Dickeya solani D s0432-1 produces an arsenal of secondary metabolites with anti-prokaryotic and anti-eukaryotic activities against bacteria, yeasts, fungi, and aphids.

Running title: *Dickeya solani* secondary metabolites

Authors: Effantin G[1], Brual T[1], Rahbé Y[1,2], Hugouvieux-Cotte-Pattat N[1] & Gueguen E[1]*.

Affiliation:

[1] Univ Lyon, Université Claude Bernard Lyon1, CNRS, INSA Lyon, UMR5240 MAP, F-69622, LYON, France

[2] INRAE, Univ. Lyon, UMR5240 MAP, F-69622, Lyon, France

*corresponding author: erwan.gueguen@univ-lyon1.fr

Orcid I.D.

G.E. 0000-0001-8784-1491

H-C-P.N 0000-0002-4322-160X

R. Y. 0000-0002-0074-4443

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SUMMARY

The necrotrophic plant pathogenic bacterium *Dickeya solani* is a new invader of potato agrosystem in Europe. All isolated strains of *D. solani* contain several large polyketide/fatty acid/non-ribosomal peptide synthetase clusters. Analogy with genes described in other bacteria, suggests that two clusters are involved in the production of secondary metabolites of the oocydin and zeamine family. In this study, we constructed by an approach of reverse genetics mutants affected in the three secondary metabolite clusters *ssm*, *ooc* and *zms* in order to compare the phenotype of the *D. solani* strain D s0432-1 with its derived mutants. We demonstrated that the zeamine cluster *zms* inhibits growth of gram-positive and gram-negative bacteria. It is also implicated in a toxicity against aphids. The oocydin cluster *ooc* inhibits growth of fungi of the phylum *Ascomycota*. Finally, we unveiled the function of a new secondary metabolite cluster *ssm* (for *solani* secondary metabolite), only conserved in some *Dickeya* species. This cluster produces a secondary metabolite inhibiting yeasts. *D. solani* therefore produces several molecules that are toxic to a wide range of living and potentially interacting organisms, from bacteria to insects. The expression of these secondary metabolite pathways could contribute to the rapid spread of *D. solani* in Europe.

INTRODUCTION

Bacterial phytopathogens of the genus *Dickeya* and *Pectobacterium* are pectinolytic necrotrophic bacteria with a broad host plant spectrum (Charkowski *et al.*, 2012; Hugouvieux-Cotte-Pattat *et al.*, 2020). These members of the family *Pectobacteriaceae* (Van Gijsegem, Toth, *et al.*, 2021) cause substantial agricultural losses worldwide by affecting many vegetables, ornamentals and crops, of which the potato is the most important economically. These bacteria are able to invade and degrade the plant tissues through the coordinated expression of genes encoding virulence factors, with a major role of pectate lyases that dissociate the plant cell wall constituents (Hugouvieux-Cotte-Pattat *et al.*, 2014).

The *Dickeya* genus was established in 2005 (Samson *et al.*, 2005), resulting from the reclassification of *Pectobacterium chrysanthemi* (formerly *Erwinia chrysanthemi*). To date, twelve species of *Dickeya* have been described, *D. aquatica*, *D. chrysanthemi*, *D. dadantii D. dianthicola* , *D. fangzhongdai*, *D. lacustris*, *D. oryzae*, *D. paradisiaca*, *D. poaceiphila*, *D. solani*, *D. undicola*, and *D. zeae* (Samson *et al.*, 2005; Brady *et al.*, 2012; Parkinson *et al.*, 2014; van der Wolf *et al.*, 2014; Tian *et al.*, 2016; Hugouvieux-Cotte-Pattat *et al.*, 2019, 2020; Oulghazi *et al.*, 2019; Wang *et al.*, 2020).

The species *D. solani* was officially established in 2014 (van der Wolf *et al.*, 2014) but *D. solani* isolates have attracted attention since its emergence on the potato agrosystem in Europe in

the early 2000s. It appeared to be highly aggressive in both subtropical and temperate climates. Recently, seed companies have adopted a zero-tolerance policy to *D. solani* due to its invasive and aggressive nature (Van Gijsegem, van der Wolf, *et al.*, 2021). Many scientific efforts have been made to provide information on this phytopathogen, resulting in 76 *D. solani* genomes available in May 2021 (Blin *et al.*, 2021). Comparative genomics was performed to identify the genetic basis for the high virulence level of *D. solani* (Garlant *et al.*, 2013; Pédron *et al.*, 2014; Khayi *et al.*, 2015; Golanowska *et al.*, 2018; Motyka-Pomagruk *et al.*, 2020; Blin *et al.*, 2021). Most *D. solani* strains Isolated from different regions show a low level of genetic variation, suggesting a clonal origin (Khayi *et al.*, 2015). The genomes of *D. solani* share a high similarity and synteny with those of the model strain *D. dadantii* 3937, prompting comparison between the two species. Only a few hundred genes were specific to each species, including a few dozen distinctive genomic regions (Garlant *et al.*, 2013; Pédron *et al.*, 2014). Three of these regions have been shown to encode polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS) and amino acid adenylation domain proteins, suggesting that they encode proteins involved in the production of secondary metabolites (Garlant *et al.*, 2013).

PKSs and NRPSs are able to synthesize molecules by sequential condensation of carboxylic acids and amino acids, respectively. PKS and NRPS modules can combine together to form hybrid PKS/NRPS systems capable of producing compounds of great structural diversity (Cane and Walsh, 1999). The molecules synthesized may have siderophore, antibiotic or phytotoxic properties that promote the virulence of a plant pathogen. Three PKS/NRPS clusters are present in all sequenced *D. solani* strains and found in a few other *Dickeya* species (Duprey *et al.*, 2019). In *Serratia plymuthica,* the cluster *ooc* is involved in the synthesis of oocydin A, a halogenated macrolide with antifungal and anti-oomycete activity (Matilla *et al.*, 2012). The cluster *zms*, previously found in the genomes of *S. plymuthica* and *Dickeya oryzae,* leads to the biosynthesis of a polyamino-amide antibiotic, zeamine (Zhou *et al.*, 2011; Masschelein *et al.*, 2013). A third cluster is found in a few *Dickeya* species but the nature and function of the molecule synthesized are unknown.

Mutagenesis is one of the most powerful genetic tools for analyzing the function of a gene and its involvement in bacterial virulence. A random mutagenesis approach was used in the *D. solani* type strain IPO2222 with a transposon Tn5 harboring a promoterless *gusA* reporter gene to obtain mutants affected under given conditions (Fikowicz-Krosko and Czajkowski, 2017; Lisicka *et al.*, 2018; Czajkowski *et al.*, 2020). Some mutants affected for a selected gene have also been constructed in various strains of *D. solani* using a generalized transduction method with phage, to transfer a mutation previously constructed in *D. dadantii* 3937 (Potrykus *et al.*, 2014, 2018). However, this method is strictly limited to genes common to *D. dadantii*

and *D. solani*. Given the need for in-depth studies on this economically important species, we have tested a general method to precisely inactivate a selected gene using the SacB-based reverse genetics method. We used this method to construct mutants affected for the three loci involved in the biosynthesis of secondary metabolites in the highly virulent *D. solani* strain Ds0432-1. This method is potentially applicable to all genes and in all strains of *D. solani*. We showed that *D. solani* Ds0432-1 clusters *zms* and *ooc,* encoding zeamine and oocydin biosynthesis, are involved in growth inhibition of bacteria and fungi, respectively. We also showed evidence that the *D. solani* Ds0432-1 cluster *ssm* (for *s*olani secondary metabolite) produces a third secondary metabolite that prevents yeast growth.

