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Cellular Reporter Screens for Inhibitors of *Burkholderia pseudomallei* Targets in *Pseudomonas aeruginosa*

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Summary

To facilitate the discovery of new therapeutics for *Burkholderia pseudomallei* infections, we have developed cellular reporter screens for inhibitors of *B. pseudomallei* targets in the surrogate host *Pseudomonas aeruginosa*. *P. aeruginosa* strains carrying deletions of essential genes were engineered to be dependent on the IPTG-regulated expression of their *B. pseudomallei* orthologs on a broad-host-range plasmid. *P. aeruginosa* genes which are upregulated in response to depletion of each target gene product were fused to the *Photorhabdus luminescens luxCDABE* operon via pGSV3-*lux*-Sp^R to generate reporter strains with increased bioluminescence upon target inhibition. A total of 11 of 19 *B. pseudomallei* genes complemented deletions of their orthologs in *P. aeruginosa*. The dependence of growth on IPTG levels varied from complete dependence (*ftsQ, gyrA, glmU, secA*), to slower growth in the absence of IPTG (*coaD, efp, mesJ*), to apparently normal growth in the absence of IPTG (*ligA, lpxA, folA, ipk*). Reporter screening strains have been constructed for three gene targets (*gyrA, glmU, secA*), and one (*gyrA*) has been applied to 68,000 compounds resulting in a primary hit rate of 0.5% and a confirmed hit rate of 0.06% including several fluoroquinolones. These results provide proof of principle for surrogate cellular reporter screens as a useful approach to identify inhibitors of essential gene products.

Keywords

bioluminescence; reporter; screen

Introduction

Burkholderia pseudomallei is a Gram-negative bacterium that causes melioidosis in humans and animals. Transmission to humans occurs through skin abrasion, ingestion, and inhalation. The disease often presents as a serious pneumonia or septicemic infection, and lethality is

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Declarations:

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typically as high as 40%, even with the use of third-generation cephalosporins (Currie *et al.*, 2000; White *et al.*, 1989).

Current therapies are based on existing drugs which happen to have a broad enough spectrum to show some efficacy against *B. pseudomallei*. Carbapenems, third generation cephalosporins, chloramphenicol, and tetracyclines are usually effective, but the best current therapies utilize combinations of antibiotics such as ceftazidime-cotrimoxazole or amoxicillin-clavulanate (Brett and Woods, 2000). Clinical isolates of *B. pseudomallei* are consistently resistant to a wide variety of antibiotics (Gilligan and Whittier, 1999; Jenney *et al.*, 2001; Piliouras *et al.*, 2002; Vorachit *et al.*, 2000; Wiedemann and Grimm, 1991). The bacterium is well-armed with drug-resistance mechanisms, including a nearly impermeable outer membrane (Burtnick and Woods, 1999) and the action of several broad-acting efflux pumps (Chan *et al.*, 2004; Moore *et al.*, 1999). The most effective antibiotics in use today for *B. pseudomallei* target only cell wall biosynthesis, protein synthesis, and co-factor biosynthesis.

In order to facilitate the discovery of new classes of anti-*B. pseudomallei* agents, we have developed a platform approach for screening *B. pseudomallei* targets directly for inhibitors by using whole cell reporter screens in the surrogate host *P. aeruginosa* (Moir *et al.*, 2007). These screens depend upon transcription and translation of a reporter as an indirect measure of target inhibition based on the response of the cellular regulatory network to depletion of the target. Suitable reporter promoters were discovered by expression profiling of engineered *P. aeruginosa* strains grown under conditions which are limiting for the *B. pseudomallei* target gene expression. Appropriate promoters were fused to the *P. luminescens luxCDABE* operon to provide a luminescence report without the need to permeabilize cells and add a substrate. *In vivo* reporter screens of this type offer substantial benefits including, for example (a) preselection for permeable compounds, (b) ability to monitor multiple metabolic steps simultaneously (e.g., pathway screens), (c) sensitivity (e.g., often superior to assays that simply detect growth inhibition), (d) applicability to biochemically intractable targets (e.g., those with no known function or functions that are difficult to assay), as well as (e) a safer approach to high throughput, cell-based screening of targets from BSL-3 organisms.

As a proof of principle, we describe here the development of *P. aeruginosa* bioluminescent reporter screening strains for inhibitors of *B. pseudomallei gyrA, glmU,* and *secA* gene products. In addition, we provide details on the implementation of a high throughput screen for *B. pseudomallei* gyrase inhibitors.

Experimental/Materials and methods

Strains, plasmids, and growth media

P. aeruginosa strains and plasmids are described in Table 1. *E. coli* TOP10 (Invitrogen®), *E. coli* DB3.1 (Gateway® host, Invitrogen®), *E. coli* SM10 (de Lorenzo and Timmis, 1994), and *E. coli* S17-1 (ATCC 47055) were used as hosts for molecular cloning. VBMMG is VBMM medium (Vogel and Bonner, 1956) containing 0.3% trisodium citrate and 30 μg/ml gentamicin. Luria-Bertani (LB) medium (liquid and agar) was purchased from Difco. LB was supplemented with 10 μg/ml gentamicin (LBG) or both 10 μg/ml gentamicin and 200 μg/ml spectinomycin (LBGS) and various concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) as indicated. Opaque, white, flat-bottom, 96-well microplates (Nunc Cat. No. 236108; VWR International) were covered with gas permeable adhesive seals (Abgene, Inc., Cat. No. AB-0718) for reporter screens. RNA Protect Bacteria Reagent was purchased from Qiagen, Inc.

PCR and Primers

Synthetic oligonucleotide primers (from Operon, Inc.) were designed using the published genome sequences for *P. aeruginosa* (Stover *et al.*, 2000) and *B. pseudomallei* (Holden *et al.*, 2004) and web-based PRIMER3 (Whitehead Institute)(see electronic Supplementary Information for a list of primer sequences). Primers were used at 10 μM in PCR amplifications with Failsafe polymerase (Epicentre®), Buffer G (Epicentre®), and 4% DMSO for *P. aeruginosa* and *B. pseudomallei* chromosomal DNA templates.

