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Title: BEEP: An assay to detect Bio-Energetic and Envelope Permeability alterations in
Pseudomonas aeruginosa

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25 **Abstract**

26 We developed an effective and rapid assay to detect both bio-energetic and envelope
27 permeability (BEEP) alterations of *Pseudomonas aeruginosa*. The assay is based on
28 quantification of extracellular ATP in bacterial cultures using luciferase as a reporter. To
29 demonstrate the validity of our assay we conducted a biased screen of a transposon insertion
30 library in *P. aeruginosa* strain PAO1 in order to expedite the isolation of mutants with defects
31 in bioenergetic pathways. We successfully isolated insertion mutants that were reduced for
32 extracellular ATP accumulation and identified the corresponding mutations that caused the
33 phenotype. Most of the genes identified from this analysis were associated with energy
34 metabolism and several appeared to be potentially novel bioenergetic targets. In addition,
35 we show that treatment of *P. aeruginosa* strain PAO1 with antibiotics that disrupt the bacterial
36 cell envelope lead to greater extracellular ATP accumulation. In summary, increases in
37 extracellular ATP accumulation above wild type levels indicated a perturbation of membrane
38 permeability while decreases in extracellular ATP accumulation indicated defects in
39 bioenergetics.

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49 **1. Introduction**

50 Very few assays have been developed to easily detect alterations in bioenergetics or
51 envelope permeability in bacteria, let alone a single assay that can do both. Bacterial
52 permeability assays generally utilize one of two common approaches, none of which have
53 become universally adopted. The first is to measure uptake of a hydrophobic compound such
54 as ethidium bromide, a fluorochrome or an antibiotic (Helander and Mattila-Sandholm, 2000,
55 Miki and Hardt, 2013, Ruiz et al., 2005). Uptake of a variety of compounds is often
56 complicated by efflux systems and therefore this approach may require blocking efflux by de-
57 energizing cells to obtain a more accurate measurement (Bengoechea et al., 1998, Sun et
58 al., 2014). One of the most widely used assays to measure permeability in bacteria is based
59 on uptake of the fluorochrome 1-*N*-phenylnaphthylamine (NPN) which fluoresces upon
60 entering the phospholipid layer of the cell membrane. However, the NPN assay requires
61 circumventing the membrane barrier by destabilizing the LPS layer with heat or chemicals
62 such as detergents or the chelating agent ethylenediaminetetraacetic acid (Helander and
63 Mattila-Sandholm, 2000, Tsuchido et al., 1989).

64 The second commonly used method to detect perturbation of membrane permeability
65 measures the release of a cellular component into the extracellular environment and some of
66 these assays have been developed into genetic screens (Aguilera et al., 2003, Ohmizo et al.,
67 2004, Orlov et al., 2002). One such screen was developed in 1972 and involves detecting
68 periplasmic RNase that creates a zone of clearance around colonies on agar plates
69 containing RNA (Lopes et al., 1972). A more recent and versatile screen for membrane
70 permeability monitored degradation of the β -galactosidase substrate chlorophenyl red- β -D-
71 galactopyranoside (CPRG) as an indicator on agar plates (Paradis-Bleau et al., 2014).
72 Hydrolysis of CPRG results when bacteria are compromised for permeability or when cells in
73 the culture lyse, a phenotype common to envelope biogenesis mutants. A limitation of this

74 screen is that development of color is directly proportional to incubation time and diffusion of
75 color requires careful monitoring. Our BEEP screen also relies upon the identification of a
76 reporter substance, adenosine triphosphate (ATP), that escapes from leaky or lysed cells.
77 The power of the assay lies in the sensitivity of ATP detection, which is as low as picomolar
78 to femtomolar concentrations. The other obvious attraction to BEEP is that it can identify
79 bioenergetic alterations in addition to envelope permeability alterations. Measurement of
80 intracellular ATP has been used to monitor bacterial bioenergetics in other studies
81 (Charpentier et al., 2009, Hong and Brown, 2009, Sanchez et al., 2010). In this study we
82 show that measurement of extracellular ATP is also effective for monitoring bacterial
83 bioenergetics.

