Experimental Evolution of the Megaplasmid pMPPla107 in Pseudomonas stutzeri Enables Identification of Genes Contributing to Sensitivity to an Inhibitory Agent

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ABSTRACT

Horizontally transferred elements such as plasmids can, at times, burden host cells with various metabolic and fitness costs. Our previous work demonstrated that acquisition of the *Pseudomonas syringae* megaplasmid pMPPla107 causes sensitivity to a growth inhibiting substance that is produced in cultures during growth under standard laboratory conditions. After 500 generations of laboratory passage of *P. stutzeri* lines containing pMPPla107, two out of six independent lines displayed resistance to this inhibitory agent. We therefore sequenced the genomes of isolates from each independent evolutionary line to identify the genetic basis of this resistance phenotype through comparative genomics. Our analysis demonstrates that two different compensatory mutations on the megaplasmid ameliorate the sensitivity phenotype: 1) a large deletion of approximately 368kb in pMPPla107 and 2) a SNP in the gene we name *skaA* for Supernatant Killing Activity. These results provide further evidence that costs associated with horizontal gene transfer can be compensated through single mutational events and emphasize the power of experimental evolution and resequencing to better understand the genetic basis of evolved phenotypes.
INTRODUCTION

Plasmids are secondary replicons that can rapidly move across bacterial genomes increasing genomic plasticity through a process known as horizontal gene transfer (HGT). Thousands of genes can be transferred via HGT in an instance allowing for the colonization of new niches by the acquisition of genes encoding for metabolism, antibiotic resistance, virulence factors, and symbiosis thus enabling colonization of new niches (1-6). While plasmids could provide advantages for a bacterial cell in a given environment, horizontal gene transfer also brings many costs that may be manifested in phenotypic changes rather than lowered fitness alone (7-11). Outside of a handful of examples, relatively little is known about general trends underlying the mechanistic basis of such costs (8, 9, 12, 13).

We have previously shown that acquisition of the *Pseudomonas syringae* megaplasmid pMPPla107 by *Pseudomonas stutzeri* sensitizes this strain background to the presence of an inhibitory agent that has bacteriostatic properties (9, 10, 14). Sensitivity is found in pMPPla107's native strain *P. syringae pv. lachrymans* 107 and can be transferred to various *Pseudomonas spp.* upon their acquisition of pMPPla107; thus, indicating the phenotype is linked to pMPPla107. Furthermore, production of the inhibitory agent is conserved across *Pseudomonas spp.* appears linked to Pseudomonas metabolism, and may be associated with an essential gene (14).
Although acquisition of plasmids by new host backgrounds often creates metabolic, physiological, and fitness costs, previous research has shown that various types of compensatory mutations occur rapidly on either the chromosome or plasmid and that such amelioration of costs enables the persistence of plasmids (15-19). Evolutionary experiments of *P. fluorescens* with the mercury resistant pQBR103 start with fitness costs in *P. fluorescens*. However, after hundreds of generations compensatory mutations in *gacA/gacS* occur in strains with and without selection using mercury, suggesting that a plasmid can exhibit parasitic behaviors influencing chromosomal mutations without any selective benefit to establish stable cohabitation (15). Furthermore, mutations in two helicases and an RNA polymerase subunit resulted in host dependence on the plasmid RP4 while also increasing the uptake of additional plasmids (16). Therefore, compensatory mutations may not only explain plasmid persistence mechanisms, but also increased plasmid promiscuity. For these reasons and to better understand the genetic basis of previously described phenotypic costs associated with pMPPla107 (9), we carried out experimental evolution of *P. stutzeri* under conditions that selected for maintenance of pMPPla107. Our goal with these passage experiments was to identify strain backgrounds that have ameliorated known costs of pMPPla107 carriage with the hope that identification of compensatory mutations would provide better understanding of the genetic basis of these costs (14).

Here we resequence genomes from single colony isolates sampled from six independently evolving lines of *P. stutzeri* containing pMPPla107 after 500
generations of passage. Two of these lines evolved resistance to a well characterized, but currently unknown, inhibitory agent produced by Pseudomonas species. We further find that two different compensatory mutations provide resistance to this inhibitory agent and that both changes were found on the megaplasmid itself. Although one of these mutations was a large deletion that eliminated many different genes, the other was a single non-synonymous nucleotide polymorphism (SNP) that occurred in a gene with no known function. Our work provides insights into the genetic basis of mutations that ameliorate fitness costs associated with plasmid acquisition but also more specifically inform our understanding of the genetic basis of sensitization of Pseudomonas strains to a currently unidentified inhibitory agent associated with maintenance of pMPPla107.

METHODS

Long Term Evolution Experiment

Six single colonies of *P. stutzeri* strain DBL408 were picked after growth on Salt Water LB (SWLB) agar (20), into independent 2mL cultures of SWLB liquid containing rifampicin (50ng/µL) and tetracycline (10ng/µL) within 5mL polypropylene tubes with caps. These cultures were grown within shaking (220rpm) at 27°C for two days at which point a subset of this culture was frozen in 40% glycerol at -80°C and labeled as “passage 0” while a 1:1000 (cells:media) dilution was also made into fresh 2mL of SW-LB. Each passage, cells were plated to SW-LB agar plates containing rifampicin (50ng/µL) and tetracycline (10ng/µL) to observe colony morphology in case of contamination. Tetracycline in the media
selected for maintenance of the megaplasmid in strain DBL408. Every 10 passages, a 750µL sample of the culture was mixed with 40% final concentration of glycerol and stored at -80°C. This process was repeated for approximately 500 generations of growth (Log2 of 1000=9.96 divisions per passage; 50 passages total).

**Genome Sequencing and Annotation**

Single colonies of each generation 500 line were picked and grown overnight in 2mL of SW-LB with rifampicin (50ng/µL) and tetracycline (10ng/µL). DNA samples were extracted from these cultures using a Promega Wizard kit. *P. stutzeri* lines 1B, 4B, 5B, and 6B were sequenced using 100bp paired end reads on an Illumina HiSeq (SRA in progress). *P. stutzeri* lines 2B and 3B were sequenced using 250bp paired end reads on an Illumina MiSeq by MicrobesNg (SRA in progress). We used Prokka(21) gene annotations of pMPPla107 from a previous publication(5) and the annotations from the *P. stutzeri* 28a24 reference sequence (Accession: CP007441.1).