Generation of complemented deletions of essential genes

Knock-out pEX18ApGW vectors were built for 19 *P. aeruginosa* loci by means of splicing by overlap extension (SOE) PCR and used successfully to create merodiploids which were resolved in the presence of a complementing gene copy on the extrachromosomal plasmid pUCP24GW (with or without the *lacI*^{Q} gene) as described previously (Moir *et al.*, 2007). The pEX18ApGW vectors carried a Tc^{R} element flanked by about 1 kb of homology on both sides of the locus to be deleted. Presumed deletions were confirmed by PCR with flanking primers outside the regions cloned into pEX18ApGW.

Expression profiling with microarrays

Complemented deletion strains were grown overnight in 5 ml of LBG with 1 mM IPTG and diluted 100-fold into 7 ml VBMMG with (≥0.1 mM) or without (≤0.025 mM) IPTG. Cells were harvested by centrifugation for 5 min at 5,000 x g about 120 minutes after detectable effects on growth were observed in the limiting IPTG cultures. Cells were resuspended in a 2:1 mixture of RNA Protect Bacteria Reagent:VBMM, incubated at room temperature for 5 min, re-centrifuged in a microfuge, decanted and stored at −70°C until used for RNA isolation. RNA isolation, cDNA production, labeling, fragmentation, hybridzation to GeneChip® *P. aeruginosa* Genome Arrays (Cat. No. 900340, Affymetrix, Inc.), washing and reading of signals were done as described by Wolfgang et al (Wolfgang *et al.*, 2003). About 80% of the genes were called “present” in each hybridization, consistent with adequate elimination of DNA in the RNA preparations. Each deleted target gene was called “absent” by the Affymetrix analysis software as expected due to negligible cross-hybridization of the complementing *B. pseudomallei* ortholog gene with the array probes. Each complemented deletion strain was analyzed in duplicate. To enable comparisons between arrays, we normalized the signal intensity data for each gene in the array to the total signal from that array and then calculated the ratio of intensity at limiting and excess IPTG levels for each locus in each complemented deletion strain.

Generation of transcriptional fusions to P. luminescens luxCDABE for reporter evaluation

Luciferase reporter fusions were added to the complemented deletions in an improved version of the method described previously (Moir *et al.*, 2007). To permit the addition of reporters directly to the complemented deletion strains which carry Tc^{R} and Gm^{R} markers, we modified pGSV3-lux (Moore *et al.*, 2004) to carry Sp^{R} instead of Gm^{R}. This was accomplished by restriction digestion of pGSV3-lux with *Sac*I, gel purification of the larger fragment followed by ligation to a Sp^{R} cassette which was PCR amplified from pVLT35 (de Lorenzo *et al.*, 1993) with primers carrying *Sac*I-tails (Spec+SacI-F/Spec+SacI-R), and cut with *Sac*I. Internal fragments from genes whose expression level was up-regulated in response to target depletion were amplified with primers carrying *Eco*RI tails, cut with *Eco*RI and ligated to *Eco*RI-cut and alkaline phosphatase-treated pGSV3-lux-Sp^{R}. Resulting gene fragment-luciferase operon transcriptional fusions on pGSV3-lux-Sp^{R} were introduced into *E. coli* SM10 by electroporation and conjugated with the complemented deletions to generate single cross-over

insertions placing the *P. luminescens luxCDABE* operon under regulation of the target-depletion-responsive gene and its promoter region. Insertion at the correct genomic locus was confirmed by PCR with a primer outside of the cloned gene fragment (see "out-F" primers for each locus in the primer list) and a primer within the *luxC* gene (LuxC-R).

Detection of bioluminescence of reporter strains

Complemented deletion strains carrying *luxCDABE* transcriptional fusions were grown overnight at 37°C on LBGS plates containing various concentrations of IPTG and then subcultured at 30°C into LBGS without IPTG (except the control which was maintained in ≥0.05 mM IPTG) at an initial OD_{600} ~0.03). Relative light units (RLU) were measured in white opaque microplates in a Perkin Elmer Envision Multilabel Reader periodically throughout a 7 hour period, and one final measurement was made the next day. In some cases, relative luminescence units (RLU) were normalized to cell number by using OD_{600} measured in a Perkin Elmer Victor^{3}V 1420 Multilabel HTS counter.

High-throughput screening with reporter strains

Screening was carried out essentially as previously described (Moir *et al.*, 2007) but with the following modifications. The screening strain, MDM981, consisted of a *gyrA* deletion in *P. aeruginosa* complemented by a *lac*-regulated copy of *B. pseudomallei gyrA* on pUCP24GW and carrying an insertion of pGSV3-lux-Sp^R at the *PA0614* locus. It was grown overnight from a frozen stock at 37°C on LB agar containing 1 mM IPTG, 10 μg/ml gentamicin and 200 μg/ml spectinomycin. Cells from the agar plate were used to inoculate liquid LB medium containing the same concentrations of IPTG, gentamicin and spectinomycin at an OD_{600} ~0.05. Cultures were grown for about two hours to an OD_{600} = 0.4, and then 200 μl of culture was added to each well of a 96-well microtiter dish containing compound to initiate the screen. Compounds for screening were purchased from the following vendors – Chembridge (San Diego, CA), Timtec (Newark, DE), and ChemDiv (San Diego, CA). Compounds were diluted in 96-well master plates at 2.5 mM in DMSO at −20°C. Master plates were thawed at room temperature on the day of the screen, and 2 μl of compound was added to the screening plates by means of a Sciclone ALH 3000 liquid handling robot (Caliper, Inc.) and a Twister II Microplate Handler (Caliper, Inc.). The first column of wells contained only culture (negative control), and the last column contained culture plus 0.5-fold MIC of ciprofloxacin (0.03 μg/ml) (positive control). Plates were sealed with a gas permeable sealant (see Materials). Luminescence was measured after 16h incubation at 30°C by using a Perkin Elmer Envision Multilabel Reader. Z′ values were calculated as previously described (Zhang *et al.*, 1999) based on the positive and negative controls in order to monitor the reproducibility of the screen; plate Z′ values were typically >0.5. A z-score for each sample was derived by subtracting the sample RLU from the mean negative control RLU and dividing the difference by the negative control standard deviation. Screening hits generated luminescence with a z-score >3. Confirmed hits exhibited luminescence with z-score >3 in at least three of four re-tests.