84 The most common way to detect mutants affected for bioenergetics is to monitor colony size
85 on agar plates or growth rate in liquid culture. An ideal assay for identifying or assessing
86 bioenergetics mutants should recognize defects in a variety of pathways including metabolic
87 pathways, the generation of the proton motive force (PMF) and the generation of ATP.
88 Because ATP is one of the major end products from these activities, it seems reasonable that
89 defects in these pathways would result in reduced accumulation of ATP. ATP is produced by
90 a variety of reactions in a bacterial cell and powers numerous cellular processes.
91 Consequently, the disruption of pathways leading to ATP synthesis is expected to have a
92 pleiotropic effect on a bacterium leading to compromised growth and survival. Similarly, the
93 PMF is essential for a bacterium to grow, replicate and survive because it is used for a variety
94 of important cellular processes including ATP synthesis, flagella-mediated motility,
95 maintenance of pH homeostasis and to drive efflux pumps. Fluorescent compounds that
96 measure components of the PMF are available and have been used in high-throughput
97 screens to detect the effect of drugs on the PMF (Farha et al., 2013, Gentry et al., 2010).

98 However, assays that broadly detect bioenergetics defects in bacteria are lacking and would
99 be powerful tools in identifying potential targets for therapeutic intervention. Recently,
100 bacterial membrane integrity and energy metabolism have emerged as new targets for the
101 development of therapeutic drugs (Cook et al., 2014, Hurdle et al., 2011, Nathan, 2004).

102
103 Drugs that target bacterial membrane permeability, menaquinone, the PMF and the F_1F_0 ATP
104 synthase are inhibitory against bacteria and some have proven to be clinically effective
105 (Balemans et al., 2012, Cook, Greening, Hards and Berney, 2014, Dadi et al., 2009, Gupta et
106 al., 2009, Hamamoto et al., 2015, Pethe et al., 2013, Symersky et al., 2012). Therefore, the
107 finding that a wide variety of Gram-positive and Gram-negative bacteria secrete ATP was
108 quite surprising (Ivanova et al., 2006, Mepin et al., 2013). The presence of extracellular
109 ATP enhances stationary survival of bacteria but the molecule is not internalized as a nutrient
110 (Mepin, Tran, Chen, Gong, Kim Ho and Lu, 2013) suggesting that extracellular ATP plays a
111 role in bacterial physiology that is not yet fully understood. A recent study showed that
112 extracellular ATP induces bacterial adhesion and biofilm formation suggesting it may act as a
113 signaling molecule (Xi and Wu, 2010). Therefore, the BEEP assay may also be useful in
114 identifying mutants that are defective in the initial stages of biofilm formation.

115 While membrane permeability and energy generation are well-studied areas of research in
116 bacteria, better methods are needed to easily screen for compounds that affect these
117 processes and to identify genes involved in these pathways in order to further our
118 understanding. We believe there are yet unidentified players in these processes that could
119 lead to important insights into *Pseudomonas aeruginosa* physiology specifically and bacterial
120 physiology in general.

121

122 2. Materials and Methods

123 2.1 Bacterial strains, plasmids, and media. Bacteria were cultured in cation-adjusted Muller-
124 Hinton II broth (MH2), Lysogeny-broth (LB) or on L-agar at 37°C and maintained as frozen
125 stock cultures at -80°C. Solidified media contained 1.5% (w/v) Bacto Agar (Difco, Becton
126 Dickinson). Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at the
127 following concentrations unless otherwise stated: 100 µg ampicillin ml⁻¹, 20 µg gentamicin ml⁻¹,
128 1, and 50 µg kanamycin ml⁻¹ for *Escherchia coli*; 100 µg gentamicin ml⁻¹, 150 µg carbenicillin
129 ml⁻¹ for *P. aeruginosa*. Growth curves were performed in 48 well microtiter plates using 0.5
130 ml MH2 broth and the OD₆₀₀ was monitored hourly at 37°C with a BioTek Synergy HT plate
131 reader over a 20 hour period (BioTek, Winooski, VT). UV-Visible absorption spectra were
132 recorded on a Shimadzu UV-1601 Spectrophotometer using 1 cm path length cells.