RESULTS

Use of the SacB-based reverse genetics method to delete genes of *D. solani*

A technique commonly used in bacterial genetics to delete a gene from a Gram-negative bacterium requires a suicide plasmid with the R6K origin of replication and the counter-selection gene *sacB1*, which confers sucrose sensitivity (Edwards *et al.*, 1998). The vector pRE112 had previously been successfully used for allelic exchange in *D. dadantii* 3937 (Koskiniemi *et al.*, 2013; Royet *et al.*, 2019). We tried to use it with *D. solani*.

To evaluate the effectiveness of the reverse genetic approach with *D. solani*, we first tried to delete the *araBA* operon of the *D. solani* strain Ds0432-1. *araA* encodes the L-arabinose isomerase and *araB* a ribulokinase. A Δ*araBA* mutant, deleted for this operon, is unable to catabolize arabinose and it forms white colonies on MacConkey-arabinose agar plates. This mutant is thus easily distinguishable from the wild-type Ara^+ strain forming red colonies. Two 1-kb fragments upstream and downstream *araBA* were assembled and cloned directly into linearized pRE112 vector (Fig. 1A). The plasmid pRE112-Δ*araBA* was transferred from *E. coli* MFDpir to *D. solani* Ds0432-1 by conjugation (Fig. 1B). Plasmid integrants were selected by their chloramphenicol resistance (Cm^R) on LB agar plates supplemented with chloramphenicol. On this selective medium, the *E. coli* donor strain cannot survive because LB agar does not contain diaminopimelic acid (DAP). The plasmid is integrated either upstream or downstream of the *araBA* locus (Fig. 1A). The integrated plasmid can be lost by homologous recombination between the tandemly duplicated *ara* sequences (Fig. 1A). Plasmid loss is stimulated in the presence of sucrose, *i.e.* by spreading the integrants onto LB agar without NaCl containing 5% sucrose. The sucrose-resistant colonies were then replica-plated onto MacConkey-arabinose agar, allowing the identification of Ara^- segregants, and on LB agar-Cm, to confirm the plasmid loss by their Cm^S phenotype (Fig. 1B). Finally, the correct

137 structure of the mutants was confirmed by colony PCR analysis (Fig. 1C). By this first
138 experiment, we were able to verify that this method works perfectly and is easy to implement
139 in *D. solani*. In addition, this method was shown to work by cloning only 500 bp DNA fragments
140 upstream and downstream of *araBA*, instead of 1000 bp. We also used this protocol to
141 construct a Δ*araBA* mutant of the *D. solani* type strain IPO2222 with comparable efficiency
142 (data not shown).

Figure 1

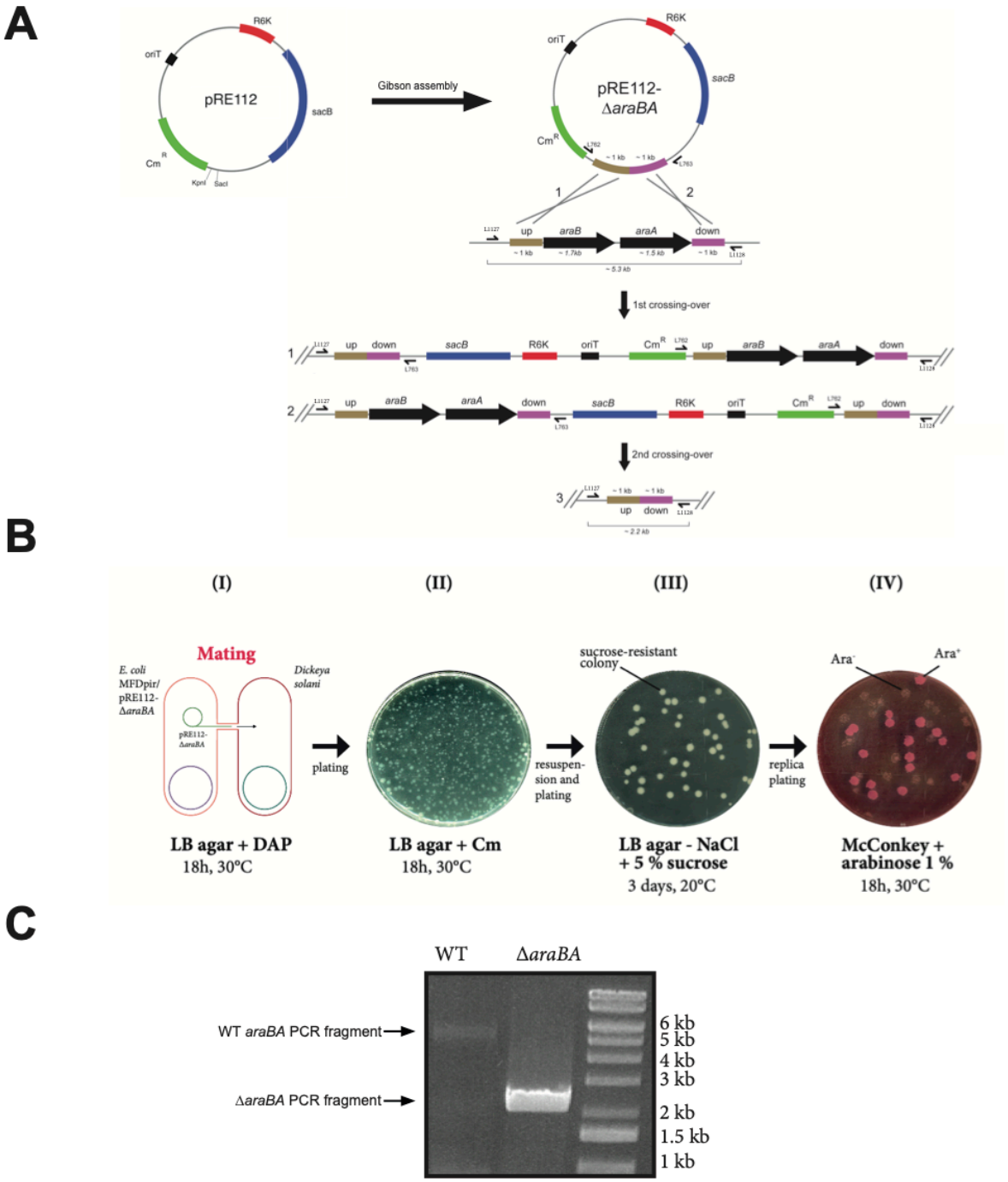

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Figure 1. Summary of the method used to obtain in-frame deletion mutants. (A) Genetic steps to obtain a Δ*araAB* deletion mutant of *Dickeya solani*. The 1 kb upstream and downstream DNA regions of the *araBA* genes are cloned between the KpnI and SacI restriction sites of the suicide plasmid pRE112 by Gibson assembly. The resulting plasmid pRE112-Δ*araBA* is then transferred into *D. solani* by conjugation. The plasmid is integrated into the chromosome by homologous recombination either upstream or downstream the *araBA* locus. The structures of both possible integrants are shown as structures 1 and 2. Next, sucrose-resistant segregants lose the integrated plasmid pRE112-ΔaraBA by homologous recombination between the tandemly duplicated upstream or downstream *ara* sequences. This either regenerates a WT *araBA* locus or an *araBA* deletion on the chromosome, shown as structure 3.
(B) Microbiological steps to obtain a Δ*araBA D. solani* mutant. The *E. coli* donor strain MFDpir(pRE112-Δ*araBA*) was mated with *D. solani* (step I). *D. solani* recombinants were selected onto LB agar medium supplemented with chloramphenicol (step II). *D. solani* recombinant were then spread onto LB agar without NaCl and supplemented with 5% sucrose. Sucrose resistant colonies (step III) were transferred onto MacConkey agar plate with 1 % arabinose (step IV). White colonies are formed by bacteria unable to catabolize arabinose because they acquired the Δ*araBA* mutation. (C) Their genotype was confirmed by PCR on colonies using oligo pairs L1127/L1128. The desired mutants give a PCR product of 2 kb, whereas PCR done on WT *D. solani* gives a PCR product > 5 kb. Detail procedure is indicated in the material and methods section.