Results

Evaluation of B. pseudomallei orthologs in P. aeruginosa

Heterospecific complementation—Predicted orthologs for 19 *P. aeruginosa* genes (TABLE 2) which had been demonstrated previously to be essential in *P. aeruginosa* or in other Gram-negative species (Gerdes *et al.*, 2003;Gerdes *et al.*, 2002;Jacobs *et al.*, 2003) were identified in the *B. pseudomallei* strain K96243 genomic sequence (Holden *et al.*, 2004) by using standard BLAST tools (Wheeler *et al.*, 2002). Knock-out pEX18ApGW vectors were constructed for all 19 *P. aeruginosa* loci and used successfully to create merodiploids in the *P. aeruginosa* strain PAO-LAC which carries a copy of the lac repressor *lacI*^{Q} at the φCTX chromosomal locus (Hoang *et al.*, 2000). All 19 *P. aeruginosa* merodiploids were

electroporated with the corresponding *B. pseudomallei* orthologs on pUCP24GW and tested for the formation of deletions by selecting for sucrose resistance and screening for carbenicillin-sensitivity and tetracycline-resistance. Presumed deletions were confirmed by PCR with outer flanking primers as described previously (Moir *et al.*, 2007). In 11 of the 19 cases, the *B. pseudomallei* ortholog complemented sufficiently to permit deletion of the *P. aeruginosa* gene (TABLE 2).

We were not able to generate complemented deletions for 8 of the 19 genes (TABLE 2) despite the fact that the complementing *B. pseudomallei* orthologs on pUCP24GW were verified to be of accurate nucleotide sequence. Two of the genes that did not support deletions (*ftsA, dnaA*) inhibited the growth of their respective merodiploids when IPTG levels were increased above 0.025 mM. While the merodiploids containing the *B. pseudomallei ftsA* and *dnaA* genes on pUCP24GW could be grown in low IPTG levels, we were not able to generate complemented deletions for either of them.

Growth dependence on ortholog expression—In order to identify suitable promoters to express the luciferase operon in reporter screens, we used expression profiling by microarrays to survey the expression response of all genes to depletion of each target. Complemented deletions were first tested for growth impairment in low IPTG concentrations as a means to assess their suitability for expression profiling. We used detectable effects on growth due to repression of the *lac* promoter driving the complementing target copy as an indication that cells were responding to target depletion in a manner that would be sensed by the cellular regulatory circuitry and revealed in expression profiling. The response to limiting IPTG concentrations varied widely, from complete dependence on IPTG for growth (*glmU, secA, gyrA, ftsQ*), to significant impairment of growth in the absence of IPTG (*coaD, efp, mesJ*), to apparently normal growth in the absence of IPTG (*ligA, lpxA, folA, ipk*) (TABLE 2). These results appear to reflect a varying requirement by the cell for these specific gene products.

Expression profiling of complemented deletions under target-depletion conditions: In order to identify target-depletion responsive genes and promoters, expression profiles were generated for four complemented deletions (*glmU, secA, coaD* and *gyrA*) which demonstrated adequate growth dependence on IPTG levels. Due to poor growth of the *B. pseudomallei gyrA* complemented strain in excess IPTG, we complemented the *gyrA* deletion with a copy of the *P. aeruginosa gyrA* gene for the purposes of expression profiling. We then used the *B. pseudomallei gyrA* gene to complement the deletion in the reporter strain used for screening (see below). Expression profiles were examined from cells grown in excess IPTG, providing nearly wild-type growth, and for cells grown in limiting IPTG, causing impaired growth and early saturation. Signals from the corresponding microarrays were processed (see Methods) and analyzed as the ratio for each gene of its signal under inducing conditions to its signal under repressing conditions.

As a control, gene expression in the wild-type parental PAO-LAC strain was profiled from cells grown in the absence of IPTG and in 0.1 mM IPTG. The signal ratios for all genes called “present” averaged 1.0 (S.D. ± 0.27) reflecting the absence of any significant effect of IPTG on gene expression in PAO-LAC. In order to prioritize genes which could represent suitable sources of reporter promoters, we required that the normalized signal intensity ratio of limiting to excess IPTG levels be >5, that the intensity in excess IPTG be >25, and that the intensity in limiting IPTG be >100. These criteria were designed to ensure that up-regulated genes were statistically significant and that signals generated by reporter fusions would be easily detectable. Next, we prioritized the genes that met these criteria for each depleted target to determine which up-regulated genes responded specifically to reduction in the amount of that target. In addition, to further ensure specificity, we compared the target depletion gene expression responses to published expression profiling results from cells grown under a variety

of conditions – hydrogen peroxide stress (Palma *et al.*, 2004); nitrosative stress (Firoved *et al.*, 2004); imipenem (Bagge *et al.*, 2004); Ca^{++}/EGTA (Wolfgang *et al.*, 2003); stationary phase (S. Lory, personal communication); iron deprivation (Ochsner *et al.*, 2002; Palma *et al.*, 2003); and biofilm/quorum sensing (Wagner *et al.*, 2004; Whiteley *et al.*, 2001).

Comparison of the gene expression profiles of *coaD, glmU, gyrA,* and *secA* complemented deletions growing at limiting versus excess IPTG concentrations revealed several *P. aeruginosa* genes whose expression levels increased significantly and specifically in response to depletion of the essential gene product (TABLE 3). Some of the responding genes appear to be in operons, for example, *PA2030* and *PA2031* for *coaD*, *PA4181* and *PA4182* for *glmU, PA2403* through *PA2410* for *secA*, and *PA0612* through *PA0648* for *gyrA*. Of particular note, the *gyrA*-depletion-responsive operon has been shown previously to respond to ciprofloxacin treatment (Brazas and Hancock, 2005;Cirz *et al.*, 2006). The present discovery that it also responds to GyrA depletion supports the hypothesis that cellular regulatory changes in response to target depletion mimic the regulatory changes expected for antibiotic inhibition of the same target. Identification of multiple genes in an apparent operon as up-regulated in response to target depletion provides assurance of the reproducibility of detection by microarray-based expression profiling. Note that none of the up-regulated genes has any obvious mechanistic involvement in the depletion of the target. In most cases, they are hypothetical or conserved hypothetical genes. However, it is not necessary that there be a mechanistic relationship between the target and responsive gene product as long as the promoter responds specifically and potently to depletion of the target.