**Mapping Reads and Calling Variants**

Illumina reads from all six evolved lines were mapped to the *P. stutzeri* 23a24 and pMPPla107 references (Accession No.: CP007441 and NZ_CP031226.1 respectively) using the Geneious11.1.3 (https://www.geneious.com/) mapper. Parameters used for the mapping step were: do not trim, gaps allowed, maximum gap per read = 10%, word length = 18, ignore words repeated more than 12 times, maximum mismatches per read = 20%, maximum gap size = 15, index word length = 13, maximum ambiguity = 4, accurately map reads with errors to repeat regions.
Additionally the sensitivity parameter was set at medium-low sensitivity with up to 5 iterations. We found that mapping at higher sensitivities did not change our outputs and therefore chose this setting. After mapping, variants were called inside and outside of coding regions with a conservative frequency filter of 0.90. Variant maximum P-values were set at $10^{-6}$ and a minimum strand-bias P-value of $10^{-5}$ was also used. Since we were interested in gene mutations only in 5B responsible for resistance to the inhibitory agent we filtered for unique SNPs by removing redundant SNPs that occurred in $> 1$ evolved lines. This gave us a set of candidate genes to then conduct genetic analyses, allowing us to confirm a causative gene for the sensitivity phenotype. In cases where genes appeared to have higher rates of variance, we pruned our SNP data by removing variant calls in high variance regions with less than 30x coverage.

**Synteny Plots**

SynMap is a web-based software found at genomeevolution.org used to build synteny plots of sequence data(22). We used SynMap2 with the LAST algorithm and default parameters to compare the sequences of ancestral pMPPla107 and pMPPla107-4B(22). DAGChainer Options were: nucleotide distance, -D = 20, and -A = 5. Tandem duplication distance was set to 10 and the C-score was set to 0.

**Inhibitory Agent Sensitivity Test**

We followed previous protocols to test the inhibitory agent against the six evolved lines, as described elsewhere(9, 14). Briefly, overlays were prepared by mixing cells
grown for four hours with 0.4% molten agar and plated on to KB plates. Overlays were allowed to solidify for approximately 15 min. The inhibitory agent was collected by growing P. stutzeri for 24-48 hrs, centrifuging cells at 10,000 × g for 5 min, and sterilizing supernatants through a 0.22µm filter. After sterilization 10µL of supernatants were spotted onto the overlay plate and allowed to dry. Overlay plates were grown at 27°C for approximately 24 hrs at which point, zones of inhibition were observed.

Conjugation of Evolved Megaplasmids into Ancestral P. stutzeri

Ancestral P. stutzeri (DBL386) was used with either evolved lines 4B or 5B to conduct a biparental conjugation by mixing 1:1 mixture of overnight cultures. Mixed cells were centrifuged at 3000 × g for 3 min and supernatants were removed without disturbing the pellet. Pellets were washed and resuspended in 1 mL of 10 mM MgCl₂. Centrifugation and washing steps were repeated once more. 10µL and 100µL of resuspended cells were spread and for 24-48 hr at 27°C on KB plates with rifampicin (50 ng/µL) and tetracycline (10 ng/µL). Resistant colonies underwent diagnostic PCR for presence of pMPPla107 using primers from Baltrus et al. 2011(23).

Gene function prediction with Phyre2 and blastx

We attempted to predict functional characteristics of skaA using the Phyre2 web server. The amino acid for skaA was used as input and the intensive setting was selected(24). We also used the nucleotide sequence of skaA as input into the NCBI
non-redundant protein sequences BLAST database using blastx using the BLOSUM62 matrix, expect threshold = 10, word size = 6, and max target sequences = 100. (date of search last search: January 21st 2019)(25).

RESULTS

Genome Sequencing Reveals 2 of 6 Evolved Lines Gain Resistance to a Previously Described Inhibitory Agent

Given our interest in a phenotype involving sensitization of Pseudomonas strains to an unknown inhibitory agent after acquisition of pMPPla107(9), we screened for the presence of inhibition in these evolved lines. Single colony isolates from two out of six lines (referred to from here on as DBL408-4BGen500 and DBL408-5BGen500) revert to the non-pMPPla107 phenotype and demonstrate resistance to this inhibitory agent (Figure 1).

In previous evolutionary studies focusing on plasmids, the burden of plasmid acquisition resulted in compensatory mutations present on host chromosomes(15, 16, 26, 27). To identify where the resistance mutations occurred, we analyzed the genomes of six laboratory passage strains of P. stutzeri after 500 generations under conditions that selected for maintenance of megaplasmid pMPPla107. Sequencing of the chromosome and pMPPla107 revealed variants across all six evolved lines (Tables 1 and 2 DOIs: doi.org/10.6084/m9.figshare.7393415 and doi.org/10.6084/m9.figshare.7393493 respectively), the majority of which (209/219) occur on the chromosome. All six evolved P. stutzeri lines also have a SNP on pMPPla107 at 731,508bp in qseF indicating this was a mutation either
occurred prior to the start of the evolutionary experiment or is a sequencing error in the reference sequence.

We found several variants to be unique in line 5B when compared to the remaining five evolved lines after 500 generations of evolution had occurred. Therefore, we were able to back track through frozen stocks to test generations 100, 200, 300, 400, and 500 of line 5B and determined that the transition from sensitivity to resistance of the inhibitory agent occurs between generations 300 and 400 (Figure 2). Sequencing of the populations at generations 300 and 400 then allowed us to narrow the scope of candidate SNPs occurring between these time points.

**Conjugation of pMPPla107 from Lines 4B and 5B Results in Resistance to the Inhibitory Agent**

The large deletion found in the 4B megaplasmid could alter how the plasmid interacts with its host in a variety of ways including the inhibitory phenotype. Therefore, we hypothesized conjugation of the evolved megaplasmids would transfer resistance to an ancestral strain. Conjugation of evolved pMPPla107 from lines 4B and 5B into a *P. stutzeri* strain containing the ancestral chromosome resulted in resistance to the inhibitory agent while conjugation of ancestral pMPPla107 resulted in sensitivity (Figure 3). Furthermore conjugation of the 5B evolved megaplasmid into *P. syringae* also caused resistance to the inhibitory agent (Supplemental Figure 1). Together these data not only suggest mutations found on pMPPla107 can transfer resistance of the inhibitory agent between *Pseudomonas*
spp., but that the underlying mechanism for sensitivity and resistance is shared by Pseudomonads.

