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# Fast and reliable screening assay developed to preselect candidate Soft Rot *Pectobacteriaceae* Tn5 mutants showing resistance to bacteriophage infection

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**Abstract** We present a simple, fast and inexpensive screening assay to preselect candidate *Pectobacterium* spp. and *Dickeya* spp. Tn5 mutants, which carry transposon insertions in genes putatively encoding proteins used by lytic bacteriophages to interact with host cells, for the follow-up studies. The proposed method is fast and cost-effective and it does not need any specialized laboratory equipment and/or technical support. The Tn5 mutants are generated using random transposon mutagenesis with the mini-Tn5 transposon. The obtained bacterial mutants are incubated in the presence of viable lytic bacteriophage particles in liquid bacterial growth medium supplemented with resazurin for 12 h at 28 °C in a 96-well microtiter plate assay. During the cultivation, the Tn5 mutants that are susceptible to phage infection are lysed. The mutants that are resistant to a viral infection (not lysed after contact with bacteriophages) irreversibly reduce the resazurin violet dye to pink/yellowish-colored resorufin indicating active bacterial metabolism (a positive reaction). The change of the culture color can be observed by eye. The Tn5 mutants that are positive in the screen are selected for sequencing of the Tn5 insertion site directly from bacterial genome. The proposed assay allows generation of a number of immediately-available Tn5 mutants expressing phage-resistant phenotypes that can be later selected for further examinations. As a proof-of-concept, we used this method to evaluate resistance to viral infection of Tn5 mutants of *Dickeya solani* strain IPO2222 and *Pectobacterium parmentieri* strain SCC3193 using lytic bacteriophages ϕD5 and ϕA38, respectively.

**Keywords** Gene fusion · Survival · *Pectobacterium* spp. · *Dickeya* spp. · Resazurin · Growth · Phage

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## Main text

Bacteriophages (phages) are obligatory bacterial parasites able to multiply exclusively in the presence of the host bacteria (Abedon 2009). The bacteriophage life cycle can be divided into several successive stages which are common to all known bacterial viruses. For lytic bacteriophages these include: adsorption (attachment), transfer of the genetic material from phage to host, expression and replication of phage genetic material inside infected cell, assembly of progeny (daughter) viral particles and their release (lysis of the host cell) to the environment and finally transmission to the new host (Clokie et al. 2011).

The accepted paradigm of the phage-host interaction states that the susceptibility of a bacterium to a particular bacteriophage is predominantly dependent on whether or not the bacteriophage can attach to specific attachment sites (so-called receptors) on the host (Bertozzi

Silva et al. 2016). All bacteriophage infections are initiated through this irreversible binding process that involves a specific and tight contact between the virus particle and components present on the host cell surface. A fundamental understanding of the adsorption (attachment) mechanism is prerequisite to establish the nature of viral infection as well as the phage receptor-finding process is crucial for the efficient propagation of the infection and it determines its fate in environment (Marsh and Wellington 1994).

Despite the fact that phage-bacterial interactions have been studied for more than a century since the first discovery of bacterial viruses, still relatively little is known about the molecular basis of attachment (Miao and Miller 1999). Only a limited number of bacteriophage receptors have been identified, crystalized and characterized in detail. The majority of those well-characterized examples came from model systems including bacteriophages: λ and T4 infecting *Escherichia coli* and/or viruses infecting human pathogenic bacteria (e.g. *Staphylococcus aureus* and *Listeria monocytogenes*) (Bertozzi Silva et al. 2016). Conversely, in the case of the other viral-host systems, e.g. environmental and/or plant associated bacterial strains, this topic has been largely ignored so far (Okabe and Goto 1963; Rakhuba et al. 2010).

We propose here an inexpensive, high-throughput assay to preselect *Pectobacterium* spp. and *Dickeya* spp. genes involved in bacteriophage adsorption to host cells with the employment of a random transposon mutagenesis using a mini-Tn5 transposon combined with a screen for the active bacterial metabolism (visualized by reduction of resazurin to pink/yellowish-colored resorufin) in the presence of infectious bacteriophage particles.