Response of *luxCDABE* fusions to target depletion: In order to convert the gene expression response to target depletion into a report for target inhibition, we created specific transcriptional fusions to the *P. luminescens luxCDABE* operon. This was accomplished by cloning internal fragments of several target-depletion responsive genes into the *Eco*RI site of pGSV3-lux-Sp^R and integrating them into the chromosome of each corresponding complemented deletion strain (Moore *et al.*, 2004). Resulting strains were grown under limiting IPTG conditions, transferred to medium lacking IPTG, and bioluminescence was measured over several hours to assess the response of the reporter construct to loss of the target gene product by dilution and by repression of further expression (see Methods). In general, bioluminescence intensity was indirectly dependent on the initial IPTG level, directly dependent on the time of growth in the absence of IPTG up to a limit of 5–7 h and specific for depletion of the target. Detailed results are shown in Fig. 1 for the GlmU-depletion-sensitive reporter fusion *PA1494-luxCDABE* in both *glmU* and *secA* complemented deletion strains MDM1142 and MDM1169, respectively, grown in limiting IPTG concentrations. Cell growth is reduced markedly by either GlmU or SecA depletion (Fig. 1A and B), but bioluminescence increases significantly only under GlmU depletion conditions (Fig. 1C). The maximum ratio of bioluminescence intensity in limiting versus excess IPTG for several reporter strains for the targets *coaD, glmU, secA,* and *gyrA* are shown in Table 3. In general, the mRNA ratios measured in the expression profiling experiments are good predictors of the bioluminescence response, but there are several exceptions presumably due to the additional steps of translation, protein stability and light production not accounted for in expression profiling. It is also interesting to note that use of an inhibitor may be more effective than limiting IPTG to deplete essential gene products from these complemented deletions. For example, the bioluminescence ratios for both the *P. aeruginosa gyrA* and the *B. pseudomallei gyrA* complemented deletions (MDM977 and MDM981, respectively) +/− limiting IPTG concentrations and +/− 0.5x MIC of ciprofloxacin are shown in Table 3. The source of the complementing gene has little effect on the RLU ratios, but addition of the known gyrase inhibitor ciprofloxacin appears to be much more effective than the use of limiting IPTG to deplete functional gyrase in both strains, producing RLU ratios over 4-fold greater than those observed +/− IPTG.

High throughput screening – *gyrA* proof of principle

For proof of principle for these reporter screens, we chose to use strain MDM981, a *P. aeruginosa gyrA* deletion strain complemented by *B. pseudomallei gyrA* on pUCP24GW and carrying a pGSV3-lux-Sp^R insertion at *PA0614*. This strain was chosen for two reasons – (a) a successful pilot screen was done previously on 2,000 compounds using a similar strain except that it was complemented by *P. aeruginosa gyrA* (Moir *et al.*, 2007), and (b) the existence of known inhibitors of GyrA, the fluoroquinolones, provided accessible positive controls. We screened 68,000 compounds using reporter strain MDM981 (see Methods) and identified 328 primary hits which exhibited z-scores >3 (compared to the negative control) for a primary hit rate of 0.48%. About 13% of the primary hits, or 43 compounds, were confirmed in quadruplicate assays with the same reporter strain. The best 34 of those, as judged by having drug-like scaffolds, were re-ordered from vendors, re-examined, and 16 exhibited z-scores >3 when re-assayed.

Examples of two chemotypes of confirmed hits are shown in Fig. 2. The screen identified 51% (20 of 39) of the fluoroquinolones in the screening library as hits. All 20 contain the piperazine ring recognized as important for anti-pseudomonas activity (Neu, 1989), while only 5 of the 19 fluoroquinolones, which were not identified as hits, contain the piperazine ring. Several nitrofurans were also identified as hits. These may be activated by a nitro-reductase in the bacterial cell to generate DNA alkylating agents as has been described previously for metronidazole (Goodwin *et al.*, 1998). Both chemotypes were expected as hits based on the known response of the reporter promoter from gene *PA0614* (Matsui *et al.*, 1993;Moir *et al.*, 2007) to DNA damage.

Discussion

In this study, heterospecific complementation of essential *P. aeruginosa* genes by *B. pseudomallei* orthologs was feasible for 11 of 19 of genes attempted, and down-regulation of the complementing ortholog affected cell growth significantly for 7 of 11 genes tested. Apparently specific effects on the regulatory circuitry of the cell were observed in expression profiles of 4 complemented deletions examined in excess and limiting inducer concentrations. These properties have permitted the development of homogeneous, whole-cell bioluminescent screening assays for inhibitors of *B. pseudomallei* targets in *P. aeruginosa*. Similar bioluminescent reporter assays have been described in *B. subtilis* based on the cellular regulatory response to antibiotic treatments and conditional mutants (Freiberg *et al.*, 2005; Hutter *et al.*, 2004). However, those assays utilized firefly luciferase as a reporter and required the addition of exogenous substrate. The assays described here are simpler and homogeneous, requiring no substrate addition, operate in a less-permeable Gram-negative species, *P. aeruginosa* and are designed to identify inhibitors of heterologous targets in a surrogate species. We discuss a few aspects of the approach below.

Complementation efficiency of orthologs

Slightly over half (11/19) of the *B. pseudomallei* genes tested were capable of complementing deletions of their *P. aeruginosa* orthologs. It is interesting to note that the success in complementation did not correlate with the overall sequence similarity (see P-value exponents in TABLE 2). However, all of these proteins are highly homologous, and sequence similarity within functional motifs might be a better predictor of cross-species complementation. There are several possible reasons for the eight complementation failures. In most cases, failure is probably because the *B. pseudomallei* gene product is not capable of performing all of the functions of the *P. aeruginosa* ortholog. We verified the accuracy of the sequences of the *B. pseudomallei* genes cloned into pUCP24GW; thus, sequence errors introduced by PCR cannot explain the complementation failures. Another possibility is that the *lac* regulatory control may

be an inadequate substitute for native regulation of these genes, and/or the fusion of the *lac* promoter to the gene fragment may have been suboptimal for expression. Other promoters could be tested, and we are interested in experimenting with other promoters for a second reason as well. We would like to reduce transcription of complementing *B. pseudomallei* genes such as *ligA, lpxA, folA* and *ipk* more completely in order to detect target-depletion-responsive genes in transcription profiling experiments with those complemented deletions.