**A 368kb Deletion Occurs in pMPPla107 Evolved Line 4B**

Analysis of the genome from isolate DBL408-4BGen500 had the lowest number of variants (20) occurring on the chromosome, while a large deletion of approximately 368kb occurred within pMPPla107 between 131-499kb (Figure 4). This deletion region includes 440 predicted genes without any known homologue and 27 genes with predicted functions (Table 3). Interestingly, there are no repetitive or overlapping sites at the ends of the deletion site suggesting it was not a single deletion event that occurred (https://genomevolution.org/r/uboj). Analysis of previous generations that gave rise to this line against the inhibitory agent indicated that this mutation occurred within the first colony selected for 4B (generation zero) indicating rapid evolutionary changes to pMPPla107 (Figure 5). Although these results indicate that the deletion in line 4B is responsible for resistance to the inhibitory agent, the large size of the deletion and the density of genes within this region make it difficult to discern which gene(s) are responsible for the resistance phenotype in this region.

**A SNP in Line 5B pMPPla107 Causes Resistance to the Inhibitory Agent**

Our results indicated that conjugation of pMPPla107 from the evolved 5B line does transfer resistance against the inhibitory agent. Therefore, the variant again, occurs on pMPPla107 (Figure 3). Additionally stated above, we were able to back track
through generations of frozen 5B isolates and identified that the resistance phenotype switches from sensitive to resistant between generations 300 and 400 (Figure 2). When comparing SNPs from all six megaplasmids the only unique SNP occurring on pMPPla107 from line 5B between generations 300 and 400 is at 57,137bp and causes a non-synonymous mutation changing a glutamate to a lysine (395 E>K) in an uncharacterized protein. Furthermore, Sanger sequencing of line 5B pMPPla107 in its evolved strain and conjugated to the ancestral P. stutzeri strain both confirm the presence of the SNP (DOI: doi.org/10.6084/m9.figshare.7268531). These data suggest that this SNP eliminates the sensitivity phenotype seen by strains that have acquired pMPPla107, thus we name this gene skaA for Supernatant Killing Activity. Given that the 5B pMPPla107 SNP occurs outside the deletion region found in the 4B megaplasmid, we also confirm that two separate compensatory strategies exist within pMPPla107 that cause resistance to the inhibitory agent.

**DISCUSSION**

We used experimental evolution to identify mutations that are associated with compensation to a unique cost associated with acquisition of megaplasmid pMPPla107. Strains of *P. stutzeri* containing pMPPla107 are sensitized to the presence of a currently unidentified inhibitory agent produced by a variety of Pseudomonas strains under normal growth conditions, and isolates from two of six experimental lines evolve resistance to this inhibition after approximately 500 generations of passage. Numerous studies have found that compensatory mutations
to plasmid carriage often occur on the chromosome, but we found that both mutations providing resistance (in lines 4B-500 and 5B-400/5B-500) occur on the megaplasmid. (15-17, 19).

Sequencing of line 4B-500 demonstrated that this line contains a 368kb deletion. This deletion occurs within the same genomic loci of a previously described region of high sequence dissimilarity between the two related plasmids pMPPla107 and pBASL58(5). This suggests a potential cargo region where genes may experience higher mutation and recombination rates resulting in genes that are expendable and provide benefits in certain environments rather than necessary genes for maintenance or transmission. Some of the genes found within this region include efflux pumps, antitoxins, and multidrug resistance proteins all of which may cause resistance to the inhibitory agent (Table 3). It is unclear which of the hundreds of genes in this region is responsible for increased sensitivity to the pseudomonas inhibitory agent, but we identify a specific region, responsible for the sensitivity phenotype.

Conjugating the evolved 5B megaplasmid into an ancestral P. stutzeri strain and P. syringae demonstrated resistance to the inhibitory agent indicating that the SNP present on pMPPla107-5B was the compensatory mutation and can be transferred across Pseudomonas spp. It is still unclear how skaA interacts with inhibitory agent or how the 395 E>K SNP changes these interactions. Protein structure and amino acid alignments using Phyre2 and blastx with the NCBI database provided results
with low confidence when attempting to identify a function for skaA (data not shown)(24, 25).

By combining comparative genomics, microbial genetics and evolutionary methodologies we identified two genetic causes for pMPPla107’s ability to sensitize recipients to a commonly produced inhibitory agent(14). Mutations occurring on the megaplasmid of separately evolved lines indicate that acquisition of pMPPla107 may create conflicts in pseudomonas cellular networks causing a once nontoxic molecule to result in toxicity, but that these mutations alleviate damaged networks. We identify a region on pMPPla107 and a SNP in the gene we now call skaA that are responsible for resistance to the pseudomonas inhibitory agent. Our data presented here is the framework on which to begin future work identifying the mechanism behind skaA and designing directed deletions within the 4B deletion that will be critical to identifying the other component regarding the inhibitory agent sensitivity phenotype associated with acquisition of pMPPla107.
Figures:

![Evolved Overlay Strain](image)