Resazurin as an indicator of cell viability has been used so far in a number of applications including assessment of bacterial susceptibility to antimicrobials (Palomino et al. 2002), studies targeting microorganisms abundance in environment (Guerin et al. 2001) or measurements of aerobic respiration in ecosystems (González-Pinzón et al. 2012). Till present, the method has not been used however neither to assess survival of plant pathogenic bacteria in the presence of lytic bacteriophages, nor to search for Soft Rot *Pectobacteriaceae* (SRP) genes encoding bacterial proteins used by bacteriophages to interact with host bacterial cells during adsorption and infection.

SRP, including *D. solani* and *P. parmentieri*, are important necrotrophic plant pathogenic bacteria infecting a number of commercially relevant plant species worldwide (Pérombelon 2002; Charkowski 2007). In Europe, *D. solani* and *P. parmentieri* are recognized as emerging pathogens of important farming crops leading to increasing losses in agriculture (Toth et al. 2011; van Der Wolf et al. 2017). Likewise, SRP are recognized among the top-ten most important plant pathogenic bacterial species worldwide (Mansfield et al. 2012). For many years SRP have been a useful model for studying different molecular aspects of the plant-bacterium interactions for plants (Hugouvieux-Cotte-Pattat et al. 1996; Charkowski et al. 2012; Reverchon and Nasser 2013), these bacteria were however not used as a model to study viral- host interactions.

The aim of this study was to develop a fast and reliable screening technique to preselect candidate *D. solani* and *P. parmentieri* genes encoding bacterial proteins used by bacteriophages to interact with host bacterial cells during adsorption and infection.

To achieve this objective we selected as a model two type strains of emerging Soft Rot *Pectobacteriaceae* in Europe viz. *D. solani* strain IPO2222 (van Der Wolf et al. 2014) and *P. parmentieri* strain SCC3193 (Pirhonen et al. 1988; Khayi et al. 2016) and two characterized in detail in our previous studies lytic bacteriophages ϕD5 (Czajkowski et al. 2013; Czajkowski et al. 2014) – infecting IPO2222 and ϕA38 (Smolarska et al. 2017) – infecting SCC3193. Unless noted otherwise, well-known molecular biology and microbiological methods were used for all described experiments (Sambrook et al. 1989).

The methodology was based on a random transposon mutagenesis of IPO2222 and SCC3193 genomes with a mini-Tn5 transposon. To mutate bacterial genomes, conjugation of *D. solani* or *P. parmentieri* with *Escherichia coli* S17 λ-pir (transposon donor) carrying pFAJ1819 (Xi et al. 1999) suicide vector with mini-Tn5 was performed as previously described (Czajkowski et al. 2017). After conjugation, the resulting *D. solani* Tn5 and *P. parmentieri* Tn5 mutants were selected as previously described (Czajkowski et al. 2011) using M9 agar plates supplemented with 50 μg $ml^{-1}$ neomycin (Sigma). All obtained IPO2222 and SCC3193 Tn5 transposon mutants were selected for assay with bacteriophages ϕD5 and ϕA38, respectively. For this, the bacteriophages ϕD5 and ϕA38 were propagated on their wild type bacterial hosts and titer as described earlier (Czajkowski et al. 2013). The adjusted stock concentration of ϕD5 and ϕA38 phage particles used in this study was $10^8$ plaque forming units (pfu) $ml^{-1}$ in tryptone soya broth (TSB; Oxoid). The individual *D. solani* and *P. parmentieri* Tn5 mutants and respective

