 567/972 - 559/972; 1(TL), 851/972	

Locus[a]	Gene name and product annotation[b]	Putative orthologous in *Pseudomonas* species[c]	Transposon insertion (N[d], IP[e])	Notes
PA1037	*yicG* - conserved hypothetical protein	Pae(12/12), Pbr, Pde, Pfl, Pen, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TA), 331/621 - 85/621; 2(TL), 258/621 - 449/621	
PA1038	hypothetical protein	Pae(12/12)	2(TA), 109/318 - 82/318; 2(TL), 112/318 - 164/318	
PA1039	*ychJ* - hypotetical protein	Pae(12/12), Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	1(TA), 230/474	
PA1040	hypothetical protein	Pae(12/12),Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	1(TA), 494/498	
PA1089	conserved hypothetical protein	Pae(4/12), Pme	1(TA), 490/603	
PA1090	conserved hypothetical protein	Pae(4/12), Pme, Pfl	1(TA), 198/663	
PA1088	hypothetical protein	Pae(4/12), Pfu, Pme	1(TA), 37/762	
PA0392	*yggT* - conserved hypothetical protein	Pae(12/12),Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	1(TA), 85/594	
PA0393	*proC* - pyrroline-5-carboxylate reductase	Pae(12/12),Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	2(TA), 610/822 -414/822; 2(TL), 744/822 -743/822	PA0393 orthologs are reported as essential in *Escherichia coli*, *Mycobacterium tuberculosis*, *Acinetobacter baylyi* [1].
PA0394	*yggS* - conserved hypothetical protein	Pae(12/12),Pbr, Pde, Pen, Pfl, Pfu, Pme, Ppo, Ppr, Ppu, Pre, Pst, Psy	1(TA), 351/693	
PA1001	*phnA* - anthranilate synthase component I	Pae(12/12)		
PA1002	*phnB* - anthranilate synthase component I	Pae(12/12)	1(TA), 184/603	

[a] *Loci* from Table 2 belonging to a single operon are in bold.

[b] Annotations according to the Pseudomonas Genome Database (www.pseudomonas.com)[3].

[c] For each hit, the computationally-predicted putative orthologs that were found in the Pseudomonas Genome Database (www.pseudomonas.com) [3] are indicated with the abbreviation of the harboring bacterial species. For *Pseudomonas aeruginosa*, the number of strains harboring orthologs over the total of 12 sequenced strains considered for the analysis at the Pseudomonas Genome Database is indicated inside parenthesis. Species name abbreviations: Pae (*P. aeruginosa*), Pen (*P. entomophila*), Pme (*P. mendocina*), Pre (*P. resinovorans*), Pfl (*P. fluorescens*), Pbr (*P. brassicacearum*), Ppr (*P. protegens*), Pde (*P. denitrificans*), Ppu (*P. putida*), Pfu (*P. fulva*), Pst (*P. stutzeri*), Psy (*P. syringae*), Ppo (*P. poae*).

[d] **N**: number of transposon insertions in the PA Two Allele Transposon Library (TA) [4] and/or in the Tn5 lux Transposon Mutant Library (TL) [5]. For all hits, a PA14 ortholog was Tn inserted in [6].

[e] **IP**: transposon insertion site given as number of base pairs from the gene 5' / total gene length.

References

1. Zhang R, Lin Y: **DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes.** *Nucleic acids research* 2009, **37:**D455-458.
2. Comolli JC, Donohue TJ: **Differences in two *Pseudomonas aeruginosa* cbb3 cytochrome oxidases.** *Molecular microbiology* 2004, **51:**1193-1203.
3. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS: **Pseudomonas Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes.** *Nucleic acids research* 2011, **39:**D596-600.
4. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, et al: **Comprehensive transposon mutant library of *Pseudomonas aeruginosa*.** *Proc Natl Acad Sci U S A* 2003, **100:**14339-14344.
5. Lewenza S, Falsafi RK, Winsor G, Gooderham WJ, McPhee JB, Brinkman FS, Hancock RE: **Construction of a mini-Tn5-luxCDABE mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes.** *Genome Res* 2005, **15:**583-589.
6. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM: **An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants.** *P Natl Acad Sci USA* 2006, **103:**2833-2838.

OPEN ACCESS Freely available online

PLOS ONE

TgpA, a Protein with a Eukaryotic-Like Transglutaminase Domain, Plays a Critical Role in the Viability of *Pseudomonas aeruginosa*

Andrea Milani, Davide Vecchietti, Ruggero Rusmini, Giovanni Bertoni*

Department of Life Sciences, Università degli Studi di Milano, Milan, Italy

Abstract

The Gram-negative bacterium *Pseudomonas aeruginosa* is an important opportunistic pathogen in compromised individuals, such as patients with cystic fibrosis, severe burns or impaired immunity. In this work we aimed to screen novel essential genes of *P. aeruginosa* by shotgun antisense identification, a technique that was developed a decade ago for the Gram-positive bacterium *Staphylococcus aureus* and was under-used in Gram-negative bacteria for a considerable period of time. Following antisense screenings in the PAO1 strain of *P. aeruginosa*, we focused on a *locus*, PA2873, which was targeted by an antisense RNA construct that can impair cell growth. The PA2873 gene product was annotated as a hypothetical membrane protein endowed with a periplasmic region harbouring a structural domain belonging to the transglutaminase-like superfamily, a group of archaeal, bacterial and eukaryotic proteins homologous to animal transglutaminases. In this study, we show that the periplasmic portion of the PA2873 protein, which we named TgpA, does possess transglutaminase activity *in vitro*. This is the first report of transglutaminase activity in *P. aeruginosa*. In addition, we have provided strong evidences that TgpA plays a critical role in the viability of *P. aeruginosa*.

Citation: Milani A, Vecchietti D, Rusmini R, Bertoni G (2012) TgpA, a Protein with a Eukaryotic-Like Transglutaminase Domain, Plays a Critical Role in the Viability of *Pseudomonas aeruginosa*. PLoS ONE 7(11): e50323. doi:10.1371/journal.pone.0050323

Editor: Gunnar F. Kaufmann, The Scripps Research Institute and Sorrento Therapeutics, Inc., United States of America

Received July 6, 2012; **Accepted** October 18, 2012; **Published** November 27, 2012

Funding: This work was funded by Italian Cystic Fibrosis Research Foundation (grants FFC#10/2004, FFC#6/2006, FFC#6/2007) and by European Commission (grant NABATIVI, EU-FP7-HEALTH-2007-B contract number 223670). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: giovanni.bertoni@unimi.it

Introduction

P. aeruginosa is a highly adaptable bacterium which thrives in a broad range of ecological niches. In addition, it can infect multiple hosts as diverse as plants, nematodes and mammals. The broad habitat and host ranges of *P. aeruginosa* reflect the large variety of structural, metabolic and virulence functions found in its large pangenome [1–4]. In humans, it is an important opportunistic pathogen in compromised individuals, such as patients with cystic fibrosis, severe burns or impaired immunity [5,6]. Unfortunately, *P. aeruginosa* is difficult to control because of its ability to develop resistances, often multiple, to all classes of clinical antibiotics [7–9]. The common *P. aeruginosa* mechanisms of antibiotic resistance often appear simultaneously and are cephalosporinase AmpC-, porin OprD- and efflux pumps-mediated [8]. Consequently, *P. aeruginosa* is a major concern to medical practitioners who increasingly face extremely-drug resistant strains (i.e. bacterial isolates susceptible to only one or two antibiotic categories) [9] which may require carefully-selected antibiotic combinations [10].

The discovery of novel essential genes or pathways that have not yet been targeted by clinical antibiotics can underlie the development of alternative effective antibacterials to overcome the extant mechanisms of resistance. We screened novel essential genes of *P. aeruginosa* by shotgun antisense identification, a technique that was developed a decade ago in *Staphylococcus aureus* [11,12]; following a period of limited success in Gram-negative bacteria [13,14], the technique has been used effectively in *E. coli* [15]. Following our shotgun antisense screenings in *P. aeruginosa* PAO1, we focused on a *locus*, PA2873, which was targeted by an antisense RNA construct that can impair cell growth. The features of the predicted protein encoded by PA2873 *locus* were intriguing. In fact, it was annotated in the Pseudomonas Genome Database [16] as a hypothetical membrane protein endowed with a periplasmic region harbouring a structural domain belonging to the transglutaminase-like superfamily, a group of archaeal, bacterial and eukaryotic proteins homologous to animal transglutaminases [17]. In this study we show that the periplasmic portion of the PA2873 protein does have transglutaminase activity *in vitro*. This is the first report of a transglutaminase activity in *P. aeruginosa*. The PA2873 gene product was called "transglutaminase protein A" (TgpA). In addition, we provided strong evidences that TgpA plays a critical role in the viability of *P. aeruginosa*.

Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table S1. pVLT31 [18] is an *E. coli/Pseudomonas* shuttle vector carrying the $lacI^q/P_{tac}$ pair and tetracycline resistance. pVI533EH [19] and pHERD20T [20], used for the construction of antisense libraries (see below), are *E. coli/Pseudomonas* shuttle vectors carrying *araC*/P_{BAD} pair and ampicillin/carbenicillin resistance. Since pHERD20T allows blue/white screening for the identification of recombinants, it was used in place of pVI533EH at later stages of

antisense libraries preparation. pVI533HE is a pVI533EH twin-plasmid carrying the polylinker sequence in opposite orientation downstream P_{BAD} promoter. pDM4 [21] is a suicide plasmid in *Pseudomonas* species carrying the chloramphenicol resistance. pVLT31-M4G6 and pVI533HE-M4G6i were obtained by cloning the *Eco*RI-*Hind*III insert of pVI533EH-M4G6, identified in the antisense libraries screenings, into pVLT31 and pVI533HE, respectively. pVLT31-M4G6i was obtained by cloning a *Hind*III-*Eco*RI fragment amplified by PCR from pVI533EH-M4G6 with primers M4G6Hind5′ and M4G6Eco3′ (Table S2) into pVLT31. PCR reactions were performed using DreamTaq Polymerase (Fermentas) with the addition of 1 M betaine (Sigma Aldrich), because of the high GC content of *P. aeruginosa* genome.

Bacteria were grown at 37°C in Luria–Bertani (LB) broth, or in M9 minimal medium supplemented with 0.2% citrate (M9-citrate). Antibiotics were added at the following concentrations (μg/ml): carbenicillin (Cb) 300; kanamycin (Km) 50; chloramphenicol (Cm) 30 and 85 for *E. coli* and *P. aeruginosa*, respectively; tetracycline (Tc) 25; gentamicin (Gm) 25 and 50 for *E. coli* and *P. aeruginosa*, respectively. Arabinose, rhamnose, glucose and IPTG were used at concentrations of 7.5 mM, 0.2%, 1%, and 1 mM, respectively. In mating experiments, exconjugant *P. aeruginosa* PAO1 clones were selected on Pseudomonas Isolation Agar (PIA; Difco) with an appropriate antibiotic or on M9-citrate in case of use of tetracycline.

Construction and screening of PAO1 shotgun antisense libraries

Shotgun antisense libraries (SALs) of *P. aeruginosa* PAO1 were constructed in *E. coli* into pVI533EH and pHERD20T as described previously [11,12]. To screen for genomic fragments interfering with PAO1 growth, SALs were mobilized from *E. coli* to PAO1 by conjugative triparental mating. PAO1 exconjugant cell spots were inspected for growth defects after 24–48 hrs of incubation at 37°C, both in the presence and in the absence of arabinose. The inserts pVI533EH and pHERD20T derivatives impairing recipient PAO1 growth were sequenced after PCR amplification using oligo pairs pVI533F/pVI533R and pHERD-F/pHERD-R for pVI533EH and pHERD20T, respectively (Table S2).

Identification of PA2873 gene product in *P. aeruginosa* membrane fractions

P. *aeruginosa* PAO1 cells, grown in LB with aeration at 37°C until OD_{600} of 0.6, were harvested by centrifugation at 4000×g for 15 min at 4°C and washed twice with PBS supplemented with 20% sucrose (TIB buffer). PAO1 cells in TIB buffer were disrupted in a French press device. To remove intact cells that had escaped lysis, crude extracts were filtered through 0.22 μm filters. Following incubation for 1 hr at room temperature with RNase A (Qiagen) and DNase I (Ambion), filtered crude extracts were ultracentrifuged at 135,000×g for 60 min at 4°C to separate total membranes from the cytoplasmic fraction. Pellets containing the membrane fraction were washed in sequence with PBS, 1 M NaCl, and water to remove the contaminants; they were treated overnight under stirring with 40 μg/ml trypsin at 37°C for shaving and the extensive peptide proteolysis required for the Multidimensional Protein Identification Technology (MudPIT) analysis. Trypsin-digested samples were centrifuged at 16,000×g for 1 hr at 4°C and the supernatants were subjected to MudPIT analysis using the ProteomeX configuration (Thermo Fisher, San Josè, CA, USA). The mass spectra produced by MudPIT analyses were correlated to *in silico* peptide sequences of non-redundant *P. aeruginosa* protein database (5753 entries) retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). Data processing of raw spectra was performed by the Bioworks 3.3.1 software (University of Washington, licensed to Thermo Fisher Scientific), based on the SEQUEST algorithm [22].

Purification of TgpA $TG_{180–544}$ domain and transglutaminase activity assay

The TgpA periplasmic domain (aa. 180–544; $TG_{180–544}$) was expressed in *E. coli* as $N(His)_{10}$-tagged protein with an improved His-tag (MGSDKIHHHHHHHHHHGV) under the control of T7 promoter in plasmid p2N[M4G6 (180–544 aa)] (PRIMM srl), and purified using standard protocols of His-tag affinity purification. The fractions containing $TG_{180–544}$ were pooled and stored at −80°C until use in aliquots at the concentration of 2.7 mg/ml and 95% purity, determined by SDS-PAGE.

Transglutaminase activity (TGase; EC 2.3.2.13) was assayed by a Transglutaminase Colorimetric Microassay Kit (Covalab) which uses immobilized N-carbobenzoxy(CBZ)-Gln-Gly as amine acceptor and biotin-conjugated cadaverine as amine donor. Protein samples were incubated in a 96-well microtiter plate coated with CBZ-Gln-Gly at 37°C for 15 min with calcium, DTT and biotinylated cadaverine. The wells were washed three times with phosphate buffer containing 0.1% Tween 20. To assay the formation by TGase of cadaverine covalently linked to CBZ-Gln-Gly (γ-glutamyl-cadaverine-biotin), the wells were filled with streptavidin-labelled horseradish peroxidase (HRP) and incubated for 15 min at 37°C; they were washed three times with phosphate buffer containing 0.1% Tween 20, filled with HRP substrate/chromogen solution containing H_2O_2 as the substrate and tetramethyl benzidine as the electron acceptor (chromogen). These were incubated for 10 min at room temperature, 50 μl of reaction blocking reagent were added and the mixture quantified by measuring OD_{450}. As references for the TGase activity, the kit included purified guinea pig TGase with a specific activity of 0.1 U/mg. By definition, 1 U of TGase catalyzes the formation of 1 μmole of hydroxamate at pH 6.0 at 37°C, using L-glutamic acid γ- monohydroxamate as the standard [23].

RT-PCR analysis of PA2873 genomic region and transcript 5′-end mapping

Total RNA was purified from *P. aeruginosa* PAO1 cells, grown in LB with aeration at 37°C until OD_{600} of 0.6 (mid-exponential phase) or of 1.2 (early stationary phase) was achieved using RNeasy Mini kits (Qiagen) that included DNase I treatment. Residual DNA was removed from purified RNA by further treatment with RNA-free DNase I (Ambion) at 37°C for 15 min, followed by DNase I inactivation with 2.5 mM EDTA at 65°C for 10 min. cDNA was generated by incubating 1 μg of RNA with Superscript II Reverse Transcriptase (RT) (Invitrogen), 100 pg of random primers (Invitrogen) and buffer supplied by the manufacturer for 50 min at 42°C. RT was inactivated by incubation at 70°C for 15 min. As a control of DNA contamination in the subsequent RT-PCR analysis, reactions were also run without RT. RT-PCR analysis was performed with the oligo pairs listed in Table S2.

For mapping 5′-ends of transcripts upstream PA2873 *locus*, a primer extension assay was performed on PAO1 total RNA with oligo 2873_PE60 (Table S2) end-labelled using [γ-^{32}P]-dATP (3000 Ci mmol^{-1}) and polynucleotide kinase (Promega). 50 μg of total RNA were mixed with 10 units of RNasin (Promega) and 1 pmol of ^{32}P-2873_PE60 in a final volume of 10 μl of SS hybridization buffer (300 mM NaCl, 10 mM Tris-HCl pH 7.5,

2 mM EDTA). Reactions were heated at 80°C for 4 min, incubated at 55°C for 2 hrs, diluted with pre-heated RT-buffer (1 mM dNTPs, 10 mM DTT, 12.5 mM Tris-HCl pH 8.0, 7.5 mM $MgCl_2$, 1 M betaine, 5 U of RNasin and 100 U of SuperScript III RT) to a final volume of 50 µl, incubated at 50°C for 30 min, and stopped with 1 µl of 0.5 M EDTA and 6 µl of 1 M NaOH at 55°C for 1 hr. Samples were neutralized with 6 µl of 1 M HCl, precipitated, resuspended in Stop Solution (50% formamide, 5 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), and electrophoresed on 50% urea, 6% acrylamide/bis-acrylamide (19:1) gels in TBE buffer. Reference DNA sequencing reaction was generated using as template a DNA fragment amplified from PAO1 genomic DNA by PCR with oligo pair SeqF/SeqR (Table S2). The DNA sequencing reaction was performed with the fmol DNA Cycle Sequencing System (Promega) with the addition of 1 M betaine.

Mutagenesis analyses

For pDM4 cointegration targeting, internal 600–800 bp DNA fragments of PA2875, PA2874, PA2873, *dnaG* and *algR*, respectively, were amplified by PCR with oligo pairs containing *Sal*I restriction sites listed in Table S2, digested with *Sal*I (New England Biolabs) and cloned into the corresponding site of pDM4. The cloning was checked by PCR with the oligo pair pDM4-ori/pDM4-cat (Table S2). This procedure gave rise to pDM4 derivatives listed in Table S1 which were transferred from *E. coli* to *P. aeruginosa* PAO1 by triparental mating (see above for details) selecting exconjugant PAO1 clones carrying pDM4 cointegration on PIA plates supplemented with Cm.

The conditional mutagenesis of PA2873 *locus* was obtained through upstream insertion of the rhamnose-induced/glucose-repressed P_{rhaB} promoter. The first 300 bp of PA2873 were amplified by PCR with TgFullFw and Tg300RevXbaI oligos (Table S2) carrying *NdeI* and *XbaI* sites, respectively. The resulting DNA fragment was digested with *NdeI* and *XbaI* (New England Biolabs) and cloned into the corresponding sites of the vector pSC200, giving rise to the plasmid pSC200-PA2873, which was mobilized to *P. aeruginosa* PAO1 by triparental mating. Exconjugant PAO1 clones carrying pSC200-PA2873 cointegration were selected on PIA plates supplemented with Gm and rhamnose. One such clone, named PAO1 P_{rhaB}::PA2873, was grown overnight in 20 ml of M9-citrate, Gm and rhamnose as the P_{rhaB} inducer at 37°C with shaking. Cells were collected through centrifugation at 13,000 rpm, washed twice with PBS, and resuspended in a suitable volume of PBS to reach an OD_{600} of 1. To test the effects of P_{rhaB} promoter modulation on growth, the cell suspension was used to inoculate, with a 10^6 -fold dilution, M9-citrate supplemented with Gm, and either with rhamnose (M9-citrate-rhamnose) or glucose (M9-citrate-glucose). 200 µl aliquots of PAO1 P_{rhaB}::PA2873-inoculated M9-citrate-rhamnose and M9-citrate-glucose were distributed in triplicates in "Well Optical Bottom" 96-wells microplates (Nunc, Thermo Fisher Scientific) and incubated for 21 hrs in a Sunrise microplate reader (TECAN Group Ltd.) at 37°C with constant shaking and real time OD measurement at 595 nm every 15 min. In parallel, to test PAO1 P_{rhaB}::PA2873 growth on a solid medium, the cell suspension in PBS at OD_{600} of 1 was serially diluted up to 10^{-7} and spotted on solid M9-citrate with Gm, in the presence of rhamnose or glucose.

Results and Discussion

Identification of an antisense construct targeting PA2873 *locus* and impairing PAO1 growth

To identify novel essential genes in *P. aeruginosa*, we constructed shotgun antisense libraries (SALs) in *E. coli* by cloning genomic DNA fragments of *P. aeruginosa* PAO1 downstream the arabinose inducible promoter P_{BAD} of pVI533EH or pHERD20T. Genomic inserts able to impair PAO1 growth, supposedly by antisense transcription effects, were screened by mating transfer of SALs from *E. coli* to PAO1 and replica-plating of exconjugants on Pseudomonas Isolation Agar (PIA) supplemented with carbenicillin, both in the absence and in the presence of arabinose. These screenings resulted in the identification of several positive pVI533EH and pHERD20T derivatives impairing PAO1 growth when transferred from *E. coli* (manuscript in preparation). As expected, some positives of this panel carried inserts corresponding to already known "essential-for-growth" genes. For instance, in the pHERD20T derivative pHERD-S2F1, in antisense orientation, we detected a 331 bp DNA fragment that spanned from coordinates 637810 to 638141 of PAO1 genome, within the PA0577 *locus* coding for DnaG primase [24].

