ARTICLE

Electroporation Inefficiency in *Caulobacter henricii* CB4

Taylor Howell Carter

All Res. J. Biol., **2022**, 13, 1-6

The publication cost of this article might be covered by external sponsors. More info for sponsors at: innovacion@sacsis.es

All your results are good results!

THE ALL RESULTS JOURNALS

Issue 1, Vol 13, 2022,1-6

Electroporation Inefficiency in *Caulobacter henricii* CB4

Taylor Howell Carter[1]

[1]University of South Carolina, Department of Biology

Abstract: *Caulobacter* is a well-studied model organism. The introduction of plasmids into *Caulobacter* by way of electroporation or conjugation has been well-documented, as well as the barrier *Caulobacter*'s S-layer has played in the efficiency of these techniques. Standard electroporation and conjugation protocols with *Caulobacter henricii* CB4 resulted in zero transformants. Various parameters have been shown to increases electroporation efficiency and these parameters were adjusted for CB4. Although, CB4 was not able to take up foreign DNA though electroporation or conjugation, a variety of parameters which increases electroporation efficiency are now located in one location.

Keywords: Electroporation*, Caulobacter,* Plasmids, EPS, S-layer

Introduction

Caulobacter crescentus is a gram-negative Alphaproteobacterium that divides asymmetrically, resulting in a stalked cell and a motile swarmer cell (1). In addition, it has a well-developed system of genetics (2) that makes it an excellent model system for the study of cellular differentiation, asymmetric division, and cell cycle transition (3,4). Unlike the *C. crescentus* strains, the wild type strain *C. henricii* CB4 has received little attention even though it houses a 93 kb plasmid, which contains 21 heavy metal resistance genes (5,6, * submitted). CB4 was isolated from pond water in 1959 displaying a vibrioid morphology and forming bright yellow colonies (7). The nucleotide sequence of the CB4 genome and its plasmid has been determined (6), and an analysis of the results indicated that the plasmid contains genes involved in conjugation so it may be able to transfer itself to other strains of *Caulobacter*.

To facilitate a test for conjugal transfer, we attempted to add a kanamycin resistance gene to the plasmid by transposon mutagenesis. Christen et al. (8) established the essential genome of *Caulobacter crescentus* utilizing transposon mutagenesis from the pXylTn5 plasmid containing a xylose inducible Tn5 transposase. The basic approach was to transfer the plasmid into *C. crescentus* from *E. coli* with selection for the kanamycin resistance conferred by the transposon. This technique proved to be an excellent way to get large numbers of *C. crescentus* mutants (8). We planned to use the pXylTn5 plasmid to transpose the gene for kanamycin resistance into the CB4 plasmid to provide a marker to select for the transfer of the CB4 plasmid transfer into a *C. crescentus* strain.

A second approach was to modify the pBR322 plasmid so that it contained sequence homology to a copper ATPase gene (*copA*) located on the CB4 plasmid. The resulting pBR322-*copA* plasmid contained the beginning and ending sequences of the *copA* gene flanking a tetracycline antibiotic gene. Since this plasmid is not stable in Caulobacters, once it was transferred to CB4, a homologous recombination event would be needed to disrupt the plasmid copper-translocating P-type ATPase gene and confer tetracycline resistance to the host cell.

Preliminary experiments employing standard electroporation or conjugation techniques did not produce any antibiotic resistant CB4 colonies even though control experiments with *C. crescentus* CB15 were successful. Although conjugation experiments generally work well, Caulobacters are difficult to electroporate due to their paracrystalline protein surface layer, or S-layer (9). Visual and centrifuge assays have shown the CB4 S-layer to be thicker than the CB15 S-layer and could be the reason for the unsuccessful electroporation. Alternatively, CB4 could house an exopolysaccharide layer, or EPS. In CB15 and CB2, the EPS is a tetrasaccharide capsule which could not be easily removed by washing (10).