133

134 2.2 DNA manipulations, transformations, and conjugations. *E. coli* strain DH10B (Grant et
135 al., 1990) was routinely utilized as a host strain for cloning. DNA was introduced into *P.*
136 *aeruginosa* strain PAO1 (Holloway et al., 1979) via conjugation as previously described (Suh
137 et al., 1999). DNA sequencing was performed by Eurofins (Huntsville, AL).

138

139 2.3 Transposon mutagenesis of *P. aeruginosa*

140 *P. aeruginosa* strain PAO1 was mutagenized with transposon Tngfp carried on plasmid
141 pAG408 (Suarez et al., 1997). pAG408 was introduced into *P. aeruginosa* via tri-parental
142 mating as previously described (Suh, Silo-Suh, Woods, Hassett, West and Ohman, 1999).
143 The conjugation mixtures were plated onto a 1:1 mixture of L-agar and Pseudomonas
144 Isolation Agar (PIA) containing gentamicin (180 µg ml⁻¹) to select for *P. aeruginosa* containing
145 transposon insertions. Colonies displaying a small colony phenotype were tested for

146 swimming motility as described by Ha et al. (Ha et al., 2014). Briefly, a colony was stabbed
147 onto L- agar plates made with 0.3% agar, incubated overnight at 37°C and the diameter of
148 bacterial growth caused by motile cells was measured. The transposon insertion sites were
149 identified as previously described (Lindsey et al., 2008).

150

151 2.4 Extracellular ATP Assay

152 Extracellular ATP concentration was measured using the ENLITEN ATP Assay (Promega,
153 Madison, WI) according to the manufacturer's instructions. The assay requires a single
154 reagent which is added in equal volume to samples, incubated for 5 minutes at room
155 temperature and then monitored for luminescence. To monitor extracellular ATP
156 accumulation over a growth cycle, PAO1 was inoculated into 15 ml of L- broth in a 125 ml
157 Erlenmeyer flask and grown at 37°C on a shaker at 220 rpm. Aliquots were taken at various
158 time points for an OD₆₀₀ reading and measuring extracellular ATP accumulation. To acquire
159 culture supernatants, cells were removed via centrifugation in a microcentrifuge for 2 minutes
160 at 14,000rpm. Twenty-five µl of culture or supernatant were inoculated into wells of a white
161 96 flat-well polystyrene microtiter plate (Thermo Fisher) and an equal volume of ATP reagent
162 was added. The plates were then incubated for 5 minutes at room temperature and light
163 production was measured in a TECAN Infinite M1000 using the luminescence mode and an
164 integration time of 1000 ms. Extracellular ATP is presented as relative light units (RLU). To
165 test cultures grown in 48 well microtiter plates, a colony from an agar plate was inoculated
166 into 0.2 ml L-broth and then incubated in the BioTek Synergy HT plate reader with shaking at
167 37°C. Statistical analyses were carried out using GraphPad Prism version 5.0d for Mac OS X.