For a number of positives to SALs screenings, growth impairment was also observed in the absence of arabinose, suggesting that basal antisense expression of the insert in PAO1, a regulatory context for P_{BAD} not as restrictive as *E. coli*, was enough to produce deleterious effects. One such pVI533EH derivative, pVI-M4G6 (Figure 1), was further characterized in this study. The insert of pVI-M4G6 was sequenced and found to correspond to a PAO1 genomic fragment spanning coordinates 3226136 to 3226491 within PA2873 *locus* (from 898 to 1252 positions; PA2873 total length: 2007 bp), which inserted downstream to the P_{BAD} promoter of pVI533EH in antisense orientation. To assess the antisense effect, the insert of pVI-M4G6 was inverted to give rise to pVI-M4G6i and then retested for PAO1 growth impairment. As shown in Figure 1, unlike pVI-M4G6, pVI-M4G6i was unable to impair PAO1 growth once it had been transferred from *E. coli*. To rule out effects of sensitization to Cb, pVI-M4G6 insert was recloned into the Tet^R vector pVLT31 [18], downstream to the $P_{trp\text{-}lac}$ promoter, both in antisense and sense orientations, giving rise to pVL-M4G6 and pVL-M4G6i, respectively. As in the case of pVI533EH, only the antisense expression of insert from pVLT31 again resulted in PAO1 growth impairment (Figure 1). These results strongly suggested that pVI-M4G6-induced growth impairment of PAO1 originated from the expression of a transcript of about 350 nt antisense to PA2873 *locus*. We therefore speculated that the PA2873 *locus* could code for a novel and uncharacterized essential function of PAO1.

Features of PA2873 gene product, *locus* and genomic region

Bioinformatic analyses, listed in Pseudomonas Genome Database (PGD) [16], on the structural features of the PA2873 product (688 aa; Figure 2A), predict that it is an inner membrane protein endowed with six transmembrane helices and a large periplasmic domain between aa 180 and 544 (Figure S3); between aa 396 and 467, it has a highly recognizable structural sub-domain belonging to the transglutaminase-like superfamily (PF01841 in PFAM database [25]), a group of archaeal, bacterial and eukaryotic proteins homologous to animal transglutaminases [17] (Figure S1). It is interesting to note that the 5 transmembrane helices spanning the first 180 aa were within the bacterial domain of unknown function DUF3488 (PF11992 in the PFAM database [25]), typically between 323 to 339 amino acids in length and found to

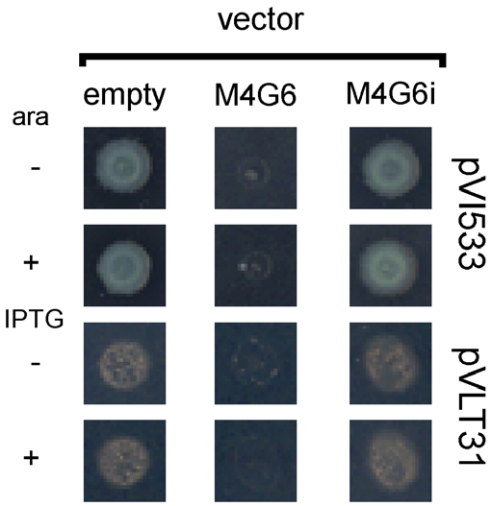


Figure 1. Analysis of the growth impairment elicited by the M4G6 insert resulting from SALs screenings. *E. coli* donors harbouring the pVI533-based vectors pVI-M4G6 and pVI-M4G6i, which carry downstream P_{BAD} promoter the M4G6 insert in antisense and sense orientation, respectively, were mated with *P. aeruginosa* PAO1 and exconjugants were spotted onto PIA to counterselect *E. coli* cells. The medium was also supplemented with carbenicillin to select for pVI533 maintenance. As a control, empty pVI533 was transferred to PAO1 with the same procedure. Induction of P_{BAD} promoter was achieved through the addition of 7.5 mM arabinose (ara). A similar protocol, with the only variant of M9-citrate for donor counterselection, was used for the transfer to PAO1 of pVLT31-based vectors pVLT31-M4G6 and pVLT31-M4G6i, and empty pVLT31. Induction of pVLT31 P_{tac} promoter was achieved through the addition of 1 mM IPTG.
doi:10.1371/journal.pone.0050323.g001

be associated with PF01841. Figure S2 illustrates the distribution among prokaryotic species of protein presenting the association of PF01841 transglutaminase domain and DUF3488.

Unfortunately, the PA2873 product was annotated in PGD as a "hypothetical protein", i.e. no experimental evidence of *in vivo* expression was available and thus its existence had only been predicted during bioinformatic genome analysis. To provide evidence of PA2873 product expression, we searched a peptide database that resulted from our recent proteomic survey in PAO1 (unpublished) and found five peptides belonging to the PA2873 periplasmic domain (Table S3), originating from the trypsin digestion of native membrane fractions. Consequently, we have provided experimental evidence that removes the PA2873 product from the *status* of "hypothetical protein". Since all five PA2873 peptides "shaved" by trypsin belonged to the predicted outside membrane domain, this strongly supports the notion that it actually emerges from membrane itself.

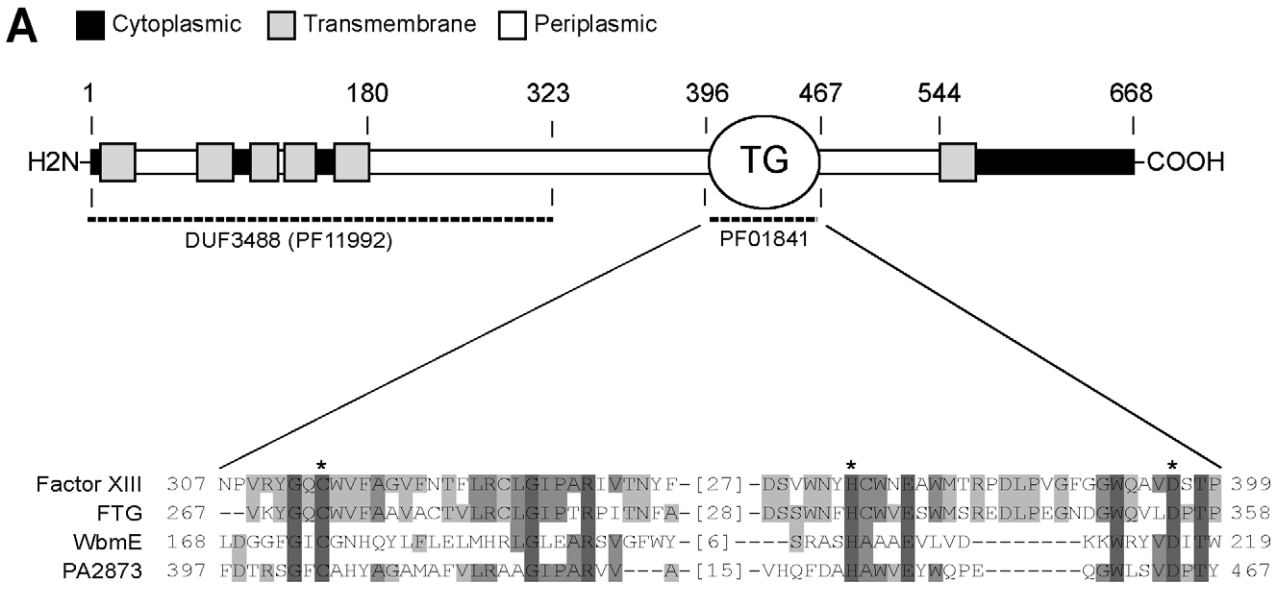
The presence of a transglutaminase-like domain (TG) in PA2873 protein was intriguing. Transglutaminases (TGases) are enzymes that establish covalent links between proteins through the acyl-transfer reaction between the γ-carboxamide group of peptide-bound glutamine and the ε-amino group of peptide-bound lysine. Catalysis of this reaction involves a catalytic triad consisting of cysteine, histidine and aspartate residues that are well-conserved in eukaryotic and prokaryotic TGases [17]. First of all, we verified the conservation of the catalytic triad in the PA2873 TG domain by sequence alignment with characterized members of the TGase superfamily: the human coagulation factor XIII conserved domain [26], TGase from red sea bream liver (fish-derived transglutaminase, FTG) [27] and WbmE, a periplasmic TGase of *Bordetella bronchiseptica* [28] involved in the post-assembly modification of LPS O-antigen. As shown in Figure 2A, the catalytic triad of PA2873 TG domain appeared to be well-conserved. To assess whether the conservation of the catalytic triad was correlated to the TGase activity, the N-$(His)_{10}$-tagged periplasmic domain (aa 180–544) of PA2873 protein ($TG_{180–544}$) was expressed in *E. coli* and subjected to affinity purification. TGase activity of the purified protein was tested through a colorimetric microassay, using 0.25 mU of purified guinea pig TGase (gpTGase) as the reference enzyme activity (Figure 2B). Negative control of TGase activity was gpTGase incubated with EDTA. $TG_{180–544}$ was positive to the TGase test. In actual fact, 2.5 μg of $TG_{180–544}$, equal to reference gpTGase, showed TGase activity that differed significantly from the negative control and was about 45% of gpTGase activity in the absence of EDTA. To test activity dependence from Ca^{2+}, essential for eukaryotic TGases [27], EDTA was added to $TG_{180–544}$. Unlike gpTGase, EDTA addition did not affect $TG_{180–544}$ activity. This suggested that PA2873 TGase activity is Ca^{2+}-independent, similar to members of the microbial transglutaminase family [29]. For the features described above, we called the PA2873 gene product "transglutaminase protein A" (TgpA).

In PGD, PA2873 *locus* was predicted to cluster in an operon with the adjacent *loci* PA2875, PA2874 and PA2872 (Figure 3A). This cluster arrangement is conserved throughout the sequenced *P. aeruginosa* strains with the exception of strain 39016, where there is a 354 bp intergenic region between PA2873 and PA2872 orthologs. When sequenced-*Pseudomonas* species in PGD were examined, it was found in *P. fulva*, *P. mendocina*, *P. syringae* and *P. stutzeri* strains. In the latter case, the PA2872 ortholog was absent.

To profile the transcription of PA2875-2874-2873-2972 predicted operon, we performed RT-PCR experiments on total RNAs purified from PAO1 cell samples, taken both at mid-exponential (OD_{600} of 0.6) and at early-stationary phase (OD_{600} of 1.2) in LB at 37°C. To detect single ORF transcription, we used oligo pairs which amplified ORF internal regions (i, iii, v, and vii; Figure 3B). Furthermore, to detect ORF cotranscription, we used oligo pairs which amplified regions spanning the 3′ to 5′ of adjacent ORFs (ii, iv, and vi; Figure 3B). As shown in Figure 3B, we could observe transcription of every ORF in both growth stages. Furthermore, our data strongly suggested that PA2875, PA2874 and PA2873 are co-transcribed and form a transcription unit, while PA2872 is transcribed independently.

Mutagenesis of the PA2875-2874-2873 gene cluster

The essential role of PA2873 *locus* (*tgpA*), suggested by the results of SALs screenings (see above), was validated by insertional mutagenesis. Consequently, we also aimed to evaluate the essential role of the co-transcribed PA2875 and PA2874 *loci*. For this reason, each gene was targeted for knock-out by homologous recombination-mediated cointegration of the suicide vector pDM4 carrying Cm resistance (Cm^R). Since pDM4 is incapable of autonomous replication in PAO1, following conjugational transfer of pDM4 from *E. coli* to PAO1, Cm^R clones can be selected only in the event of pDM4 cointegration with the chromosome. The *dnaG* gene for DNA primase [24] and *algR* gene for a LytTR-type two-component response regulator [30] were used as positive and negative controls of essentiality, respectively. For cointegration targeting, we cloned internal 600–800 bp fragments of PA2875, PA2874, *tgpA*, *dnaG* and *algR* respectively, into pDM4. The resulting constructs were transferred from *E. coli* S17-λpir to PAO1 by conjugation, selecting cointegration events by plating the conjugation mixtures on PIA supplemented with Cm. Three independent conjugation experiments were performed for each gene involved. To take fluctuations of conjugation efficiency into



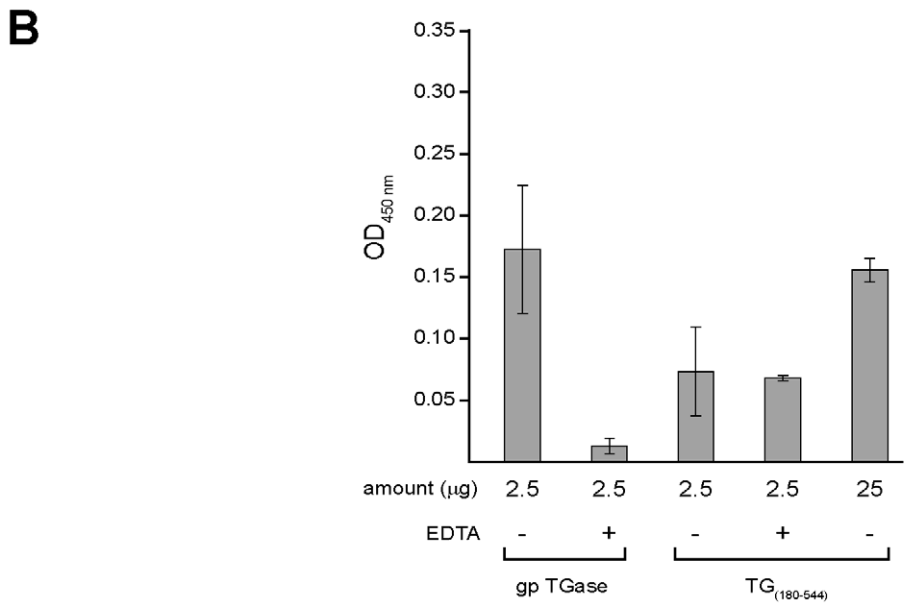


Figure 2. Predicted domain organization and transglutaminase activity of TgpA protein. (A) Map of the predicted domains DUF3488 (PF11992) and TG (PF01841) along the primary sequence of the PA2873 gene product, called TgpA. The sequence of TgpA spanning aa 396 to 467 of the TG domain is highlighted and aligned to homologous functional TG domains of human coagulation Factor XIII, fish-derived transglutaminase (FTG) and WbmE protein from *B. bronchiseptica*. Conserved aminoacids of catalytic triad are indicated by an asterisk. (B) Colorimetric assay of transglutaminase activity of purified TgpA $TG_{180–544}$ domain by Transglutaminase Colorimetric Microassay Kit (TCM kit; Covalab). TCM kit uses immobilized N-carbobenzoxy(CBZ)-Gln-Gly as the amine acceptor and biotin-conjugated cadaverine as the amine donor. The indicated amounts of purified TgpA $TG_{180–544}$ (stock: 2.7 mg/ml, 95% purity) were incubated in 96-well microtiter plate coated with CBZ-Gln-Gly at 37°C for 15 min with calcium, DTT and biotinylated cadaverine, both in the presence and the absence of EDTA supplied in the kit. As a reference for TGase activity, the indicated amounts of kit-included purified guinea pig TGase with specific activity of 0.1 U/mg were incubated under the same conditions. The wells were washed extensively and filled with streptavidin-labelled horseradish peroxidase (HRP) to assay the formation of immobilized γ-glutamyl-cadaverine-biotin by OD_{450} measurement of HRP activity using H_2O_2 as substrate and tetramethyl benzidine as electron acceptor (chromogen).
doi:10.1371/journal.pone.0050323.g002

account, the results of replicate experiments were expressed as a percentage of Cm^R ex-conjugant clones relative to the negative control *algR*. As shown in Figure 4A, targeting pDM4 cointegration to both PA2874 and *tgpA* gave rise to a dramatic decrease in the yield of Cm^R ex-conjugant clones, comparable to *dnaG* inactivation. On the contrary, targeting pDM4 cointegration to PA2875 resulted in a yield of Cm^R ex-conjugants that was even higher than the control *algR*.

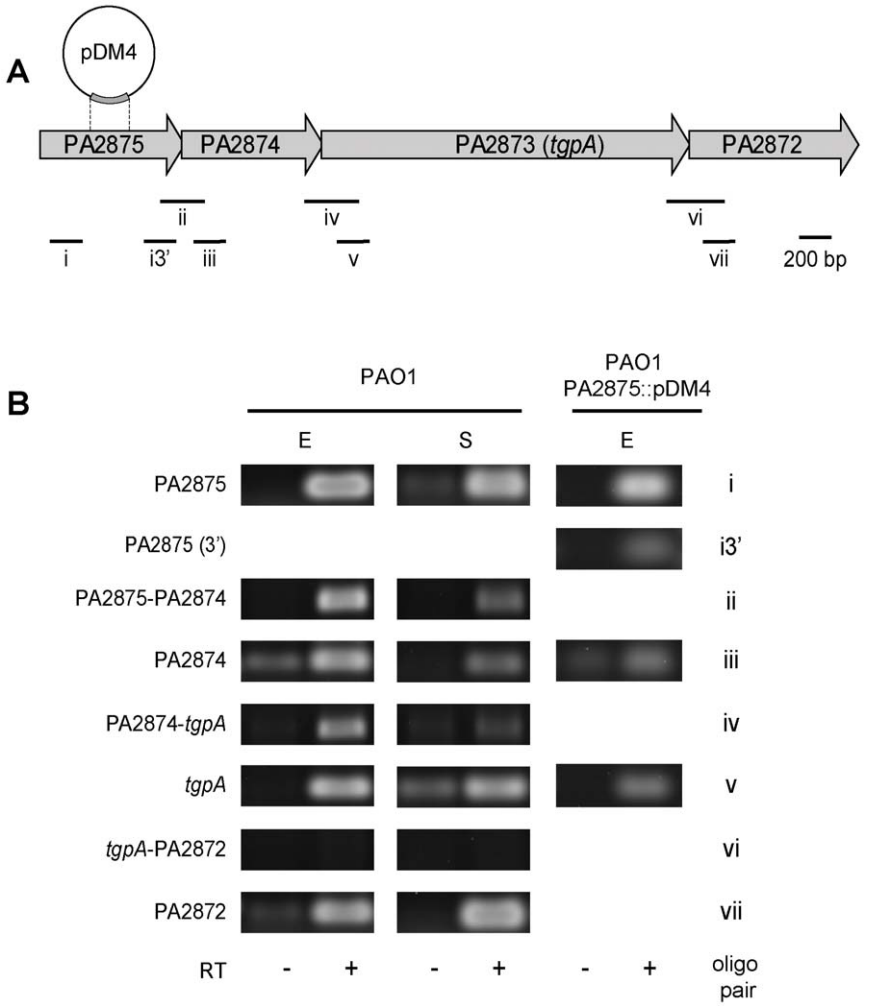


Figure 3. Genetic organization and transcription analysis of the genomic region including PA2873 *locus* (*tgpA*). (A) PA2875-2874-2873-2872 gene cluster is represented according to visualization by GBrowse in Pseudomonas Genome Database. Locations of fragments amplified by the oligo pairs (Roman numbers) used in RT-PCR-based transcription analysis (B) are shown along the region map. The position of plasmid pDM4 cointegration in PAO1 PA2875::pDM4 strain is indicated. (B) Total RNA was extracted from PAO1 and PAO1 PA2875::pDM4 cells in both exponential (E) and stationary (S) phases, and analyzed by RT-PCR. RT untreated samples (RT−) as controls of genomic DNA contamination were included in the analysis.
doi:10.1371/journal.pone.0050323.g003

These results strongly support the notion that *tgpA* is essential. In the case of PA2874, the above results were not conclusive. In actual fact, the low yield of Cm^R ex-conjugant clones may result either from the essential role of PA2874 or from polar effects of the cointegration in PA2874 on the expression of *tgpA*. We were unable to discriminate these two possibilities by targeting pDM4 cointegration within PA2874 in a PAO1 strain expressing *tgpA* from a plasmid vector. Several attempts to introduce the *tgpA*-expressing vector pVLT31-PA2873 into PAO1 were unsuccessful. Therefore, we suggested that unbalancing the *tgpA* expression may be deleterious. Finally, PA2875 would appear to be non-essential.

