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The pea aphid as both host and vector for the phytopathogenic bacterium, *Pseudomonas syringae*

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Abstract

Aphids are widespread agricultural pests that are capable of disseminating plant viral diseases; but despite coming into frequent contact with epiphytic bacteria, aphids are considered as having no role in bacterial transmission. Here we demonstrate the ability of pea aphids to vector the phytopathogen *Pseudomonas syringae* pv. syringae B728a (PsyB728a). While feeding on plants colonized by epiphytic bacteria, aphids acquire the bacteria, which colonize the digestive tract, multiply and are excreted in the aphid honeydew, resulting in inoculation of the phyllosphere with up to 10^7 phytopathogenic bacteria per cm^2. Within days of ingesting bacteria, aphids succumb to bacterial sepsis, indicating that aphids serve as an alternative, non-plant host for PsyB728a. The related strain *Pseudomonas syringae* pv. tomato DC3000 is >1000-fold less virulent than PsyB728a in the pea aphid, suggesting that PsyB728a possesses strain-specific pathogenicity factors that allow it to exploit aphids as hosts. To identify these factors, we performed a mutagenesis screen and recovered PsyB728a mutants that were hypovirulent, including one defective in a gene required for flagellar formation and motility. These interactions illustrate that aphids can also vector bacterial pathogens and that even seemingly host-restricted pathogens can have alternative host specificities and lifestyles.

Introduction

Aphids are prolific insect pests that impact numerous agricultural crop plants (8). Although direct parasitization of their hosts can reduce plant biomass, crop quality, and crop yield, aphids are of particular importance for their role in the dissemination of plant disease (24, 30). Phytopathogenic viruses can be acquired by aphids as they probe infected plant hosts with their stylet (29, 30), with the subsequent movement of infected aphids to healthy plants contributing significantly to the dissemination of plant disease (29). Surprisingly, however, aphids are considered strictly vectors of phytopathogenic viruses, despite their frequent physical contact with a variety of bacterial epiphytes (10).

Upon landing on a potential host, the aphid begins to explore the plant, probing the plant tissues with its stylet, and puncturing through the epidermal layers to locate and access the phloem sieve tube elements to feed (3, 18, 24). As it traverses the plant surface, the aphid continually evaluates the suitability of the host, with some aphid species sampling plant fluids with droplets of saliva that are expelled and reingested (3). This saliva can contact the plant surface where many bacterial epiphytes reside, such as the ubiquitous phytopathogens, *Pantoea agglomerans* and *Pseudomonas syringae* (4, 5, 10, 21, 22, 32).

The pea aphid, *Acyrthosiphon pisum*, feeds on a variety of agriculturally relevant crop plants in the Fabaceae, including soybean (*Glycine max*), fava/broad bean (*Vicia faba*), pea (*Pisum sativum*) and snap bean (*Phaseolus vulgaris*) (15, 28, 34-36), and has been shown to vector bean yellow mosaic virus between these hosts (15, 34-36). Several of these plants are also primary hosts for the highly aggressive bean pathogen *Pseudomonas syringae* pv. syringae B728a (PsyB728a), which establishes epiphytically

and can invade the plant and cause disease (4, 5, 17). Although PsyB728a generally exhibits low survivorship on plant surfaces due to limitations of carbohydrate and water (23), it can form aggregates on plant surfaces, which can be further dispersed by rainsplash (16, 17, 26, 37). Ultimately, under optimal environmental conditions, high titres of bacteria increase the likelihood of plant disease (17).

In this study, we describe the vectoring of the phytopathogenic bacterium *P. syringae* by the pea aphid. We illustrate infection of the aphid by epiphytic populations of PsyB728a, which multiply in the aphid and are deposited onto the plant surface in high titre via the aphid honeydew. In addition, we provide an account of *P. syringae* infecting a non-plant host, and identify a PsyB728a mutant that is hypovirulent in *A. pisum*.