**Figure 1: Testing inhibitory agent on all six evolved lines reveals two resistant lines.** Six lines carrying pMPPla107 were evolved for 500 generations and tested for sensitivity against inhibitory agent found in *Pseudomonas spp.* supernatants. Lines 4B and 5B revert to a non-pMPPla107 phenotype where a zone of inhibition is not present indicating resistance to the inhibitory agent. All overlays were plated after 4 hours of growth in KB and spotted with 10µL of *P. stutzeri* filter sterilized supernatants. The number (1, 2, 3...) indicates the individual lines and B indicates the second of two isolates taken at Generation 500. All images are representative of three biological replicates.
Figure 2: The switch from sensitivity to resistance occurs between generations 300 and 400 in line 5B. We tested generations frozen at various time points of the evolution experiment and found that between generations 300 and 400 line 5B regains resistance to the inhibitory agent produced by *Pseudomonas* spp. A zone of inhibition can be seen when treated with IA (inhibitory agent) at generation 300, and this zone is no longer present at generation 400. Likewise, the negative control (KB media) demonstrates no zones of inhibition as expected. All overlays were plated after 4 hours of growth in KB and spotted with 10µL of *P. stutzeri* filter sterilized supernatants. All images are represented of three biological replicates.
Figure 3: Conjugating evolved pMPPla107 from Lines 4 and 5 into ancestral chromosomal background transfers resistance to the inhibitory agent. Given that Lines 4 and 5 were known to have resistance against the inhibitory agent and chromosomal SNPs did not appear to have any effect we conjugated evolved pMPPla107 into the ancestral chromosomal background (*P. stutzeri* DBL386). We conjugated and used the 3B megaplasmid as a sensitive (positive) control as we knew this evolved strain was still sensitive. When evolved megaplasmids were conjugated into the ancestral background 3B demonstrated sensitivity to the inhibitory agent as expected while 4B and 5B showed resistance. EV = evolved for 500 generations, pMP = pMPPla107. All overlays were plated after 4 hours of growth n KB and spotted with 10µL of *P. stutzeri* filter sterilized supernatants. All images are represented of three biological replicates.
Figure 4: A 368kb occurs in the evolved line 4B megaplasmid. SynMap dotplot visualizes the large deletion occurring from 131-499kb in the evolved 4B pMPPla107 as a large shift across the x-axis. The remaining portions of the sequences maintain perfect synteny indicating a clean deletion occurred. The x-axis is ancestral pMPPla107 gene order where $x_{1...N} = gene_{1...N}$ and the y-axis is the line 4B evolved pMPPla107 gene order where $y_{1...N} = gene_{1...N}$. 
Figure 5: The large deletion in line 4B occurred within the first passage of *P. stutzeri* with pMPPla107. To determine when the deletion occurred we tested frozen generations for sensitivity to the inhibitory agent and found that the deletion was present in the first passage of the evolved line at generation zero. This deletion is maintained in generation 100 (shown) through generation 500 and is the only unique mutation in pMPPla107 other than a synonymous SNP (See Table 2). All overlays were plated after 4 hours of growth n KB and spotted with 10µL of *P. stutzeri* filter sterilized supernatants. All images are represented of three biological replicates.
Table 1: Variants present in the chromosomes of *P. stutzeri* evolved lines 1-6 after 500 generations of evolution. The table can be found at Figshare (DOI: doi.org/10.6084/m9.figshare.7393415)
Table 2: Variants present on pMPPla107 in *P stutzeri* evolved lines 1-6 after 500 generations of evolution. The table can be found on Figshare (DOI: doi.org/10.6084/m9.figshare.7393493)
| Name  | Type | Minimum | Maximum | Length | Direction |
|-------|------|---------|---------|--------|-----------|
| CDS   | CDS  | 130243  | 131439  | 1197   | reverse   |
| CDS   | CDS  | 131589  | 132254  | 666    | reverse   |
| CDS   | CDS  | 132320  | 132703  | 384    | reverse   |
| CDS   | CDS  | 133286  | 133642  | 357    | reverse   |
| CDS   | CDS  | 133741  | 135102  | 1362   | reverse   |
| CDS   | CDS  | 135202  | 136674  | 1473   | reverse   |
| CDS   | CDS  | 136674  | 137732  | 1059   | reverse   |
| CDS   | CDS  | 137832  | 138527  | 696    | reverse   |
| CDS   | CDS  | 138541  | 138840  | 300    | reverse   |
| CDS   | CDS  | 139401  | 139859  | 459    | reverse   |
| dsbA  | CDS  | 139852  | 140547  | 696    | reverse   |
| CDS   | CDS  | 140571  | 141056  | 486    | reverse   |
| CDS   | CDS  | 141346  | 141708  | 363    | forward   |
| CDS   | CDS  | 141698  | 142231  | 534    | forward   |
| nrbB  | CDS  | 142392  | 143558  | 1167   | forward   |
| CDS   | CDS  | 143616  | 144122  | 507    | reverse   |
| CDS   | CDS  | 144218  | 145108  | 891    | reverse   |
| CDS   | CDS  | 145159  | 145689  | 531    | reverse   |
| CDS   | CDS  | 145837  | 146367  | 531    | reverse   |
| CDS   | CDS  | 146497  | 146670  | 174    | reverse   |
| CDS   | CDS  | 146798  | 147337  | 540    | reverse   |
| CDS   | CDS  | 147420  | 147860  | 441    | forward   |
| CDS   | CDS  | 147871  | 148110  | 240    | forward   |
| CDS   | CDS  | 148117  | 148422  | 306    | forward   |
| CDS   | CDS  | 148444  | 148680  | 237    | forward   |
| CDS   | CDS  | 148682  | 148918  | 237    | forward   |
| dns   | CDS  | 149053  | 149961  | 909    | forward   |
| rtcB  | CDS  | 149987  | 151210  | 1224   | forward   |
| CDS   | CDS  | 151295  | 151477  | 183    | forward   |
| CDS   | CDS  | 151474  | 151821  | 348    | forward   |
| CDS   | CDS  | 151811  | 152374  | 564    | forward   |
| CDS   | CDS  | 152401  | 152748  | 348    | reverse   |
| CDS   | CDS  | 152996  | 153736  | 741    | forward   |
| CDS   | CDS  | 153778  | 154545  | 768    | reverse   |
| CDS   | CDS  | 154820  | 155503  | 684    | forward   |
| CDS   | CDS  | 155663  | 156148  | 486    | forward   |
| CDS   | CDS  | 156227  | 156604  | 378    | forward   |
| CDS   | CDS  | 156830  | 157216  | 387    | forward   |
| CDS   | CDS  | 157295  | 157480  | 186    | forward   |
| CDS   | CDS  | 157702  | 158103  | 402    | forward   |
| CDS   | CDS  | 158353  | 158742  | 390    | forward   |
| CDS   | CDS  | 158832  | 159416  | 585    | forward   |
| CDS | CDS | Start Base | End Base | Length | Orientation |
|-----|-----|------------|----------|--------|-------------|
|    |    | 159735     | 159986   | 252    | forward     |
|    |    | 160023     | 160295   | 273    | forward     |
|    |    | 160601     | 160750   | 150    | forward     |
|    |    | 160868     | 161353   | 486    | forward     |
|    |    | 161350     | 161484   | 135    | forward     |
|    |    | 161484     | 162068   | 585    | forward     |
|    |    | 162068     | 162529   | 462    | forward     |
|    |    | 162687     | 162827   | 141    | forward     |
| tRNA | tRNA | 162927     | 163003   | 77     | forward     |
|    |    | 163027     | 163431   | 405    | forward     |
|    |    | 163574     | 164611   | 1038   | forward     |
|    |    | 164684     | 165424   | 741    | forward     |
|    |    | 165504     | 165890   | 387    | reverse     |
|    |    | 166010     | 166447   | 438    | forward     |
|    |    | 166715     | 168022   | 1308   | reverse     |
|    |    | 168296     | 169561   | 1266   | forward     |
|    |    | 169572     | 170141   | 570    | reverse     |
|    |    | 170289     | 173108   | 2820   | forward     |
|    |    | 173419     | 174288   | 870    | forward     |
|    |    | 174362     | 175588   | 1227   | reverse     |
|    |    | 175620     | 176885   | 1266   | reverse     |
|    |    | 176958     | 178229   | 1272   | reverse     |
|    |    | 178291     | 179547   | 1257   | reverse     |
|    |    | 179644     | 180141   | 498    | reverse     |
|    |    | 180131     | 180466   | 336    | reverse     |
|    |    | 180470     | 182548   | 2079   | reverse     |
|    |    | 182578     | 182919   | 342    | reverse     |
|    |    | 183043     | 183255   | 213    | reverse     |
|    |    | 183361     | 184566   | 1206   | reverse     |
|    |    | 184737     | 185102   | 366    | forward     |
|    |    | 185106     | 185834   | 729    | forward     |
|    |    | 185861     | 186391   | 531    | reverse     |
|    |    | 186505     | 186951   | 447    | reverse     |
|    |    | 186941     | 188131   | 1191   | reverse     |
|    |    | 188251     | 189444   | 1194   | reverse     |
|    |    | 189528     | 190739   | 1212   | reverse     |
|    |    | 190978     | 192180   | 1203   | forward     |
|    |    | 192219     | 193448   | 1230   | reverse     |
|    |    | 193616     | 194422   | 807    | reverse     |
|    |    | 194642     | 195829   | 1188   | reverse     |
|    |    | 195911     | 197113   | 1203   | reverse     |
|    |    | 197140     | 198348   | 1209   | reverse     |
|    |    | 198506     | 199702   | 1197   | reverse     |