Using this reverse genetic approach, we decided to study the three secondary metabolite clusters of *D. solani* which are absent in *D. dadantii*. We focused our work on *D. solani* D s0432-1 since it is one of the most aggressive *D. solani* strain (Khayi *et al.*, 2015; Golanowska *et al.*, 2018). This strategy should allow us to assess the contribution of these three clusters on the competitiveness of *D. solani* against eukaryotic and prokaryotic organisms.

Description of the three selected PKS/NRPS secondary metabolite clusters of *D. solani*.

The clusters A, B and C encoding complex NRPS and PKS involved in the production of secondary metabolites were named *ssm*, *ooc* and *zms,* respectively (Fig.2).
The ~42-kbp cluster A contains the 12 genes *ssmABCDEFGHIJKL* (Fig.2). It is widely conserved in the genus *Dickeya*, *i.e.*, in all sequenced *D. solani, D. aquatica, D. fangzhondai, D. poaceiphila* and *D. zeae* genomes, in some *D. dadantii* strains (NCPPB 898, NCPPB 3537, but not the model strain 3937), in some *D. undicola* strains (FVG1, FVG10), and in some *D. oryzae* strains (EC2, NCPPB 3531 and CSLRW192). The role and the structure of the metabolite produced from this cluster have not been elucidated yet.
The ~80-kbp cluster B is highly similar to the *oocBCDEFGJKLMNOPQRSTUVW* cluster of *S. plymuthica* A153. It is present in all the sequenced genomes of *D. solani* and *D. dianthicola*, in four *D. oryzae* strains (ZYY5, EC1, DZ2Q and ZJU1202), and in two *D. paradisiaca* strains, Ech703 and NCPPB2511. In *S. plymuthica* A153, disruption of this gene cluster abolished bioactivity against the fungi *Verticillium dahliae* and the oomycetes *Pythium ultimum*. This

cluster produces oocydin A (Matilla *et al.*, 2012), a chlorinated macrolide, powerfully active against plant pathogenic oomycetes (Strobel *et al.*, 1999). Since various *D. solani* strains inhibit *V. dahliae* and *P. ultimum* growth (Matilla *et al.*, 2015), it was suggested, on the basis of gene sequence homologies and similar cluster organization, that *D. solani* also produces oocydin A.

The ~55-kbp cluster C encodes mixed fatty acid synthase (FAS)/PKS and hybrid NRPS/PKS enzymes. Its genomic organization is identical to the *D. oryzae* EC1 zeamine cluster and related to the *S. plymuthica* AS12 zeamine cluster (Zhou *et al.*, 2011, 2015). The *zms* cluster is conserved in all the sequenced *D. solani* and *D. fangzhongdai* genomes. After reclassification of several *D.zeae* strains in the novel species *D. oryzae* (Wang *et al.*, 2020), this cluster appeared to be absent in the genomes of true *D. zeae* strains. It is present in some, but not all, *D. oryzae* rice strains (ZYY5, EC1, DZ2Q and ZJU1202). The zeamine biosynthetic clusters from *D. oryzae* EC1 and *D. solani* Ds0432-1 share from 59 to 94% identity at individual protein level (Zhou *et al.*, 2015).

Figure 2

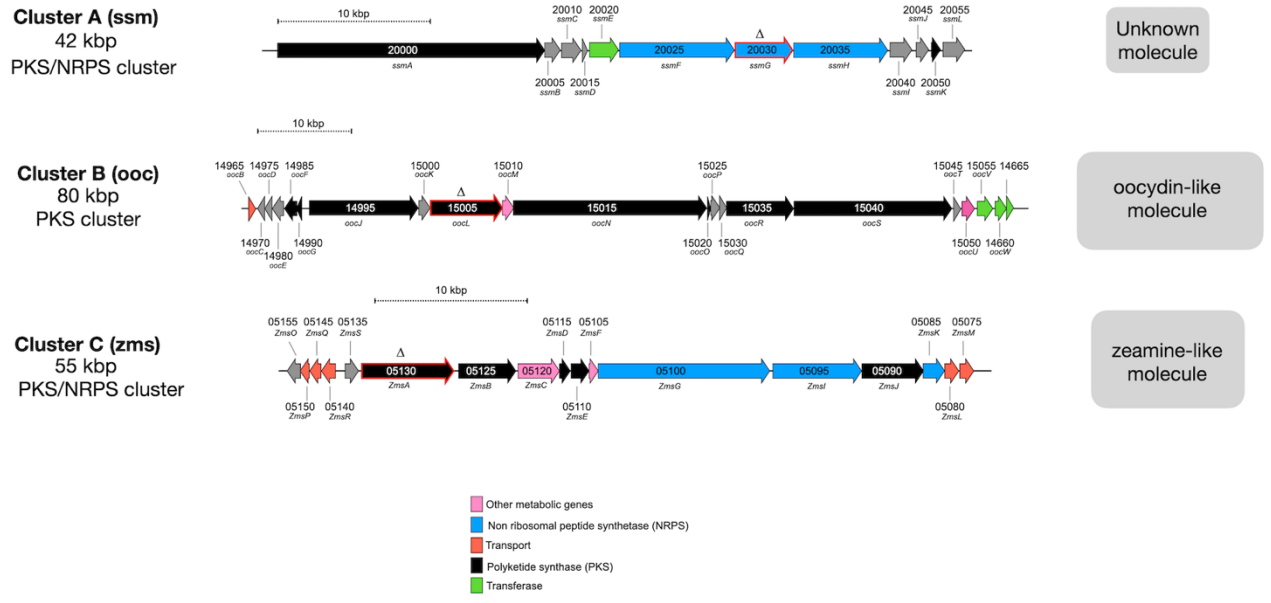


Figure 2. **Organization of the secondary metabolite clusters of *D. solani* D s0432-1.** Genes are indicated using the NCBI nomenclature of the NCBI reference genome sequence NZ_CP017453.1 of *D. solani* D s0432-1. XXXXX are the digital number in BJD21_RSXXXXX, which corresponds to the locus tag. Arrowheads show gene orientations. Color code indicates gene function. The red-framed arrows indicate genes targeted for in-frame deletion performed in this study. Cluster function is noted on the right.