Using RT-PCR, we profiled the PA2875-2874-*tgpA* gene cluster transcription in a Cm^R ex-conjugant strain PAO1 PA2875::pDM4, both upstream and downstream of the site of pDM4 insertion within PA2875. As shown in Figure 3B, we could detect transcription both upstream (i; PA2875) and downstream of pDM4 cointegration in PA2875 (i3′), in PA2874 (iii) and in *tgpA* (v), respectively. Therefore, we validated the lack of polar effects of the pDM4 cointegration in PA2875. Furthermore, these results strongly suggested the presence of promoter(s) downstream of the site of pDM4 insertion within PA2875. By primer extension analysis with an oligo annealing with 5′ region of *tgpA* (not shown), we could detect 5′ transcript ends located at −31, around −280 and −335 from the translation start site of *tgpA*.

The essentiality of *tgpA* was further confirmed by conditional mutagenesis. The first 300 bp of *tgpA* were cloned into the suicide vector pSC200 [31] downstream of P_{rhaB}, a rhamnose inducible/glucose repressible promoter, to give rise to pSC200-PA2873. Upon transfer to PAO1, pSC200-PA2873 cointegration event placed *tgpA* under P_{rhaB} control. In both liquid and solid media, the PAO1 P_{rhaB}::*tgpA* strain showed a specific conditional growth phenotype (Figure 4B), i.e. repression of P_{rhaB} by glucose strongly impairs growth, while rhamnose addition allows normal growth. Due to the low number of cells in the inoculum of liquid media, growth rate was undetectable by microtiter reader during the initial half-time of the experiments (Figure 4B). The growth of PAO1 P_{rhaB}::*tgpA* in liquid M9-citrate supplemented with either rhamnose or glucose was monitored for 7 hrs from inoculum by titration of colony forming units per ml (CFU/ml) on LB plates. As shown in Figure 4C, PAO1 P_{rhaB}::*tgpA* in rhamnose started

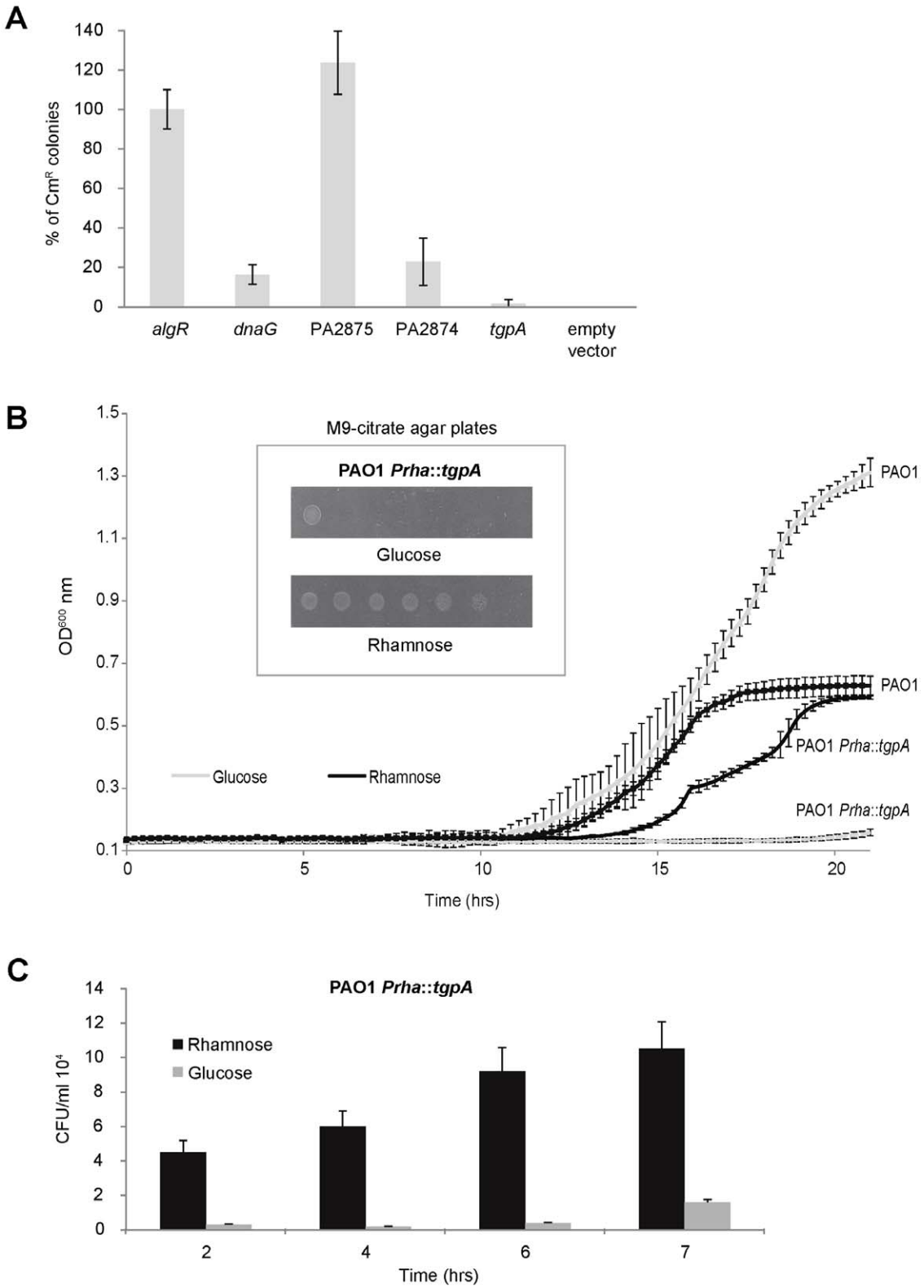


Figure 4. Mutagenesis analysis of the PA2875-2874- *tgpA* gene cluster. (A) Each indicated *locus* was targeted for knock-out by homologous recombination-mediated cointegration of the suicide vector pDM4 carrying chloramphenicol resistance (Cm^R). The *dnaG* gene for DNA primase and the *algR* gene for a LytTR-type two-component response regulator were used respectively as positive and negative controls of essentiality. Cointegration targeting was achieved by cloning internal 600–800 bp fragments of PA2875, PA2874, *tgpA*, *dnaG* and *algR*, respectively, into pDM4. The resulting constructs were transferred from *E. coli* S17-λpir to PAO1 by conjugation, selecting cointegration events by plating the conjugation mixtures on PIA supplemented with chloramphenicol. Three independent conjugation experiments were performed. Efficiency of cointegration in a given *locus* is expressed as a percentage of Cm^R ex-conjugant colonies relative to the negative control *algR*. (B) The rhamnose inducible/glucose

repressible promoter P_{rhaB} was inserted upstream to *tgpA* giving rise to PAO1 P_{rhaB}::*tgpA* strain. To test the repression effects of glucose on growth rate, overnight cultures of PAO1 P_{rhaB}:: *tgpA* in M9-citrate supplemented with rhamnose were diluted to $OD_{600} = 10^{-6}$ and inoculated in microtiter wells filled with 200 µl of M9-citrate supplemented with either rhamnose or glucose. Culture growth at 37° with stirring was monitored in real-time by OD_{600} measurement in a microtiter reader for 21 hrs. Specificity of glucose/rhamnose effects on the growth of PAO1 P_{rhaB}::*tgpA* was assessed by monitoring the PAO1 cultures in M9-citrate supplemented with either rhamnose or glucose. Note the opposite effects of glucose on growth of PAO1 P_{rhaB}::PA2873 and PAO1, respectively. In the insert, overnight cultures of PAO1 P_{rhaB}::*tgpA* in M9-citrate supplemented with rhamnose were also tested for growth on solid M9-citrate supplemented with either rhamnose or glucose, by spotting 2 µl of 10-fold serial dilutions, from $OD_{600} = 1$ (left) to $OD_{600} = 10^{-6}$ (right). (C) During the first 7 hrs from inoculum, a time window in which growth rate was undetectable by microtiter reader, the growth of PAO1 P_{rhaB}::*tgpA* in liquid M9-citrate supplemented with either rhamnose or glucose was monitored by titration of colony-forming units per ml (CFU/ml) on LB plates.
doi:10.1371/journal.pone.0050323.g004

growing and the number of cells was detectable 11 hrs from inoculum (Figure 4B). On the contrary, for PAO1 P_{rhaB}::*tgpA* in glucose only residual growth was observed (Figure 4C). In this case, the threshold of cell number detection by the microtiter reader was barely achieved in the 21 hrs of analysis (Figure 4B). Some escape from glucose repression and/or the activity of TgpA, which might have a low turn-over rate, may account for the residual growth of the inoculum cells in a glucose-supplemented medium.

Conclusions

Overall, the results presented above strongly indicate that TgpA plays a critical role in the viability of *P. aeruginosa*. The unambiguous prediction of the presence of six transmembrane helices (Figure S3), our detection in membrane fractions (see above) and the export across inner membrane found by PhoA fusion screen [32] would strongly suggest that TgpA acts at a cytoplasmic membrane level. Figure S3 shows that the TgpA region $TG_{180–544}$ containing the functional TG domain has a high probability of being exposed on the outward face of cytoplasmic membrane, i.e. to protrude into the periplasmic space. Therefore, we suggest that TgpA takes part in an essential function linked to the cell wall. Specific but non-essential functions at cell wall level have been shown for some prokaryotic proteins endowed with TG domains. In the *Methanothermobacter* species, prophage proteins PeiW and PeiP act as pseudomurein endoisopeptidases [33,34]. In the periplasm of *B. bronchiseptica*, WbmE protein catalyzes the deamidation of uronamide-rich O chains of lipopolysaccharide (LPS) [28]. It is conceivable that TgpA activity can participate in cell wall functions such as i) assembly of peptidoglycan structures ii) maturation/secretion of key periplasmic proteins iii) assembly of surface polypeptide structures iv) biogenesis/maturation of LPS.

Supporting Information

Figure S1 Graphical representation of the distribution across species of the structural TGase domain belonging to the transglutaminase-like superfamily [17] (PF01841 in PFAM database [25]). The radius of the arc, i.e. distance from the root node at the center of the sunburst, shows the taxonomic level (''superkingdom'', ''kingdom'', etc). The length of the arc represents the number of domains at a given level. Among the 1752 species represented in the figure, we found 4842 sequences containing the TGase domain. 265 sequences, belonging to 238 prokaryotic species, present a specific association of TGase domain in front of the domain of unknown function DUF3488 (PF11992 in PFAM database [25]) containing typically 6 transmembrane helices.
(TIF)

Figure S2 Graphical representation of the distribution of DUF3488 across prokaryotic species. The radius of the arc, i.e. distance from the root node at the center of the sunburst, shows the taxonomic level (''superkingdom'', ''kingdom'', etc). The length of the arc represents the number of domains represented at a given level. Among the 238 species represented in the figure, 234 present the specific architecture of the DUF3488 domain followed by TGase domain.
(TIF)

Figure S3 Prediction of transmembrane helices in TgpA (PA2873). Transmembrane helice prediction along the 668 aa sequence of PA2873 was performed by TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) [35]. TMHMM supplies some statistics and a list of the locations of the predicted transmembrane helices and the predicted location of the intervening loop regions. The prediction gives the most probable location and orientation of transmembrane helices in the sequence. It is found by an algorithm called N-best that sums over all paths through the model with the same location and direction of the helices. Some statistics are given as follows. Length: the length of the protein sequence; Number of predicted TMHs: the number of predicted transmembrane helices; Exp number of AAs in TMHs: the expected number of amino acids in transmembrane helices. If this number is larger than 18 it is very likely to be a transmembrane protein (OR have a signal peptide); Exp number, first 60 AAs: the expected number of amino acids in transmembrane helices in the first 60 amino acids of the protein; Total prob of N-in: The total probability that the N-term is on the cytoplasmic side of the membrane; POSSIBLE N-term signal sequence: a warning that is produced when ''Exp number, first 60 AAs'' is larger than 10. The plot shows the posterior probabilities of inside/outside/TM helix. At the top of the plot (between 1 and 1.2) the N-best prediction is shown. Note the high probability of outside location of the region spanning aa 180 to 544 containing the functional TG domain.
(TIF)

Table S1 List of bacterial strains and plasmids.
(PDF)

Table S2 Oligonucleotides.
(PDF)

Table S3 TgpA peptides detected through MudPIT analysis of membrane fractions.
(PDF)

Acknowledgments

The authors are grateful to all members of the lab for their helpful discussions and technical support, D. Haas for the critical discussion and experimental hints, P. Mauri and D. Di Silvestre for the MudPIT analysis, F. Bonomi and S. Iametti for the biochemistry support.

Author Contributions

Conceived and designed the experiments: AM DV RR GB. Performed the experiments: AM DV RR. Analyzed the data: AM DV RR GB. Wrote the paper: AM DV GB.

References

1. Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, et al. (2008) Dynamics of *Pseudomonas aeruginosa* genome evolution. Proc Natl Acad Sci U S A 105: 3100–3105.
2. Kung VL, Ozer EA, Hauser AR (2010) The accessory genome of *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev 74: 621–641.
3. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tummler B (2011) *Pseudomonas aeruginosa* Genomic Structure and Diversity. Front Microbiol 2: 150.
4. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW (2011) *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol Rev 35: 652–680.
5. Pier GB, Ramphal R (2005) *Pseudomonas aeruginosa*. In: Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. Philadelphia, PA: Elsevier Churchill Livingstone. pp. 2587–2615.
6. Wagner VE, Filiatrault MJ, Picardo KF, Iglewski BH (2008) *Pseudomonas aeruginosa* virulence and pathogenesis issues. In: Cornelis P, editor. *Pseudomonas* genomics and molecular biology. Norfolk: Caister Academic Press. pp. 129–158.
7. Bonomo RA, Szabo D (2006) Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. Clin Infect Dis 43: S49–S56.
8. Lister PD, Wolter DJ, Hanson ND (2009) Antibacterial-Resistant *Pseudomonas aeruginosa*: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. Clin Microbiol Rev 22: 582–610.
9. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, et al. (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infec 18: 268–281.
10. Lim TP, Lee W, Tan TY, Sasikala S, Teo J, et al. (2011) Effective Antibiotics in Combination against Extreme Drug-Resistant *Pseudomonas aeruginosa* with Decreased Susceptibility to Polymyxin B. Plos One 6: e28177.
11. Forsyth RA, Haselbeck RJ, Ohlsen KL, Yamamoto RT, Xu H, et al. (2002) A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*. 43: 1387–1400.
12. Ji Y, Zhang B, Van SF, Horn, Warren P, et al. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. 293: 2266–2269.
13. Engdahl HM, Lindell M, Wagner EGH (2001) Introduction of an RNA stability element at the 5′-end of an antisense RNA cassette increases the inhibition of target RNA translation. Antisense Nucleic A 11: 29–40.
14. Wagner EGH, Flardh K (2002) Antisense RNAs everywhere? Trends Genet 18: 223–226.
15. Meng J, Kanzaki G, Meas D, Lam CK, Crummer H, et al. (2012) A genome-wide inducible phenotypic screen identifies antisense RNA constructs silencing *Escherichia coli* essential genes. Fems Microbiol Lett 329: 45–53.
16. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, et al. (2011) *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res 39: D596–600.
17. Makarova KS, Aravind L, Koonin EV (1999) A superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases. 8: 1714–1719.
18. de Lorenzo V, Eltis L, Kessler B, Timmis KN (1993) Analysis of *Pseudomonas* gene products using lacIq/Ptrp-lac plasmids and transposons that confer conditional phenotypes. 123: 17–24.
19. Sze CC, Shingler V (1999) The alarmone (p)ppGpp mediates physiological-responsive control at the sigma 54-dependent Po promoter. 31: 1217–1228.
20. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD (2008) PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. 74: 7422–7426.
21. Milton DL, O"Toole R, Horstedt P, Wolf-Watz H (1996) Flagellin A is essential for the virulence of *Vibrio anguillarum*. 178: 1310–1319.
22. Ducret A, Van Oostveen I, Eng JK, Yates JR, Aebersold R (1998) High throughput protein characterization by automated reverse-phase chromatography electrospray tandem mass spectrometry. Protein Sci 7: 706–719.
23. Folk JE, Cole PW (1966) Identification of a functional cysteine essential for the activity of guinea pig liver transglutaminase. J Biol Chem 241: 3238–3240.
24. Frick DN, Richardson CC (2001) DNA primases. Annu Rev Biochem 70: 39–80.
25. Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, et al. (2012) The Pfam protein families database. Nucleic Acids Res 40: D290–D301.
26. Pedersen LC, Yee VC, Bishop PD, Le TI, Teller DC, et al. (1994) Transglutaminase factor XIII uses proteinase-like catalytic triad to crosslink macromolecules. Protein Sci 3: 1131–1135.
27. Noguchi K, Ishikawa K, Yokoyama K, Ohtsuka T, Nio N, et al. (2001) Crystal structure of red sea bream transglutaminase. J Biol Chem 276: 12055–12059.
28. King JD, Vinogradov E, Preston A, Li J, Maskell DJ (2009) Post-assembly modification of *Bordetella bronchiseptica* O polysaccharide by a novel periplasmic enzyme encoded by wbmE. 284: 1474–1483.
29. Kashiwagi T, Yokoyama K, Ishikawa K, Ono K, Ejima D, et al. (2002) Crystal structure of microbial transglutaminase from *Streptoverticillium mobaraense*. 277: 44252–44260.
30. Deretic V, Dikshit R, Konyecsni WM, Chakrabarty AM, Misra TK (1989) The algR gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. 171: 1278–1283.
31. Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, et al. (2007) A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. 189: 3639–3644.
32. Lewenza S, Gardy JL, Brinkman FS, Hancock RE (2005) Genome-wide identification of *Pseudomonas aeruginosa* exported proteins using a consensus computational strategy combined with a laboratory-based PhoA fusion screen. 15: 321–329.
33. Luo Y, Pfister P, Leisinger T, Wasserfallen A (2002) Pseudomurein endoisopeptidases PeiW and PeiP, two moderately related members of a novel family of proteases produced in *Methanothermobacter* strains. 208: 47–51.
34. Steenbakkers PJ, Geerts WJ, Ayman-Oz NA, Keltjens JT (2006) Identification of pseudomurein cell wall binding domains. 62: 1618–1630.
35. Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. 305: 567–580.

SUPPLEMENTARY FIGURES

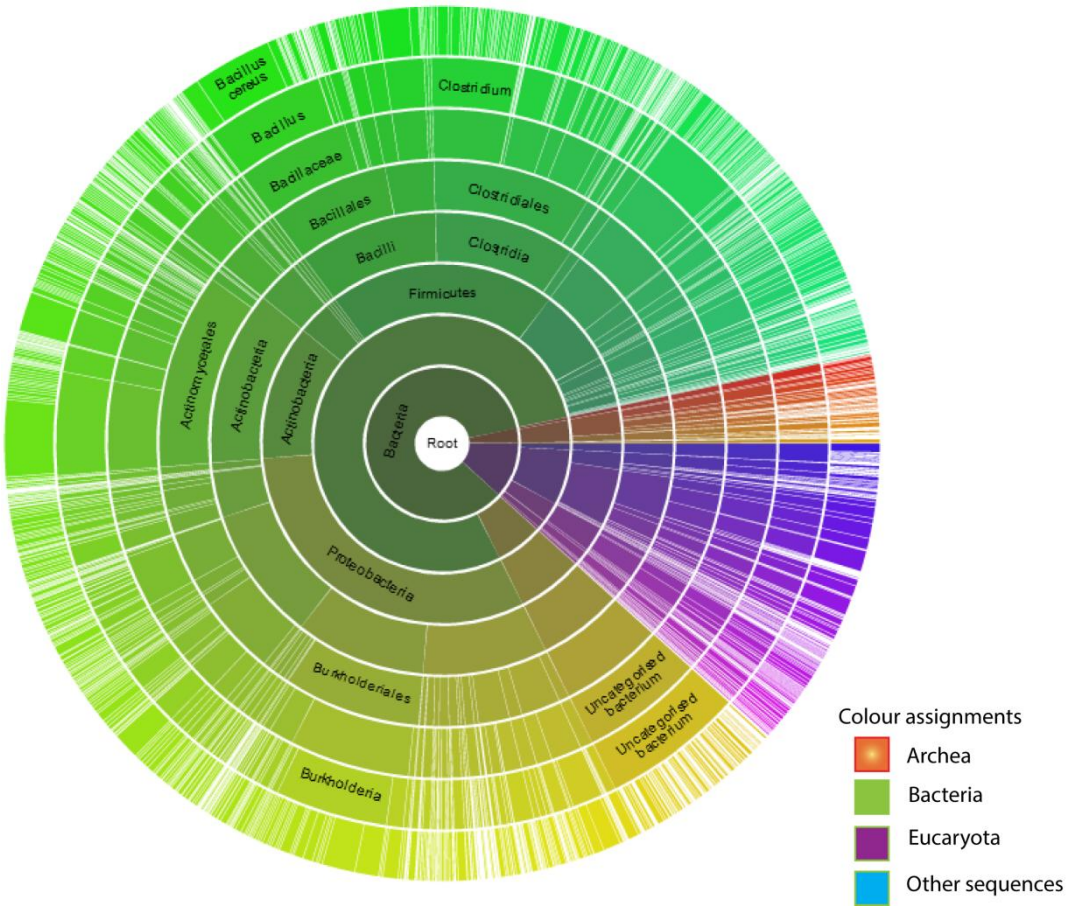


Figure S1 Graphical representation of the distribution across species of the structural TGase domain belonging to the transglutaminase-like superfamily [17] (PF01841 in PFAM database [25]). The radius of the arc, i.e. distance from the root node at the center of the sunburst, shows the taxonomic level ("superkingdom", "kingdom", etc). The length of the arc represents the number of domains at a given level. Among the 1752 species represented in the figure, we found 4842 sequences containing the TGase domain. 265 sequences, belonging to 238 prokaryotic species, present a specific association of TGase domain in front of the domain of unknown function DUF3488 (PF11992 in PFAM database [25]) containing typically 6 transmembrane helices.