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| CDS | CDS | Start | End   | Length | Direction |
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| 200975 | 202225 | 1251   | reverse |
| 202247 | 203494 | 1248   | reverse |
| 203965 | 205173 | 1209   | reverse |
| 205262 | 208562 | 1230   | forward |
| 206350 | 207243 | 894    | forward |
| 207366 | 208565 | 1200   | forward |
| 208562 | 209887 | 1326   | forward |
| 209962 | 211191 | 1230   | forward |
| 211264 | 212496 | 1233   | forward |
| 212569 | 213807 | 1239   | forward |
| 213980 | 215764 | 780    | reverse |
| 214222 | 216527 | 306    | reverse |
| 216789 | 219734 | 2946   | forward |
| 220194 | 221039 | 846    | reverse |
| 221363 | 222097 | 735    | reverse |
| 222422 | 223198 | 777    | reverse |
| 223316 | 224041 | 726    | reverse |
| 224048 | 224287 | 240    | reverse |
| 224484 | 224738 | 255    | reverse |
| 224754 | 225293 | 540    | reverse |
| 225408 | 226187 | 780    | reverse |
| 226273 | 226719 | 447    | reverse |
| 226824 | 227030 | 207    | forward |
| 227208 | 227495 | 288    | reverse |
| 227502 | 227957 | 456    | reverse |
| 227981 | 228340 | 360    | reverse |
| 228385 | 228606 | 222    | reverse |
| 228614 | 228805 | 192    | reverse |
| 228820 | 229128 | 309    | reverse |
| 229118 | 229327 | 210    | reverse |
| 229346 | 229918 | 573    | reverse |
| 229915 | 230247 | 333    | reverse |
| 230244 | 230681 | 438    | reverse |
| 230819 | 231289 | 471    | reverse |
| 231356 | 231796 | 441    | reverse |
| 231838 | 232377 | 540    | reverse |
| 232488 | 232922 | 435    | reverse |
| 232919 | 233605 | 687    | reverse |
| 233671 | 233949 | 279    | reverse |
| 234029 | 234247 | 219    | reverse |
| CDS | CDS | Start  | End   | Length | Orientation |
|-----|-----|--------|-------|--------|-------------|
| CDS | CDS | 234244 | 234498| 255    | reverse     |
| CDS | CDS | 234495 | 234719| 225    | reverse     |
| CDS | CDS | 234716 | 235495| 780    | reverse     |
| CDS | CDS | 235553 | 235843| 291    | reverse     |
| CDS | CDS | 235866 | 236060| 195    | reverse     |
| CDS | CDS | 236063 | 236779| 717    | reverse     |
| CDS | CDS | 236783 | 237502| 720    | reverse     |
| CDS | CDS | 237755 | 238744| 990    | reverse     |
| CDS | CDS | 238923 | 239786| 864    | forward     |
| CDS | CDS | 239898 | 240428| 531    | forward     |
| CDS | CDS | 240512 | 241015| 504    | forward     |
| CDS | CDS | 241031 | 241657| 627    | reverse     |
| CDS | CDS | 241737 | 242537| 801    | reverse     |
| CDS | CDS | 242947 | 243948| 1011   | forward     |
| CDS | CDS | 244514 | 246097| 1584   | forward     |
| CDS | CDS | 246097 | 246201| 105    | forward     |
| CDS | CDS | 246164 | 247747| 1584   | forward     |
| CDS | CDS | 247758 | 248222| 465    | forward     |
| CDS | CDS | 248409 | 249053| 645    | forward     |
| CDS | CDS | 249099 | 250118| 1020   | reverse     |
| CDS | CDS | 250312 | 251355| 1044   | forward     |
| CDS | CDS | 251412 | 251744| 333    | forward     |
| CDS | CDS | 251806 | 253131| 1326   | reverse     |
| CDS | CDS | 253201 | 254529| 1329   | reverse     |
| CDS | CDS | 254601 | 255965| 1365   | reverse     |
| CDS | CDS | 256050 | 257399| 1350   | reverse     |
| CDS | CDS | 257533 | 257742| 210    | forward     |
| CDS | CDS | 257838 | 259172| 1335   | reverse     |
| CDS | CDS | 259333 | 260640| 1308   | forward     |
| CDS | CDS | 260830 | 262212| 1383   | reverse     |
| CDS | CDS | 262325 | 263539| 1215   | reverse     |
| CDS | CDS | 263593 | 263838| 246    | reverse     |
| CDS | CDS | 263835 | 264032| 198    | reverse     |
| CDS | CDS | 264044 | 264280| 237    | reverse     |
| CDS | CDS | 264289 | 264612| 324    | reverse     |
| CDS | CDS | 264622 | 264993| 372    | reverse     |
| CDS | CDS | 265033 | 265557| 525    | reverse     |
| CDS | CDS | 265587 | 266216| 630    | reverse     |
| CDS | CDS | 266719 | 267924| 1206   | reverse     |
| CDS | CDS | 267953 | 269122| 1170   | reverse     |
|   |   |   |   |   |
|---|---|---|---|---|
| CDS | CDS | 269304 | 269519 | 216 forward |
| CDS | CDS | 269566 | 270537 | 972 forward |
| CDS | CDS | 270716 | 272461 | 1746 forward |
| CDS | CDS | 272554 | 273768 | 1215 forward |
| fabH | CDS | 273913 | 275001 | 1089 forward |
| CDS | CDS | 274998 | 275414 | 417 forward |
| CDS | CDS | 275411 | 276382 | 972 forward |
| CDS | CDS | 276375 | 277217 | 843 forward |
| CDS | CDS | 277214 | 277798 | 585 forward |
| CDS | CDS | 277813 | 278277 | 465 forward |
| CDS | CDS | 278308 | 279537 | 1230 reverse |
| CDS | CDS | 279617 | 280825 | 1209 reverse |
| CDS | CDS | 280890 | 282149 | 1260 reverse |
| tRNA | tRNA | 282629 | 282713 | 85 forward |
| CDS | CDS | 282798 | 283166 | 369 forward |
| CDS | CDS | 283196 | 283462 | 267 forward |
| CDS | CDS | 283525 | 283722 | 198 forward |
| CDS | CDS | 283753 | 284139 | 387 forward |
| hipB | CDS | 284257 | 284511 | 255 forward |