168

169 2.5 Antibiotic Assays

170 To test for the effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) on extracellular
171 ATP accumulation, a PAO1 culture was grown to an OD₆₀₀ of ~1.0 at which point the cells
172 were centrifuged and the supernatant discarded to remove extracellular ATP. The cells were
173 then resuspended in an equal volume of fresh L-broth. One ml aliquots were distributed into
174 test tubes with, or without, CCCP and incubated for 30 minutes at 37°C with shaking. This
175 incubation time allowed the cells to recover from centrifuging and initiate growth, but not
176 enough time to divide. To test for the effect of polymyxin B on extracellular ATP
177 accumulation, PAO1 was scraped from an agar plate and diluted to approximately 5 x 10⁵
178 cells/ml in MH2 broth. The culture was grown to an OD₆₀₀ of approximately 1.0, aliquoted
179 into tubes containing different concentrations of the drug, incubated for 10 minutes with
180 shaking and assayed for extracellular ATP. To test for the effect of other antibiotics on
181 extracellular ATP accumulation, PAO1 was scraped from an agar plate and diluted to
182 approximately 5 x 10⁵ cells/ml in MH2 broth and then 0.5 ml of the culture was distributed to
183 48 well microtiter plates containing antibiotics. For the purpose of this study, sub-inhibitory
184 concentrations of antibiotics are defined as the highest concentration of drug that does not
185 alter the growth curve of PAO1.

186

187 **3. Results**

188 3.1 Extracellular ATP accumulation by *P. aeruginosa* strain PAO1

189 Extracellular ATP has been correlated with respiration and metabolic activity in *E. coli* and
190 *Salmonella enterica* suggesting that extracellular ATP can be used to identify energy deficient
191 mutants (Hironaka et al., 2013, Mempin, Tran, Chen, Gong, Kim Ho and Lu, 2013). In order
192 to identify an optimal time point for extracellular ATP detection, we monitored its
193 accumulation over a PAO1 growth curve (Figure 1). We also compared extracellular ATP
194 accumulation from cultures and supernatants. Because the ENLITEN ATP assay (Promega,

195 WI) does not contain chemicals to lyse bacterial cells, cultures (cells plus supernatant) should
196 contain the same amount of extracellular ATP as supernatants unless the cells interfere with
197 luminescence.

198

199 As shown in Figure 1, *P. aeruginosa* accumulates extracellular ATP throughout its growth
200 curve with peak accumulation occurring at the transition from the log phase to the stationary
201 phase of growth, and then declining in stationary phase. This pattern of extracellular ATP
202 accumulation mirrors the metabolic activity of the cells in liquid culture with extracellular ATP
203 declining in the absence of new growth. Extracellular ATP accumulation from cultures and
204 supernatants was similar with slightly more detected in cultures. Therefore, bacterial cells
205 may interfere with the luciferase assay. However, the pattern of extracellular ATP
206 accumulation is similar for both cultures and supernatants indicating that the interference is
207 similar for all of the time points. In addition, filter sterilizing the supernatants did not greatly
208 alter extracellular ATP accumulation (data not shown). Therefore, cultures can be used to
209 assess relative extracellular ATP between isolates or conditions.

210

211 3.2 Dissipation of the PMF reduces extracellular ATP accumulation

212 Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is a protonophore that disrupts the PMF
213 by dissipating the H⁺ gradient and thus reduces the activity of ATP synthase. Treatment of
214 PAO1 cells with increasing concentrations of CCCP correlated with reduced extracellular
215 ATP accumulation (Figure 2) indicating that extracellular ATP reflects intracellular ATP
216 production and is affected by dissipating the PMF. All of the CCCP concentrations used
217 prolonged the lag phase but did alter the growth rate of PAO1 in log phase (data not shown).