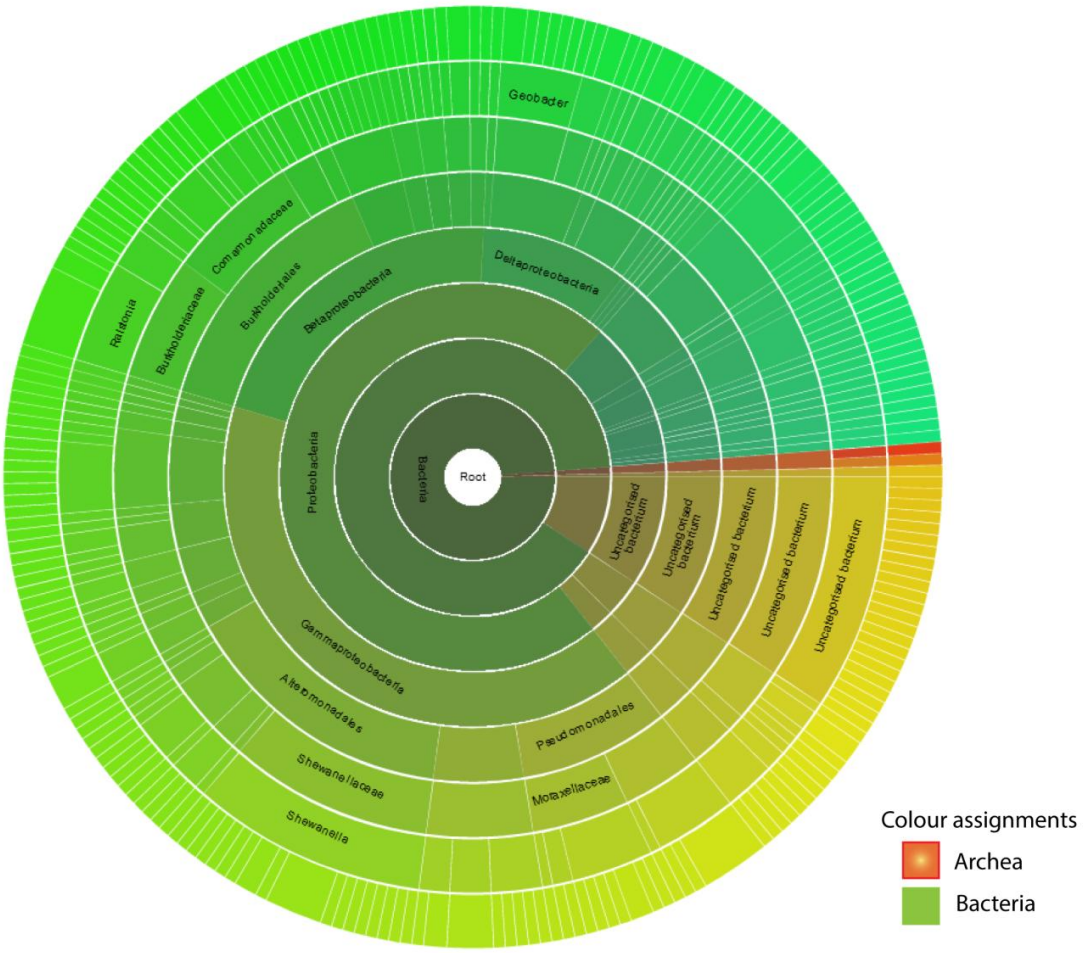


Figure S2 Graphical representation of the distribution of DUF3488 across prokaryotic species. The radius of the arc, i.e. distance from the root node at the center of the sunburst, shows the taxonomic level ("superkingdom", "kingdom", etc). The length of the arc represents the number of domains represented at a given level. Among the 238 species represented in the figure, 234 present the specific architecture of the DUF3488 domain followed by TGase domain**.**

```
> INPUT SEQUENCE PA2873
MNAIPRVALVWLLVAQVLVILPHLAYMPLWIAAMWLGCAAWRVQVFRMRAGYPRAWVKLALALLAGAGVWLSRGSLVGLDAGAVLLIAAFILKLVEMKTR
RDALVLVFLGFFAVVVGYLFDDGFLAALYSLLPVTALLAALIGLQQSAFASRPWPTLRLAGGLLLQALPLMLLLFLFFPRLGPLWSLPMPGNKGVTGLSE
SMAPGDIAELGRSAELAFRVRFEGALPPREQLYWRALTMERFDGRRWAQAPQWSGEDALHWQKRGPELRYDVIMQPSSQPWLFALDVAQTDQTDTRLMSD
FHLQRRQPVEQRLFYRVSSWPQALRESSIDPRTRWRNLQLPMHGNPRARALADELRQAHAQPQALVAALLQRFNHEPFAYTLKPPATGADGVDDFLFDTR
SGFCAHYAGAMAFVLRAAGIPARVVAGYQGGELNPAGNYLLVHQFDAHAWVEYWQPEQGWLSVDPTYQVAPERIEQGLEQALAGDSEYLADAPLSPLRYR
GLPWLNDMRLAWDSLNYGWQRWVLAYQGEQQGAFLQRWFGGLDPTRLGLLLGAAAILSVGLLALFLLKPWQGRGDLRSRQLRRFERLLEMHGLRRSPGEG
LRSYGERAARVLPAQAPAIAAFVGAFEAQRYGHGGADDPGLRLRALRRALPWRLVRTPTRDGRGEEQA
```

```
# Sequence Length: 668
# Sequence Number of predicted TMHs:  6
# Sequence Exp number of AAs in TMHs: 142.34241
# Sequence Exp number, first 60 AAs:  31.47924
# Sequence Total prob of N-in:        0.68659
# Sequence POSSIBLE N-term signal sequence
Sequence    TMHMM2.0    inside           1     6
Sequence    TMHMM2.0    TMhelix          7    29
Sequence    TMHMM2.0    outside         30    68
Sequence    TMHMM2.0    TMhelix         69    91
Sequence    TMHMM2.0    inside          92   102
Sequence    TMHMM2.0    TMhelix        103   120
Sequence    TMHMM2.0    outside        121   124
Sequence    TMHMM2.0    TMhelix        125   144
Sequence    TMHMM2.0    inside         145   156
Sequence    TMHMM2.0    TMhelix        157   179
Sequence    TMHMM2.0    outside        180   544
Sequence    TMHMM2.0    TMhelix        545   567
Sequence    TMHMM2.0    inside         568   668
```

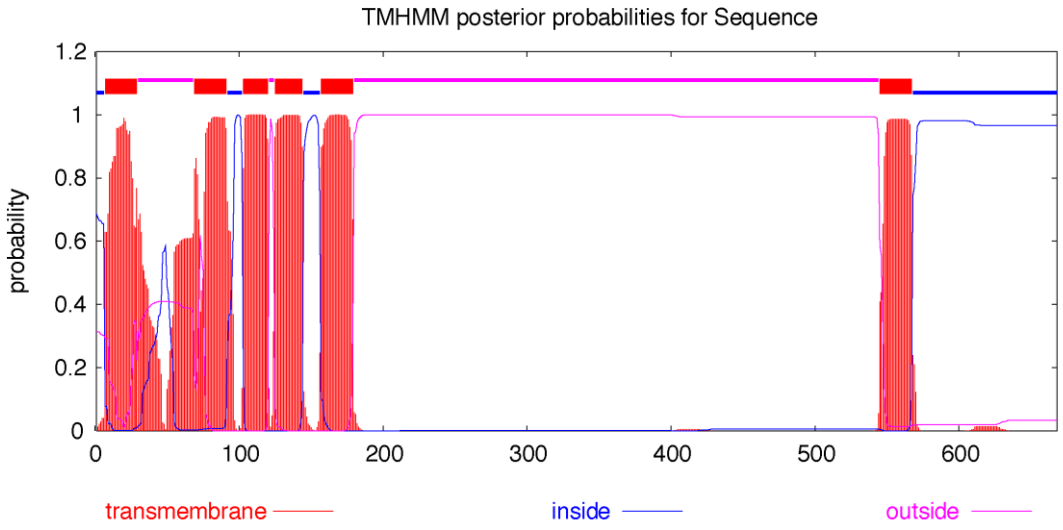


Figure S3 Prediction of transmembrane helices in TgpA (PA2873). Transmembrane helice prediction along the 668 aa sequence of PA2873 was performed by TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) [35]. TMHMM supplies some statistics and a list of the locations of the predicted transmembrane helices and the predicted location of the intervening loop regions. The prediction gives the most probable location and orientation of transmembrane helices in the sequence. It is found by an algorithm called N-best that sums over all paths through the model with the same location and direction of the helices. Some statistics are given as follows. Length: the length of the protein sequence; Number of predicted TMHs: the number of predicted transmembrane helices; Exp number of AAs in TMHs: the expected number of amino acids in transmembrane helices. If this number is larger than 18 it is very likely to be a transmembrane protein (OR have a signal peptide); Exp number, first 60 AAs: the expected number of amino acids in transmembrane helices in the first 60 amino acids of the protein; Total prob of N-in: The total probability that the N-term is on the cytoplasmic side of the membrane; POSSIBLE N-term signal sequence: a warning that is produced when "Exp number, first 60 AAs" is larger than 10. The plot shows the posterior probabilities of inside/outside/TM helix. At the top of the plot (between 1 and 1.2) the N-best prediction is shown. Note the high probability of outside location of the region spanning aa 180 to 544 containing the functional TG domain.

Table S1: List of bacterial strains and plasmids.

Strains or plasmids	Genotype or description	Reference
Pseudomonas aeruginosa		
PAO1		[1]
PAO1 PA2875*::*pDM4	PA2875 disrupted by cointegration of pDM4	This work
PAO1 P_{rhaB}*::tgpA*	PA2873 (*tgpA*) under the control of the rhamnose-dependent promoter P_{rhaB}	This work
Escherichia coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44, relA1,* Δ*(lac-proAB)* F' *(traD36, proAB+ lacIqZ* ΔM15)	[2]
HB101 [RK2013]	*recA, thi, hsdR, hsdM, proA, leu, strA*	[3]
S17-λpir	Tp^R Sm^R *hsdR pro recA* RP4-2-Tc::Mu-Km::Tn7 (λpir)	[4]
C118-λpir	*araD*139 Δ(*ara leu*)7697 Δ*lacX*74 *phoA*20 *galE galK thi rpsE rpoB araEam recA1* (λpir)	[5]
Plasmids		
pHERD20T	Ap^R, Cb^R broad-host-range *araC-*P_{bad} expression vector; blue/white screening for recombinants	[6]
pVI533EH	Ap^R, Cb^R broad-host-range *araC-*P_{bad} expression vector	[7]
pVI533EH-M4G6	pVI533EH harbouring a fragment spanning position 898 to 1252 of PA2873 cloned in antisense direction.	This work
pVI533HE	As pVI533EH with inverted MCS	[7]
pVI533HE-M4G6i	pVI533HE harbouring a fragment spanning position 898 to 1252 of PA2873 cloned in sense direction.	This work
pVLT31	Tc^R, RSF1010-*lacI*^{q}/P_{tac},expression vector with MCS of pUC18	[8]
pVLT31-M4G6	pVLT31 harbouring a fragment spanning position 898 to 1252 of PA2873 cloned in antisense direction, subcloned from pVI533EH-M4G6	This work
pVLT31-M4G6i	pVLT31 harbouring a fragment spanning position 898 to 1252 of PA2873 cloned in sense direction.	This work
pDM4	Cm^R, suicide plasmid in *Pseudomonas spp*, pBR322 origin of replication, *sacB*	[9]
pDM4-*algR*	pDM4 harbouring position 22 to 610 of *algR* within the MCS	This work
pDM4-*dnaG*	pDM4 harbouring position 12 to 422 of *dnaG* within the MCS	This work
pDM4-PA2875	pDM4 harbouring position 242 to 825 of PA2875 within the MCS	This work
pDM4-PA2874	pDM4 harbouring position 156 to 883 of PA2874 within the MCS	This work
pDM4-PA2873	pDM4 harbouring position 590 to 1392 of PA2873 within the MCS	This work

pSC200	Gm^R, ori_{R6K}, P_{rhaB} rhamnose-inducible promoter, mob^+	[10]
pSC200-PA2873	pSC200 harbouring the first 300 bp of PA2873 downstream the P_{rhaB} promoter	This work
p2N[M4G6 (180-544 aa)]	Ap^R, T7 promoter-based vector espressing the $N(His)_{10}$-tagged periplasmic domain (aa 180-544) of TgpA	This work

References

1. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406: 959-964.
2. Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. 33: 103-119.
3. Boyer HW, Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. 41: 459-472.
4. de Lorenzo V, Herrero M, Jakubzik U, Timmis KN (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. 172: 6568-6572.
5. de Lorenzo V, Timmis KN (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. 235: 386-405.
6. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD (2008) PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. 74: 7422-7426.
7. Sze CC, Shingler V (1999) The alarmone (p)ppGpp mediates physiological-responsive control at the sigma 54-dependent Po promoter. 31: 1217-1228.
8. de Lorenzo V, Eltis L, Kessler B, Timmis KN (1993) Analysis of *Pseudomonas* gene products using lacIq/Ptrp-lac plasmids and transposons that confer conditional phenotypes. 123: 17-24.
9. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H (1996) Flagellin A is essential for the virulence of *Vibrio anguillarum*. 178: 1310-1319.
10. Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, et al. (2007) A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. 189: 3639-3644.

Table S2. Oligonucleotides

Oligo name	Sequence[a] (5'→3')	
RT-PCR		oligo pair[b]
2875fwRT	CTGGAAGCCTGCCTGAAGGC	i
2875revRT	GGTACCGAGGATATCGCCCG	
2875fw_3'	GGACGCGACTATGTGATTCC	i3'
2875rev_3'	CAACAGCCATTGCACCAG	
2875-2874fw	AGCAGCAGGAAGGTCAAC	ii
2875-2874rev	GACGCGACTATGTGATTCC	
2874fwRT	ACGGATACCGCCGGCGAG	iii
2874revRT	GAACACCGGGTCCGCTCC	
2874-2873fw	TTCCACCAGTTTGAGGATGA	iv
2874-2873rev	CATGCAGTCCTGGAACTCT	
2873fwRT	GGTGGCGCAGGTACTGGTG	v
2873revRT	CGCGGCGGGTCTTCATTTCC	
2873-2872fw	ATGATCCAGGGTTGCGTCT	vi
2873-2872rev	CCAGACGTCCAGGCAAAG	
2872fwRT	GGTGGCGTTGATGTCGGCG	vii
2872revRT	GCATCCCGCAGCCCTTCG	
Primer extension		
2873_PE60	ATCCACAGCGGCATGTAGGC	
Seq_Fw	GAACGCTCGCTGTTGCTC	
Seq_Rev	CGCGGCGGGTCTTCATTTCC	
Antisense libraries construction and screening		
M4G6Hind5'	CCCAAGCTTCGGCGCGCAGGACGAAAG	
M4G6Eco3'	CCGGAATTCCCGATTTCCACCTGCAGCG	
pVI533F	ATCACGGCAGAAAAGTCCAC	
pVI533R	CTTCTCTCATCCGCCAAAAC	
pHERD-F	ATCGCAACTCTCTACTGTTTCT	
pHERD-R	TGCAAGGCGATTAAGTTGGGT	
RSP	AGCGGATAACAATTTCACACAGGA	
VLT31-rev	AATTGGGGACCCTAGAGGTCC	
pDM4-derivatives construction		
algRfwSalI	GAAAGTCGACGAACCTCTGGCGCG	
algRrevSalI	GAAAGTCGACCAGCGCGTTGCGG	
dnaGfwSalI	GAAAGTCGACGATACCGCAAAGCTTCATCG	
dnaGrevSalI	GAAAGTCGACGGCCCTTCAGGTAGTTCACC	
2875fwSalI	GAAAGTCGACAAGGACAGCGGGC	
2875revSalI	GAAAGTCGACCGCCGGCAACACC	
2874fwSalI	GAAAGTCGACGATCAACTACCAGAACAGC	
2874revSalI	GAAAGTCGACAGTTCCAGGACTGCAT	
2873fwSalI	GAAAGTCGACTCTCGGAGAGCATGGC	
2873revSalI	GAACGTCGACGCTCAGCCAGCC	
2872fwSalI	GAACGTCGACATCGATGAACTGCTG	
2872revSalI	GAACGTCGACAGCCACTGTTCGAG	
pDM4-ori	GTGACACAGGAACACTTAACG	
pDM4-cat	TGTCCCTCCTGTTCAGCTAC	
pSC200-PA2873 construction		
TgFullFw	CGGCGGCATATGAACGCGATTCCGC	
Tg300RevXbaI	AACTGCTCTAGACGCATCGCGGCGGGTC	
pSC200-824(fw)	GCCCATTTTCCTGTCAGTAACGAGA	
pSC200-1400(rev)	TAACGGTTGTGGACAACAGCCAGGG	

[a] Restriction enzyme sites are underlined
[b] Oligo pairs used in the experiments shown in Figure 3.

Table S3. TgpA peptides detected through MudPIT analysis of membrane fractions

Peptide	From residue	To residue
APGDIAELGR	203	212
APQWSGEDALHWQK	250	263
LDVAQTDQTDTR	285	296
HEPFAYTLKPPATGADGVDDFLFDTR	375	400
KPPATGADGVDDFLFDTR	383	400

The conserved protein Gcp is essential for the viability of *Pseudomonas aeruginosa*

Ruggero Rusmini, Davide Vecchietti, and Giovanni Bertoni*

Department of Biosciences,
Università degli Studi di Milano
Via Celoria, 26
20133 Milan, Italy

*corresponding author:
E-mail: giovanni.bertoni@unimi.it (GB)

Abstract

The Gram-negative *Pseudomonas aeruginosa* is a metabolically versatile bacterium and one of the most deadly pathogens in compromised individuals, such as patients with cystic fibrosis, severe burns or impaired immunity. Compared with other pathogens, *P. aeruginosa* is very difficult to eradicate as it displays high intrinsic resistance to almost all antibiotics in clinical use and treatment of its infections is a growing challenge. The discovery of novel essential functions of *P. aeruginosa* could drive the development of alternative and effective anti-*Pseudomonas* molecules to overcome the extant mechanisms of resistance.

In this work, we focused on the characterization of the PA0580 *locus* encoding the protein Gcp belonging to the Kae1/Qri7/YgjD family, which was one of the essential candidates resulting from antisense screenings that we performed previously in the *P. aeruginosa* strain PAO1. Combining several genetic approaches, we showed that Gcp is essential for *P. aeruginosa*. In addition, we focused on the product of PA3685 *locus*, the YeaZ protein, a putative partner of Gcp. We obtained evidence that YeaZ also plays a critical role in *P. aeruginosa* viability.

Introduction

Pseudomonas aeruginosa is a versatile bacterium that can adapt to a broad range of ecological niches [1,2]. In addition, it can infect multiple hosts as diverse as plants, nematodes and mammals [3]. In humans is the third most common cause of nosocomial infections, such as chronic infection of the lungs in cystic fibrosis (CF) patients, urinary tract infections resulting from catheterization, burn wounds and keratitis [4–6]. *P. aeruginosa* is resistant to virtually all classes of antibiotics in clinical use and has consequently joined the ranks of "Superbugs" [7,8]. *P. aeruginosa* infections are difficult to eradicate due to its high intrinsic resistance, together with its ability to develop resistance, often multiples, through adaptation and mutation [9]. Therefore, *P. aeruginosa* is a major concern in hospital settings and its multi-drug resistance nature incredibly limited the number and effectiveness of therapeutic options [10].

The discovery of novel essential genes or pathways that have not yet been targeted by clinical antibiotics can underlie the development of alternative effective antibacterials to overcome the extant mechanisms of resistance. We recently published an antisense-mediated strategy [11], established previously in *S. aureus* [12,13], for the identification of novel essential genes of *P. aeruginosa*. Among several growth-impairing genomic inserts that resulted from our antisense screenings, we focused on one that targeted the *locus* PA0580.