wild type strains (IPO2222 and SCC3193) were grown individually in 10 ml of TSB supplemented with 50 μg $ml^{-1}$ of neomycin at 28 °C for 16 h with shaking (150 rpm) and after this time the bacterial cultures (containing ca. $10^8$ colony forming units (cfu) $ml^{-1}$) were diluted 100 times in the same fresh medium but not supplemented with the antibiotic. Such freshly diluted bacterial cultures contained ca. $10^6$ cfu $ml^{-1}$. Per Tn5 mutant to be analyzed, the wells of 96-well microtiter plate were inoculated with 50 μl of individual IPO2222 or SCC3193 Tn5 mutant culture (final bacterial density ca. $5 \times 10^4$ cfu per well) and subsequently were filled with 200 μl of bacteriophage solution (final phage particle concentration ca. $2 \times 10^7$ pfu per well). Each Tn5 mutant was analyzed in duplicates. To each well of the microtiter plate 5 μl of 0.22 μm-filter-sterilized 0.7% resazurin (Sigma) solution in demineralized water was added. As a control, 200 μl of sterile TSB instead of bacteriophage suspension was added to the wells inoculated with Tn5 mutants or wild type strains. Culture of a ϕD5 and ϕA38-resitant *Escherichia coli* strain DH5α (ThermoFisher Scientific) (Woodcock et al. 1989) grown in TSB at 28 °C for 24 h with shaking (150 rpm) and treated similarly as described above in case for *Pectobacterium* spp. and *Dickeya* spp. mutants and wild type strains, was used as an external assay control. After inoculation, 96-well plates were sealed with optically transparent sealing tape (Sarstedt) as suggested earlier (Lisicka et al. 2018) to prevent from contamination and evaporation of bacterial cultures during incubation. The plates were incubated for up to 12 h at 28 °C with shaking (150 rpm). After this time the development of the pink/yellowish color (positive reaction) in the inoculated wells was determined by eye (Fig. 1). The positive reaction indicates the growth of the Tn5 mutant in the presence of viable bacteriophages (which is equal to phage resistance) (Fig. 2). The experiment was performed twice with the same setup.

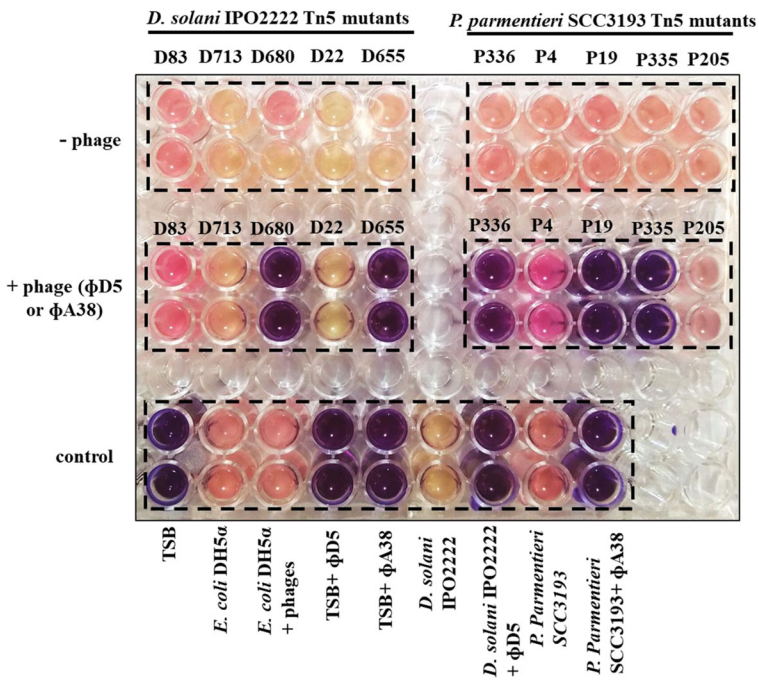


**Fig. 1** Example results of the fast screening assay to preselect *Dickeya solani* and *Pectobacterium parmentieri* Tn5 mutants in genes coding for proteins used by lytic bacteriophages ϕD5 and ϕA38 as receptors. Per Tn5 IPO2222 or SCC3193 mutant to be analyzed, in duplicates, the wells of 96-well microtiter plate were inoculated with 50 μl of individual IPO2222 or SCC3193 freshly-prepared Tn5 mutant culture in TSB (final bacterial density ca. $5 \times 10^4$ cfu per well) and subsequently with 200 μl of bacteriophage solution (final phage particle concentration ca. $2 \times 10^7$ pfu per well) and 5 μl of 0.7% resazurin (Sigma) solution in demineralized water. For control, per mutant, to the wells inoculated with bacterial Tn5 mutants or wild type strains as described above, 200 μl of the sterile TSB instead of bacteriophage suspension was added. The external assay control was culture of *E. coli* DH5α grow and propagated under the same conditions as described for IPO2222 and SCC3193. The pink/yellowish color indicates a positive reaction – bacterial growth in the presence of bacteriophage (phage resistance), whereas violet color indicates a negative reaction – no bacterial growth in the presence of bacteriophage (phage susceptibility)