Materials and Methods

Aphid lines, bacterial strains and growth conditions

Isolates of *Pseudomonas syringae* were grown overnight with continuous shaking in 5 ml LB medium at 25°C. *Escherichia coli* DH5a and RK600 were grown in 5 ml LB medium at 37°C. When applicable, antibiotics were supplemented at the following concentrations: chloramphenicol (64 μg/ml), kanamycin (50 μg/ml), rifampicin (50 μg/ml), and ampicillin (150 μg/ml). The pea aphid, *Acyrthosiphon pisum* strain 5A, was maintained at 20°C on fava bean plants. For screening and growth assays, a modified aphid artificial diet containing essential amino acids, vitamins, and sucrose was used (11). Fava bean plants were grown by sowing fava bean seeds in 1:1 ratio of Sunshine Mix to vermiculite. Approximately 11-day-old seedlings were used in all assays.

Transposon mutagenesis and screening

Transposon mutagenesis of PsyB728a was performed via triparental mating, whereby a helper strain containing a self-transmissible plasmid assists in mobilizing a plasmid from a donor to the recipient strain. The mini-Tn5 vector pBSL118 in *E. coli* VPE42 (carrying kanamycin and ampicillin resistance) was used as donor, *E. coli* RK600 (chloramphenicol resistance) used as helper, with PsyB728a as the intended recipient. The three parental strains were combined in a 1:1:1 ratio, spotted on LB medium without antibiotics, and incubated overnight at 28°C-30°C. Bacterial lawns were resuspended in 10mM $MgSO_4$, and serial dilutions plated on LB medium containing rifampicin and kanamycin. Single transconjugants were dipped into each well of a 96-well microtitre plate that contained 350 µL of artificial aphid diet. Positive (wildtype PsyB728a) and negative (no bacteria) controls were included on each plate. Plates were sealed with parafilm, allowing the aphid diet in each chamber to contact the parafilm. These plates were each placed on top of a second plate that contained a single second or third aphid instar in each well, so that the wells aligned, allowing the aphids to feed on diet containing a single transconjugant. Plates were kept at ambient temperature and scored after 3-4 days. Transconjugants were recovered from those wells in which the aphid survived longer than the wildtype control. Positives were then confirmed to be *P. syringae* via both blue/white screening and PCR, and retested eight times. Approximately 1500 mutants were screened.

Mutated genes were identified by inverse PCR (31). Briefly, genomic DNA from each mutant was extracted using the Gentra Puregene Kit (Qiagen, California, USA) according to manufacturer's instructions. Between 1-2 µg of gDNA were digested using *HincII* and *EcoRI* in a 20 µl volume. After heat inactivation, 10 µl of each digest was

used in a unimolecular ligation, consisting of 15 units of T4 DNA ligase (Fermentas, Maryland, USA) in a final volume of 200 µl. After overnight incubation at 16°C, the ligation was purified using the Qiagen PCR purification kit (Qiagen, California, USA), eluting in a final volume of 30 µl. Individual PCRs were performed on 2, 5, and 10 µl of the purified product, using primers npt-41 (5'-AGC CGA ATA GCC TCT CCA CCC AAG-3') and npt+772 (5'-TTC GCA GCG CAT CGC CTT CTA TC-3').