218

219 3.3 Identification of mutants affected for bioenergetics via BEEP

220 In order to demonstrate that reduced extracellular ATP accumulation can be used to identify
221 *P. aeruginosa* mutants affected for bioenergetics we conducted a biased screen of a
222 transposon insertion library in strain PAO1. We reasoned that a subset of bioenergetic
223 mutants would manifest as small colony-variants (SCV) because these isolates often contain
224 defects in electron transport pathways (Proctor et al., 2006). We mutagenized PAO1 with
225 *Tngfp* (Suarez, Guttler, Stratz, Staendner, Timmis and Guzman, 1997) and then isolated the
226 smallest colonies after plating the cells onto agar plates containing antibiotics that select for
227 cells harboring a *Tngfp* insertion. To further increase the probability of identifying mutants
228 defective in bioenergetics we imposed a secondary screen for reduced swimming motility.
229 Because flagellar-mediated swimming motility is powered by PMF in bacteria we reasoned
230 that SCV that were reduced for swimming (and therefore reduced for PMF) would exhibit a
231 more drastic reduction in extracellular ATP accumulation than SCV that were swim proficient.
232 As expected, some of the swim deficient SCV proved to be reduced for extracellular ATP
233 accumulation compared to PAO1 (Figure 3A and 3B). While both cultures and supernatants
234 gave similar results, the difference between PAO1 and the SCV mutants was more
235 pronounced with supernatant testing. We also determined that a random set of transposon
236 insertion mutants was not reduced for extracellular ATP accumulation compared to PAO1.
237 With this group of mutants, supernatants clearly grouped with PAO1 for extracellular ATP
238 accumulation and gave more consistent results than the cultures (Figure 3C and 3D).
239 We identified the transposon insertion site for eleven of the SCV mutants that are reduced for
240 extracellular ATP (Table 1). Three of the disrupted genes representing 4 of the mutants have
241 previously been linked with SCV phenotypes and are involved in ATP production. These
242 mutants include PA4214 (*phzE1*), PA1903 (*phzE2*) and PA4973 (*thiC*) (Glasser et al., 2014,
243 Proctor, von Eiff, Kahl, Becker, McNamara, Herrmann and Peters, 2006). A fourth gene,

244 PA5203, encodes for a protein that is required for thiamine biosynthesis in *S. enterica*
245 serovar Typhimurium (Gralnick et al., 2000) and is likely involved in energy metabolism
246 similar to ThiC. Three additional genes appear to be involved in nutrient acquisition. PA1569
247 and PA4067 encode for proteins involved in the transport of small charged molecules while
248 PA2343 has homology to carbohydrate kinases. PA1569, which was picked up three times in
249 our analysis, exhibits a high degree of similarity to the *E. coli* glucose-6-phosphate sensor.
250 The final mutant has an insertion in PA1547, which is the last gene in an uncharacterized 5-
251 gene operon. PA1547 has homology to cytochrome C assembly proteins and therefore likely
252 plays a role in respiration. Taken together the results validate our approach to identify known
253 and novel genes that play a role in energy metabolism in *P. aeruginosa* by measuring
254 extracellular ATP accumulation.

255

256 3.4 Adjusting BEEP for Genetic Screens

257 In order for BEEP to be a useful tool for genetic screens to identify potential mutants that are
258 defective for envelope permeability or bioenergetics, it should be applicable to microtiter plate
259 grown cultures and preferably with a single time-point to be high throughput. Our initial
260 concern with moving the assay to microtiter plates was the lack of oxygenation required for
261 vigorous aerobic respiration during growth. Not surprisingly, we were unable to detect a
262 difference in extracellular ATP accumulation between most of the SCV mutants and PAO1
263 using 48 or 96 well microtiter plates containing either 0.2 ml and 0.1 ml respectively of MH2
264 broth/well (Figure 4A and data not shown). The only mutant that consistently showed
265 reduced extracellular ATP accumulation under either growth condition was SCV11, which
266 contains an insertion in a gene (PA1547) predicted to encode for a protein involved in
267 cytochrome C assembly. The random transposon insertion mutants were similar to PAO1 for
268 extracellular ATP accumulation using 48 well microtiter plates (Figure 4B). Taken together,

269 microtiter grown cultures can be used as a screening platform to distinguish only the most
270 severe bioenergetic mutants.