PA0580, annotated in the Pseudomonas Genome Database (PGD) [14] as *gcp* or *ygjD*, encodes a protein, Gcp, which belongs to Kae1/Qri7/YgjD family (COG0533) including top-10 universal proteins of unknown function awaiting characterization [15,16]. Members of this family have been studied in terms of overall cellular effects mainly by modulation of protein expression studies. In yeast, Kae1 is not essential but forms part of the KEOPS complex involved in transcription, maintenance of telomeres, and segregation of chromosomes [17–19]. In prokaryotes, YgjD has been shown to be an essential gene product in many different species such as *Staphylococcus aureus* [20], *Streptococcus pneumonia* [21], *Escherichia coli* [22], *Bacillus subtilis* [23] *Francisella novicida* [24].

Several functions have been proposed for YgjD orthologs and their various partner proteins. A YgjD ortholog was annotated first 20 years ago as Gcp for *O*-sialo*g*ly*c*oprotein-endo*p*eptidase in *Mannheimia* (formerly *Pasteurella*) *haemolytica* [25,26]. The endopeptidase activity of Gcp was never confirmed [19]. More recently, it has been shown that YgjD orthologs are involved in the generation of N^6-threonyl-carbamoyl-adenosine (t^6A), an universal modification found at position 37 of tRNAs decoding ANN codons [27,28] (Figure S1). In *E. coli*, four proteins TsaB, TsaC, TsaD, and TsaE (formely YeaZ, YrdC, YgjD, and YjeE respectively) have been reported to be

‘necessary and sufficient’ for t^6A synthesis *in vitro* [29]. Moreover, it has been published that the *E. coli* YgjD (TsaD) form a complex with YeaZ (TsaB) which is also essential for *E. coli* growth [22]. Although YeaZ and YgjD share 30% identity within their first 100 amino acids, YeaZ is only found in bacteria and was assigned to COG1214 which is annotated as “Inactive homolog of metal-dependent proteases, putative molecular chaperone.”[19]. Recently, the structure of the *Salmonella typhimurium* YgjD ortholog was solved in complex with YeaZ to 2.3 Å resolution showing the typical ASKHA superfamily fold, with the protein organized into 2 lobes as an atypical DNA binding protein [30].

In this study, combining several genetic approaches, we showed that Gcp is essential for *P. aeruginosa*. In addition, we focused on the product of PA3685 *locus*, a YeaZ ortholog. Our results suggest that this protein also plays a critical role in *P. aeruginosa* viability.

Methods and Materials

Bacterial strains, plasmids and growth conditions

The *P. aeruginosa* and *E. coli* strains, as well as the plasmids used in this study, are listed in Table S1. pHERD20T [31], used for the cloning procedures and for the construction of antisense libraries [11], is a *E. coli*/*Pseudomonas* shuttle vector carrying *araC*/P_{BAD} pair and ampicillin/carbenicillin resistance. pHERD-E2-A*gcp* was obtained by cloning the *Eco*RI-*Hind*III fragment amplified by PCR from pHERD-S1E2 with primers AgcpFw and AgcpRev (Table S2) into pHERD20T digested with the same enzymes. pDM4 [32] is a suicide plasmid in *Pseudomonas* species carrying the chloramphenicol resistance. Plasmid pQE30 was purchased from QIAGEN and used for recombinant protein expression. Plasmid pSC200 carrying gentamicin resistance [33] enables the delivery of the rhamnose-inducible P_{rhaB} promoter into the chromosome to drive the expression of a targeted gene.

Bacteria were grown at 37 °C in Luria–Bertani (LB) broth, or in M9 minimal medium supplemented with 0.2% citrate (M9-citrate). Antibiotics were added at the following concentrations (µg/ml): carbenicillin (Cb) 300; ampicillin (Amp) 100; kanamycin (Km) 50; chloramphenicol (Cm) 30 and 85 for *E. coli* and *P. aeruginosa*, respectively; 25; gentamicin (Gm) 25 and 50 for *E. coli* and *P. aeruginosa*, respectively. Arabinose, rhamnose, glucose and isopropyl-β-D-Thiogalactoside (IPTG) were used at concentrations of 7.5 mM, 0.2%, 1%, and 0.1 mM, respectively. In mating experiments, exconjugant *P. aeruginosa* PAO1 clones were selected on Pseudomonas Isolation Agar (PIA; Difco) with an appropriate antibiotic.

Plasmids were mobilized from *E. coli* to *P. aeruginosa* PAO1 by conjugative triparental mating [34]. *E. coli* donor strains were grown overnight in LB broth supplemented with appropriate antibiotic. The recipient *P. aeruginosa* PAO1 and helper *E. coli* HB101/pRK2013 strains were grown overnight in LB broth at 42 °C and 37 °C respectively. Fifty microliters each of helper, recipient, and donor strains were mixed in 5 ml of sterile $MgSO_4$ solution. After mixing, each mixture was filtered on 13 mm filters (containing pores of 0.45 µm in diameter; Millipore, Bedford, Mass., USA) and placed on nutrient agar plates. The plates were incubated at 37 °C overnight and then bacteria grown on filter were resuspended in 3 ml of a sterile $MgSO_4$ solution. 100 µl aliquots of resuspended cells were plated onto nutrient agar plates containing appropriate antibiotic.

Cloning, expression and Gcp, YeaZ purification

pQE30 clonings and construction pHR6HT-Gcp plasmid. The *gcp* open reading frame (ORF) was amplified from PAO1 genomic DNA by PCR with the forward primer GcpFULLfw and the reverse primer GcpFULLrev (Table S2). PCR product was digested with *BamH*I and *Hind*III and inserted into the commercial expression pQE30 (Qiagen) digested with the same enzymes to generate the Gcp expression plasmid pQE30-Gcp for expression of the 6xHis tagged protein.

The PA3685 coding sequence was amplified from PAO1 genomic DNA by PCR with oligo pairs YeaZFULLfw/YeaZFULLrev, then PCR product followed the same protocol described above obtaining the plasmid pQE30-YeaZ for expression of the 6xHis tagged YeaZ protein.

pHR6HT-Gcp was obtained by cloning *Eco*RI/*Sal*I the fragment amplified by PCR with primers pQE30-RBS-Fw and pQE30-Sal-rev (Table S2) from pQE30-Gcp into pHERD20T digested with the same enzymes. This construct contains the synthetic ribosomal binding site of pQE30 for high translation rates, the 6xHis-tagged coding sequence of *gcp* and strong transcriptional terminator t_0 from phage lambda [35] and allows the expression of Gcp-6xHis-tagged protein directly in *P. aeruginosa.* PCR reactions were performed using DreamTaq Polymerase (Fermentas) with the addition of 1 M betaine (Sigma Aldrich), because of the high GC content of *P. aeruginosa* genome.

Expression and purification of Gcp and YeaZ. M15[pREP4] transformed with pQE30-Gcp or pQE30-YeaZ were grown in LB medium supplemented with Amp and Km at 37°C until the logarithmic phase (at OD_{600} of 0.6) and induced by isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 0.1 mM for 5 hrs at 30 °C. PAO1 carrying pHR6HT-Gcp was cultured in LB medium supplemented with Cb for growth at 37°C until the logarithmic phase (at OD_{600} of 0.6) and induced by arabinose at a final concentration of 7.5 mM for 4 hrs at 37 °C.

Cells were harvested and resuspended in resuspension buffer (50 mM Phosphate Buffer, 150 mM NaCl, 10 mM imidazole, pH 7.4). Cells were disrupted by French Press device (20 bar pressure for *E. coli* and 25 bar for *P. aeruginosa*) then cellular debris was removed by centrifugation at 7000 rpm for 20 min at 4°C. Protein extracts were incubated with one tablet of protease inhibitor Complete Mini EDTA-free (Roche) 10 µg/ml DNase I (New England Biolabs) 5 µg/ml Ribonuclease A (Sigma) for 30 minutes at 4 °C. Extracts were centrifuged at 13000 rpm, for 45 minutes at 4 °C then soluble fraction was collected and filtered (0.22 µm).

FPLC was performed with 1 ml Ni-NTA Superflow (Qiagen), previously equilibrated with 20 ml of Phosphate Buffer. Column was washed with 50 ml of wash buffer (50 mM Phosphate Buffer, 150 mM NaCl, 30 mM Imidazole, pH 7.4) before the bound His-tagged proteins were eluted by the addition of elution buffer (50 mM Phosphate Buffer, 150 mM NaCl, 200 mM Imidazole). Eluted

fractions were collected together using Pierce Protein Concentrators, 9K MWCO (Thermo Scientific) to remove imidazole and concentrate the sample that was subsequently loaded on a HiLoad 16/600 Superdex 75 pg (GE Healthcare) column to perform gel filtration chromatography analysis.

Gel filtration chromatography. Analytical gel-filtration experiments were performed in an ÄKTA Purifier–10 system (GE Healthcare) with a HiLoad 16/600 Superdex 75 pg (GE Healthcare) column. A 2 ml aliquot of purified $(His)_6$-Gcp/YeaZ proteins were eluted using 50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 1 mM β-mercaptoethanol. Elution volumes were collected and protein was detected by absorbance at 280 nm. Molecular mass values for $(His)_6$-Gcp/YeaZ were interpolated from a calibration curve using Aldolase (158 kDa), Conalbumin (75 kDa), Ovalbumin (44 kDa), Carbonic anhydrase (29 kDa), and Ribonuclease A (13.7 kDa) as standards (GE Healthcare).

In vitro activity assays

Protease activity assay. Detection of Gcp protease activity was carried out with EnzChek Protease Assay Kit (E6638, Molecular Probes, Life Technologies) that provides a FRET (fluorescence resonance energy transfer)-based method for the simple and accurate quantification of a wide range of protease activities The EnzChek Protease Assay Kit was used essentially according to manufacturer's instructions. Gcp purified protein was mixed in a total volume of 200 µl with 1 mg/ml bovine serum albumin (SIGMA) and 5 µg/ml BODIPY-FL labeled casein substrate in digestion buffer (10 mM Tris-HCl pH 7.8, 100 µM sodium azide) in a 96-well microtiter plate. The plate was incubated for 1h at 37 °C and fluorescence was measured at 485 nm excitation/525 nm emission in an Infinite M200 microplate reader (TECAN Group Ltd.). Trypsin (Sigma) 1 mg/ml was used as a control.

ATPase activity assays. Gcp ATPase activity was determined using adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma FL-AA). This kit allows quantitative bioluminescent determination of ATP through a reaction that emits light proportional to the ATP present. The adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit was used following the manufacturer′s protocol. 10 µl solutions with all the compounds necessary (Mg^{2+} 1 mM, Na_2CO_3 10 mM, Threonine 10 mM, ATP 100 mM, purified Gcp 2 mg/ml) have been set up along with the control with the denatured protein (warmed at 95 °C for 15 minutes) and incubated at 25 °C for 1 hour.

Then 180 µl of the assay kit were added and measurements were made on a TD 20/20 luminometer (Turner Designs).

RT-PCR analysis of PA3685 genomic region

Total RNA was purified from *P. aeruginosa* PAO1 cells, grown in LB with aeration at 37 °C until OD_{600} of 0.6 (mid-exponential phase) was achieved using RNeasy Mini kits (Qiagen) that included DNase I treatment. Residual DNA was removed from purified RNA by further treatment with RNA-free DNase I (New England Biolabs) at 37 °C for 15 min, followed by DNase I inactivation with 2.5 mM EDTA at 65°C for 10 min. cDNA was generated by incubating 1 µg of RNA with Superscript II Reverse Transcriptase (RT) (Invitrogen), 100 pg of random primers (Invitrogen) and buffer supplied by the manufacturer for 50 min at 42 °C. RT was inactivated by incubation at 70 °C for 15 min. As a control of DNA contamination in the subsequent RT-PCR analysis, reactions were also run without RT. RT-PCR analysis was performed with the oligo pairs listed in Table S2.

Mutagenesis analyses

Insertional mutagenesis assay. DNA fragments about 300-500 bp of PA0580, PA2873, PA3685, *dnaG* and *algR*, respectively, were amplified by PCR with oligo pairs containing *Sal*I restriction sites listed in Table S2, digested with *Sal*I (New England Biolabs) and cloned into the corresponding site of pDM4. The cloning was checked by PCR with the oligo pair pDM4-ori/pDM4-cat (Table S2). This procedure gave rise to pDM4 derivatives listed in Table S1 which were transferred from *E. coli* to *P. aeruginosa* PAO1 by triparental mating (see above for details) selecting exconjugant PAO1 clones carrying pDM4 cointegration on PIA plates supplemented with Cm.

Generation of rhamnose-dependent conditional mutant. An approximately 300 bp fragment of PA0580 starting at the start codon was amplified by PCR with gcp-psc-fw and gcp-psc-rev oligos (Table S2) carrying *Nde*I and *Xba*I sites, respectively. PCR product was purified with QIAquick PCR Purification Kit (Qiagen), digested with *Nde*I and *Xba*I (New England Biolabs) and cloned into the corresponding sites of the vector pSC200.
pSC200-PA0580 plasmid generated in *E. coli* JM109 was mobilized to *P. aeruginosa* PAO1 by triparental mating (see above). Exconjugant PAO1 clones carrying pSC200-PA0580 cointegration were selected on PIA plates supplemented with Gm and rhamnose. A clone, identified as PAO1

P_{rhaB}*::gcp* , was checked by PCR with oligos annealing at both sides of pSC200 MCS and stored for subsequently analysis.

Growth assay of conditional mutant. The PAO1 P_{rhaB}*::gcp* clone, was grown overnight at 37 °C with shaking in 20 ml of M9-citrate, supplemented with Gm and rhamnose. 2 ml aliquots of the overnight culture were centrifuged at 13.000 rpm, the pellet was washed several times with PBS. Bacterial cells were adjusted to an optical density OD_{600} of 1.0 and serially diluted up to 10^{-6}. To investigate the effects of P_{rhaB} promoter modulation on growth, the cell suspension was used to inoculate M9-citrate supplemented with Gm, and either with rhamnose (M9-citrate-rhamnose) or glucose (M9-citrate-glucose). 200 µl aliquots of PAO1 P_{rhaB}*::gcp* inoculated in M9-citrate-rhamnose and M9-citrate-glucose were distributed in triplicates in "Well Optical Bottom" 96-wells microplates (Nunc, Thermo Fisher Scientific) and incubated for 22 hrs in a Sunrise microplate reader (TECAN Group Ltd.) at 37 °C with constant shaking and real time OD measurement at 595 nm every 15 min. In parallel, to investigate the growth defect of essential gene conditional mutants in solid media, the cell suspension in PBS at OD_{600} of 1 was serially diluted up to 10^{-7} and spotted on solid M9-citrate with Gm, in the presence of rhamnose or glucose and incubated for 12 hrs at 37 °C.

Complementation of conditional mutant PAO1 P_{rhaB}*::gcp.* The recombinant plasmid pHR6HT-Gcp was introduced into PAO1 P_{rhaB}*::gcp* by triparental mating (see above) and used for complementation *in trans* of conditional mutant. The mutant so obtained PAO1*gcp*C was selected on PIA medium supplemented with rhamnose, Cb and Gm. PAO1*gcp*C was grown overnight at 37 °C with shaking in 20 ml of LD, supplemented with Cb, Gm and rhamnose. Overnight culture was centrifuged at 13.000 rpm and the pellet was washed three times with PBS. Bacterial cells were adjusted to an optical density OD_{600} of 1.0 and serially diluted up to 10^{-7}: 2 µl drops from each dilution were transferred on M9-citrate media supplemented with arabinose and with glucose or rhamnose, and incubated for 12 hrs at 37 °C.

Transmission electron microscopy analysis

Transmission electron microscopy analysis of PAO1 P_{rhaB}*::gcp* strain was carried out essentially as described by Stanley *et al.* [36]. For TEM observation of Epon resin-embedded samples, overnight cultures were centrifuged 30 minutes at 6000 *g*, resuspended in 2.5% gluteraldehyde in 0.1 M sodium cacodylate, incubated at ambient temperature for 15 minutes, centrifuged at 20000 *g* for 10 minutes. After an overnight incubation at 4°C, samples were treated with 1% (wt/vol) OsO_4 in

distilled water for 30 min on ice in darkness. The osmium fixation was followed by three 10 min washes in distilled water before initiation of the ethanol dehydration series. Finally, cells were dehydrated in a graded ethanol series (70, 80, 90, 96, and 100% ethanol), gradually infiltrated with Epon resin sections were made on an Ultracut E microtome (Reichert). Ultrathin sections of 80 nm, made with a diamond knife (Diatome), were collected on 200 mesh formvar/carbon copper grids, double stained with uranyl acetate and lead citrate and examined at an EFTEM Leo912ab transmission electron microscope (Zeiss) at 80 kV. Digital images were acquired by Esivision CCD-BM/1K system. Images were processed with Adobe Photoshop for contrast adjustment (Image-adjustments-autocontrast).

Statistical analysis

If not otherwise stated, all experiments were performed at least in triplicate and the data were analysed by one-way analysis of variance, with a *p*-value of 0.05 being significant, using the statistical software package R (R Core Team, 2013).

Results and Discussion

Antisense targeting of PA0580 *locus*

In a shotgun antisense approach to the identification of novel essential genes in *P. aeruginosa* [11], we identified 43 growth-impairing inserts carrying multiple *loci*. One such insert of 692 bps, named S1E2, corresponded to a PAO1 genomic region (coordinates 640687 to 639995) including the 3' portions of two contiguous inverted ORFs (Figure S2): PA0581 (coordinates 640420 – 640989; - strand), from 302 to 569 positions, and PA0580 (639316 – 640341; + strand) from 680 to 1026 positions. S1E2 insert orientation placed in the S1E2-carrying plasmid pHERD-S1E2 the 3' portions of PA0581 and PA0580 downstream the P_{BAD} promoter in sense and antisense direction, respectively. We speculated that the growth-impairment mediated by S1E2 was elicited by antisense effect on PA0580 because we found in the Database of Essential Genes (DEG) [37] several orthologs of PA0580. To test this hypothesis, a DNA fragment spanning 680 to 1026 of PA0580 was cloned downstream to the P_{BAD} promoter of pHERD20T in antisense orientation to generate pHERD-E2-A*gcp*. Similarly to pHERD-S1E2, pHERD-E2-A*gcp* was able to impair PAO1 growth once it had been transferred from *E. coli*. These results strongly suggested that the product of PA0580 *locus*, the Gcp protein, could play a novel and uncharacterized essential role in *P. aeruginosa*.

Validation of PA0580 essentiality

The essential role of PA0580 was validated by insertional mutagenesis. PA0580 was targeted for knock-out by homologous recombination-mediated cointegration of the suicide vector pDM4 carrying Cm resistance (Cm^R). Since pDM4 is unable of autonomous replication in PAO1, following conjugal transfer from *E. coli* to PAO1, Cm^R clones can be selected only if pDM4 cointegrates with the PAO1 chromosome. The *dnaG* gene for DNA primase, a single-strand DNA (ssDNA)-dependent RNA polymerase that plays a key role in DNA synthesis [38], and *algR* gene for a LytTR-type two-component response regulator [39] were used as positive and negative controls of essentiality, respectively. An additional positive control of essentiality was the *tgpA* gene that we recently characterized as essential [40].

Briefly, approximately 500 bp fragments of PA0580 (*gcp*), *tgpA*, *dnaG* and *algR*, were cloned into pDM4 and the resulting constructs were transferred from *E. coli* CC118-λpir to PAO1 by triparental mating. Cointegration events were selected by plating the conjugation mixtures on PIA supplemented with Cm. Three independent conjugation experiments were performed for each gene

and the results were expressed as a percentage of Cm^R ex-conjugant clones relative to the negative control *algR*. As shown in Figure 1, targeting pDM4 cointegration to PA0580 gave rise to a resounding decrease (more than 99%) in the yield of Cm^R ex-conjugant clones, comparable to *tgpA* inactivation and even lower than the results obtained for the positive control *dnaG*.

To further assess the essentiality of PA0580, we performed conditional mutagenesis through the insertion of the rhamnose-induced and glucose-repressed P_{rhaB} promoter upstream PA0580. In this approach, a fragment of approximately 300 bp spanning the 5' region of PA0580 was cloned into the suicide vector pSC200 and the resulting recombinant plasmid pSC200-PA0580 was transferred into *P. aeruginosa* PAO1. Following cointegration of pSC200-PA0580 with the PAO1 chromosome by homologous recombination, PA0580 was located downstream P_{rhaB} and thus its expression could be stringently controlled by the amounts of rhamnose and glucose in the growth medium. The *P. aeruginosa* strain resulting from pSC200-PA0580 cointegration was named PAO1 P_{rhaB}::*gcp*. To test the essentiality of PA0580, PAO1 P_{rhaB}::*gcp* was inoculated in M9-citrate medium supplemented with either 0.2% rhamnose (permissive condition) or 1% glucose (non-permissive condition). As shown in Figure 2A and B, the PAO1 P_{rhaB}::*gcp* strain showed a conditional rhamnose-dependent growth in both liquid and solid media. The strong impairing effects of glucose on growth were specific for PAO1 P_{rhaB}::*gcp* since PAO1 *wt* grew efficiently on glucose (Figure 2A). Due to the low number of cells in the inoculum, growth was expected to be initially undetectable by microtiter reader (Figure 2A). Therefore, the growth of PAO1 P_{rhaB}::*gcp* was monitored for 6 hours from inoculum also by titration of colony forming units per ml (CFU/ml) on LB plates. As shown in Figure 2C, PAO1 P_{rhaB}::*gcp* in rhamnose started growing and the number of cells was detectable about 12 hours from inoculum (Figure 2A). On the contrary, for PAO1 P_{rhaB}::*gcp* in glucose only residual growth was initially observed (Figure 2C) and the threshold of cell number detection was never reached (Figure 2A). Plasmid pHR6HT-Gcp, a pHERD20T derivative expressing PA0580 under the control of the P_{BAD} promoter was introduced into PAO1 P_{rhaB}::*gcp* and growth in the presence of glucose was tested on solid medium. As shown in Figure 2B, pHR6HT-Gcp, and not pHERD20T, could restore growth of PAO1 P_{rhaB}::*gcp* in the presence of glucose. Overall, the results presented above strongly indicated that PA0580 product Gcp plays a critical role in the viability of *P. aeruginosa*.