Site-directed mutagenesis and homologous integration

Genes targeted for disruption included YP_237273.1 (Psy4205), a 7509-bp gene with homology to the TcdA1 toxin complex gene of *Photorhabdus luminescens* (AAF05542.1) but lacking any major protein domains, and YP_237102.1 (Psy4034), a 2946-bp gene with homology to the TccA3 toxin complex gene of *Photorhabdus luminescens* (NP_928150.1), which is also lacks any informative protein folds or domains. A *trp* terminator was introduced into pBluescript (Stratagene, California, USA) by the ligation of oligos 5'-AAT TGA GCC CGC CTA ATG AGC GGG CTT TTT TTT G - 3' and 5'-AAT TCA AAA AAA AGC CCG CTC ATT AGG CGG GCT C - 3' into the EcoRI restriction site, creating pBluescript-trp. A 500-bp portion of Psy4034 was amplified from *P. syringae* pv. syringae B728a using primers Psy4034+1 (5'-ATG ACC GAG CAA CCC TTC TCC C-3') and Psy4034-568 (5'-GAT TGA CGA TGT GCA GCG TAG GC - 3'), which was subsequently ligated into pBluescript-trp. A fragment of Psy4205 was amplified using Psy4205+64 (5'- GCT GCA CAG GCG TTG ATA TCC C - 3') and Psy4205-549 (5'- CGT TAC ATC CAG CTT CAG CGA CTC TC - 3'), and also ligated into pBluescript-trp. Both constructs were introduced into *P. syringae* pv.

syringae B728a independently by electroporation in 0.2 cm cuvettes using a Micropulser electroporator (Biorad) with a setting of 2.5 kV.

Bacterial growth assays

Second and third instar aphids were allowed to feed for 14 hours on aphid artificial diet containing 10^7 cfu/ml of either *P. syringae* pv. syringae B728a or *P. syringae* pv. syringae DC3000, after which, aphids were transferred to fresh diet. Three aphids were sampled at 12-hour intervals, macerated in 100 µl of 10mM $MgSO_4$, and dilutions plated on LB containing rifampicin. Growth assays were performed three times with similar results.

Vectoring experiment

Second and third instars were allowed to feed for 14 hours on artificial diet containing 10^7 cfu/ml of *P. syringae* pv. syringae B728a. Up to 20 aphids were transferred to fava bean plants and allowed to incubate for seven days at 24°C under ambient light. After seven days, leaves were harvested, washed in 10mM $MgSO_4$, and the wash was then plated on LB containing rifampicin. Colony counts were performed after 24-48 hours. Leaf surface area was calculated by tracing leaves on grid paper. Experiments were performed twice, with at least five plants used per experiment.

Aphid infection from epiphytic bacterial populations

Fava bean leaves and stems were brushed with PsyB728a, resuspended in 10 mM $MgSO_4$ (OD_{600} = 1.8), and second and third instars of *A. pisum* 5A were introduced at the base of the plant. After 24 hours in ambient light, all aphids on the plant were collected, and each was placed into an individual 1.5 ml microfuge tube containing 100 µl of 10 mM $MgSO_4$. Surface bacteria were washed from the aphid by agitation, and 10 µl of the

wash plated on LB plates containing rifampicin. Each aphid was then macerated with a pestle in the remaining 90 µl, and 10 µl of the homogenate plated on LB containing rifampicin. Bacterial growth was examined after 24-36 hrs. Aphids were scored as being infected if bacteria were completely absent from the wash but at least 10 cfu were present on plates containing the homogenate. Experiments were repeated twice, each with 30-80 aphids screened from each of three plants.

Swimming and swarming assays

Swimming and swarming assays of *P. syringae* strains were conducted by inoculating 5 µl of an overnight culture ($OD_{600} > 1$) in the center of 0.2%-0.6% LB agar plates and incubating at room temperature (25°C) overnight.

Results

Infected aphid honeydew inoculates plant surfaces.

To determine if aphids become infected from plants colonized epiphytically with PsyB728a, healthy aphids were introduced onto fava bean plants that were surface-inoculated with PsyB728a. Recovered aphids surveyed for the presence of ingested bacteria revealed an infection rate of 15%, with infection rates of up to 29% in some experiments. As a result of these studies, we found that aphid honeydew can contain high titres of bacterial inoculum, as assayed by a plating method. Because of this, we postulated that infected aphids contribute to the inoculation of plants with PsyB728a. To quantify the relative size of the PsyB728a inoculum that can be introduced through aphid honeydew onto the leaf surfaces, infected aphids were transferred to healthy fava bean plants, and leaves assayed for the presence of bacteria. Leaves contained bacterial titres averaging 3.6×10^8 colony forming units (cfu) per leaf (4.5×10^7 cfu/cm^2) , with some

leaves harboring titres of up to 1.6 x 10^9 cfu (1.6 x 10^8 cfu/cm^2). On average, each aphid was responsible for depositing 2 x 10^7 cfu on leaves over the duration of the experiment.