| CDS | CDS | 284528 | 284839 | 312 forward |
| CDS | CDS | 284869 | 285198 | 330 forward |
| CDS | CDS | 285376 | 285927 | 552 forward |
| CDS | CDS | 285988 | 286584 | 597 forward |
| CDS | CDS | 286682 | 287764 | 1083 reverse |
| CDS | CDS | 287876 | 288199 | 324 forward |
| CDS | CDS | 288202 | 288801 | 600 forward |
| CDS | CDS | 288804 | 289397 | 594 forward |
| CDS | CDS | 289430 | 290050 | 621 forward |
| CDS | CDS | 290088 | 290642 | 555 forward |
| CDS | CDS | 290688 | 291260 | 573 forward |
| CDS | CDS | 291332 | 291718 | 387 reverse |
| CDS | CDS | 291843 | 292373 | 531 reverse |
| CDS | CDS | 292503 | 293810 | 1308 forward |
| CDS | CDS | 293862 | 294389 | 528 forward |
| CDS | CDS | 294542 | 294733 | 192 forward |
| CDS | CDS | 294730 | 296100 | 1371 forward |
| CDS | CDS | 296093 | 296755 | 663 forward |
| CDS | CDS | 296755 | 297102 | 348 forward |
| CDS | CDS | 297102 | 297524 | 423 forward |
| CDS | CDS | 297519 | 298157 | 639 reverse |
| CDS | CDS | 298190 | 298504 | 315 reverse |
| CDS | CDS | 298616 | 299653 | 1038 reverse |
| CDS | CDS | 299946 | 300050 | 105 forward |
CDS  CDS  300025  300390  366  reverse
CDS  CDS  300539  300877  339  forward
CDS  CDS  300971  302272  1302  reverse
CDS  CDS  302307  303518  1212  reverse
CDS  CDS  303520  304737  1218  reverse
CDS  CDS  304934  305476  543  forward
CDS  CDS  305517  306764  1248  reverse
CDS  CDS  306899  307255  357  forward
CDS  CDS  307342  308259  918  forward
CDS  CDS  308441  309052  615  forward
CDS  CDS  309062  309592  531  reverse
CDS  CDS  310460  311263  804  reverse
CDS  CDS  311319  312125  807  reverse
ttgC  CDS  312173  313537  1365  reverse
CDS  CDS  313760  315067  1308  forward
CDS  CDS  315094  315534  441  forward
CDS  CDS  315513  316037  525  reverse
CDS  CDS  316058  316573  516  reverse
CDS  CDS  316826  317344  519  reverse
CDS  CDS  317492  317845  354  reverse
CDS  CDS  317925  318233  309  forward
CDS  CDS  318252  318563  312  forward
CDS  CDS  318556  319140  585  forward
CDS  CDS  319219  319530  312  forward
CDS  CDS  319798  320346  549  forward
CDS  CDS  320454  320834  381  forward
CDS  CDS  320886  321659  774  reverse
CDS  CDS  321954  322724  771  forward
CDS  CDS  322699  322950  252  reverse
CDS  CDS  323096  323401  306  forward
CDS  CDS  323438  324460  1023  reverse
CDS  CDS  324614  324892  279  forward
CDS  CDS  324955  325800  846  forward
CDS  CDS  325846  326271  426  reverse
CDS  CDS  326393  326689  297  reverse
CDS  CDS  326884  327282  399  forward
CDS  CDS  327279  327638  360  forward
CDS  CDS  327715  328134  420  forward
parE  CDS  328197  330101  1905  forward
CDS  CDS  330098  330379  282  forward
CDS  CDS  330387  330545  159  forward
CDS  CDS  330591  330887  297  forward
| Gene | Start Position | End Position | Length | Strand |
|------|----------------|--------------|--------|--------|
| parC | 330900         | 333152       | 2253   | forward |
| CDS  | 333202         | 333522       | 321    | forward |
| CDS  | 333561         | 333896       | 336    | forward |
| CDS  | 333924         | 334235       | 312    | reverse |
| CDS  | 334362         | 334547       | 186    | reverse |
| CDS  | 334761         | 335630       | 870    | reverse |
| CDS  | 335703         | 335954       | 252    | forward |
| CDS  | 335955         | 336419       | 465    | reverse |
| CDS  | 336567         | 337994       | 1428   | forward |
| CDS  | 338158         | 339000       | 843    | forward |
| CDS  | 339011         | 340855       | 1845   | forward |
| CDS  | 340867         | 342075       | 1209   | forward |
| CDS  | 342159         | 342839       | 681    | reverse |
| CDS  | 342895         | 343218       | 324    | reverse |
| CDS  | 343582         | 343863       | 282    | reverse |
| CDS  | 344110         | 344715       | 606    | forward |
| CDS  | 345001         | 345387       | 387    | reverse |
| CDS  | 345416         | 345796       | 381    | reverse |
| CDS  | 345903         | 346604       | 702    | reverse |
| CDS  | 346793         | 346963       | 171    | forward |
| CDS  | 347025         | 347645       | 621    | forward |
| CDS  | 347645         | 348913       | 1269   | forward |
| CDS  | 348994         | 350577       | 1584   | forward |
| CDS  | 350632         | 351204       | 573    | reverse |
| CDS  | 351207         | 351830       | 624    | reverse |
| CDS  | 351827         | 352141       | 315    | reverse |
| CDS  | 352270         | 353301       | 1032   | forward |
| CDS  | 353360         | 353944       | 585    | reverse |
| CDS  | 354123         | 354566       | 444    | reverse |
| CDS  | 354607         | 355089       | 483    | reverse |
| CDS  | 355140         | 355478       | 339    | reverse |
| CDS  | 355541         | 356005       | 465    | forward |
| CDS  | 356031         | 356447       | 417    | reverse |
| CDS  | 356685         | 356936       | 252    | reverse |