We investigated at cellular level the consequences of Gcp depletion. PAO1 P_{rhaB}*::gcp* cells were inoculated as above in M9-citrate medium supplemented with either 0.2% rhamnose or 1% glucose and incubated overnight at 37° C. PAO1 P_{rhaB}*::gcp* cells were then collected and processed for TEM observation. As shown in Figure 3, depletion of Gcp in the presence of glucose resulted in pleiotropic effects with unusual cellular morphologies, i.e, cell elongation and branching,

concatenated cells, anomalies in septation, “rough” outer membranes, lack of OM vescicles, etc. Similar effects were already described following YgjD depletion in *E. coli* [22].

Gcp protein forms homodimers and shows residual peptidase and ATPase activities

Gcp is a 36.6 kDa protein annotated in PGD [14] as a O-sialoglycoprotein endopeptidase with a predicted extracellular localization. Different structural features can be predicted: the Pfam protein families database [42] recognizes, between aa 23 and 311, a conserved domain belonging to glycoprotease family (Peptidase_M22, PF00814) while TIGRFAMs database [43] identifies, between aa 3 and 318, a domain belonging to tRNA threonylcarbamoyl adenosine modification. These conflicting annotations regarding its function reflect 20 years of studies and attempts of characterization resulting in sometimes elusive data [17–19,22,25,44,45].

N-terminal $(His)_6$-tagged full length Gcp was expressed in *E. coli* and purified as described in the Materials and Methods section. Unfortunately, as described previously for YgjD [22], we observed that Gcp was subjected to degradation during the purification steps. To overcome this problem, we performed the purification in *P. aeruginosa*. To this purpose, the pHERD20T shuttle vector has been engineered as described in the Materials and Methods section to give rise to pHR6HT-Gcp.

Analytical gel filtration experiments were performed on purified Gcp. As shown in Figure 4, Gcp eluted as a single peak corresponding to a molecular weight of 75 kDa. This correlates with the estimated mass of the dimeric form of Gcp (74.5 kDa). Interestingly, our results are consistent with those proposed for the *E. coli* Gcp ortholog YgjD [22] while in *S. typhimurium* only the monomeric form was described [30].

The Gcp protein was first described in *Mannheimia haemolytica* as a secreted protein endowed with glycopeptidase activity. To assess this proteolytic activity, the purified Gcp protein was tested through a fluorescence-based microassay at pH 7. As shown in Figure 5A, Gcp showed residual proteolytic activity. The assay was repeated at different pH values, ranging from 5.5 to 8.5. No increase of proteolytic activity was observed in this pH range. This results are consistent with those proposed previously by several research groups [28,46] that could not confirm the evidence for glycopeptidase activity which was initially claimed.

More recently, it has been shown that YgjD orthologs are involved in N^6-threonyl-carbamoyl-adenosine (t^6A), an universal modification found at position 37 of tRNAs decoding ANN codons [27,28] through a potential biochemical pathway and intermediates showed in Figure S1. In this context, it was proposed that YgjD could be an ATP-dependent protein [27,47] potentially endowed with ATPase. Therefore, we tested Gcp for ATPase activity through a bioluminescent assay. The experiments were performed in the presence of the putative cofactors [29], threonine and

bicarbonate, and using as control the same amount of inactivated protein. As shown in Figure 5B, Gcp showed residual ATPase activity.

Putative partners of Gcp in *P. aeruginosa*

It has been recently published [29] that in *E. coli* YgjD, renamed TsaD, interacts with other three proteins TsaB, TsaC and TsaE (formely YeaZ, YrdC, and YjeE, respectively) for t^6A synthesis *in vitro*. We speculated the presence of these partners in *P. aeruginosa*. Sequence alignment analysis strongly suggested that the *P. aeruginosa* hypothetical proteins PA3685 (YeaZ), PA0022 (YrdC) and PA4948 (YjeE) are orthologs of *E. coli* TsaB, TsaC and TsaE, respectively (Table S3; Figure S3). We focused our attention on PA3685, since it was recently shown that physically interacts with Gcp in *S. typhimurium* and crystal structure of Gcp-YeaZ eterodimer was resolved [30]. PA3685 encodes a small highly conserved protein unique to bacteria; it was found in all bacterial genomes sequenced to date, with the exceptions of the highly reduced genomes of the obligate endosymbionts *Carsonella ruddii* and *Sulcia muelleri* [48,49]. Like Gcp, with whom shares 30% identity within their first 100 amino acids, also YeaZ has been shown to be essential in many different species such as *S. aureus* [20], *S. pneumonia* [21], *E. coli* [22], *B. subtilis* [23] *F. novicida* [24]. First, we set out to validate the essential role in *P. aeruginosa* through the insertional mutagenesis approach described above. As shown in Figure 6A, targeting pDM4 cointegration to PA3685 gave rise to dramatic decrease in the yield of Cm^R ex-conjugant clones, comparable with the results obtained for the positive control *dnaG*. However, since in PGD PA3685 *locus* was predicted to be the first ORF of an operon which spans up to PA3679, it is possible that the results of insertional mutagenesis reflected a polar effect on downstream ORFs. The cluster arrangement annotated in PGD is not classified similarly in EcoCyc database [50] which instead indicates that there is no high-quality evidence to confirm the extent of this transcription unit. To clarify this annotation and to profile the co-transcription of the first three ORFs (PA3685-3684-3683), we performed RT-PCR experiments on total RNAs purified from PAO1 cell samples with oligo pairs which amplified regions spanning the 3' to 5' of adjacent ORFs. As shown in Figure 6B, we could observe co-transcription for PA3685-3684 and PA3684-3683 pairs but not for PA3685-3683. These results suggested that PA3685 and PA3683 do not belong to the same transcription unit. In this scenario, the insertional mutagenesis experiment described above suggests the essentiality of PA3685. However, polar effects on PA3684 of insertions within PA3685 cannot be ruled out.

Finally, an N-$(His)_6$-tagged YeaZ was purified and analytical gel filtration experiments were performed. As shown in Figure 6C, YeaZ eluted as single peak of 51 kDa that correlates with the

estimated molecular weight of the dimeric form (49.7 kDa). These results are consistent with the previous characterization of YeaZ orthologs in *E.coli* and *S. typhimurium* [22,30].

Conclusions

In this work, we showed the essentiality of Gcp, the gene product encoded by PA0580 and showed that its depletion resulted in pleiotropic effects with unusual cellular morphologies. We speculated that the role of Gcp in *P. aeruginosa* could be the same as YgjD in *E.coli* [29], i.e. an involvement in producing the t^6A modification. This can be crucial for translation of several proteins and Gcp depletion could lead to an impairment of cell cycle progression. Moreover, the results presented above suggest that also YeaZ, a supposed partner of Gcp, plays a critical role in the viability of *P. aeruginosa*. To our knowledge, this represents the first report where both *P. aeruginosa* Gcp and YeaZ proteins were purified and their homodimeric nature was described. Further investigations will be required to unravel the role of Gcp and YeaZ in *P. aeruginosa*. YeaZ and Gcp are sequence-related proteins, sharing 30% identity within their first 100 amino acids. While at least one copy of the *gcp* gene is found in the genome of every sequenced organism to date, *yeaZ* shows a different distribution, being present uniquely in bacteria. Therefore, YeaZ by itself, or its interaction with Gcp, could better represent a novel target for the development of innovative anti-*Pseudomonas* agents.

Acknowledgments

The authors are grateful to Nadia Santo (Interdepartimental Center of Advanced Microscopy, Università degli Studi di Milano) for electron microscopy analysis.

References

1. Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, et al. (2008) Dynamics of Pseudomonas aeruginosa genome evolution. Proc Natl Acad Sci U S A 105: 3100–3105. doi:10.1073/pnas.0711982105.

2. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tümmler B (2011) Pseudomonas aeruginosa Genomic Structure and Diversity. Front Microbiol 2: 150. doi:10.3389/fmicb.2011.00150.

3. Mahajan-Miklos S, Rahme LG, Ausubel FM (2000) Elucidating the molecular mechanisms of bacterial virulence using non-mammalian hosts. Mol Microbiol 37: 981–988. Available: http://www.ncbi.nlm.nih.gov/pubmed/10972817. Accessed 6 April 2014.

4. Mittal R, Aggarwal S, Sharma S, Chhibber S, Harjai K (2009) Urinary tract infections caused by Pseudomonas aeruginosa: A minireview. J Infect Public Health 2: 101–111. doi:10.1016/j.jiph.2009.08.003.

5. Hazlett LD (2004) Corneal response to Pseudomonas aeruginosa infection. Prog Retin Eye Res 23: 1–30. doi:10.1016/j.preteyeres.2003.10.002.

6. Azzopardi EA, Azzopardi E, Camilleri L, Villapalos J, Boyce DE, et al. (2014) Gram negative wound infection in hospitalised adult burn patients-systematic review and metanalysis-. PLoS One 9: e95042. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3994014&tool=pmcentrez&rendertype=abstract. Accessed 30 April 2014.

7. Strateva T, Yordanov D (2009) Pseudomonas aeruginosa - a phenomenon of bacterial resistance. J Med Microbiol 58: 1133–1148. doi:10.1099/jmm.0.009142-0.

8. Poole K (2011) Pseudomonas aeruginosa: resistance to the max. Front Microbiol 2: 65. doi:10.3389/fmicb.2011.00065.

9. Breidenstein EBM, de la Fuente-Núñez C, Hancock REW (2011) Pseudomonas aeruginosa: All roads lead to resistance. Trends Microbiol 19: 419–426. doi:10.1016/j.tim.2011.04.005.

10. Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y (2006) Multidrug-resistant Pseudomonas aeruginosa: risk factors and clinical impact. Antimicrob Agents Chemother 50: 43–48. doi:10.1128/AAC.50.1.43-48.2006.

11. Rusmini R, Vecchietti D, Macchi R, Vidal-Aroca F, Bertoni G (2014) A shotgun antisense approach to the identification of novel essential genes in Pseudomonas aeruginosa. BMC Microbiol 14: 24. Available: http://www.ncbi.nlm.nih.gov/pubmed/24499134.

12. Ji Y, Zhang B, Van SF, Horn, Warren P, et al. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. Science 293: 2266–2269. doi:10.1126/science.1063566.

13. Forsyth RA, Haselbeck RJ, Ohlsen KL, Yamamoto RT, Xu H, et al. (2002) A genome-wide strategy for the identification of essential genes in Staphylococcus aureus. Mol Microbiol 43: 1387–1400. doi:10.1046/j.1365-2958.2002.02832.x.

14. Winsor GL, Lam DKW, Fleming L, Lo R, Whiteside MD, et al. (2011) Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. Nucleic Acids Res 39: D596–D600. doi:10.1093/nar/gkq869.

15. Galperin MY, Koonin E V (2004) "Conserved hypothetical" proteins: prioritization of targets for experimental study. Nucleic Acids Res 32: 5452–5463. doi:10.1093/nar/gkh885.

16. Galperin MY, Koonin E V. (2010) From complete genome sequence to "complete" understanding? Trends Biotechnol 28: 398–406. doi:10.1016/j.tibtech.2010.05.006.

17. Kisseleva-Romanova E, Lopreiato R, Baudin-Baillieu A, Rousselle J-C, Ilan L, et al. (2006) Yeast homolog of a cancer-testis antigen defines a new transcription complex. EMBO J 25: 3576–3585. doi:10.1038/sj.emboj.7601235.

18. Downey M, Houlsworth R, Maringele L, Rollie A, Brehme M, et al. (2006) A Genome-Wide Screen Identifies the Evolutionarily Conserved KEOPS Complex as a Telomere Regulator. Cell 124: 1155–1168. doi:10.1016/j.cell.2005.12.044.

19. Hecker A, Leulliot N, Gadelle D, Graille M, Justome A, et al. (2007) An archaeal orthologue of the universal protein Kae1 is an iron metalloprotein which exhibits atypical DNA-binding properties and apurinic-endonuclease activity in vitro. Nucleic Acids Res 35: 6042–6051. doi:10.1093/nar/gkm554.

20. Lei T, Liang X, Yang J, Yan M, Zheng L, et al. (2011) The C-terminal domain of the novel essential protein gcp is critical for interaction with another essential protein yeaZ of staphylococcus aureus. PLoS One 6. doi:10.1371/journal.pone.0020163.

21. Zalacain M, Biswas S, Ingraham KA, Ambrad J, Bryant A, et al. (2003) A global approach to identify novel broad-spectrum antibacterial targets among proteins of unknown function. J Mol Microbiol Biotechnol 6: 109–126. doi:10.1159/000076741.

22. Handford JI, Ize B, Buchanan G, Butland GP, Greenblatt J, et al. (2009) Conserved network of proteins essential for bacterial viability. J Bacteriol 191: 4732–4749. doi:10.1128/JB.00136-09.

23. Kobayashi K, Ehrlich S, Albertini A, Amati G, Andersen K, et al. (2003) Essential Bacillus subtilis genes. Proc … 100: 4678–4683. Available: http://www.ncbi.nlm.nih.gov/pubmed/23420519\nhttp://www.pnas.org/content/100/8/4678.short.

24. Gallagher LA, Ramage E, Jacobs MA, Kaul R, Brittnacher M, et al. (2007) A comprehensive transposon mutant library of Francisella novicida, a bioweapon surrogate. Proc Natl Acad Sci U S A 104: 1009–1014. doi:10.1073/pnas.0606713104.

25. Abdullah KM, Lo RY, Mellors A (1991) Cloning, nucleotide sequence, and expression of the Pasteurella haemolytica A1 glycoprotease gene. J Bacteriol 173: 5597–5603.

26. Abdullah KM, Udoh EA, Shewen PE, Mellors A (1992) A neutral glycoprotease of Pasteurella haemolytica A1 specifically cleaves O-sialoglycoproteins. Infect Immun 60: 56–62.

27. El Yacoubi B, Hatin I, Deutsch C, Kahveci T, Rousset J-P, et al. (2011) A role for the universal Kae1/Qri7/YgjD (COG0533) family in tRNA modification. EMBO J 30: 882–893. doi:10.1038/emboj.2010.363.

28. Srinivasan M, Mehta P, Yu Y, Prugar E, Koonin E V, et al. (2011) The highly conserved KEOPS/EKC complex is essential for a universal tRNA modification, t6A. EMBO J 30: 873–881. doi:10.1038/emboj.2010.343.

29. Deutsch C, El Yacoubi B, de Crecy-Lagard V, Iwata-Reuyl D (2012) Biosynthesis of Threonylcarbamoyl Adenosine (t6A), a Universal tRNA Nucleoside. J Biol Chem 287: 13666–13673. doi:10.1074/jbc.M112.344028.

30. Nichols CE, Lamb HK, Thompson P, El Omari K, Lockyer M, et al. (2013) Crystal structure of the dimer of two essential Salmonella typhimurium proteins, YgjD & YeaZ and calorimetric evidence for the formation of a ternary YgjD-YeaZ-YjeE complex. Protein Sci 22: 628–640. Available: http://www.ncbi.nlm.nih.gov/pubmed/23471679.

31. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD (2008) PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in Pseudomonas and Burkholderia spp. and other bacteria. Appl Environ Microbiol 74: 7422–7426. doi:10.1128/AEM.01369-08.

32. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H (1996) Flagellin A is essential for the virulence of Vibrio anguillarum. J Bacteriol 178: 1310–1319.

33. Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, et al. (2007) A putative gene cluster for aminoarabinose biosynthesis is essential for Burkholderia cenocepacia viability. J Bacteriol 189: 3639–3644. doi:10.1128/JB.00153-07.

34. Simon R, Priefer U, Pühler A (1983) A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. Bio/Technology 1: 784–791. doi:10.1038/nbt1183-784.

35. Schwarz E, Scherer G, Hobom G, Kössel H (1978) Nucleotide sequence of cro, cII and part of the O gene in phage lambda DNA. Nature 272: 410–414. doi:10.1038/272410a0.

36. Stanley NR, Findlay K, Berks BC, Palmer T (2001) Escherichia coli strains blocked in Tat-dependent protein export exhibit pleiotropic defects in the cell envelope. J Bacteriol 183: 139–144. doi:10.1128/JB.183.1.139-144.2001.

37. Luo H, Lin Y, Gao F, Zhang C-T, 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: D574–80. Available: http://www.ncbi.nlm.nih.gov/pubmed/24243843.

38. Kuron A, Korycka-Machala M, Brzostek A, Nowosielski M, Doherty A, et al. (2014) Evaluation of DNA primase DnaG as a potential target for antibiotics. Antimicrob Agents Chemother 58: 1699–1706. Available: http://aac.asm.org.pros.lib.unimi.it/content/58/3/1699.full. Accessed 8 May 2014.

39. Deretic V, Dikshit R, Konyecsni WM, Chakrabarty AM, Misra TK (1989) The algR gene, which regulates mucoidy in Pseudomonas aeruginosa, belongs to a class of environmentally responsive genes. J Bacteriol 171: 1278–1283.

40. Milani A, Vecchietti D, Rusmini R, Bertoni G (2012) TgpA, a Protein with a Eukaryotic-Like Transglutaminase Domain, Plays a Critical Role in the Viability of Pseudomonas aeruginosa. PLoS One 7: e50323.

41. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, et al. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2: 2006.0008. doi:10.1038/msb4100050.

42. Finn RD, Mistry J, Tate J, Coggill P, Heger A, et al. (2010) The Pfam protein families database. Nucleic Acids Res 38: D211–D222. doi:10.1093/nar/gkm960.

43. Haft DH, Selengut JD, Richter RA, Harkins D, Basu MK, et al. (2013) TIGRFAMs and Genome Properties in 2013. Nucleic Acids Res 41: D387–95. doi:10.1093/nar/gks1234.

44. Katz C, Cohen-Or I, Gophna U, Ron EZ (2010) The ubiquitous conserved glycopeptidase Gcp prevents accumulation of toxic glycated proteins. MBio 1. doi:10.1128/mBio.00195-10.

45. Lei T, Yang J, Zheng L, Markowski T, Witthuhn BA, et al. (2012) The Essentiality of Staphylococcal Gcp Is Independent of Its Repression of Branched-Chain Amino Acids Biosynthesis. PLoS One 7. doi:10.1371/journal.pone.0046836.

46. Hecker A, Leulliot N, Gadelle D, Graille M, Justome A, et al. (2007) An archaeal orthologue of the universal protein Kae1 is an iron metalloprotein which exhibits atypical DNA-binding properties and apurinic-endonuclease activity in vitro. Nucleic Acids Res 35: 6042–6051. doi:10.1093/nar/gkm554.

47. Srinivasan M, Mehta P, Yu Y, Prugar E, Koonin E V, et al. (2011) The highly conserved KEOPS/EKC complex is essential for a universal tRNA modification, t6A. EMBO J 30: 873–881. doi:10.1038/emboj.2010.343.

48. McCutcheon JP, Moran NA (2007) Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci U S A 104: 19392–19397. doi:10.1073/pnas.0708855104.

49. Nakabachi A, Yamashita A, Toh H, Ishikawa H, Dunbar HE, et al. (2006) The 160-kilobase genome of the bacterial endosymbiont Carsonella. Science 314: 267. doi:10.1126/science.1134196.

50. Keseler IM, Mackie A, Peralta-Gil M, Santos-Zavaleta A, Gama-Castro S, et al. (2013) EcoCyc: fusing model organism databases with systems biology. Nucleic Acids Res 41: D605–12. Available: http://nar.oxfordjournals.org/content/41/D1/D605.