The S-layer is comprised of a 98 kDa RsaA protein that assembles into six subunit hexagons that combine with other hexagon subunits to create a two-dimensional hexagonal array (11). Ca^{2+} is required for the proper crystallization of the RsaA protein, and the removal of Ca^{2+} has been shown to disrupt the crystallization (12). S-layers are involved in cellular protection and stabilization (13). In *Caulobacter*, the S-layer is hypothesized to be involved in both ion sensing and allowing *Caulobacter* to live in calcium

deficient environments (14). S-layer deficient CB2 and CB15 *Caulobacter* strains were found to have a 10 times higher electroporation efficiency compared to their S-layer containing counterparts (9).

Various techniques to destabilize the S-layer have been described. For example, a $LiCl_2$ wash has been shown to decrease the calcium levels destabilizing the S-layer and increasing electroporation efficiency (15). Increasing the resistance utilized during electroporation has been demonstrated to increase electroporation efficiency in S-layer *Caulobacter* strains as well (9). Unrelated to the S-layer, it has been shown that growing the bacterium in glycine can improve electroporation efficiency in various bacteria (16, 17). Finally, unmethylated plasmids were shown to incorporate at a higher frequency than methylated plasmids (18). Since our initial attempts to move plasmids into CB4 were not successful, these techniques were used in combination and independently in an attempt to improve electroporation efficiency in CB4 with no success.

Methods

Plasmids

The pXylTn5 plasmid (8) was obtained from Dr. Beat Christen (ETH Zurich). To construct the pBR322-*copA* plasmid (Figure 1), primers for the two flanking regions of the *copA* CB4 plasmid gene were designed with restriction sites added on the end of the primers and used to amplify the beginning and end regions of the *copA* gene with PCR (Supplementary Table 1). Concurrently, the pBR322 plasmid was cut with *Bsm*I and *Ava*I (Fig. 1) and the resulting fragments were separated by agarose gel electrophoresis. The larger section of the cleaved pBR322 plasmid was extracted, purified, and ligated to the *copA* BsmF2 and AvaR2 PCR fragment. The ligated plasmid was transformed into *E. coli* S17 (19) and then plated on LB (20) tetracycline (1 ug/ml) plates. A tetracycline resistant colony was purified, grown overnight in LB, and the pBR322 plasmid containing the distal part of the *copA* gene was isolated. The plasmid was digested with *Bsm*I and *Ava*I to confirm the presence of the *copA* gene fragment. The newly constructed plasmid was then digested with *Eco*R1 and *Cla*I, purified as above, and ligated with the *copA* EcoF1 and ClaR1 PCR fragment. After the ligated construct was transformed into *E. coli*, purified and re-isolated, the proper configuration of the pBR322-*copA* plasmid was confirmed by PCR using the EcoF1 and AvaR2 primers. The pBR322-*cop*A plasmid also was transformed into a DCM^-/DAM^- *E.coli* strain (ZYMO mix n go), and both the methylated and unmethylated forms of pBR322-*cop*A were used for the remainder of the experiments.

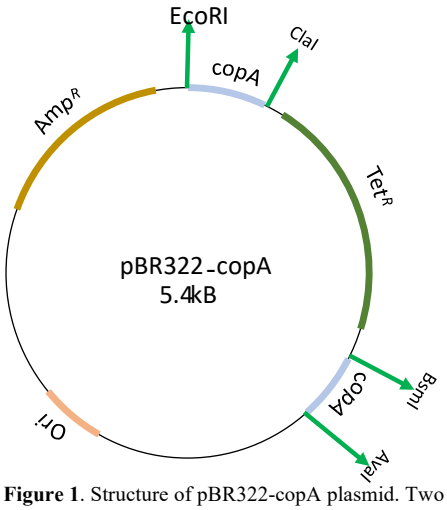

Figure 1. Structure of pBR322-copA plasmid. Two ~250 bp segments from the CB4 plasmid *copA* gene were ligated in at *Eco*RI/*Cla*I and *Bsm*I/*Ava*I cut sites.