Over-expression lethals

Over-expression of the *B. pseudomallei dnaA* and *ftsA* genes in *P. aeruginosa* inhibited the growth of the merodiploids carrying them, judging from the fact that *P. aeruginosa* cells carrying these genes on pUCP24GW grew normally in LB medium containing ≤0.025 mM IPTG but failed to grow in LB with more IPTG. Over-expression of the *E. coli dnaA* gene in *E. coli* has been reported as lethal (Weigel *et al.*, 1999). In contrast, the *P. aeruginosa ftsA* could be over-expressed in *E. coli*, but its accumulation as inclusion bodies does not indicate whether functional FtsA can be over-produced in *E. coli* (Paradis-Bleau *et al*., 2005). We note that over-expression growth inhibition might be used to select for compounds which inhibit the offending gene product, and this is an area of current research.

Dependence of growth on IPTG as a validation of drug targets

The sensitivity of cell growth to reduction of *lac*-regulated *B. pseudomallei gyrA, secA, glmU, efp, coaD, ftsQ* and *mesJ* gene expression when IPTG levels are reduced suggests that cells require a relatively high minimal level of expression of these genes for normal growth. The most dramatic cases are the *glmU* and *secA* complemented deletion strains which fail to grow at all in the absence of IPTG. Apparently, substantial quantities of these gene products are required for growth, certainly more than the basal level produced from the uninduced *lac* promoter. Thus, even inefficient inhibition of these gene products should be adequate to arrest cell growth, making these attractive drug discovery targets. By contrast, Korycka-Machala *et al.* (Korycka-Machala *et al.*, 2007) demonstrated that the minimum amount of DNA ligase required for growth of *Mycobacterium smegmatis* is quite low, suggesting that this gene product is not an ideal target for drug discovery.

Specificity of reporter strains

Our confidence in the specificity of the up-regulation is limited to the range of conditions included in our analysis, but the inclusion of a variety of stresses from published sources insures that we will avoid selecting reporter fusions responding to these common perturbations. Clearly, secondary assays will be required to further qualify the hits from the reporter screens. Nevertheless, the reporter screens provide a sensitive means to detect inhibitors of targets inside intact cells, which is quite useful since many biochemical screening hits fail at the level of inhibiting whole cells (Payne *et al.*, 2007). We observed some lack of specificity for GyrA depletion -- a partial overlap in response from depletion of GyrA and treatment with ciprofloxacin (DTM and MD, unpublished observations, and TABLE 3). The overlap includes the *PA0614* gene, which is the source of the promoter used for the reporter strain, and this supports the hypothesis that depletion of an antibiotic target will cause a gene expression response similar to that of inhibition of the target with a drug. The lack of a complete overlap in the responses probably reflects the fact that ciprofloxacin acts on an additional target in *P. aeruginosa* – topoisomerase IV (*parE + parC* gene product)(Lee *et al.*, 2005; Oh *et al.*, 2003) as well as gyrase, and the *lac*-regulated control of *gyrA* may not reduce GyrA activity as substantially as does treatment with ciprofloxacin.

Essentiality of target genes in *B. pseudomallei*

Several of these gene targets including *gyrA*, *glmU*, *secA* and *coaD*, have been shown to be essential in *B. pseudomallei* by the failure to resolve pKAS46-generated merodiploids into deletions (RAM and DEW, unpublished observations). Thus, the reporter screens described here offer an opportunity to identify novel anti-*B. pseudomallei* compounds for further development as antibacterials.

Conclusions

This study demonstrates the feasibility of constructing and using whole cell reporter screens to identify inhibitors of targets from a BSL-3 species, *B. pseudomallei*, in a surrogate BSL-2 species, *P. aeruginosa*. The principles are generally transferable to other related sets of species, and the resulting reporter strains provide a relatively safe and inexpensive means for high throughput screening for inhibitors of biothreat species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Bpseu
: *B. pseudomallei*

Paer
: *P. aeruginosa*

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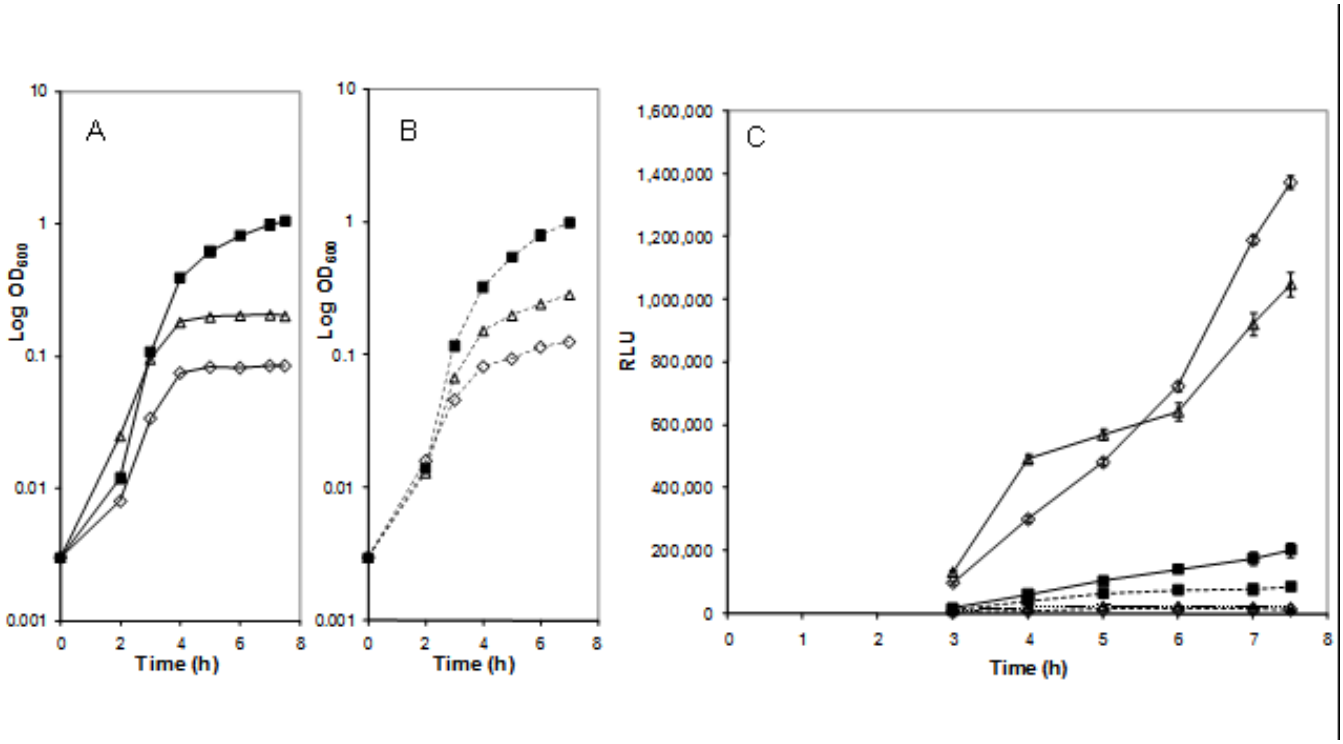