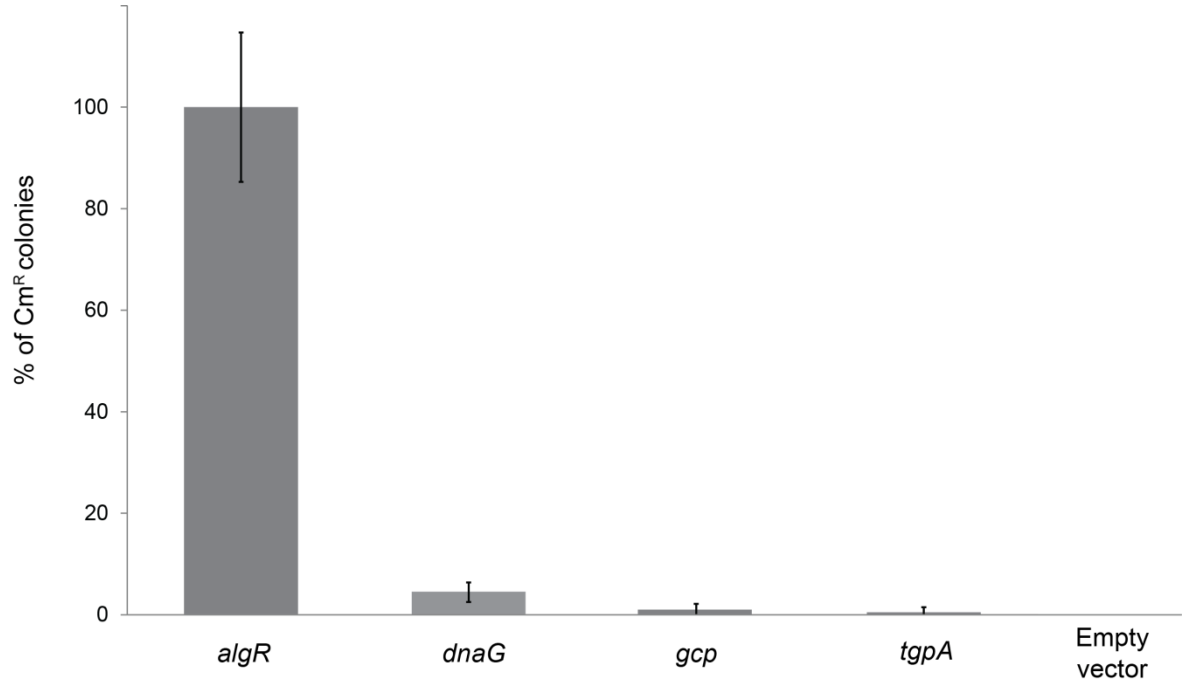


Figure 1. Insertional mutagenesis assay. Each indicated *locus* was targeted for knock-out by homologous recombination-mediated cointegration of the suicide vector pDM4 carrying chloramphenicol resistance (Cm^R). The *dnaG* gene and the *algR* gene were used respectively as positive and negative controls of essentiality. DNA fragments about 300-500 bp of *gcp* (PA0580), *tgpA*, *dnaG* and *algR*, respectively, were amplified by PCR and cloned into the corresponding site of pDM4. pDM4 derivatives obtained were transferred from *E. coli* CC118-λpir to *P. aeruginosa* PAO1 by triparental mating selecting exconjugant PAO1 clones carrying pDM4 cointegration on PIA plates supplemented with Cm. Three independent conjugation experiments were performed. Efficiency of cointegration in a given *locus* is expressed as a percentage of Cm^R ex-conjugant colonies relative to the negative control *algR*.

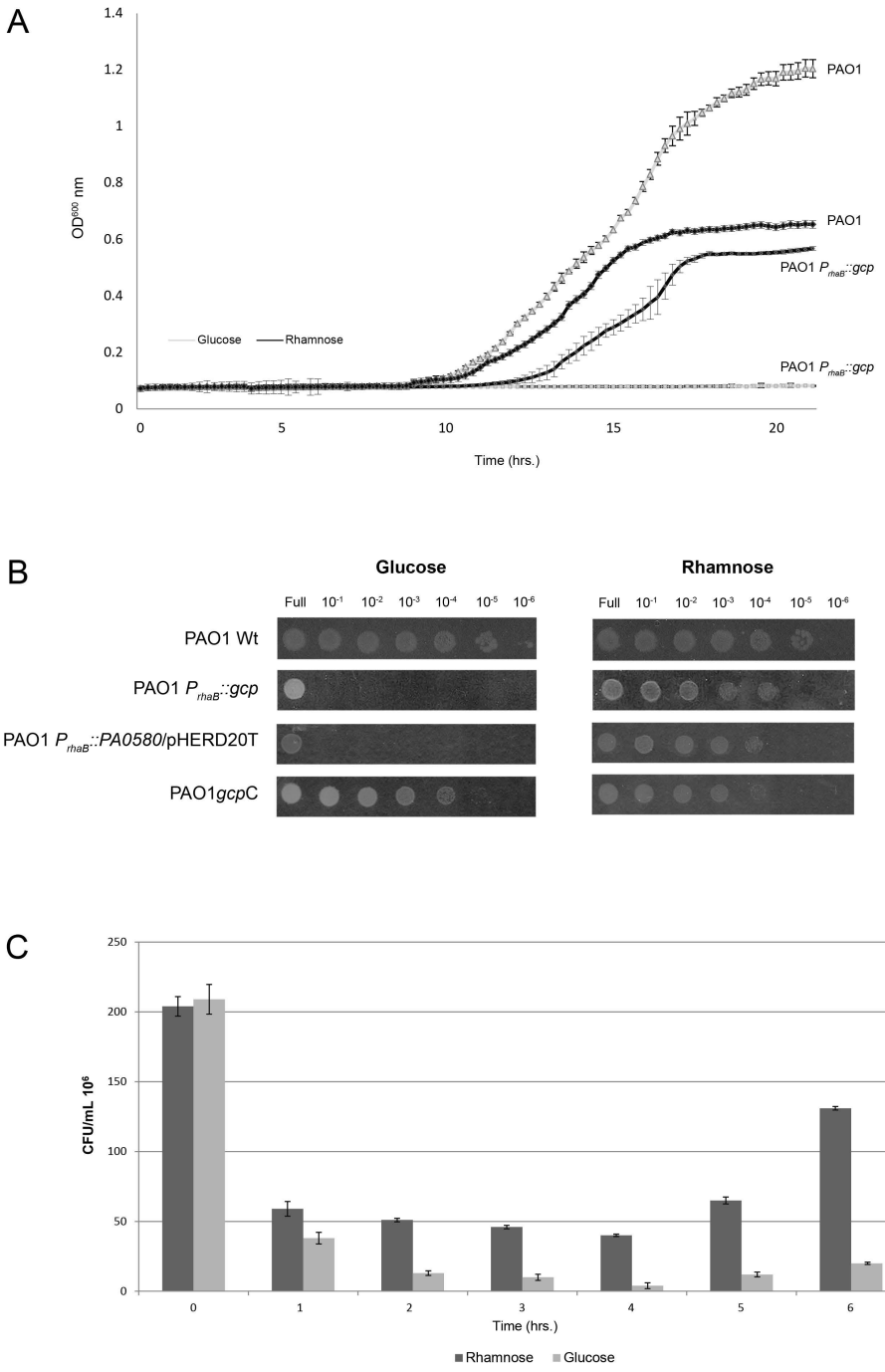


Figure 2. Conditional lethal phenotype of the rhamnose-dependent mutant PAO1 *P_{rhaB}*::*gcp*.
(A) The rhamnose inducible/glucose repressible promoter *P_{rhaB}* was inserted upstream to *gcp* giving rise to PAO1 *P_{rhaB}*::*gcp* strain. To test the repression effects of glucose on growth rate, overnight cultures of PAO1 *P_{rhaB}*::*gcp* in M9-citrate supplemented with rhamnose were centrifuged, pellet washed, resuspended and bacterial cells were adjusted to an optical density OD_{600} of 1.0 and serially diluted up to 10^{-6}. 200 µl aliquots of PAO1 *P_{rhaB}*::*gcp* were inoculated in microtiter wells in M9-citrate-rhamnose and M9-citrate-glucose and incubated for 22 hours. Culture growth at 37° C with stirring was monitored in real-time by OD_{600} measurement in a microtiter reader every 15 minutes. Note specificity of glucose/rhamnose effects on the growth of PAO1 *P_{rhaB}*::*gcp* and

especially how glucose does not interfere in any way with the growth of wild type strain while, on the contrary, completely inhibits growth of PAO1 P_{rhaB}*::gcp*. **(B)** Overnight cultures of PAO1 P_{rhaB}::*gcp* in M9-citrate supplemented with rhamnose were also tested for growth on solid M9-citrate supplemented with either rhamnose or glucose, by spotting 2 µl of 10-fold serial dilutions, from OD_{600} = 1 (left) to $OD_{600} = 10^{-6}$ (right). In the first lane is shown the control strain PAO1 wild type that, as in the previous experiment in liquid medium, is not influenced by the presence of glucose. Conversely, PAO1 P_{rhaB}*::gcp* is able to grow only in plates supplemented with rhamnose but not with glucose as expected for a mutant with essential gene under the control of rhamnose promoter. Complementation *in trans* of mutant PAO1 P_{rhaB}*::gcp* with recombinant plasmid pHR6HT-Gcp (PAO1*gcp*C) restored its ability to grow on glucose while the same mutant carrying the empty vector pHERD20T could not grow in the presence of repressor. **(C)** During the first 6 hrs from inoculum, a time window in which growth rate was undetectable by microtiter reader, the growth of PAO1 P_{rhaB}::*gcp* in liquid M9-citrate supplemented with either rhamnose or glucose was monitored by titration of colony-forming units per ml (CFU/ml) on LB plates.

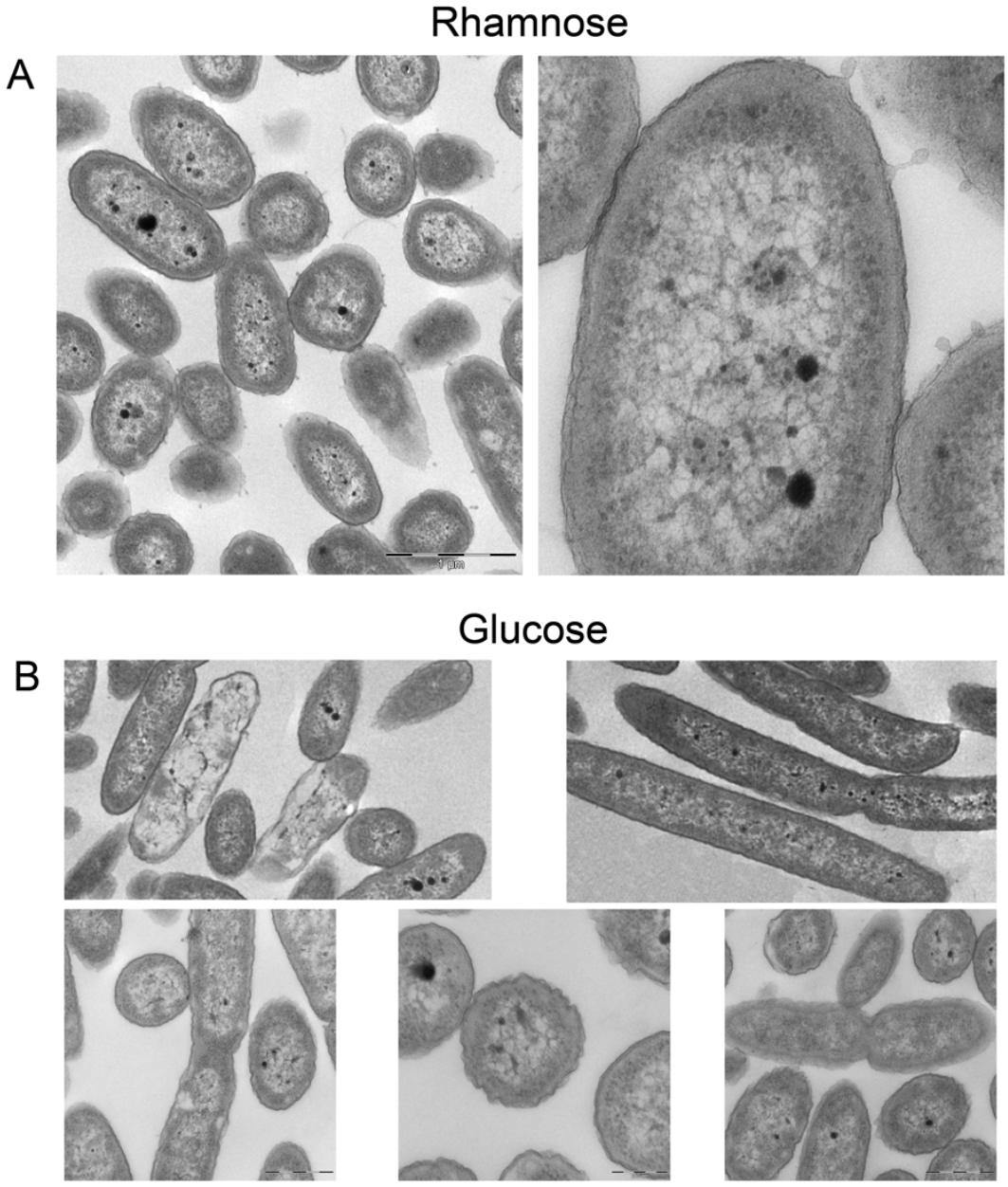


Figure 3. Cells depleted of Gcp display unusual morphologies. (A) Transmission electron micrographs of strain PAO1 P_{rhaB}*::gcp* after growth in LB containing rhamnose. Note that, as expected, no unusual morphologies are displayed. **(B)** Transmission electron micrographs of strain PAO1 P_{rhaB}*::gcp* after growth in LB containing glucose. Several growth defects are shown such as dead empty cells, elongated cells, anomalies in septum formation, "rough" outer membrane and lack of outer membrane vesicles.

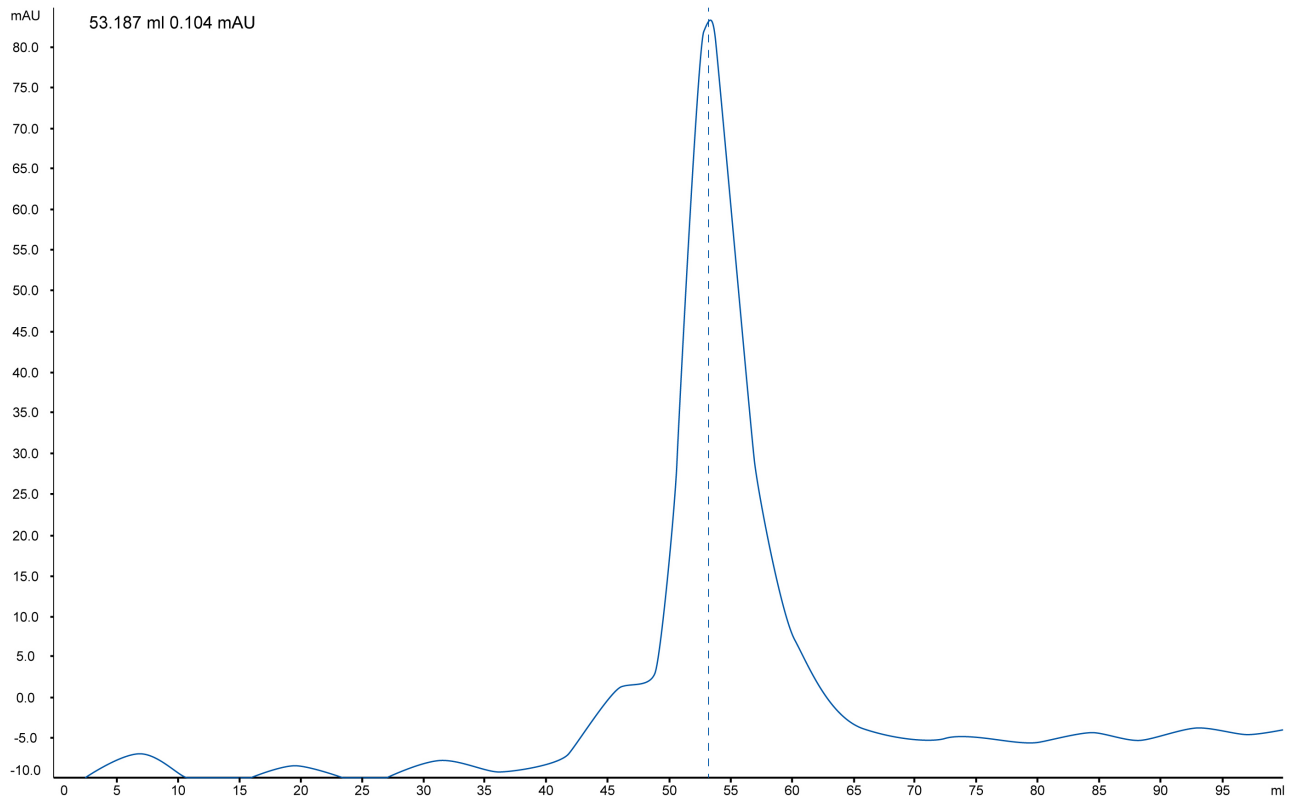


Figure 4. Analytical gel-filtration assay. A 2 ml aliquot of purified Gcp was analyzed with a HiLoad 16/600 Superdex 75 pg (GE Healthcare) column. The protein eluted predominantly as a single peak after gel filtration, corresponding to molecular masses of 75 kDa which was really close to the estimated masses for dimeric (74,5 kDa) form of N-$(His)_6$-tagged Gcp.

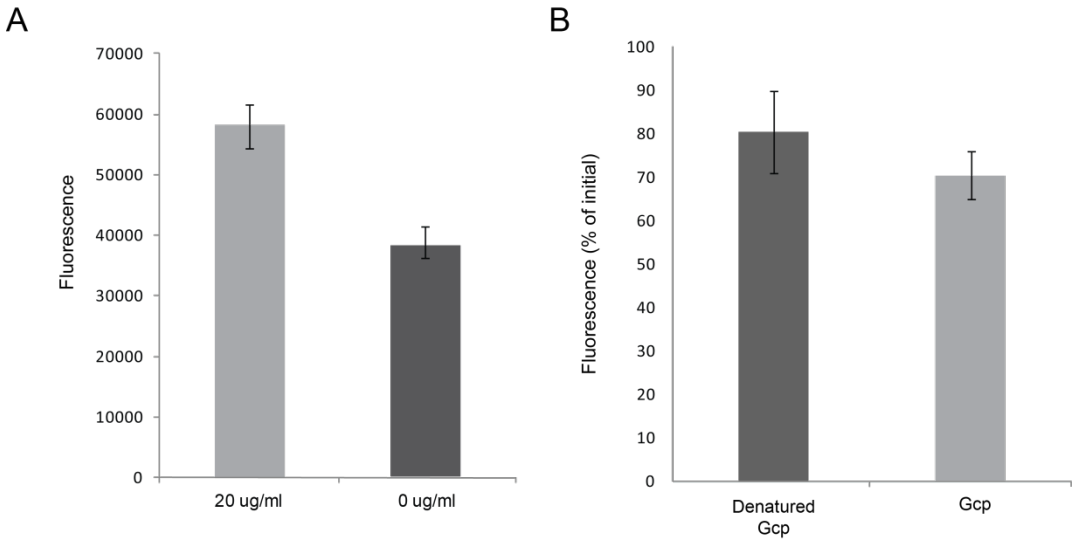


Figure 5. Protease and ATPase activity of Gcp purified protein. (A) $(His)_6$-Gcp purified protein, (20 µg/ml), has been tested for its protease activity at pH7.0. The assay provides a FRET-based method for the simple and accurate quantitation of a wide range of protease activities. The magnitude of the resultant signal (fluorescence at 485/525 nm) is proportional to the degree of substrate cleavage, and can therefore be used to quantitate the enzyme activity. **(B)** The ATPase activity was assessed using a commercial kit that, also in this case, allows quantification of the ATP present in the reaction correlating it with the fluorescence emitted (fluorescence at 485/525 nm). As a consequence, lower fluorescence correspond to higher ATPase activity. Gcp, compared to the control in which it was previously denatured and inactivated, shows greater activity estimated at approximately 15%.