| CDS  | 357007         | 358215       | 1209   | reverse |
| CDS  | 358704         | 359468       | 765    | forward |
| CDS  | 359452         | 359700       | 249    | forward |
| CDS  | 359702         | 359953       | 252    | forward |
| CDS  | 359957         | 360364       | 408    | forward |
| CDS  | 360361         | 361239       | 879    | forward |
| CDS  | 361244         | 361747       | 504    | reverse |
| CDS  | 361737         | 362117       | 381    | reverse |
| CDS  | 362148         | 362543       | 396    | reverse |
| Start Base | End Base | Length | Orientation |
|------------|----------|--------|-------------|
| 362530     | 362769   | 240    | reverse     |
| 362766     | 363083   | 318    | reverse     |
| 363092     | 363286   | 195    | reverse     |
| 363280     | 363792   | 513    | reverse     |
| 363802     | 364878   | 1077   | reverse     |
| 364912     | 365415   | 504    | reverse     |
| 365430     | 365963   | 534    | reverse     |
| 366029     | 366613   | 585    | reverse     |
| 366942     | 367082   | 141    | reverse     |
| 367082     | 367516   | 435    | reverse     |
| 367569     | 368159   | 591    | reverse     |
| 368277     | 368594   | 318    | reverse     |
| 368728     | 370662   | 1935   | reverse     |
| 370844     | 371395   | 552    | reverse     |
| 371699     | 372124   | 426    | forward     |
| 372348     | 372581   | 234    | forward     |
| 373295     | 374560   | 1266   | reverse     |
| 375046     | 375759   | 714    | forward     |
| 375872     | 376132   | 261    | forward     |
| 376242     | 376409   | 168    | reverse     |
| 376520     | 377086   | 567    | forward     |
| 377009     | 377185   | 177    | reverse     |
| 377210     | 377620   | 411    | forward     |
| 377721     | 378359   | 639    | forward     |
| 378522     | 379406   | 885    | forward     |
| 379865     | 380323   | 459    | reverse     |
| 380409     | 380891   | 483    | reverse     |
| 380954     | 381412   | 459    | reverse     |
| 381565     | 382674   | 1110   | forward     |
| 382748     | 383311   | 564    | reverse     |
| 383456     | 384043   | 588    | forward     |
| 384058     | 384492   | 435    | forward     |
| 384507     | 385454   | 948    | reverse     |
| 385954     | 386391   | 438    | reverse     |
| 386541     | 386786   | 246    | reverse     |
| 386789     | 387235   | 447    | reverse     |
| 387252     | 387701   | 450    | reverse     |
| 387705     | 388100   | 396    | reverse     |
| 388110     | 388442   | 333    | reverse     |
| 388439     | 388642   | 204    | reverse     |
| 388639     | 389058   | 420    | reverse     |
| 389240     | 389596   | 357    | reverse     |
| 389698     | 391275   | 1578   | reverse     |
CDS CDS 391362 392561 1200 reverse
CDS CDS 392612 393799 1188 reverse
CDS CDS 393871 394482 612 reverse
CDS CDS 394486 395811 1326 reverse
CDS CDS 395901 396041 141 reverse
CDS CDS 396079 396471 393 reverse
CDS CDS 396619 397377 759 reverse
CDS CDS 397  398066 288 forward
CDS CDS 398102 399460 1359 reverse
CDS CDS 399457 400764 1308 reverse
CDS CDS 400767 402110 1344 reverse
CDS CDS 402241 403656 1410 forward
CDS CDS 403681 405477 1797 forward
CDS CDS 405552 406880 1329 reverse
CDS CDS 406959 407210 252 reverse
CDS CDS 407207 408373 1167 reverse
CDS CDS 408576 409007 432 forward
CDS CDS 409428 410651 1224 forward
CDS CDS 410907 411344 438 reverse
CDS CDS 411439 412797 1359 reverse
CDS CDS 412763 414067 1305 reverse
CDS CDS 414281 415690 1410 forward
CDS CDS 415714 416247 534 forward
CDS CDS 416959 417165 207 forward
CDS CDS 417162 418361 1200 reverse
CDS CDS 418358 419710 1353 reverse
CDS CDS 419712 420980 1269 reverse
CDS CDS 421156 421947 792 forward
CDS CDS 422090 422686 597 reverse
CDS CDS 422894 423568 675 reverse
CDS CDS 423568 423894 327 reverse
CDS CDS 424029 424790 762 forward
CDS CDS 424874 425152 279 forward
CDS CDS 425300 425968 669 reverse
CDS CDS 425965 426633 669 reverse
CDS CDS 426630 427856 1227 reverse
CDS CDS 427921 429219 1299 reverse
CDS CDS 429273 429872 600 reverse
CDS CDS 429883 430479 597 reverse
CDS CDS 430549 431145 597 reverse
CDS CDS 431223 432578 1356 reverse
CDS CDS 432807 433238 432 forward
CDS CDS 433226 434356 1131 reverse
| Gene | CDS | Start | End   | Length | Orientation |
|------|-----|-------|-------|--------|-------------|
| CDS  | CDS | 434460| 435155| 696    | forward     |
| CDS  | CDS | 435229| 435954| 726    | forward     |
| CDS  | CDS | 435941| 436195| 255    | forward     |
| CDS  | CDS | 436550| 437497| 948    | forward     |
| CDS  | CDS | 437619| 439064| 1446   | reverse     |
| CDS  | CDS | 439118| 440578| 1461   | reverse     |
| recX | CDS | 441216| 44169  | 5      | forward     |
| CDS  | CDS | 441729| 442802| 1074   | reverse     |
| CDS  | CDS | 442955| 443662| 708    | forward     |
| CDS  | CDS | 443674| 444744| 1071   | reverse     |