While the *zmsABCDEFGIJKLMNPQRS* cluster directing zeamine biosynthesis is present only in some *D. oryzae* strains isolated from rice, it is conserved in all *D. fangzhongdai* and *D. solani* strains, which suggest secondary acquisition by horizontal gene transfer in *D. oryzae* (Zhou *et al.*, 2015; Duprey *et al.*, 2019). Zeamine-related antibiotics are polyamino-amide molecules toxic to a wide range of pro- and eukaryotic organisms such as bacteria, fungi, oomycetes, plants, and nematodes (Masschelein *et al.*, 2017). Mutation of the zeamine synthase gene *zmsA* in *D. oryzae* EC1 attenuates the inhibition of rice seed germination (Zhou *et al.*, 2011) and suppresses antibacterial activity against *E. coli* (Zhou *et al.*, 2011). Zeamine produced by *S. plymuthica* kills nematodes and yeast (Hellberg *et al.*, 2015). *D. solani* IPO2222 can also kill *C. elegans* but not as quickly as *S. plymuthica* (Hellberg *et al.*, 2015).
To interrupt the synthesis of the secondary molecules produced by these three clusters, we constructed in-frame deletion mutants inactivating a key gene of each cluster. The mutants Δ*ssmG* (cluster A), Δ*oocL* (oocydin cluster B) and Δ*zmnA* (zeamine cluster C) were constructed using the described reverse genetic approach with the pRE112 suicide plasmid. Inhibitory effects of these mutants against fungi, bacteria, yeasts, aphids and plants were compared with that of the wild-type strain.

The *D. solani* oocydin cluster inhibits *Ascomycota* growth

We compared the ability of the *D. solani* Ds0432-1 DS58 strain (a Nal^R Gm^R derivative of the WT strain) and its derived mutants to inhibit the growth of *Botrytis cinerea*, *Magnaporthe oryzae* and *Sclerotinia sclerotiorum*, three fungi-like eukaryotes of the phylum *Ascomycota*. A potato dextrose agar (PDA) plate was inoculated at the periphery of the Petri dish with fungal mycelium and 10 µl of overnight bacterial culture of each *D. solani* strain was deposited at the center of the plate (Fig. 3). After incubation at 25°C for several days, we observed a growth inhibition of the three fungi by the *D. solani* WT and the Δ*ssmG* and Δ*zmsA* mutants. In contrast, the Δ*oocL* mutant did not inhibit mycelium growth (Fig. 3). Thus, we conclude that the gene *oocL* is involved in the production of an oocydin-like secondary metabolite that has an anti-fungal activity.

Figure 3

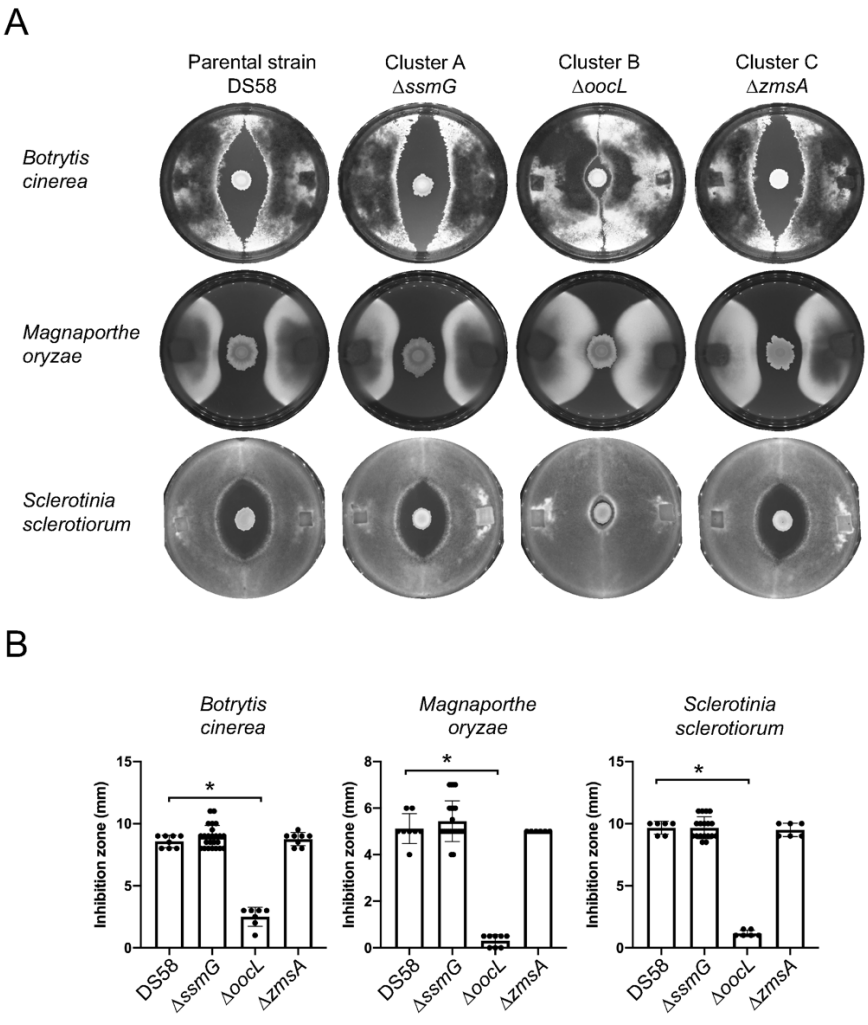


Figure 3. Radial diffusion assays to assess fungal growth inhibition on PDA plates with *D. solani* D s0432-1 or derived mutants. 5 µl of *D. solani* D s0432-1 at OD_{600nm} of 0.1 was deposited in the center of a 90 mm Petri dish inoculated with plugs of *Sclerotinia sclerotiorum*, *Botrytis cinerea* or *Magnaporthe oryzae* mycelium. Plates were incubated at 25 °C until the mycelium covers the plate. Lengths of fungi inhibition zone were measured in 3 independent experiments. A statistical difference was significant only between the WT and Δ*oocL* mutant (Mann-Whitney test; * P value < 0.05).

The *D. solani* zeamine cluster inhibits bacterial growth.

The zeamine cluster of *D. oryzae* EC1 is responsible of the bactericidal activity against *E. coli* DH5α (Zhou *et al.*, 2011). Because *D. oryzae* EC1 and *D. solani* zeamine biosynthetic genes share a high degree of similarity (Zhou *et al.*, 2015), we evaluated the capacity of *D. solani* WT DS58 and mutants to inhibit the growth of gram-positive and gram-negative bacteria. It was previously shown that D. *oryzae* EC1 produced 128 µg.mL^{-1} of zeamine in the minimum medium LS4+yeast extract, while zeamine production was not detected in LB (Liao *et al.*, 2014). Based on this work, we first used LS4+yeast extract medium for production of zeamine

by *D. solani*. The *D. solani* strains were grown in LS4+yeast extract minimal medium before addition of 5 µl of the overnight culture onto a LB agar plate pre-inoculated with *B. subtilis*. Only a very slight zone of *B. subtilis* inhibition was observed around the drop of *D. solani* in our bioassay (Fig. S1). We then tested *D. solani* growth in minimal medium M63 supplemented with sucrose and yeast extract. A large diameter of inhibition was observed, allowing differentiation between strains capable or not of inhibiting *B. subtilis* growth (Fig. S1). Thus, we used this medium for overnight growth of *D. solani* in all the following experiments. The *D. solani* WT strain DS58 and the three mutants were tested (Fig. 4A). Only the Δ*zmnA* mutant was unable to inhibit *B. subtilis* growth, indicating that *D. solani* produces an active zeamine antibiotic (Fig 4A). We also tested the ability of *D. solani* to inhibit growth of other bacteria (Fig 4A) such as *E. coli* DH5α, *Streptomyces scabiei* CFBP 4517, *D. dadantii* 3937 and *P. atrosepticum* SCRI 1043. The *D. solani* zeamine cluster was also the sole responsible for the growth inhibition of *S. scabiei*, a plant pathogen causing the potato disease common scab (Loria *et al.*, 2006) (Fig 4A). The *zms* cluster also inhibits the growth of *E. coli*, but with less efficiency than that observed for the two Gram$^+$ bacteria tested. No inhibition of the two other pectinolytic bacteria *D. dadantii* and *P. atrosepticum* growth was observed. In conclusion, out of the three clusters analyzed, only the zeamine-like molecule has an anti-bacterial activity.