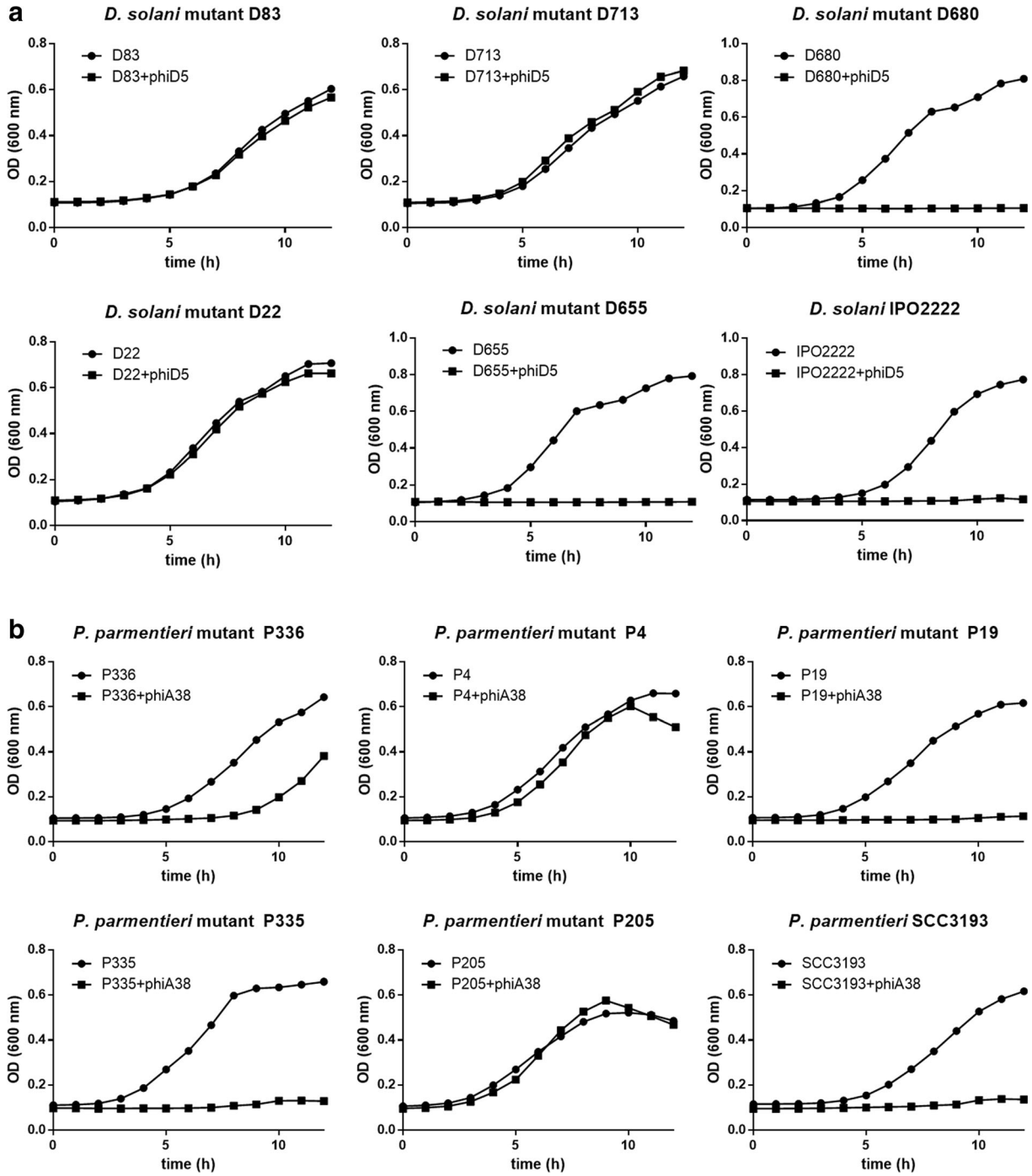


**Fig. 2** Host challenge assay of the parental (*D. solani* IPO2222 and *P. parmentieri* SCC3193) strains and selected Tn5 IPO2222 (A) and Tn SCC 3193 (B) mutants in the absence and presence of lytic bacteriophages ϕD5 and ϕA38, infecting *D. solani* IPO2222 and *P. parmentieri* SCC3193, respectively. Per mutant to be analyzed, in duplicates, the wells of the 96-wells plate were inoculated with 50 μl of freshly prepared bacterial cultures in TSB (final cfu $ml^{-1}$ of $5 \times 10^4$). Subsequently, 200 μl of bacteriophage suspension in TSB was added per well (final pfu $ml^{-1}$ of $2 \times 10^7$). Inoculated plates were incubated at 28 °C with shaking (150 rpm). For control instead of bacteriophage suspensions, the sterile TSB was added. *E. coli* strain DH5α, known to be resistant to lytic bacteriophages ϕD5 and ϕA38, was used as an external assay control. Growth rate was measured as a change in the optic density (OD) of the bacterial cultures at 600 nm wavelength for the total period of 12 h every 0.5 h using Epoch2 Microplate Spectrophotometer (BioTek Instruments)

**Table 1** Plaque formation on lawns of *D. solani* IPO2222 Tn5 mutants and *P. parmentieri* SCC3193 Tn5 mutants

| Strain | Plaque formation with phiD5 phage | Strain | Plaque formation w ith phiA38 phage |
|---|---|---|---|
| IPO2222 | + [A] | SCC3193 | + |
| D83 | – [B] | P336 | + |
| D713 | – | P4 | +/– [C] |
| D680 | + | P19 | + |
| D22 | – | P335 | + |
| D655 | + | P205 | – |

[A] indicates formation of a bacteriophage plaque (region of host destruction) on the susceptible host; [B] indicates no plaque formation on resistant host; [C] indicates a significant drop of the number of formed plaques on the mutant lawn in comparison with the number of formed plaques on a susceptible wild type strain (IPO2222 or SCC3193)

The candidate phage resistant Tn5 mutants selected in the experiments with resazurin were additionally screened using plaque formation assay and host challenge assay with the respective bacterial hosts (IPO2222 and SCC3193) (Clokie and Kropinski 2009) to confirm the phenotype of the each individual tested Tn5 mutant (Table 1).