PsyB728a, but not PtoDC3000, is pathogenic to the pea aphid.

We conducted pathogenicity assays with the bean pathogen PsyB728a and the tomato pathogen *Pseudomonas syringae* pv. tomato DC3000 (PtoDC3000) to determine if entomopathogenicity is either strain- or species-specific. After feeding on an artificial diet supplemented with bacteria, the PsyB728a titre in aphids was approximately 6 x 10^3 cfu/aphid, increasing to 3 x 10^6 cfu/aphid after 24 hours (Figure 1). Titres remained at this level through to 36 hours, after which, mortality set in, with only 5-10% of aphids surviving 48 hours. By comparison, titres of PtoDC3000 at transfer were 8 x 10^2 cfu/aphid, which increased exponentially to 1 x 10^9 cfu/aphid by 72 hours, exceeding the lethal PsyB728a concentration of 3 x 10^6 cfu/aphid (Figure 1).

Putative insecticidal genes of PsyB728a are not responsible for virulence in pea aphids.

Interspecific genomic analysis of PsyB728a revealed the presence of several conserved genes implicated in entomotoxicity in other systems. These include homologs of the *Photorhabdus luminescens* toxin complex (tc) genes, of which PsyB728a retains a cluster containing at least one homolog of *tcaA*, *tccC*, *tcdA*, and *tcdB* (Figure 2). A genomic comparison of PsyB728a and PtoDC3000 exposed disruptions or deletions of some of these genes in PtoDC3000 that remained intact in PsyB728a (Figure 2), possibly accounting for the observed differences in entomopathogenicity in the aphid pathosystem. To test the involvement of these genes in pathogenicity, we disrupted the open reading frames of Psy4034 and Psy4205 in PsyB728a independently by single

crossover homologous recombination (Figure 2); however, each mutant retained full virulence, as determined by a qualitative lethality assay (data not shown).

A PsyB728a *fliL* mutant is attenuated in virulence.

A transposon-mutagenesis screen designed to recover genetic loci contributing to PsyB728a pathogenicity in the pea aphid identified one hypovirulent mutant that was disrupted in the flagellar basal body-associated protein, *fliL*. A qualitative pathogenicity assay of this mutant indicated significantly reduced virulence, with aphids surviving for up to 120 hours (data not shown), unlike wildtype PsyB728a, which caused almost complete aphid mortality in 36 hours.

To determine the relative growth rate of this mutant during aphid colonization, we assayed bacterial titres over a 72-hour period. The PsyB728a (*fliL*::Tn5) mutant began at lower titres at transfer ($<10^2$ cfu/aphid), but grew exponentially, reaching titres of 4×10^7 cfu/aphid by 48 hours, which fell to 3×10^6 cfu/aphid by 72 hours. The *fliL* mutant was able to reach and surpass the lethal dose of the wildtype PsyB728a concentration of 3×10^6 cfu/aphid, closely resembling the growth curve of the avirulent PtoDC3000 (Figure 1). Given that the *fliL*-mutant bacteria were able to achieve even higher densities than wildtype PsyB728a, and the prominent role of *fliL* in bacterial motility, we decided to evaluate swimming and swarming proficiency. Swarming assays of PtoDC3000, PsyB728a and PsyB728a (*fliL*::Tn5) revealed that mutation in the *fliL* gene reduced swimming ability (Figure 3A) and eliminated swarming ability (Figure 3B). Wildtype PtoDC3000, which has been shown to be capable of swarming (7, 9), also exhibited swarming proficiency in our experiments (not shown).