The *D. solani* cluster *sms* but not the zeamine cluster inhibits yeast growth.

Since zeamine produced by *S. plymuthica* A153 has previously been shown to be toxic to the ascomycete yeast *Saccharomyces cerevisiae* (Hellberg *et al.*, 2015), we tested the capacity of *D. solani* D s0432-1 and its mutants to inhibit the growth of the yeasts *S. cerevisiae* and *Kluyveromyces lactis*. *K. lactis* has been isolated from milk and constitutes the predominant eukaryote during cheese productions (Rodicio and Heinisch, 2013). *S. cerevisiae* and *K. lactis* were grown in the rich medium YPD. We observed the inhibition of *S. cerevisiae* and *K. lactis* growths by the *D. solani* WT strain DS58 (Fig. 4B), grown either in M63+sucrose+yeast extract or in YPD medium (Fig S1). The anti-eukaryotic activity against these yeasts was caused by the cluster *sms* since only the Δ*smsG* mutant did not inhibit yeast growth (Fig 4B). Therefore, it appears that zeamine is not the primary factor responsible for yeast inhibition in *D. solani* D s0432-1. Conversely, we identified a novel secondary metabolite cluster that has never been studied previously and produces a molecule with anti-eukaryotic activity.

Figure 4

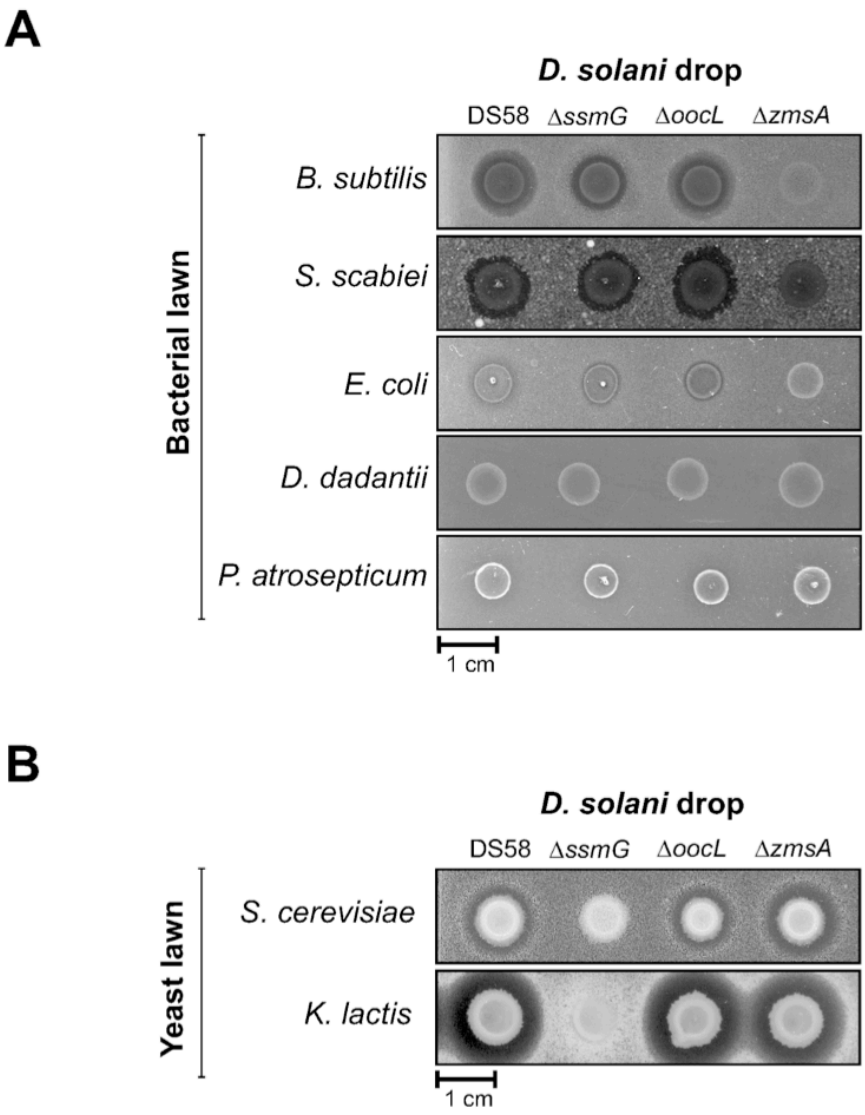


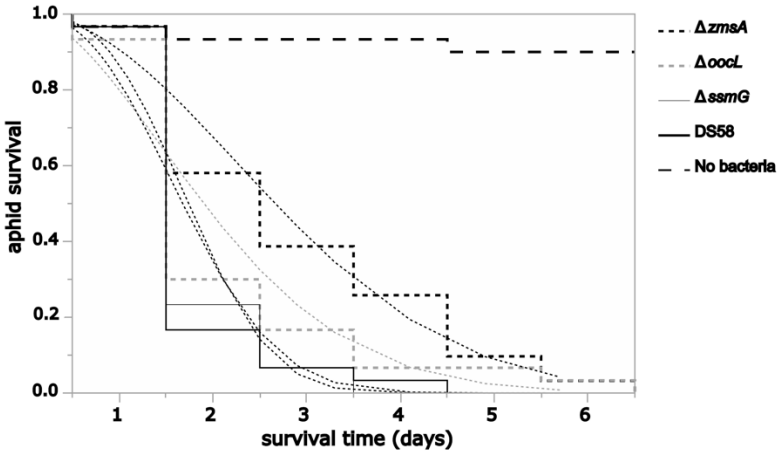
Figure 4. Plate-based bioassays for metabolite production by *D. solani* and mutant derivatives. Bioassay plates were prepared by mixing different bacteria culture (*B. subtilis, S. scabiei, E. coli, D. dadantii* or *P. atrosepticum*) with warmed LB agar. (A) 5 µl of overnight bacteria culture from *D. solani* D s0432-1 or derivative mutants were spotted onto the bioassay plate. (B) Bioassay with the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* in YPD agar plates. The experiment was repeated at least three times. The photograph was taken 24 h after incubation at 30°C.

303 **The *D. solani* zeamine cluster contributes to the killing of aphid.**

304

305 Since *D. dadantii* 3937 was previously shown to be able to kill aphids (Grenier *et al.*, 2006),
306 we tested the effect of *D. solani* D s0432-1 on these insects. Aphid survival was heavily
307 affected by infection with *D.solani*, similarly to what was observed with *D.dadantii*. Among the
308 three mutants analyzed, the mutant Δ*zmsA* was clearly altered in its virulence towards the pea
309 aphid, with a significant survival outcome over the WT strain (p=0.0008, Log-rank test) (Fig.
310 5). Mean lethal time was increased from 1.68 days ± [1.41-1.99] (mean ± *conf. interv.*) to 2.66
311 days ± [2.16-3.28]. The mutant Δ*oocL* seemed slightly affected for aphid survival, (Fig. 5), but
312 the results were not significant with the non-parametric tests used throughout (p=0.23), only
313 showing significance for a global Weibull model (p=0.027), with a mean survival time increased
314 to 1.90 days ± [1.50-2.41]. The mutant Δ*ssmG* was not affected in its capacity to kill aphids.
315 Globally however, and as this occurred for the *cyt* proteins toxin cluster in *D. dadantii* 3937
316 (Costechareyre *et al.*, 2010), the capacity of both mutants Δ*zmsA* and Δ*oocL* to kill aphids
317 remained high as all aphids were dead at the end of the 7 days period. Thus, the analyzed
318 clusters only supported partial causality for the aphid-killing phenotype harbored by *D. solani*
319 D s0432-1. In conclusion, these results showed that the zeamine cluster is partially implicated
320 in aphid killing by *D. solani* D s0432-1.
321

Figure 5



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323 **Figure 5. Bioactivity assay on pea aphids.** Survival curves of pea aphid individuals (n=30)
324 after 24h exposure to *D. solani* WT and mutants. Control survival with no infection, in green
325 (top). Curves fitted with Weibull distributions (dashed lines), showing Δ*zmsA* mutant with
326 significantly altered infection capacity (see text for statistical data).