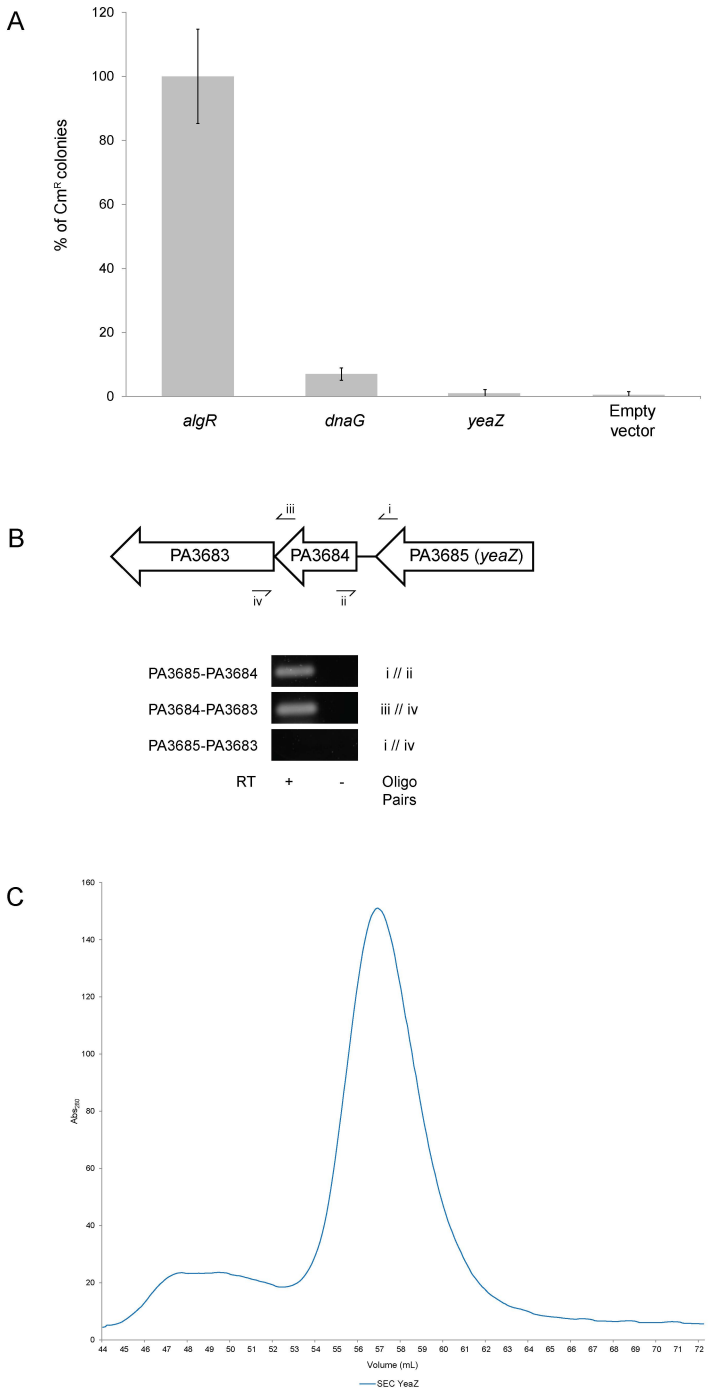


Figure 6. Characterization of *yeaZ* (PA3685): a Gcp putative partner. (A) *Insertional mutagenesis assay***.** Each indicated *locus* was targeted for knock-out by homologous recombination-mediated cointegration of the suicide vector pDM4 carrying chloramphenicol resistance (Cm^R). The *dnaG* gene and the *algR* gene were used respectively as positive and negative controls of essentiality. Three independent conjugation experiments were performed. Efficiency of cointegration in a given *locus* is expressed as a percentage of Cm^R ex-conjugant colonies relative to the negative control *algR*. **(B)** *Genetic organization and transcription analysis of the genomic region including PA3685 locus.* PA3685-3684-3683 gene cluster is represented according to visualization by GBrowse in PGD. Locations of oligo pairs (Roman numbers) used in RT-PCR-based transcription analysis are shown along the region map. Total RNA extracted from PAO1 exponential phase was

analyzed by RT-PCR. RT untreated samples (RT-) as controls of genomic DNA contamination were included in the analysis. **(C)** *Analytical gel-filtration assay***.** A 2 ml aliquot of purified YeaZ was analyzed with a HiLoad 16/600 Superdex 75 pg (GE Healthcare) column. The protein eluted showed a single peak elution, corresponding to molecular masses of 51 kDa which was close to the estimated masses for dimeric (49.7 kDa) form of N-$(His)_6$-tagged YeaZ.

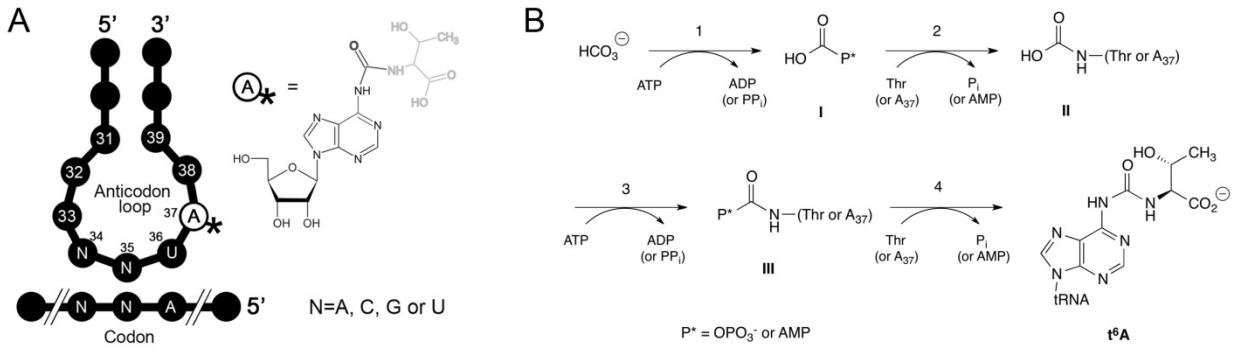


Figure S1. (A) *Schematic representation t6A modification.* On the left is a pictured an anticodon loop of tRNA carrying NNU anticodon and an mRNA molecule carrying ANN codon. Adenosine at position 37 is indicated in white circle. On the right is the chemical structure of t6A-modified nucleoside. Chemical groups originating from threonine or carbonate are indicated in grey and bold, respectively. **(B)** *Proposed reaction scheme leading to* t^6A *biosynthesis.* First step requires activation of bicarbonate to carboxy-AMP, then a condensation of carboxy-AMP with threonine occours to generate carbamoylthreonine. Carbamoylthreonine is subsequently activated to threonylcarbamoyladenylate and transfered to Gcp active site where second condensation reaction occurs to form t^6A-modified tRNA.

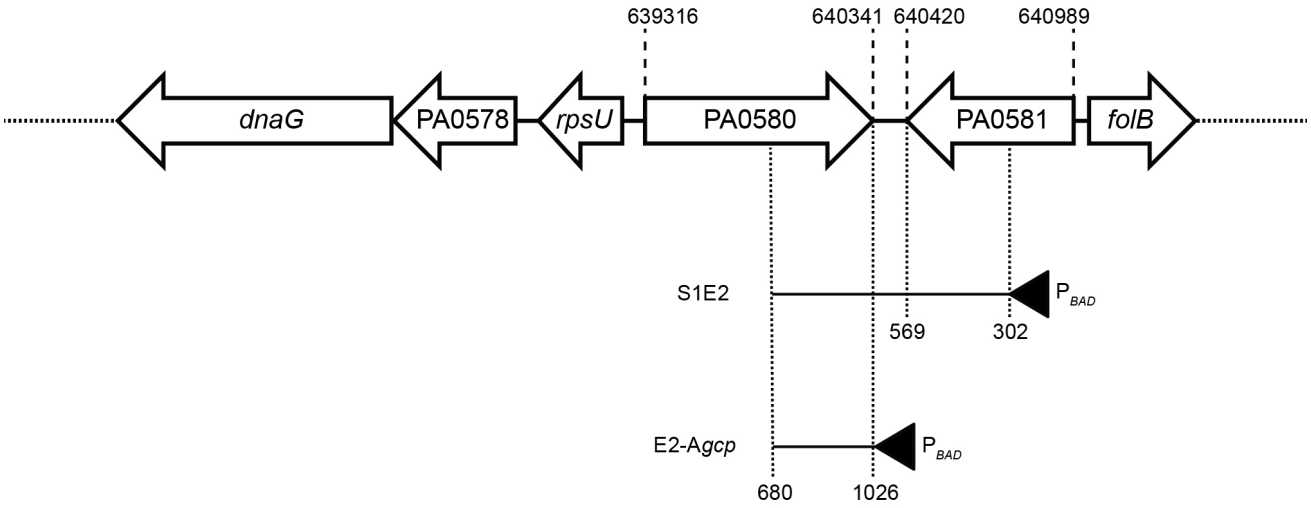


Figure S2. **Inserts orientation and organization**. PAO1 genomic region targeted (coordinates 640687 to 639995) by insert carried by pHERD-S1E2 is pictured as annotated in PGD GBrowse. In the first row below the genomic region is illustrated S1E2 insert. It targets in sense PA0581 (from 569 to 302 positions) and in antisense PA0580 (from 1026 to 680 positions). In the second row is illustrated the sub-cloned E2-A*gcp* insert. It was cloned downstream to the P_{BAD} promoter of pHERD20T in antisense orientation spanning between 680 and 1026 bps of PA0580.

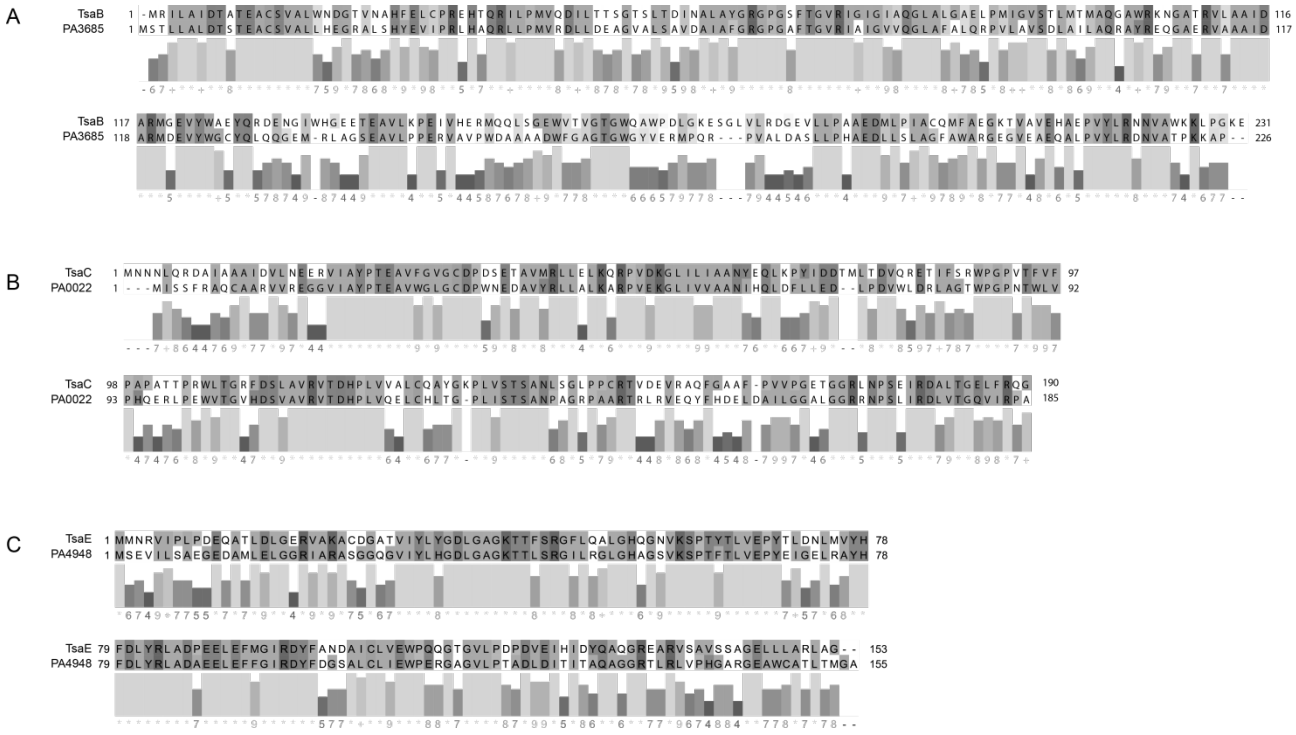


Figure S3. Sequence alignment analysis of Gcp putative partners in *P. aeruginosa*. TsaB, TsaC and TsaE protein sequences were obtained from EcoCyc database and aligned in the NCBI BlastP suite (Organism = *Pseudomonas aeruginosa* PAO1 - taxid:208964) to trace back the corresponding PAO orthologs. Sequence alignments were performed with Clustal Omega and output files analyzed with Jalview generating alignment images for TsaB **(A)** TsaC **(B)** and TsaE **(C)**. Conservation is measured as a numerical index reflecting the conservation of physico-chemical properties in the alignment: Conservation is visualised as a histogram giving the score (from 0 to 11) for each column below them. Conserved columns are indicated by '*' (score of 11) and columns with mutations where all properties are conserved are marked with a '+' (score of 10, indicating all properties are conserved).

Table S1: List of bacterial strains and plasmids.

Strains or plasmids	Genotype or description	Reference
Pseudomonas aeruginosa		
PAO1		[1]
PAO1 P_{rhaB}*::gcp*	PA0580(*gcp*) under the control of the rhamnose-dependent promoter P_{rhaB}	This work
PAO1*gcp*C	PAO1 P_{rhaB}*::gcp* complemented with pHR6HT-Gcp	This work
Escherichia coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44, relA1,* Δ*(lac-proAB)* F' *(traD36, proAB+ lacIqZ* ΔM15)	[2]
HB101 [RK2013]	*recA, thi, hsdR, hsdM, proA, leu, strA*	[3]
S17-λpir	Tp^R Sm^R *hsdR pro recA* RP4-2-Tc::Mu-Km::Tn7 (λpir)	[4]
M15 [pREP4]	Nal^S,Str^S,Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+	[5]
C118-λpir	*araD*139 Δ(*ara leu*)7697 Δ*lacX*74 *phoA*20 *galE galK thi rpsE rpoB araEam recA1* (λpir)	[6]
Plasmids		
pHERD20T	Amp^R, Cb^R broad-host-range *araC-*P_{bad} expression vector; blue/white screening for recombinants	[7]
pHERD-S1E2	pHERD20T harbouring a fragment spanning coordinates 640687 to 639995 of PAO1 chromosome	[8]
pHERD-E2-A*gcp*	pHERD20T harbouring a fragment spanning position 680 to 1026 of PA0580 cloned in antisense direction	This work
pHR6HT-Gcp	pHERD20T harbouring 6xHis-tagged Gcp protein, RBS and terminator from pQE30	This work
pQE30	Amp^R, Cm^R ,expression vector with phage T5 promoter, N-terminus 6xHis-tag, two strong transcriptional terminators	Qiagen
pQE30-Gcp	pQE30 harbouring position 1 to 1026 of *gcp* within the MCS	This work
pQE30-YeaZ	pQE30 harbouring position 1 to 888 of PA3685 within the MCS	This work
pDM4	Cm^R, suicide plasmid in *Pseudomonas spp*, pBR322 origin of replication, *sacB*	[9]
pDM4-*algR*	pDM4 harbouring position 22 to 610 of *algR* within the MCS	[10]
pDM4-*dnaG*	pDM4 harbouring position 12 to 422 of *dnaG* within the MCS	[10]
pDM4-PA2873	pDM4 harbouring position 590 to 1392 of PA2873 within the MCS	[10]
pDM4-PA0580	pDM4 harbouring position 20 to 345 of PA0580 within the MCS	This work
pDM4-PA3685	pDM4 harbouring position 22 to 282 of PA3685 within the MCS	This work

pSC200	Gm^R, ori_{R6K}, P_{rhaB} rhamnose-inducible promoter, mob^+	[11]
pSC200-PA0580	pSC200 harbouring the first 300 bp of PA0580 downstream the P_{rhaB} promoter	This work

References

1. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, et al. (2000) Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature 406: 959–964. doi:10.1038/35023079.

2. Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103–119. doi:10.1016/0378-1119(85)90120-9.

3. Boyer HW, Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in Escherichia coli. J Mol Biol 41: 459–472. doi:10.1016/0022-2836(69)90288-5.

4. De Lorenzo V, Herrero M, Jakubzik U, Timmis KN (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J Bacteriol 172: 6568–6572. doi:0021-9193/90/116568-05.

5. Villarejo MR, Zabin I (1974) Beta-galactosidase from termination and deletion mutant strains. J Bacteriol 120: 466–474.

6. Herrero M, de Lorenzo V, Timmis KN (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol 172: 6557–6567.

7. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD (2008) PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in Pseudomonas and Burkholderia spp. and other bacteria. Appl Environ Microbiol 74: 7422–7426. doi:10.1128/AEM.01369-08.

8. Rusmini R, Vecchietti D, Macchi R, Vidal-Aroca F, Bertoni G (2014) A shotgun antisense approach to the identification of novel essential genes in Pseudomonas aeruginosa. BMC Microbiol 14: 24. Available: http://www.ncbi.nlm.nih.gov/pubmed/24499134.

9. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H (1996) Flagellin A is essential for the virulence of Vibrio anguillarum. J Bacteriol 178: 1310–1319.

10. Milani A, Vecchietti D, Rusmini R, Bertoni G (2012) TgpA, a Protein with a Eukaryotic-Like Transglutaminase Domain, Plays a Critical Role in the Viability of Pseudomonas aeruginosa. PLoS One 7: e50323.

11. Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, et al. (2007) A putative gene cluster for aminoarabinose biosynthesis is essential for Burkholderia cenocepacia viability. J Bacteriol 189: 3639–3644. doi:10.1128/JB.00153-07.

Table S2. Oligonucleotides.

Oligo name	Sequence[a] (5'→3')	
RT-PCR		oligo pair[b]
PA3685/84 fw	GAAGGCGTGGAGGCGGAA	i // ii
PA3685/84 rev	CGTCGCGATACGGCGTGA	
PA3684/83 fw	GATGTCCTCGGCCTCGACCT	iii // iv
PA3684/83 rev	GGCATAGAAGGTGGTATTGC	
PA3685 Fw	AGTCACTACGAGGTGATCCCG	**
PA3683 Rev	ATGCAGGAATACGTATGGAGTC	
PA3684 Rev	TGGCGGTGGAGTTGTAGCTGG	
InsertPA3684Fw	ATGTTAGTCGACGGCTAGAGTGTGGAG	
InsertPA3684Rev	CAATAGTCGACAGGGCCATCGCCGCA	
PA3685 Fw	AGTCACTACGAGGTGATCCCG	
pHERD-E2-Agcp construction and screening		
AgcpFw	CGCGAATTCTCAGACCGCCGGCAGCGAC	
AgcpRev	CGCAAGCTTCTGGCAGCGTTGCGTCGA	
pHERD-Fw	ATCGCAACTCTCTACTGTTTCT	
pHERD-Rev	TGCAAGGCGATTAAGTTGGGT	
pQE30-ORFs construction and screening		
GcpFULLfw	CGGACTGGATCCCGCGTGCTGGGACTG	
GcpFULLrev	CGGACTAAGCTTTCAGACCGCCGGCAG	
YeaZFULLfw	CTAAGGATCCTCCACTCTGTTGGCCC	
YeaZFULLrev	CGACAAGCTTTCAGGGCGCTTTTTC	
pQE30-RBS-Fw	GTGAGCGGATAACAATTTCAC	
pQE30-Sal-rev	GTAACTGTCGACGCTCCTGAAAATCTC	
pQE30-fw	CCCGAAAAGTGCCACCTG	
pQE30-rev	GTTCTGAGGTCATTACTGG	
pDM4-ORFs construction		
pDM4-0580-fw	CGAATCGTCGACAAACGTCCTGCGACGAA	
pDM4-0580-rev	TCATAGGTCGACGTGCCCTTCCATGTGGT	
pDM4-3685-fw	CGAAGTCGACGATACCTCCACCGAA	
pDM4-3685-rev	CGAAGTCGACGGAGACCGCCAGTAC	
pDM4-2873-fw	GAAAGTCGACTCTCGGAGAGCATGGC	
pDM4-2873-rev	GAACGTCGACGCTCAGCCAGCC	
pDM4-algR-fw	GAAAGTCGACGAACCTCTGGCGCG	
pDM4-algR-rev	GAAAGTCGACCAGCGCGTTGCGG	
pDM4-dnaG-fw	GAAAGTCGACGATACCGCAAAGCTTCATCG	
pDM4-dnaG-rev	GAAAGTCGACGGCCCTTCAGGTAGTTCACC	
pDM4-ori	GTGACACAGGAACACTTAACG	
pDM4-cat	TGTCCCTCCTGTTCAGCTAC	
pSC200-PA0580 construction		
gcp-psc-fw	CCGGCACATATGCGCGTGCTGGGA	
gcp-psc-rev	CCGGCATCTAGAGAAGGCCATCGC	
pSC200-824(fw)	GCCCATTTTCCTGTCAGTAACGAGA	
pSC200-1400(rev)	TAACGGTTGTGGACAACAGCCAGGG	

[a] Restriction enzyme sites are underlined
[b] Oligo pairs used in the experiments shown in Figure 6.

Table S3. Sequence alignment analysis and relative scores of putative *P.aeruginosa* Gcp partners.

Strain		Score		
E.coli	***P.aeruginosa***	**Coverage**	**E-Value**	**Identities**
TsaB	hypothetical protein PA3685	96%	6,00E-63	49%
TsaC	hypothetical protein PA0022	92%	3,00E-50	52%
TsaE	hypothetical protein PA4948	98%	6,00E-58	58%