Figure 1.

Comparison of growth and bioluminescence of Δ*glmU* and Δ*secA* strains carrying the GlmU-depletion responsive promoter from *PA1494* fused to *P. luminescens luxCDABE*. Bioluminescent reporter strains MDM1142 (Δ*glmU*) and MDM1169 (Δ*secA*), both containing the *PA1494-luxCDABE* element, were grown overnight in LBGS containing 0.05 mM IPTG (■)(control culture), 0.025 mM ITPG (△), or 0.0125 mM IPTG (◇), then subcultured into LBGS without IPTG, except for the control, which was maintained at 0.05 mM IPTG. Growth (OD_{600}) was followed for 7.5 h for **(A)** MDM1142 (solid lines) and **(B)** MDM1169 (dashed lines). **(C)** Cells (200 μl) from each culture were added to 96-well opaque white microplates after 3 h, and relative light units (RLU) were measured for an additional 4.5 h.

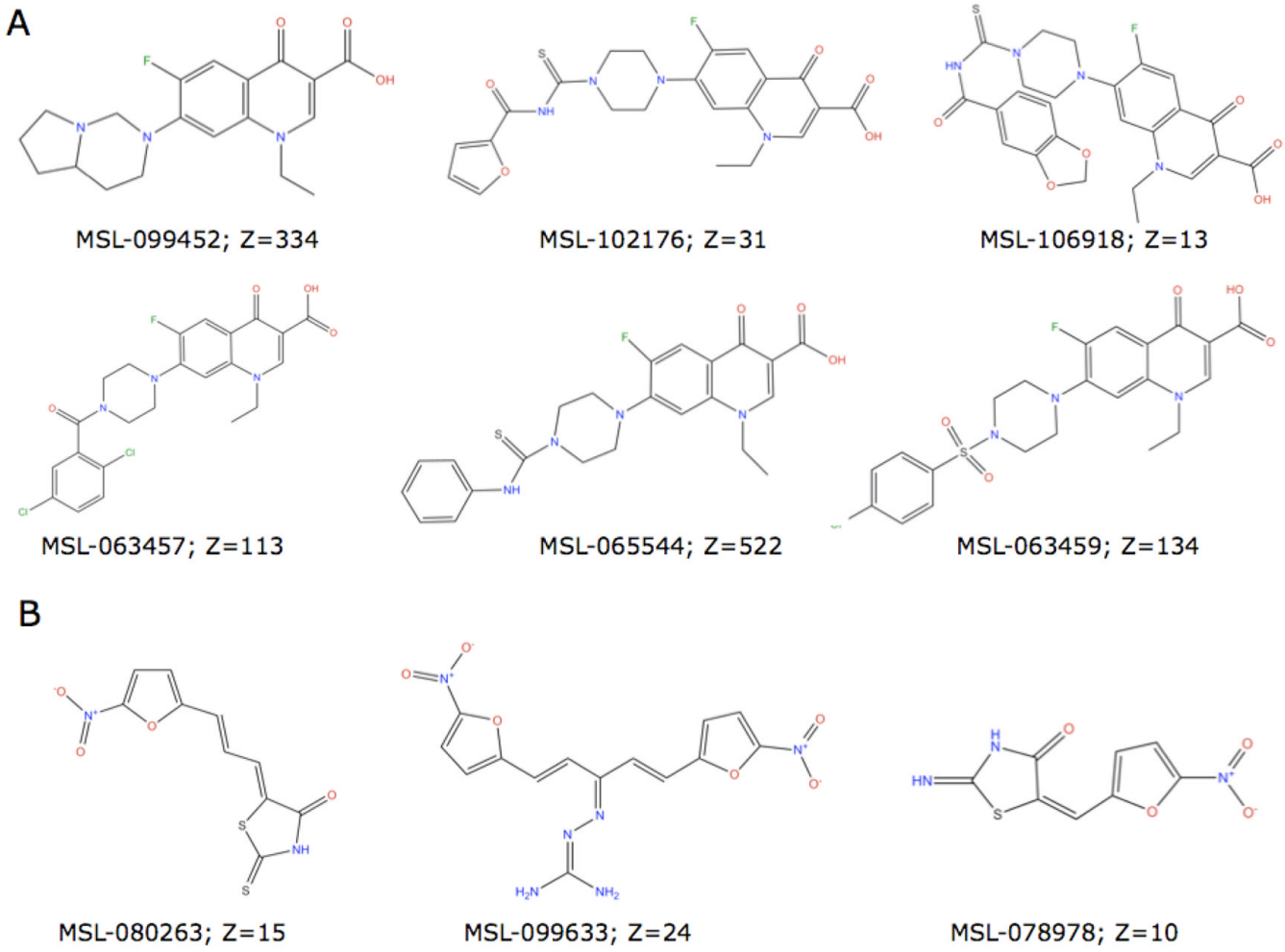

Figure 2.

Chemical structures of representative **(A)** fluoroquinolones and **(B)** nitrofurans identified in a high throughput screen with reporter strain MDM981. The corporate compound identifier (MSL#) and the z-score are shown for each confirmed hit.