Plant cell wall maceration capacity of the *D. solani* mutants affected for secondary metabolite production .

In *D. oryzae* EC1, the mutation of *zmsA* attenuates the inhibitory activity observed on rice seed germination (Zhou *et al.*, 2011). Thus, zeamine was suggested to be a phytotoxin that could affect virulence against plants.

We examined the role of clusters *ssm*, *ooc*, and *zms* on the ability of *D. solani* to infect chicory leaves. The *D. solani* WT strain and its derived mutants were inoculated on a small wound made on a chicory leaf. After overnight incubation, no difference in maceration was observed (Fig. 6), suggesting that the wild-type strain of *D. solani* does not require any of the three PKS/NRPS secondary metabolites to efficiently infect plants.

Figure 6

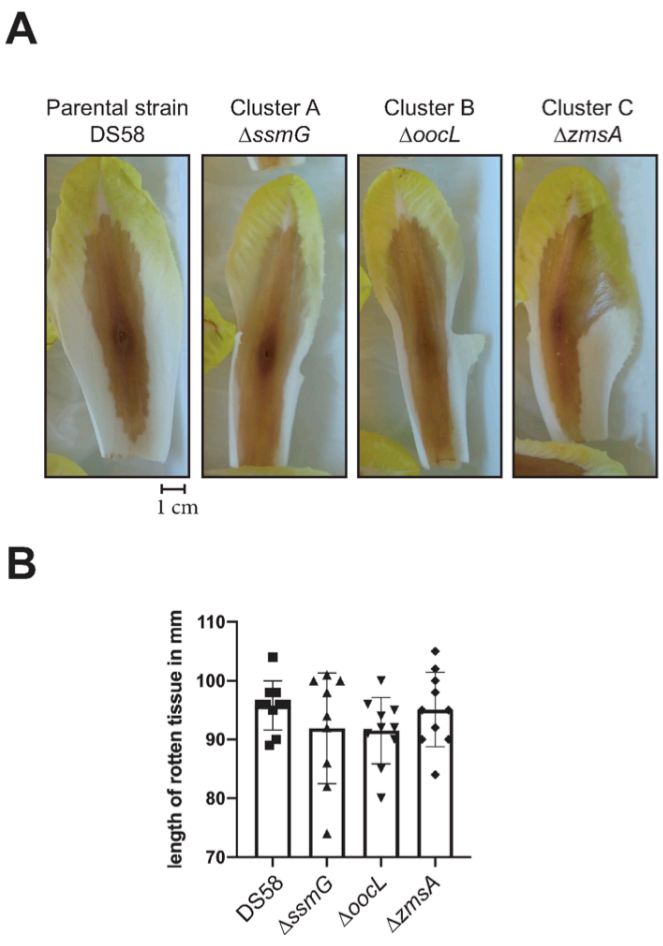

Figure 6. Maceration of chicory leaves by *D. solani* and mutant derivatives. (A) The photograph was taken 24 h after incubation at 30°C. (B) The length of rotten tissue was measured from 9 infected leaves. No statistical difference was measured between the different strains (Mann–Whitney U-test).

DISCUSSION

Secondary metabolite pathways are a great source of molecules with anti-eukaryotic or anti-prokaryotic activity giving the bacteria that synthesize them a competitive advantage over other organisms. The development of targeted mutagenesis of the *D. solani* chromosome has allowed us to specifically study the involvement of three secondary metabolite synthesis clusters encoded by all the *D. solani* strains for which genome sequence is available. We focused our work on D s0432-1, one of the most virulent *D. solani* strains (Golanowska *et al.*, 2018). We have demonstrated that this *D. solani* strain is able to inhibit the growth of a variety of living organisms. We tested bio-activities against Gram-negative bacteria (*E. coli*, *P. atrosepticum*, *D. dadantii*), Gram-positive bacteria (*B. subtilis*, *S. scabiei*), yeasts (*S. cerevisiae*, *K. lactis*), fungi (*B. cinerea*, *M. oryzae* and *S. sclerotiorum*), and pea aphids (*A. pisum*). The targeted mutagenesis approach revealed that each of the three secondary metabolite synthesis pathways studied has the capacity of inhibiting a particular type of organism. Cluster B , or *ooc*, encodes an enzymatic pathway that produces an oocydin-like molecule, a chlorinated macrocyclic lactone molecule having antifungal, anti-oomycete and antitumor activities in *S. plymuthica* (Matilla *et al.*, 2012, 2015). In contact with fungi *B. cinerea*, *M. oryzae* and *S. sclerotiorum*, *D. solani* D s0432-1 prevents their growth. It was previously shown that the *D. solani* strains MK10, MK16, IPO 2222, 3337, D-s0432-1 and GBBC 2040, all encoding the oocydin cluster, inhibit the growth of the fungus *V. dahliae* and the oomycete *P. ultimum* (Matilla *et al.*, 2015). Our study showed that inactivation of the *oocL* gene of this cluster in *D. solani* D-s0432-1 suppressed the inhibition of the three fungi but it had no apparent effect on the other organisms tested.

Our data also demonstrated that *D. solani* D s0432-1 has an anti-bacterial activity linked to cluster C, or *zsm*, which encodes a zeamine biosynthetic pathway. Zeamine produced by *S. plymuthica* kills *B. subtilis,* the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe,* and the nematode *C. elegans* (Hellberg *et al.*, 2015). In our study, we showed that *D. solani* D s0432-1, but not the *zmsA* mutant, can clearly inhibit the growth of two Gram-positive bacteria, *B. subtilis* and *S. scabies*. We tested *S. scabiei* because it is the principal causal agent of the common scab disease of potato (Loria *et al.*, 2006). Since *D. solani*, is also a potato pathogen, *D. solani* and *S. scabiei* might be in competition for the same ecological niche. It is thus interesting to note that *D. solani* can inhibit the growth of another potato pathogen. We also observed a slight inhibition exerted by *D. solani* D s0432-1 against *E. coli*, but no inhibition towards *D. dadantii* and *P. atrosepticum*. Zeamine resistance of *D. dadantii* could be explained by the presence in its genome of the genes *desAB* encoding the RDN pump DesAB involved

in zeamine efflux (Liang *et al.*, 2019). In-frame deletion of *desA* or *desB* in *D. oryzae* EC1 leads to a zeamine sensitive phenotype (Liang *et al.*, 2019). In *in planta* Tn-seq experiments with *D. dadantii* 3937 (Royet *et al.*, 2019), mutants of the genes *desA* and *desB* (Dda3937_00787 and Dda3937_00786 respectively) did not display a significant negative or positive variation (Log2 fold-changes -0.09 and +0.44, respectively), suggesting that this RND efflux pump does not play a significant role during *D. dadantii* plant infection, contrary to the *D. dadanti* RND efflux pump AcrAB that appeared to be essential for virulence (Royet *et al.*, 2019). *P. atrosepticum* does not have the genes *desAB* but it is not inhibited by *D. solani* D s0432-1. Another RND efflux pump could allow zeamine efflux, or zeamine resistance could be provided by a different mechanism.