Electroporation

To prepare electrocompetent cells, CB4 and NA1000 were grown in 100 mL PYE (2) to mid-log phase. The samples were divided into 50 mL centrifuge tubes and spun at 7000 x g for 5 minutes, and the resulting cell pellets were resuspended in 20 mL of sterile deionized water. The samples were then spun and resuspended as before. The final pellets were resuspended in 2 mL of 10% glycerol, spun at 4000 x g for 5 minutes, and resuspended in 400 ul of 10% glycerol. Finally, 200 ul aliquots were prepared with half used right away and half stored in the -70°C freezer.

For electroporation, 40 ul of the CB4 electrocompetent cells were placed in a 0.5 mL tube, and 2 ul plasmid DNA was added. Subsequently, 40 ul of the CB4/plasmid mixture was placed in a 0.2 cm Bio Rad (Hercules, CA) Gene Pulser cuvette and subjected to a 2.5-kV shock with the capacitor set at 25 uF (microfarad) and the resistance at 200 ohms in a Bio Rad Gene Pulser electroporator. With a 0.2 cm cuvette gap, a 4.5 to 5 msec time constraint was expected at 200 ohms. After the shock, the contents of the cuvette were mixed with 1 ml PYE, transferred to a sterile test tube, and incubated at 30°C for 2 hours before 200 ul of each sample was plated on PYE plates containing the appropriate antibiotic. When using the pXylTn5 plasmid, the plates also contained 100 ul of a 10% xylose solution to induce transposition. Altered electroporation parameters can be found in Supplementary Table 2.

Conjugation procedure

The *E. coli* BC1490 which houses the pXylTn5 plasmid and CB4 cultures were grown overnight in LB or PYE medium, respectively. To initiate conjugation, one mL of the CB4 culture was gently mixed with 0.1 mL of the *E. coli* BC1490 culture. After the mixture was filtered through a Millipore HA 0.45 uM filter, the filter was then placed on a PYE plate and incubated overnight at room temperature. After the incubation, the filter was placed in sterile test tube with 500 uL PYE, and the bacteria were resuspended by vortexing. Subsequently 200 uL of the bacteria suspension was plated on each of two PYE plates containing the

appropriate antibiotic to select for the presence of the plasmid and nalidixic acid (20 ug/mL) to select against the *E. coli* donor strain. Each mating experiment was performed twice for replication of results. Plates were then incubated at 30°C for 2-3 days until colonies appeared.

Results

The pBR322-*copA* plasmid contains two sections of the copper-translocating P-type ATPase *cop*A gene (approximately 250 bp each) that flank a tetracycline gene and are identical to the corresponding regions of the CB4 plasmid (Fig. 1). Since the pBR322-*copA* plasmid cannot replicate in *Caulobacter*, homologous recombination would be needed for the host bacterium to acquire tetracycline resistance after the introduction of the plasmid. Initially, the standard electroporation procedure was used in an unsuccessful attempt to get the plasmid into CB4. Of note, CB4 tended to aggregate into clumps that were difficult to disperse during the preparation of the electrocompetent cells, in comparison to NA1000 where the bacteria go back into solution from the pelleted state without much agitation. CB4 gave a time constraint of 3.4-3.6 msec at the standard electroporation settings compared to the 4.6-4.8 msec obtained with other *Caulobacter* strains. Each experiment was run in duplicate and then it was replicated 4 separate times. No CB4 transformants were obtained from any of these experiments.

To further investigate, we attempted to transform the pXylTn5 plasmid into both CB4 and NA1000. The standard protocol was followed and approximately 100 transformants were obtained with NA1000, and no transformants were obtained with CB4. The experiment was replicated with the same results. Since the standard procedures were not working, we decided to alter both the variables involved in the preparation of the electrocompetent CB4 cells and the electroporation procedure itself as described below.