| gltA  | CDS | 444888| 446483| 1596   | reverse     |
| CDS  | CDS | 446762| 447022| 261    | forward     |
| CDS  | CDS | 447085| 447279| 195    | reverse     |
| CDS  | CDS | 447276| 447629| 354    | reverse     |
| CDS  | CDS | 447672| 448202| 531    | reverse     |
| CDS  | CDS | 448397| 448729| 333    | forward     |
| CDS  | CDS | 448809| 449360| 552    | reverse     |
| CDS  | CDS | 449427| 449975| 549    | reverse     |
| trpS  | CDS | 450046| 451407| 1362   | reverse     |
| CDS  | CDS | 451621| 452031| 411    | forward     |
| CDS  | CDS | 452134| 452475| 342    | forward     |
| CDS  | CDS | 452760| 456818| 4059   | forward     |
| CDS  | CDS | 456850| 457173| 324    | reverse     |
| CDS  | CDS | 457427| 457759| 333    | forward     |
| CDS  | CDS | 457888| 459423| 1536   | reverse     |
| CDS  | CDS | 459482| 459799| 318    | reverse     |
| CDS  | CDS | 459811| 460053| 243    | reverse     |
| CDS  | CDS | 460215| 461711| 1497   | reverse     |
| CDS  | CDS | 461746| 462339| 594    | reverse     |
| CDS  | CDS | 462339| 464006| 1668   | reverse     |
| CDS  | CDS | 464009| 464293| 285    | reverse     |
| CDS  | CDS | 464507| 464881| 375    | forward     |
| CDS  | CDS | 464896| 465156| 261    | reverse     |
| CDS  | CDS | 465185| 465322| 138    | forward     |
| CDS  | CDS | 465376| 466332| 957    | forward     |
| CDS  | CDS | 466405| 467592| 1188   | reverse     |
| CDS  | CDS | 467807| 468211| 405    | forward     |
| CDS  | CDS | 468304| 468615| 312    | reverse     |
| CDS  | CDS | 468612| 469550| 939    | reverse     |
| CDS  | CDS | 469659| 470201| 543    | reverse     |
| CDS  | CDS | 470211| 470642| 432    | reverse     |
| CDS  | CDS | 470705| 472204| 1500   | reverse     |
| Gene | CDS   | Start  | End    | Length | Orientation |
|------|-------|--------|--------|--------|-------------|
|    | CDS   | 472323 | 473720 | 1398   | reverse     |
|    | CDS   | 473955 | 474710 | 756    | reverse     |
|    | CDS   | 474886 | 475155 | 270    | reverse     |
| bepE | CDS   | 475218 | 478319 | 3102   | reverse     |
| mdtE | CDS   | 478326 | 479429 | 1104   | reverse     |
|    | CDS   | 479618 | 480385 | 768    | forward     |
|    | CDS   | 480389 | 480847 | 459    | forward     |
|    | CDS   | 480944 | 481756 | 813    | forward     |
|    | CDS   | 481847 | 482416 | 570    | forward     |
|    | CDS   | 482463 | 482978 | 516    | forward     |
|    | CDS   | 483040 | 483612 | 573    | forward     |
|    | CDS   | 483637 | 483996 | 360    | forward     |
|    | CDS   | 484103 | 484336 | 234    | forward     |
|    | CDS   | 484347 | 484649 | 303    | forward     |
|    | CDS   | 484638 | 485081 | 444    | reverse     |
|    | CDS   | 485175 | 486176 | 1002   | forward     |
|    | CDS   | 486196 | 486399 | 204    | reverse     |
|    | CDS   | 486670 | 486930 | 261    | reverse     |
| rdgB | CDS   | 487114 | 487686 | 573    | forward     |
|    | CDS   | 487834 | 488244 | 411    | forward     |
|    | CDS   | 488336 | 488689 | 354    | reverse     |
|    | CDS   | 488819 | 489085 | 267    | forward     |
| yedY | CDS   | 489082 | 489852 | 771    | forward     |
|    | CDS   | 490007 | 490567 | 561    | forward     |
|    | CDS   | 490713 | 492845 | 2133   | forward     |
|    | CDS   | 493011 | 493460 | 450    | forward     |
|    | CDS   | 493512 | 494291 | 780    | reverse     |
|    | CDS   | 494352 | 495422 | 1071   | reverse     |
|    | CDS   | 495743 | 496135 | 393    | forward     |
|    | CDS   | 496135 | 496548 | 414    | forward     |
|    | CDS   | 496671 | 497045 | 375    | forward     |
|    | CDS   | 497048 | 497662 | 615    | forward     |
|    | CDS   | 497693 | 498157 | 465    | forward     |
|    | CDS   | 498138 | 498380 | 243    | reverse     |
|    | CDS   | 498595 | 498849 | 255    | forward     |
|    | CDS   | 498981 | 499583 | 603    | forward     |
|    | CDS   | 499635 | 500537 | 903    | reverse     |

Table 3: Predicted genes present in the large deletion of pMPPla107 in line 4B.
Supplemental Figures

Overlay Strain

Supplemental Figure 1: Resistance to the inhibitory agent on the 5B megaplasmid can be transferred to *P. syringae*. Various types of pMPPla107 were conjugated to *P. syringae* Pla YM8003 and then tested for sensitivity to the inhibitory agent on a bacterial overlay. Overlays above are pMP = ancestral pMPPla107, 3B-pMP = evolved line 3B pMPPla107, and 4B-pMP = evolved line 5B pMPPla107. Ancestral and line 3B megaplasmids both transfer sensitivity while line 4B’s megaplasmid transfers resistance to the inhibitory agent. Clearing is less contrasted in overlays with *P. syringae* when compared with *P. stutzeri* overlays due to growth differences (pigment, density) between species. All overlays were plated after 4 hours of growth n KB and spotted with 10µL of *P. stutzeri* filter sterilized supernatants.
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