TABLE 1

Strains and Plasmids

Strain[a] or Plasmid	Relevant Genotype[b]	Source
P. aeruginosa strains:		
PAO-LAC	Wild type strain PAO1 with *lacI*^{Q} lacZ′ inserted at *attB* in chromosome via mini-CTX-lacM15	(Hoang *et al.*, 2000)
Complemented *P. aeruginosa* deletions:		
MDM271	PAO-LAC Δ *gyrA*::Tc^{R} / pUCP24GW-*lacPO-Pae-gyrA*	This study
MDM275	PAO-LAC Δ *coaD*::Tc^{R} / pUCP24GW-*lacPO-Bpseu-coaD*	This study
MDM339	PAO-LAC Δ *ftsQ*::Tc^{R} / pUCP24GW-*lacPOBpseu-ftsQ*	This study
MDM349	PAO-LAC Δ *gyrA*::Tc^{R} / pUCP24GW-*lacPO-Bpseu-gyrA*	This study
MDM359	PAO-LAC Δ *lpxA*::Tc^{R} / pUCP24GW-*lacPO-Bpseu-lpxA*	This study
MDM363	PAO-LAC Δ *mesJ*::Tc^{R} / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-mesJ*	This study
MDM498	PAO-LAC Δ *efp*::Tc^{R} / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-efp*	This study
MDM502	PAO-LAC Δ *glmU*::Tc^{R} / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-glmU*	This study
MDM504	PAO-LAC Δ *ligA*::Tc^{R} / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-ligA*	This study
MDM510	PAO-LAC Δ *secA*::Tc^{R} / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-secA*	This study
MDM882	PAO-LAC Δ *folA*::Tc^{R} / pUCP24GW-*lacPO-Bpseu-folA*	This study
MDM949	PAO-LAC Δ *ipk*::Tc^{R} / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-ipk*	This study
MDM366	PAO-LAC Δ *dnaA*::Tc^{R} / pUCP24GW-*lacPO-Bpseu-dnaA*	This study
MDM924	PAO-LAC Δ *ftsA*::Tc^{R} / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-ftsA*	This study
Reporter strains and complemented *P. aeruginosa* deletion reporter strains:		
MDM977	PAO-LAC Δ *gyrA*::Tc^{R}::pGSV3-‘*PA0614’-luxCDABE* / pUCP24GW-*lacPO-Pae-gyrA*	This study
MDM981	PAO-LAC Δ *gyrA*::Tc^{R}::pGSV3-‘*PA0614’-luxCDABE* / pUCP24GW-*lacPO-Bpseu-gyrA*	This study
MDM1141	PAO-LAC Δ *coaD*::Tc^{R}::pGSV3-‘*PA4607’-luxCDABE* / pUCP24GW-*lacPO-Bpseu-coaD*	This study
MDM1142	PAO-LAC Δ *glmU*::Tc^{R}::pGSV3-‘*PA1494’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-glmU*	This study
MDM1143	PAO-LAC Δ *secA*::Tc^{R}::pGSV3-‘*PA2403’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-secA*	This study
MDM1144	PAO-LAC Δ *secA*::Tc^{R}::pGSV3-‘*PA2408’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-secA*	This study
MDM1145	PAO-LAC Δ *secA*::Tc^{R}::pGSV3-‘*PA1365’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-secA*	This study
MDM1164	PAO-LAC Δ *glmU*::Tc^{R}::pGSV3-‘*PA1471’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-glmU*	This study
MDM1166	PAO-LAC Δ *glmU*::Tc^{R}::pGSV3-‘*PA2358’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-glmU*	This study
MDM1167	PAO-LAC Δ *secA*::Tc^{R}::pGSV3-‘*PA2404’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-secA*	This study
MDM1168	PAO-LAC Δ *coaD*::Tc^{R}::pGSV3-‘*PA2030’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-coaD*	This study
MDM1169	PAO-LAC Δ *secA*::Tc^{R}::pGSV3-‘*PA1494’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-secA*	This study
MDM1170	PAO-LAC Δ *glmU*::Tc^{R}::pGSV3-‘*PA1365’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-glmU*	This study
MDM1184	PAO-LAC Δ *secA*::Tc^{R}::pGSV3-‘*PA1471’-luxCDABE* / pUCP24GW-*lacI*^{Q}-*lacPO-Bpseu-secA*	This study
Plasmids:		
pEX18ApGW	pEX18Ap modified with Invitrogen Gateway Vector Conversion kit	(Choi and Schweizer, 2005)
pUCP24GW	pUCP24 modified with Invitrogen Gateway Vector Conversion Kit	(Moir *et al.*, 2007)
pUCP24GW-lacI^{Q}	pUCP24GW modified by addition of *lacI*^{Q} at *Bsp*HI site	(Moir *et al.*, 2007)
pGSV3-lux-Sp^{R}	pGSV3-lux modified by replacement of Gm^{R} with Sp^{R} element at *Sac*I sites	This study

[a] Commonly used *E. coli* hosts and cloning vectors are described and referenced in Materials and Methods

[b] Ap^{R}, Sp^{R}, Gm^{R}, and Tc^{R}: ampicillin, spectinomycin, gentamicin, and tetracycline resistance, respectively.