Varying resistance during electroporation

Gilchrist and Smit (9) demonstrated that increasing the resistance from 200 or 400 to 600 or 800 ohms, respectively, increased the number of transformants for S-layer containing Caulobacters. Also, Spath et al. (18) showed that the use of an unmethylated plasmid can increase transformation efficiency. Therefore, CB4 electrocompetent cells were prepared according to standard protocol and electroporation experiments were performed at 200, 400, 600, and 800 ohms. For each level of resistance, transformations with both a methylated and an unmethylated plasmid were attempted in duplicate, and the time constraints did increase in relation to the increase in resistance. Although increasing the resistance had previously increased the number of transformants in S-layer containing Caulobacters (9), no transformants were observed for any of the 16 samples. As a control an additional aliquot was plated on antibiotic-free control plate to verify bacterial viability.

$LiCl_2$ Wash

A $LiCl_2$ wash has been shown to disrupt the Ca^{2+} component of the S-layer and increase electroporation efficiency (15). Therefore, a $LiCl_2$ wash was added after the initial pelleting of CB4. The pelleted CB4 cells were resuspended in 5 mL of 5 M $LiCl_2$ and held on ice for 30 minutes, pelleted, then resuspended in 5 M $LiCl_2$ and held on ice for an additional 30 minutes. The $LiCl_2$ procedure called for the use of SMEB as the resuspension and electroporation buffer instead of using the 10% glycerol as called for by the standard procedure. Using the SMEB after the $LiCl_2$ resulted in a 0.1 msec time constraint and an electrical arc of the cuvette. One cause of this is can be a high salt concentration. To bypass this problem, a 10% glycerol solution was used during the wash and final preparation of the electrocompetent cells. This modified procedure resulted in the expected time constraints at the 200 and 400 ohms resistance. Again, the experiment was performed with both a methylated and unmethylated plasmid at both 200 and 400 ohms, and no transformants were obtained. To confirm cell viability, 400 ul of one of the $LiCl_2$ washed electroporation samples was plated on a PYE with no antibiotics and cell growth occurred at the expected rate.

Glycine Growth Media

In other bacteria, adding glycine to the growth medium has been shown to increase electroporation efficiency (16,17). CB4 was unable to grow in the previously established 1% glycine concentration which had been shown to increase electroporation efficiency. The highest concentration of glycine that allowed CB4 to grow was 0.1%, and electrocompetent cells were prepared from CB4 cells grown in the presence of 0.1% glycine. For electroporation, both the methylated and unmethylated pBR322-cop plasmids were used at 200 and 400 ohms resistance. The experiment was performed in duplicate and the expected time constraints were obtained, yet none of the runs resulted in any transformants.

EPS

Caulobacter can possess an exopolysaccharide, or EPS layer than acts as another physical barrier to the cell (10). Electron microscopy showed that an EPS layer was present on CB4 cells, as did the clumping of cells during the cell preparation procedure (Fig. 2). Growing bacteria in the presence of 0.7 mM EDTA has been shown to disrupt the EPS and increase electroporation efficiency in other gram-negative bacteria (21), but CB4 did not grow at that concentration of EDTA. *Caulobacter* requires 0.5 mM Ca++ for optimal growth (22) and is sensitive to calcium levels when the S-layer is disrupted (14). CB4 was able to grow at 0.3 mM and 0.4 mM, but not 0.7 mM, EDTA. Therefore, 30 mL cultured of CB4 with 0.3 mM or 0.4 mM EDTA were grown to 30 Kletts, spun down, and washed with 10% glycerol four times. The cells were then pelleted and resuspended in 160 ul of 10% glycerol. Subsequently, 40 ul of the electrocompetent cells were mixed with 1 ul of methylated or unmethylated pBR322-copA and electroporated with the standard procedure. All runs resulted in zero transformants. A control experiment with electrocompetent cells created from CB4 grown in both PYE and PYE with 0.4 mM EDTA resulted in $1.25x10^5$ CFU/mL

of the PYE-grown CB4 competent cells, and no colonies were observed when 20 ul of the EDTA-grown CB4 competent cells were spread on PYE plates. Therefore, we concluded that growth in EDTA lowered the survival rate during the preparation of electrocompetent cells to the point that no surviving cells were present.