Discussion

The current model of *P. syringae* dissemination onto and between host plants involves rainsplash-mediated inoculation from infected to uninfected plants, facilitated largely by the aggressive epiphytic and aggregation capabilities of *P. syringae* (17, 25, 27). We have extended this model by showing that aphids contribute directly to pathogen amplification and inoculation of the plant phyllosphere following aphid infection through contact with epiphytic populations of PsyB728a (Figure 4). Transient ingestion of surface bacteria by aphids likely occurs during stylet-mediated plant host probing, which is consistent with the observation that aphids probe candidate host plants repeatedly during initial colonization (3, 18). Ingested bacteria migrate through the aphid digestive tract, establish in the gut (unpublished data), multiply, and are eventually deposited onto leaves in the aphid honeydew. As the bacterial load in the aphid approaches lethal titres, aphids stop feeding and begin to wander, continually depositing infected honeydew over plant surfaces in the process. Moreover, because aphid honeydew is carbohydrate-rich, deposited bacteria are effectively suspended in a nutrient-laden droplet that may enhance their survival on the leaf surface. Epiphytic bacterial populations typically have high mortality in the phyllosphere, with 75% of the epiphytic population becoming non-viable after 80 hours (38, 39); thus, the deposition in a carbohydrate-rich droplet suspension likely contributes to the persistence of the bacteria on the leaf surface and is also consistent with previous findings of sugar availability in the phyllosphere enhancing bacterial colonization of host plants (23).

The dispersal of *P. syringae* by an insect vector is not only consistent with its epiphytic life stages and likelihood of encountering insects in a foliar ecosystem, but also with the presence of many genetic loci commonly attributed to insect association and

250 entomopathogenicity in *P. syringae* genomes (20). Although many of these attributes are
251 present in other pseudomonads, only one other documented case of insect-mediated
252 dispersal of a *Pseudomonas* species has been documented. The rhizobacterium
253 *Pseudomonas chlororaphis* is vectored by the spotted cucumber beetle, *Diambrotica*
254 *undecimpunctata*, following infection through feeding on infected roots or foliage (33).
255 Other reports highlight indirect dispersal of pseudomonads, in which the insect does not
256 serve as host, but merely carries the bacteria externally (17, 19). In the case of the *P.*
257 *syringae*-aphid interaction, the bacteria are not only internalized, but can also replicate in
258 their insect vector prior to dispersal.

259 Although typically considered a dedicated plant pathogen, PsyB728a clearly
260 exploits the aphid as a secondary host, since it can support pathogen replication, escape,
261 and dispersal. The drastic difference between the pathogenic abilities of PtoDC3000 and
262 PsyB728a in this pathosystem suggests strain-specific pathogenicity determinants that
263 enable PsyB728a to colonize the pea aphid. This difference is not due to variability in
264 survival or growth rates between the PsyB728a and PtoDC3000 in artificial diet, or to a
265 greater propensity for wildtype PsyB728a to be ingested during the initial feeding. In
266 fact, similar initial titres and growth rates were observed for both strains, yet aphids were
267 unaffected by titres of PtoDC3000 that were lethal with PsyB728a.

268 We had predicted based on genomic comparisons that the *tc* genes, whose
269 homologs are known to contribute to *Photorhabdus luminescens* entomopathogenicity,
270 might be at least partly responsible for the observed differences in virulence phenotype
271 (12); however, these genes appear to have no role in this pathosystem. Instead, the
272 isolation and characterization of a hypovirulent *fliL* mutant suggests an active virulence