271

272 3.5 Perturbation of cell wall envelope leads to increased extracellular ATP accumulation

273 Bacterial cells that are compromised for cell envelope integrity have been shown to leak
274 cellular products including ATP (Chen and Cooper, 2002). The remarkable sensitivity of the
275 extracellular ATP assay prompted us to question whether a loss in membrane integrity would
276 lead to higher extracellular ATP accumulation. Therefore, we treated a growing culture of *P.*
277 *aeruginosa* strain PAO1 with polymyxin B, an antibiotic that disrupts both the outer and inner
278 membranes of gram-negative bacteria (Daugelavicius et al., 2000). As expected, increasing
279 concentrations of polymyxin B led to increased extracellular ATP (Figure 5A). We also tested
280 whether carbenicillin, a compound that inhibits bacterial cell wall synthesis affects
281 extracellular ATP accumulation by *P. aeruginosa*. As shown in Figure 5B, treatment of
282 PAO1 with carbenicillin, but not antibiotics that affect protein synthesis (i.e. gentamicin and
283 tetracycline), led to increased extracellular ATP accumulation. Because we used sublethal
284 concentrations of the antibiotics that do not affect the growth of the bacterium for this
285 experiment the results suggest that even a small perturbation of membrane integrity can be
286 detected using the extracellular ATP assay. Moreover, the carbenicillin effect was observed
287 using 48 well microtiter dishes with 0.4 ml MH2 broth/well, suggesting that aeration is less of
288 a factor when monitoring membrane integrity than with bioenergetics.

289

290 **4. Discussion**

291 In this study we developed and demonstrated that the BEEP assay is a sensitive, relatively
292 simple, and broadly effective method for identifying *P. aeruginosa* bioenergetics mutants and
293 detecting perturbations in membrane integrity. Clinical SCV isolates of *Staphylococcus*

294 *aureus* tend to have mutations affecting energy generating pathways (McNamara and
295 Proctor, 2000, Proctor, von Eiff, Kahl, Becker, McNamara, Herrmann and Peters, 2006),
296 which is the rationale we used for isolating SCV *P. aeruginosa* for this study. Remarkably,
297 most of the *P. aeruginosa* SCV that have been characterized to date do not have defects in
298 energetic pathways, but rather have defects that result in the accumulation of intracellular
299 cyclic-di-GMP (for a review see (Malone, 2015)). We suspect we were able to isolate SCV
300 mutants of PAO1, a wound isolate (Holloway, Krishnapillai and Morgan, 1979) with defects in
301 energetic pathways because our isolates were identified using an in vitro assay, whereas the
302 bulk of previously characterized SCV from *P. aeruginosa* are clinical isolates associated with
303 chronic infections which often harbor multiple mutations (Hausssler et al., 1999, Hogardt et al.,
304 2007, Malone, 2015, Reinhardt et al., 2007). Based on these results, it is tempting to
305 speculate that some *P. aeruginosa* isolates that are defective for energetic pathways are
306 unable to survive long term within the host. Importantly, none of the mutations associated
307 with the SCV phenotype identified in this study were located in genes known to be involved in
308 cyclic-di-GMP metabolism. Our current attempts to modify the assay for microtiter grown
309 cultures permitted the identification of only the most severely affected mutants for reduced
310 extracellular ATP accumulation.

311 In addition, we showed that an antibiotic that perturbs the *P. aeruginosa* envelope leads to
312 increased extracellular ATP accumulation compared to the untreated control or treatment
313 with antibiotics that do not affect the envelope. It is important to note that antibiotics that
314 affect the envelope, and mutations that affect envelope biogenesis, will eventually cause cell
315 lysis, which would raise extracellular ATP (Nicolaes et al., 2014, Paradis-Bleau, Kritikos,
316 Orlova, Typas and Bernhardt, 2014). In this study, we showed increased extracellular ATP
317 accumulation by strain PAO1 using sub-inhibitory concentrations of carbenicillin that do not
318 affect the growth curve. Because a minimum inhibitory concentration of carbenicillin did not