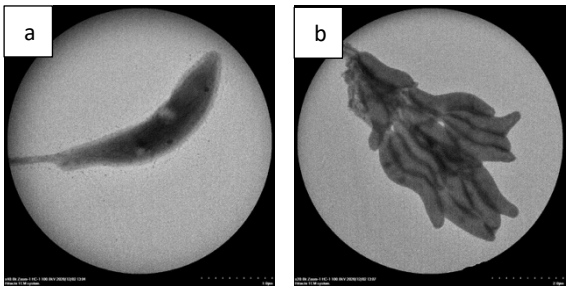

Figure 2. Electron microscope images of *C. henricii* CB4. Fig. 2a) The EPS layer surrounding the CB4 bacterium is visible as a halo. Fig. 2b) A clump of CB4 cells.

Table 1. Description of altered variables to increase electroporation efficiency.

Electroporation Variables	**Description**	**Source**
Increased Resistance	Increasing the resistance to 600 or 800 ohms has been shown to increase electroporation efficiency in Caulobacters with S-layers.	**[9]**
Unmethylated Plasmids	Unmethylated plasmids (DCM^-/DAM^-) have been shown to increase electroporation efficiency.	**[18]**
$LiCl_2$ Wash	$LiCl_2$ wash used to disrupt Ca^{2+} and RsaA interaction and destabilize the S-Layer	**[15]**
Glycine Growth Media	Glycine added to growth media has been shown to increase electroporation efficiency.	**[16,17]**
EPS Disruption	Bacteria grown in the presence of EDTA has been shown to disrupt the exopolysaccharide layer.	**[21]**

Conjugation experiments

Conjugation can also be used to transfer a plasmid from *E. coli* to *Caulobacter* (23). Therefore, conjugation experiments were carried out using the same NA1000 and CB4 bacteria strains with *E. coli* BC1490 which houses the pXylTn5 plasmid. Approximately 400 colonies were obtained with NA1000 as a recipient and no colonies were obtained with CB4 as a recipient. This experiment was repeated with *C. crescentus* strain SC1004 and CB4 with similar results (Fig. 3). Alternatively, conjugation experiments were carried out with CB4 and *E. coli* HB101 which houses the pRK290 plasmid. The pRK290 plasmid houses a RK2 replicon which has been shown to be stable in *Caulobacter* (25), but again, no colonies were obtained from the conjugation with CB4.

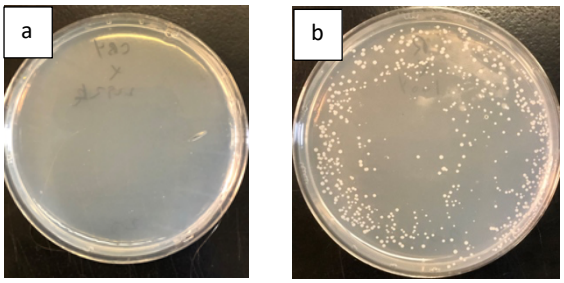

Figure 3. Conjugation results with CB4 and BC1490 (a), and *C. crescentus* SC1004 and BC1490 (b).

Discussion

Electroporation is a useful technique for the introduction of foreign DNA into a bacterium. Previous work has demonstrated that getting DNA into *C. crescentus* electroporation is more difficult due to its S-layer (9). The S-layer is a crystalline layer consisting of RsaA protein subunits and Ca^{2+} ions. Gilchrist and Smit (9) demonstrated that increasing the resistance increased electroporation efficiency in S-layer containing *C. crescentus* strains. However, our use of this procedure did not result in any CB4 transformants. Other researchers increased bacterial electroporation efficiency by using unmethylated plasmids (17), or growth in glycine (16, 17). However, neither of these procedures was successful with CB4. Also, a $LiCl_2$ wash during the preparation of electrocompetent cells has been shown to disrupt the S-layer by removing the Ca^{2+} ions (15), but this technique also did not result in any CB4 transformants. In addition, we were unable to transfer plasmids into CB4 using standard conjugation techniques.