Table 2

Complementation of *P. aeruginosa* Genes with *B. pseudomallei* Orthologs

Gene Name	Paer PAO1 ID#[a]	Bpseu K96243 ID#[b]	Functional Annotation (Pathway)	Similarity[c]	Complementation	Strain	Growth Dependence on Inducer
gyrA	*PA3168*	*BPSL2521*	DNA gyrase subunit A (DNA replication)	0	yes	MDM271	complete
secA	*PA4403*	*BPSL3016*	Secretion protein SecA (Protein secretion)	0	yes	MDM410	complete
ligA	*PA1529*	*BPSL2164*	DNA ligase (DNA replication)	−213	yes	MDM504	no detectable dependence
glmU	*PA5552*	*BPSL0313*	Glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate uridyltransferasen (Cell wall biosynthesis)	−169	yes	MDM502	complete
ftsA	*PA4408*	*BPSL3021*	Cell division protein FtsA (Cell division)	−163	unknown	MDM924	over-expression sensitive
dnaA	*PA0001*	*BPSL0075*	Chromosomal replication initiator protein DnaA (DNA replication)	−150	unknown	MDM366	over-expression sensitive
accA	*PA3639*	*BPSL2241*	Acetyl-coenzyme A carboxylase carboxyl transferase (alpha subunit) (Fatty acid synthesis)	−130	no		n/a
accD	*PA3112*	*BPSS1696*	Acetyl-CoA carboxylase beta subunit (Fatty acid synthesis)	−123	no		n/a
lpxA	*PA3644*	*BPSL2147*	UDP-N-acetylglucosamine acyltransferase (Fatty acid synthesis)	−83	yes	MDM359	no detectable dependence
ipk	*PA4669*	*BPSL0523*	Isopentenyl monophosphate kinase (ipk, ispE) (Cofactor biosynthesis)	−76	yes	MDM949	no detectable dependence
efp	*PA2851*	*BPSL2422*	Translation elongation factor P (Translation)	−73	yes	MDM498	partial
coaD	*PA0363*	*BPSL0516*	Phosphopantetheine adenylyltransferase (Cofactor biosynthesis)	−62	yes	MDM275	partial
ftsW	*PA4413*	*BPSL3026*	Cell division protein FtsW (Cell division0	−56	no		n/a
holA	*PA3989*	*BPSL2936*	DNA polymerase III, delta subunit (DNA replication)	−49	no		n/a
fabZ	*PA3645*	*BPSL2148*	(3R)-Hydroxymyristoyl-[acyl carrier protein] dehydratase (Fatty acid synthesis)	−44	no		n/a
folA	*PA0350*	*BPSL2476*	Dihydrofolate reductase (Cofactor biosynthesis)	−33	yes	MDM882	no detectable dependence
ftsQ	*PA4409*	*BPSL3022*	Cell division protein FtsQ (Cell division)	−27	yes	MDM339	complete
lolA	*PA2614*	*BPSL2603*	OM lipoproteins carrier protein (Chaperones)	−22	no		n/a
mesJ	*PA3638*	*BPSL2240*	Conserved hypothetical protein (Hypothetical, unknown)	−17	yes	MDM363	partial

[a] Paer, Pseudomonas aeruginosa; genes numbered according to (Stover et al., 2000)

[b] Bpseu, Burkholderia pseudomallei; genes numbered according to (Holden et al., 2004)

[c] The value of the exponent of the P-value from the BLAST analysis is shown

TABLE 3

Promoter Response to Target Depletion

Gene Source of *luxCDABE* Promoter	Promoter Gene Annotation	coaD Signal Ratio[a]	glmU Signal Ratio[a]	secA Signal Ratio[a]	gyrA Signal Ratio[a]	Ciprofloxacin Signal Ratio[b]	Reporter Strain RLU Signal Ratio	Reporter Strain	Control RLU Ratio	Control Strain & Condition
PA2030	hypothetical protein	**10.59**	1.23	1.33	0.45	0.73	**7.4**	MDM1168		
PA2031	hypothetical protein	**12.53**	1.82	1.48	0.60	0.72	n.d.			
PA3361	hypothetical protein	**15.68**	0.20	0.44	0.14	0.64	n.d.			
PA4607	hypothetical protein	**8.46**	0.71	1.14	0.21	1.12	**2.5**	MDM1141		
PA1471	hypothetical protein	3.10	**12.35**	1.16	0.93	0.78	**12.7**	MDM1164	**0.7**	MDM1184; limiting [IPTG]
PA1494	conserved hypothetical protein	1.64	**18.55**	0.51	0.94	1.10	**8.0**	MDM1142	**0.5**	MDM1169; limiting [IPTG]
PA2358	hypothetical protein	0.40	**6.17**	1.50	0.77	1.99	**16.0**	MDM1166		
PA4181	hypothetical protein	1.40	**15.28**	3.03	1.03	1.07	n.d.			
PA4182	hypothetical protein	1.92	**10.64**	3.93	0.82	0.91	n.d.			
PA1365	probable siderophore receptor	0.94	0.66	**16.69**	0.74	1.44	**7.9**	MDM1145	**0.3**	MDM1170; limiting [IPTG]
PA2403	hypothetical protein	0.36	2.52	**6.87**	0.07	1.07	**1.9**	MDM1143		
PA2404	hypothetical protein	0.53	1.61	**7.93**	0.06	0.71	**3.3**	MDM1167		
PA2405	hypothetical protein	0.45	1.69	**8.22**	0.03	1.03	n.d.			
PA2406	hypothetical protein	0.28	1.40	**4.88**	0.07	1.52	n.d.			
PA2407	probable adhesion protein	0.35	1.30	**5.92**	0.07	0.92	n.d.			
PA2408	probable component of ABC transporter	0.56	1.31	**9.85**	0.07	1.17	**5.6**	MDM1144		
PA2409	probable permease of ABC transporter	0.44	1.05	**6.16**	0.07	0.66	n.d.			
PA2410	hypothetical protein	0.62	1.15	**5.09**	0.11	0.99	n.d.			
PA0612	hypothetical protein	0.78	1.54	1.37	**6.00**	**4.43**	n.d.			
PA0613	hypothetical protein	1.84	1.74	1.17	**6.94**	**3.79**	n.d.			
PA0614	hypothetical protein	1.64	0.45	0.72	**7.39**	**5.44**	**6.0/7.5**	MDM981/MDM977	**25/43**	MDM981/MDM977 +/− 0.03 μg/ml FQ[c]
PA0615	hypothetical protein	3.32	0.33	0.91	**6.09**	**3.11**	n.d.			
PA0616	hypothetical protein	1.69	0.39	0.93	**7.18**	**6.51**	n.d.			
PA0617	probable bacteriophage protein	1.20	0.39	0.58	**8.09**	**7.49**	n.d.			

[a] Ratio of normalized signal intensities in limiting IPTG/excess IPTG

[b] Ratio of normalized signal intensities from growth in 0.006 μg/ml ciprofloxacin (~0.1x MIC) vs. growth in the absence of ciprofloxacin

[c] FQ, fluoroquinolone (ciprofloxacin was used in these experiments at ~0.5x MIC)