The Tn5 mutants in genes coding for putative bacteriophage receptors and other proteins facilitating bacteriophage interaction with host cell were selected for sequencing the transposon insertion sites as previously described (Lisicka et al. 2018). In the preliminary screening, we selected the first five hundred *D. solani* Tn5 mutants and first 300 *P. parmentieri* Tn5 mutants for testing them for the resistance in the presence of lytic bacteriophages ϕD5 and ϕA38, respectively. From these, 3 (0.6%) and 3 (1%) of the tested *D. solani* and *P. parmentieri* Tn5 mutants, respectively, showed a bacteriophage resistant phenotype in assays with ϕD5 and ϕA38. Example results are shown in Figs. 1 and 2 and Table 1.

The proposed fast screening of bacteriophage-resistant bacterial mutants allows direct pre-selection of candidate mutants for the follow-up studies. These studies may include the analyses of interactions of the phage-resistant *D. solani* and/or *P. parmentieri* Tn5 mutant with host plants and the impact of various biotic and abiotic stresses and environmental conditions on phage-resistance bacterial mutants.

The main advantage of the suggested method is that the assay can be considered as a definitely fast preliminary screening (the total assay time is ca. 12 h) for the identification of the genes coding for bacterial proteins used by bacteriophage to interact with host cells. What is more, such Tn5 mutants may be subsequently tested against several other factors of interest. The method does not require any expensive laboratory tools and consumables and therefore it may be performed in virtually every microbiological laboratory possessing standard microbiological equipment.

Likewise, it has to be noted here that the proposed fast screening assay may be used not only to evaluate the resistance/susceptibility of a particular Tn5 bacterial mutant for phage infection but also in ecological studies to screen a number of environmental bacterial isolates against a collection of different viruses to assess the host range of a particular phage of interest.

We postulate that due to the universal nature of this screening method, it can be easily adapted to study interaction of other bacterial species and other lytic bacteriophages of interest.

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**Compliance with ethical standards**

**Ethical statement** The results presented in this manuscript did not involve any protected and/or endangered species, field studies, human participants, specimens or tissue samples, or vertebrate animals, embryos or tissues.



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