We also showed that zeamine is involved in insect killing. We tested pea aphids because they are susceptible to infection by *D. dadantii* (Grenier *et al.*, 2006). The *D. solani* strain D s0432-1 is also able to kill pea aphids and the zeamine synthesis pathway (absent in *D. dadantii*) is partially implicated in pea aphid killing. We did not test inhibition of other multicellular organisms. Hellberg *et al.* tested nematodes with *S. plymuthica* A153 and a few *D. solani* strains (Hellberg *et al.*, 2015), they showed that *S. plymuthica* A153 but not its zeamine-deficient mutants can kill *C. elegans* in a few hours. In contrast, *D. solani* strains MK10, MK16, and IPO2222 that also carry the zeamine cluster behaves like the zeamine-deficient mutants of *S. plymuthica*. The authors suggested the *D. solani* zeamine cluster might be cryptic under their conditions or that it produces a zeamine molecule with somewhat different biological properties (Hellberg *et al.*, 2015).

Zeamine produced by *S. plymuthica* A153 is bioactive against *S. cerevisiae* and *S. pombe* (Hellberg *et al.*, 2015). We observed that *D. solani* D s0432-1 is bioactive against *S. cerevisiae* and *K. lactis*. However, the zeamine-deficient mutant of *D. solani* behaves exactly like the WT strain, ruling out the involvement of zeamine in yeast inhibition. By testing other mutants of the secondary metabolites clusters, we observed that the mutant s*smG* was totally unable to prevent the growth of *S. cerevisiae* and *K. lactis*. Therefore, the *ssm* cluster appears to be responsible for yeast inhibition. This cluster was not required for bioactivities against bacteria, aphids, and fungi. This result opens the way to the discovery of a new molecule with very specific activity against yeasts. The *ssm* cluster is only conserved in a few *Dickeya* genomes but not in other bacterial genera. The structure of the molecule and its target remains to be elucidated.

Finally, we assessed the virulence of the *D. solani* mutants Δ*ssmG*, Δ*oocL* and Δ*zsmA* by testing the maceration of chicory leaves. No difference was observed between the WT strain D s0432-1 DS58 and the mutants affected for the production of a secondary metabolite, indicating that the three secondary metabolites tested play no significant role in the degradation of the plant cell wall. Zeamine was described as a major virulence determinant of

D. oryzae since it is involved in inhibition of rice seed germination (Zhou *et al.*, 2011). Zeamine could have a phytotoxic activity mostly efficient at the level of seed germination.

In conclusion, *D. solani* D s0432-1 produces an arsenal of bioactive secondary metabolites against a variety of living organisms. The cluster *ssm* produces an unkown molecule active against yeasts; the cluster *ooc* produces an oocidin-like molecule active against fungi and the cluster *zms* produces a zeamine-like molecule active against bacteria and insects. The complementary activities of this set of molecules may have favored the rapid spread of *D. solani* in Europe by giving the capacity to compete with other microorganisms.

EXPERIMENTAL PROCEDURES

Bacterial and fungal strains, plasmids and growth conditions.

The *E. coli* and *Dickeya* bacterial strains, plasmids and oligonucleotides used in this study are described in Table S1 and S2. The genome accession number of *D. solani* D S0432-1 is NZ_CP017453. The following strains have been also used in the study: *Sclerotinia sclerotiorum* S5, *Botitris cinerea* B05.10, *Magnaporthe oryzae* strain Guy11, *Saccharomyces cerevisiae* BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0)*, *Kluyveromyces lactis* MWL9S1 (Wésolowski-Louvel, 2011), *Dickeya dadantii* 3937, *Pectobacterium atrosepticum* SCRI1043*, Streptomyces scabiei* CFBP4517, *Bacillus subtilis* PY79. *E. coli* was grown routinely at 37°C in LB. Fungus strains were grown at 25°C onto Potato Dextrose Agar (PDA). *S. scabiei* was cultivated in tryptic soy broth (TSB) medium at 28°C. *B. subtilis*, *P. atrosepticum* and the *Dickeya* strains were cultivated in LB unless specified. Yeast cells were grown at 30°C in rich medium consisting of complete yeast extract-peptone (YP) medium containing 1% Bacto yeast extract, 1% Bacto peptone (Difco) supplemented with 2% glucose (yeast extract-peptone-dextrose [YPD] medium). For the bacteria and yeast inhibition assay, M63 medium supplemented with sucrose and yeast extract (2 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, 2.5 mg $FeSO_47H_2O$, 0.2 g $MgSO_47H_2O$, 10 g sucrose, 0.1 g yeast extract, per liter) or LS4 medium supplemented with yeast extract (9.25 g K_2HPO_4, 3.3 g KH_2PO_4, 1.4 g NH_4NO_3, 12.7 g sucrose, 1 g KCl, 0.1 g yeast extract and 0.25 g $MgSO_4$, pH 7.0, per liter) (Liao *et al.*, 2014) were employed to grow the *D. solani* strains.

When required, antibiotics were added at the following concentrations: ampicillin (Amp), 100 µg/L; kanamycin (Kan), 50 µg/mL; nalidixic acid (Nal), 10 µg/mL; chloramphenicol (Cm), 20 µg/mL or 4 µg/mL for *E. coli* or *D. solani,* respectively. Diaminopimelic acid (DAP) (57 µg/mL) was added for the growth of the *E. coli* MFDpir strain. Media were solidified with 12 g/L agar.

PCR conditions

To amplify DNA for cloning, a bacterial suspension of the *D. solani* was prepared by boiling an isolated colony resuspended into 40 µl of sterilized water for 10 minutes at 95°C. The PCR was carried out in a 50 µl reaction mix containing 1 µl of bacterial suspension, 1.5 µl of 10 µM of each primer (Eurofins), 25 µL of primestar master mix 2x (Takara) and demineralized water. Thermocycling for amplification of a 1 kb upstream and downstream fragment of operon *araBA* consisted of 34 cycles of 98°C for 15 seconds, 55°C for 15 seconds and 72°C for 15 seconds. To check the correct deletion of a gene of interest in *D. solani*, a bacterial suspension was made by boiling an isolated colony resuspended into 40 µl of sterilized water for 10 minutes at 95°C. The suspension was centifuged 1min at 12000 g to remove cellular debris. The supernatant was used as a template for PCR. To amplify up to 3-kb DNA fragments, colony PCR was performed with DreamTaq Green PCR Master mix (2X) (Thermofisher, Waltham, MA, USA). However, the amplification from colonies with the dreamtaq kit did not work when the amplified fragments was larger than 3-kb. In this case the colony PCR was carried out with the Q5 High-Fidelity DNA Polymerase (Biolabs) by following manufacturer recommendations.

Construction of the Δ*araBA D. solani* mutants.