mechanism that is linked to bacterial motility, and more specifically, bacterial swarming. Because *fliL* is the leading gene of the FliL operon, transposon disruption may have had polar effects, impacting the proper assembly of the flagellum. The PsyB728a *fliL* mutant was slightly defective in swimming ability and completely defective in swarming ability, consistent with observations of both *Salmonella* and *E. coli fliL* mutants (2); however, swarming alone was not sufficient for virulence, as wildtype PtoDC3000, which is swarming proficient, is avirulent in the aphid. This suggests that *fliL* and the associated swarming phenotype have a role in the regulation of other PsyB728a-specific virulence factors. In *Proteus mirabilis*, *fliL* is responsible for inducing swarmer cell differentiation and regulating virulence gene expression, including heamolysin, urease and protease (1, 6), whereas swarming in *Vibrio cholerae* is linked to the induction of a variety of virulence-associated factors, including pili formation, and the production of hemagglutinin, hemolysins, adhesins, and cholera toxin (13). Thus, in *P. syringae*, the differentiation of vegetative cells into swarm cells may induce virulence gene expression that contributes to aphid colonization and death. The pathogenicity of *Dickeya dadantii* toward the pea aphid is mediated by Cyt-like toxin genes (14); however, homologs of these genes are not present in *P. syringae*, suggesting the involvement of other, as-yet unidentified, strain-specific virulence factors. The identity of such factors could prove useful for the development of effective pest-control strategies.

Pathogens often reach a virulence optimum that maximizes both their aggressiveness and transmission potential. Because a highly aggressive pathogen may kill its host before it has an opportunity to disperse, natural selection will favor a reduction in aggressiveness to increase host survivorship and enhance pathogen

296 dissemination (40). In the case of the interaction between *P. syringae* and the pea aphid,
297 pathogen aggressiveness appears to be extremely high, such that infection results in rapid
298 aphid mortality; however, because *P. syringae* has a direct and continual escape route
299 from its host, it can exploit the aphid maximally by replicating rapidly without the
300 tradeoff of host survivorship. Thus, the interaction between the
301 phytopathogenic/entomopathogenic PsyB728a and the pea aphid represents a novel host-
302 pathogen relationship in which the pathogen has achieved a virulence optimum that is
303 less dependent on host survivorship, and which simultaneously reduces competition for
304 resources, and enhances pathogen dispersal and fitness in a foliar environment.
305
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425 **Figure Legends**

426 **Figure 1.** Growth of bacteria in the aphid. Titres of pathogenic *P. syringae* pv. syringae
427 B728a, non-pathogenic *P. syringae* pv. tomato DC3000, and the *fliL* mutant in the pea
428 aphid following an initial feeding for 14 hours on artificial diet containing bacteria. All
429 aphids feeding on wildtype *P. syringae* pv. syringae B728a were dead within 36 hours.
430 Control aphids were fed 10mM $MgSO_4$.

431

432 **Figure 2.** Genomic comparison of toxin complex (tc) genes between pathogenic *P.*
433 *syringae* pv. syringae B728a and non-pathogenic *P. syringae* pv. tomato DC3000. In *P.*
434 *syringae* pv. tomato DC3000, the Psy4205 homolog is disrupted by two IS elements,
435 whereas the Psy4034 homolog contains a frameshift mutation. Gene knockouts of
436 Psy4205 and Psy4034 in *P. syringae* pv. syringae B728a were achieved through
437 homologous recombination of a gene fragment having a *trp* terminator (stem-loop
438 structure). The corresponding *Photorhabdus luminescens* W14 homologs are indicated
439 below each gene.

440

441 **Figure 3.** Motility assays of *fliL* mutant. A. Colony morphology of wildtype *P. syringae*
442 pv. syringae B728a and *fliL* mutant on 0.3% LB agar. B. Comparison of swarming
443 ability of wildtype and *fliL* mutant on 0.4% LB agar. Tentacle-like extensions projecting
444 outward from the growing edge of colonies were seen only on plates containing wildtype
445 B728a.