319 lyse *P. aeruginosa* cells within the first 7 hours of incubation (Ellis et al., 1976), we believe
320 our assay is detecting a membrane integrity defect in response to the low concentrations of
321 the antibiotic. For comparison, a MIC concentration of carbenicillin in our assay would be 8.3
322 fold greater (100 µg/ml) than the subinhibitory concentration used for the extracellular ATP
323 measurement. In summary, perturbation of envelope permeability may lead to lysis of
324 bacteria. While the BEEP assay may not distinguish between increased permeability and cell
325 lysis, our results show its utility in identifying mutants under conditions that reflect either of
326 these cellular outcomes. Furthermore, we suggest that mutations in targets that compromise
327 permeability or that cause cell lysis would be of interest as potential therapeutic targets.

328

329 The gram-negative cell envelope is a formidable barrier that must be circumvented in order to
330 better treat infections caused by multi-drug resistant pathogens. This study provides a useful
331 tool for monitoring defects in the gram-negative cell envelope in order to identify novel targets
332 for drug therapy.

333

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Figure 1. Extracellular ATP accumulation by PAO1. Panel A shows the growth curve of PAO1 in MH2 broth. Panel B shows extracellular ATP accumulation at the same time points used for the growth curve. Open circles indicate extracellular ATP from cultures while triangles depict extracellular ATP from supernatants. The results are representative of two independent experiments conducted in duplicate with standard error. Extracellular ATP is presented as relative light units (RLU).

Figure 2. Effect of CCCP on extracellular ATP accumulation by PAO1. PAO1 log phase cells were treated with increasing concentrations of CCCP. The data shown are representative of two independent experiments conducted in triplicate. Values with different letters differ significantly ($P < 0.05$).

Figure 3: Extracellular ATP accumulation by SCV and random transposon insertion mutants. Extracellular ATP accumulation was measured from flask grown cells. Panels A and B show extracellular ATP accumulation from SCV cultures and supernatants, respectively. Panels C and D show extracellular ATP accumulation from random transposon insertion mutant cultures and supernatants, respectively. The results are representative of three independent experiments. ATP concentration is presented as relative light units (RLU).

Figure 4: Extracellular ATP accumulation using 48 well microtiter plates. Panel A and Panel B show extracellular ATP accumulation by the SCV mutants and random transposon insertion mutants respectively. The results are representative of two independent experiments. ATP is presented in relative light units (RLU).

Figure 5. Disruption of the cell envelope increases extracellular ATP accumulation. **A.** PAO1 cells in log phase were treated with the indicated concentrations of polymyxin B for 10 minutes and then assayed for extracellular ATP accumulation. The results are representative of 2 experiments conducted in triplicate $\pm$ standard deviation. **B.** Extracellular ATP accumulation was measured using 48 well microtiter grown cultures. PAO1 was treated with each antibiotic at three different concentrations with the two lowest concentrations at sub-inhibitory levels. C = carbenicillin, G = gentamicin, T = tetracycline. The experiment was conducted in triplicate $\pm$ standard deviation and is representative of two independent experiments.

SCV #	Gene	Gene product /description
SCV3	PA4973	ThiC, thiamine biosynthesis
SCV6	PA4214	PhzE1, phenazine biosynthesis
SCV8 SCV9 SCV16	PA1569	Hypothetical protein, probable major facilitator superfamily (MFS) transporter
SCV10	PA4067	Outer membrane protein, OprG. Transport of cations
SCV11	PA1547	Hypothetical protein, membrane protein involved in oxidation reduction, cytochrome complex assembly
SCV18	PA2343	Mtly, carbon utilization, glycerol-lipid metabolism
SCV21 SCV34	PA1903	PhzE2, phenazine biosynthesis
SCVA	PA5203	GshA, glutathione synthetase

Table 1. Transposon insertion mutants identified with the BEEP assay for reduced extracellular ATP accumulation.