To construct the in-frame deletion Δ*araBA* of *D. solani*, the *sacB* counter-selection method was used (Edwards *et al.*, 1998). pRE112 is an R6K-based suicide plasmid carrying the *sacB* gene and the cat gene (Cm^R). The plasmid pRE112-Δ*araBA* was constructed by cloning simultaneously two PCR fragments corresponding to the upstream and downstream 1-kbp DNA of the *araBA* genes into SacI/KpnI digested pRE112 using the Gibson's assembly method. Chemical ultracompetent DH5α λpir cells were prepared with the Mix & Go! *E.coli* Transformation Kit using standard procedures (Zymo Research). They were transformed with 5 µl of the Gibson reaction. Transformants were selected onto LB plate supplemented with chloramphenicol. Transformants with the correct plasmid were found by colony PCR with oligo pairs L762/L763 and Dreamtaq DNA polymerase. Plasmids were extracted with the NucleoSpin Plasmid kit (Macherey-Nagel) and checked by restriction digestion (NEB) and sequencing (Eurofins).

Then, plasmids were transferred into competent *E. coli* strain MFDpir (Ferrières *et al.*, 2010) prepared with the TSS method (Chung *et al.*, 1989). *E. coli MFDpir* produces the RP4 conjugation machinery, which allows the transfer of the suicide plasmid into *D. solani* by conjugation. To do that, a few colonies of *D. solani* and MFDpir were mixed in the same proportion in 500 µl LB and centrifuged for 2 min at 8000 rpm. The pellet was resuspended in 90 µl LB with 5 µl DAP at 57 mg/mL, and deposited onto a LB agar plate incubated at 30°C. After 18h, the bacteria were resuspended in 1 ml LB, diluted in 10-fold series from 10^{-1} to 10^{-7} and spread onto LB agar supplemented with chloramphenicol at 4 µg/l to select the first event

of recombination. Transconjugants re-isolated on this medium were then spread onto LB agar without NaCl supplemented with 5% sucrose and incubated at 19°C for 2-3 days to allow the second event of recombination. Sucrose-resistant colonies were then replicated onto Mac Conkey medium supplemented with 1% arabinose incubated for 18h at 30°C to check the arabinose catabolism, and LB-Cm plate to check plasmid loss.

Construction of the secondary metabolite in-frame deletion mutants of *D. solani*.

A spontaneous mutant of *D. solani* D s0432-1 resistant to nalidixic acid (Nal^R) was obtained by growing the WT strain at 30°C in LB medium supplemented with Nal at 5 µg/mL for 18 h before spreading the liquid culture onto LB agar plates with Nal at 10 µg/mL. The Nal^R strain was named DS50 (Table S1). Then to discriminate in-frame deletion mutants from the wild-type DS50 strain, a Gm^R derivative of strain DS50 was constructed by insertion of a mini-Tn7-Gm cassette into the attTn7 site of *D. solani,* as previously performed with *D. dadantii* 3937 (Royet *et al.*, 2019) by using the plasmids pTn7-M (Zobel *et al.*, 2015) and pTnS3 (Choi *et al.*, 2008). The correct integration of the Gm^R cassette was then checked by colony PCR using oligo pairs L365/L848 (amplification of 350 bp for the correct integration) (Table S2). This gave the Nal^R Gm^R strain DS58. Subsequently, the in-frame deletion mutations Δ*ssmG* (NCBI Reference Sequence: WP_022634121.1; cluster A), Δ*oocL* (NCBI Reference Sequence: WP_023638021.1; cluster B) and Δ*zsmA* (NCBI Reference Sequence: WP_022632849.1; cluster C) were constructed in strain DS58. Derivatives of the pRE112 suicide plasmids containing upstream and downstream 500-bp DNA of the gene to delete were also constructed (Table S2). In-frame deletions were checked by PCR as previously described (Table S2).

Growth inhibition assay of bacteria and yeast.

First, *B. subtilis*, *E. coli*, *D. dadantii* and *P. atrosepticum* were grown overnight in LB at 30°C with shaking. LB agar was cooled until the temperature reached about 50°C (just before the agar re-solidified). The OD_{600} of the overnight culture of *B. subtilis*, *E. coli*, *D. dadantii* or *P. atrosepticum* were adjusted to 1 with fresh LB. 100 mL of the LB agar in surfusion were mixed with 100 µl of the OD_{600} 1 culture of *B. subtilis*, *E. coli*, *D. dadantii* or *P. atrosepticum* . 25 mL of inoculated LB agar was poured in 10- by 10-cm square plates. The plates were dried for a few hours. *D. solani* strains were grown for 24h at 30°C with shaking in M63 medium supplemented with sucrose and yeast extract or in LS4 medium supplemented with yeast extract. The OD_{600} of the *D. solani* cultures were adjusted to 1 and 5 µl were spotted onto the inoculated square plates which were incubated at 30°C for 24-48 h before visualization of the inhibition zone.

The same experiment was conducted with *Streptomyces scabiei*, except that it was grown for 3 days in TSB at 28°C and TSB agar was poured in the square plates. With the yeasts *S. cerevisiae* and *K. lactis*, YPD medium was used.

Growth inhibition of fungal strains.

S. sclerotiorum, *B. cinerea* and *M. oryzae* were grown onto PDA plate at 25°C for 5, 7 and 10 days respectively. At the start of the experiments, Colonies of *D. solani* D s0432-1 strains were resuspended in M63 medium and OD_{600} was adjusted to 0.1. Then, 10 µl of the bacterial suspensions were deposited at the center of a 90-mm Petri dish with two 5 mm agar plugs of fungus at 4.5-cm around. The radius of fungus inhibition zone was measured.

Pea aphid survival assay

Aphid survival assays were performed at 20-25°C as previously described (Grenier *et al.*, 2006). Pea aphid (*Acyrthosiphon pisum*, LL01 clone, alfalfa strain reared on faba bean) individuals aged 0-24 h were transferred on Ap3 diet (Febvay *et al.*, 1988) loaded with 10^7/mL cells of *D. solani* D S0432-1 or its corresponding mutants. After a full day on diet, aphids were transferred on single *V. faba* plantlets and scored daily for survival up to the 7^{th} day. Survival data were analysed with JMP software (SAS Inc) and its survival platform, and data adjusted through Kaplan-Meier graphing and analysis, followed by a Weibull fit (best of tested log-normal or exponential fits). Data are fully compatible with previously published work on *D. dadantii* (Costechareyre *et al.*, 2010), except that temperature was less stringently controlled.

Chicory inoculation experiments

The *D. solani* WT strain and the different mutants were grown overnight in LB medium. Bacteria were washed in M63 medium and OD_{600} was adjusted to 1. One microliter of the bacterial suspension was inoculated into a small hole made into the leaves by a yellow pipette tip. The wound was covered with mineral oil and the leaves were incubated at 30°C at high humidity for 24 h. The length of rotten tissue was measured.

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568

569 **AUTHOR CONTRIBUTION**

570

571 E.G., B.T, R.Y and G.E carried out the experiments. G.E and H-C-P.N wrote the manuscript
572 with support from E.G, B.T and R.Y. H-C-P.N and G.E conceived the original idea. G.E
573 supervised the project. All authors provided critical feedback and helped shape the research,
574 analysis and manuscript.

575

576 **CONFLICT OF INTEREST**

577 The authors state that the research was conducted in the absence of any commercial or
578 financial relationship that could be interpreted as a potential conflict of interest.

579

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583

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SUPPLEMENTAL DATA

Figure S1. Plate-based bioassays against *B. subtilis* and *K. lactis* for metabolite production by *Dickeya solani* WT cultivated in various medium.

Table S1. Strains and plasmids used in the study.

Table S2. oligonucleotides used in the study.