It is possible that the CB4 S-layer is thick enough so that these techniques do not disrupt it sufficiently to allow for the uptake of foreign DNA. In addition, CB4 also has an extracellular polysaccharide (EPS) layer that may block DNA uptake as well. It also is possible that CB4 produces an endonuclease that cleaves extracellular DNA during the electroporation procedure. However, the inability to transfer a plasmid via conjugation where exposure endonuclease activity should not occur indicates that the primary issue is likely some type of external barrier(s) that prevents both conjugation and electroporation from occurring. Further evidence for this barrier is that CB4 tends to form clumps of cells during growth (Fig. 2b), and CB4 cell pellets are difficult to resuspend after centrifugation. Thus, CB4 cells tend to stick to each other in ways that are not observed with *C. crescentus* cells. The role of calcium in S-layer and EPS disruption was also found to decrease cell viability after electroporation. This creates a difficult situation in which disrupting the EPS and S-layer to allow foreign DNA to enter decreases cell viability. Further studies could be conducted to find an equilibrium of depleting the EPS and S-layer while maintaining cell viability.

References

1. Skerker, J. M., & Laub, M. T. (2004). Cell-cycle progression and the generation of asymmetry in *Caulobacter crescentus*. *Nature Reviews Microbiology*, *2*(4), 325-337.
2. Ely, B. (1991). Genetics of *Caulobacter crescentus*. In *Methods in enzymology* (Vol. 204, pp. 372-384). Academic Press.
3. Nierman, W. C., Feldblyum, T. V., Laub, M. T., Paulsen, I. T., Nelson, K. E., Eisen, J., ... & Fraser, C. M. (2001). Complete genome sequence of *Caulobacter crescentus*. *Proceedings of the National Academy of Sciences*, *98*(7), 4136-4141.
4. Govers, S. K., & Jacobs-Wagner, C. (2020). *Caulobacter crescentus*: model system extraordinaire. *Current Biology*, *30*(19), R1151-R1158.
5. Schoenlein, P. V., & Ely, B. (1983). Plasmids and bacteriocins in *Caulobacter* species. *Journal of bacteriology*, *153*(2), 1092.
6. Scott, D., & Ely, B. (2016). Conservation of the essential genome among *Caulobacter* and Brevundimonas species. *Current microbiology*, *72*(5), 503-510.
7. Poindexter, J. S. (1964). Biological properties and classification of the *Caulobacter* group. *Bacteriological reviews*, *28*(3), 231.
8. Christen, B., Abeliuk, E., Collier, J. M., Kalogeraki, V. S., Passarelli, B., Coller, J. A., ... & Shapiro, L. (2011). The essential genome of a bacterium. *Molecular systems biology*, *7*(1), 528.
9. Gilchrist, A., & Smit, J, (1991). Transformation of freshwater and marine caulobacters by electroporation. *Journal of bacteriology*, *173*(2), 921-925.
10. Herr, K. L., Carey, A. M., Heckman, T. I., Chávez, J. L., Johnson, C. N., Harvey, E., ... & Marks, M. E. (2018). Exopolysaccharide production in *Caulobacter crescentus*: A resource allocation trade-off between protection and proliferation. *PLoS One*, *13*(1), e0190371.
11. Smit, J., Engelhardt, H., Volker, S., Smith, S. H., & Baumeister, W. (1992). The S-layer of *Caulobacter crescentus*: three-dimensional image reconstruction and structure analysis by electron microscopy. *Journal of bacteriology*, *174*(20), 6527-6538.