446

Figure 4. Model of aphid-mediated dissemination of *P. syringae*. Aphids may become infected after feeding on plant tissues colonized with epiphytic populations of *P. syringae* (yellow dots). As bacterial titres in infected aphids increase, droplets of bacteria-laden honeydew inoculate the plant surface, with movement of aphids to other hosts resulting in the dissemination of *P. syringae* and plant disease.

Figure 1

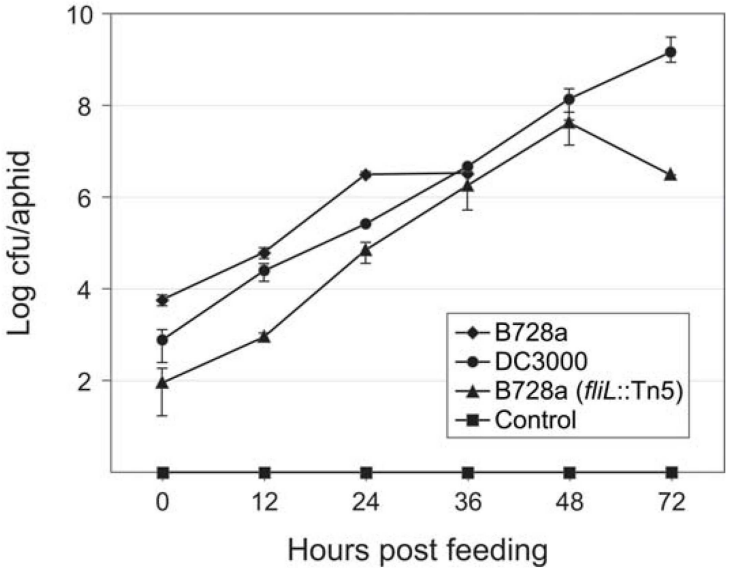

Figure 2

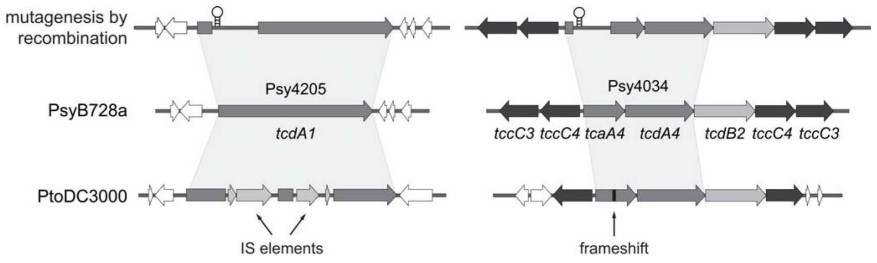

Figure 3

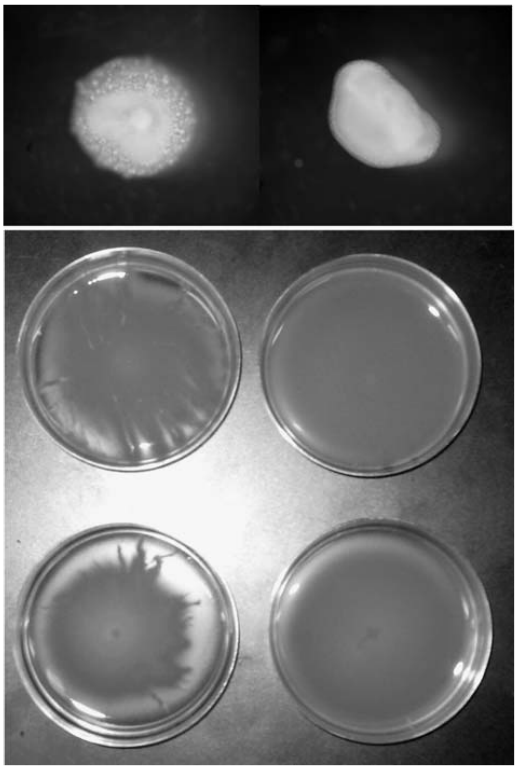

Figure 4