12. Walker, S. G., Smith, S. H., & Smit, J. (1992). Isolation and comparison of the paracrystalline surface layer proteins of freshwater caulobacters. *Journal of bacteriology*, *174*(6), 1783-1792.
13. Sleytr, U. B., & Beveridge, T. J. (1999). Bacterial S-layers. *Trends in microbiology*, *7*(6), 253-260.
14. Herrmann, J., Jabbarpour, F., Bargar, P. G., Nomellini, J. F., Li, P. N., Lane, T. J., ... & Wakatsuki, S. (2017). Environmental calcium controls alternate physical states of the *Caulobacter* surface layer. *Biophysical journal*, *112*(9), 1841-1851.
15. Walker, D. C., Aoyama, K., & Klaenhammer, T. R. (1996). Electrotransformation of Lactobacillus acidophilus group A1. *FEMS microbiology letters*, *138*(2-3), 233-237.
16. Hermans, J., Boschloo, J. G., & De Bont, J. A. M. (1990). Transformation of Mycobacterium aurum by electroporation: the use of glycine, lysozyme and isonicotinic acid hydrazide in enhancing transformation efficiency. *FEMS Microbiology letters*, *72*(1-2), 221-224.
17. Holo, H., & Nes, I. F. (1989). High-frequency transformation, by electroporation, of Lactococcus lactis subsp. cremoris grown with glycine in osmotically stabilized media. *Applied and environmental microbiology*, *55*(12), 3119-3123.
18. Spath, K., Heinl, S., & Grabherr, R. (2012). Direct cloning in Lactobacillus plantarum: electroporation with non-methylated plasmid DNA enhances transformation efficiency and makes shuttle vectors obsolete. *Microbial cell factories*, *11*(1), 1-8.
19. Singh, M., Yadav, A., Ma, X., & Amoah, E. (2010). Plasmid DNA transformation in Escherichia Coli: effect of heat shock temperature, duration, and cold incubation of CaCl2 treated cells. *International Journal of Biotechnology and Biochemistry*, *6*(4), 561-568.
20. Bertani, G. (1951). Studies on lysogenesis I.: the mode of phage liberation by lysogenic Escherichia coli1. *Journal of bacteriology*, *62*(3), 293.
21. Fournet-Fayard, S., Joly, B., & Forestier, C. (1995). Transformation of wild type Klebsiella pneumoniae with plasmid DNA by electroporation. *Journal of microbiological methods*, *24*(1), 49-54.
22. Ely, B., & Johnson, R. C. (1977). Generalized transduction in *Caulobacter crescentus*. *Genetics*, *87*(3), 391-399.
23. Ely, B. (1979). Transfer of drug resistance factors to the dimorphic bacterium *Caulobacter crescentus*. *Genetics*, *91*(3), 371-380.
24. O'Neill, E. A., Berlinberg, C., & Bender, R. A. (1983). Activity of plasmid replicons in *Caulobacter crescentus*: RP4 and ColE1. *Genetics*, *103*(4), 593-604.

Supplementary Table

Primer	Nucleotide sequence
EcoF1	TTTTTTGAATTCCAATCGACGAGTCTATGGTCAC
ClaR1	TTTTTTATCGATAGGATCGCCGAGAGGATAA
BsmF2	TTTTTTGAATGCGGGTTGACGAAACCGATCTACTC
AvaR2	TTTTTTCCCGAGGGCCTGTTCCACCATGAA

Supplementary Table 1. Primers for flanking regions of copper-translocating P-type ATPase CB4 plasmid gene

	Parameters	Methylated/ Unmethylated Plasmids	Control Bacterial Lawn	Transformants
Increased Resistance	200 Ohms, 400 Ohms, 600 Ohms, 800 Ohms	+	+	0
$LiCl_2$ Wash	Two 30-minute washes with 5.0mL of 5.0M $LiCl_2$	+	+	0
Glycine Wash	.5% Glycine growth media	+	+	0
EPS Disruption	.3-.5mM EDTA added to growth media	+	-	0

Supplementary Table 2. Parameters changed during Electroporation. (+) is present and (-) is not present. Each variable was replicated at least 4 times.