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# *Pseudomonas putida* KT2440 causes induced systemic resistance and changes in Arabidopsis root exudation

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## Summary

***Pseudomonas putida* KT2440 is an efficient colonizer of the rhizosphere of plants of agronomical and basic interest. We have demonstrated that KT2440 can protect the model plant *Arabidopsis thaliana* against infection by the phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000. *P. putida* extracellular haem-peroxidase (PP2561) was found to be important for competitive colonization and essential for the induction of plant systemic resistance. Root exudates of plants elicited by KT2440 exhibited distinct patterns of metabolites compared with those of non-elicited plants. The levels of some of these compounds were dramatically reduced in axenic plants or plants colonized by a mutant defective in PP2561, which has increased sensitiveness to oxidative stress with respect to the wild type. Thus high-level oxidative stress resistance is a bacterial driving force in the rhizosphere for efficient colonization and to induce systemic resistance. These results provide important new insight into the complex events that occur in order for plants to attain resistance against foliar pathogens.**

*Pseudomonas putida* KT2440 is used as a model bacterium in biodegradation studies. KT2440 was initially derived from the laboratory domesticated strain *P. putida (arvilla)* mt-2, which is the natural host for the catabolic pWW0 plasmid. The metabolic versatility and its vast ability to scavenge iron have been considered, among other features, to be crucial for KT2440 to adapt to multiple niches (Martínez-Bueno *et al.*, 2002; Dos Santos *et al.*, 2004). Recent studies involving KT2440 have demonstrated it to be capable of an efficient colonization of the spermosphere (Espinosa-Urgel *et al.*, 2000) and rhizosphere of plants of agronomical interest (Molina *et al.*, 2000). In fact, Nakazawa (2002) reported that this bacterium was initially isolated from soil taken from a vegetable garden. This origin provides an explanation for the ability of KT2440 to colonize roots and also suggests that the diversity of nutrients and plant secondary metabolites present in root exudates provide a satisfactory habitat for this bacterium. Therefore, exploration of the use of the agrobiotechnological potential of this strain, i.e. biocontrol, is of 'a priori' interest. Efficient colonization of the rhizosphere appears to be a requirement for an effective display of bacterial biocontrol properties as antagonism (Chin-A-Woeng *et al.*, 2000). Besides exhibiting direct mechanisms against plant pathogens, such as niche and resource competition (Lemanceau *et al.*, 1992; Bolwerk *et al.*, 2003), some specific non-pathogenic bacteria, while colonizing the root, can protect plants from pathogen infections in leaf tissues (van Peer *et al.*, 1991; Wei *et al.*, 1991; Kloepper *et al.*, 1992; van Loon *et al.*, 1998; Bakker *et al.*, 2003). This type of resistance is termed induced systemic resistance (ISR). In Arabidopsis, rhizobacteria-mediated ISR requires intact responses to jasmonic acid (JA) and ethylene (ET) (Pieterse *et al.*, 1998). Another type of systemic resistance known as systemic acquired resistance (SAR) (Ross, 1961) is induced by pathogens. SAR is characterized by an accumulation of endogenously synthesized salicylic acid (SA) (Malamy *et al.*, 1990; Métraux *et al.*, 1990) and the expression of pathogenesis-related proteins (van Loon and van Strien, 1999).

## *Pseudomonas putida* KT2440 induces systemic resistance in Arabidopsis

In this work we have evaluated the potential of KT2440 to promote systemic resistance in plants and further investigated the so far unknown contribution of certain bacterial root colonization traits to elicitation of ISR. For studying ISR, the model plant pathogen system *Arabidopsis*

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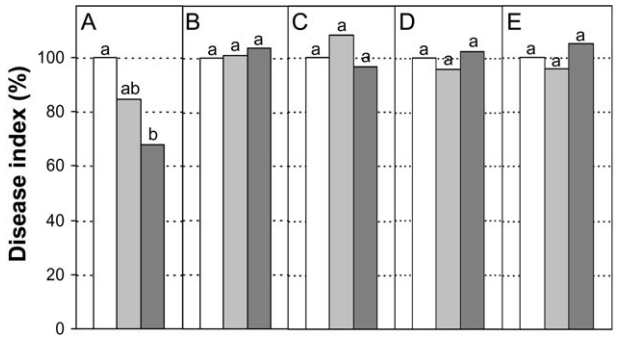


**Fig. 1.** Induced resistance capacity of KT2440 in different genotypes of *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). For the characterization of KT2440-mediated achievement of ISR, several strains of Arabidopsis were used: A, wild-type ecotype Columbia (Col-0); B, mutant *npr1*; C, mutant *jar1*; D, mutant *etr1*; and E, transgenic NahG plants (Table 1). Two-week seedlings were transplanted to soil inoculated with *P. putida* KT2440R (KTR) at final densities of $5 \times 10^3$ cfu $g^{-1}$ (pale grey) and $5 \times 10^7$ cfu $g^{-1}$ (dark grey). In control plants (white) substrate was supplemented with $MgSO_4$ 10 mM. Six-week-old plants were challenged by dipping the leaves in a suspension of *Pst* DC3000 containing $2.5 \times 10^7$ cfu $ml^{-1}$. A detailed experimental procedure is described in Appendix S1. Disease severity was determined 4–5 days after infection by calculating the percentage of diseased leaves per plant. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis as described before (Pieterse *et al.*, 1996). Rate of diseased leaves per plant line differed as follows: 58% (Col-0), 72% (*npr1*), 52% (*jar1*), 66% (*etr1*) and 66% (NahG). Data were statistically analysed using one-way analysis of variance followed by Fisher's LSD test ($n = 20$ plants; $\alpha = 0.05$). Different letters indicate significant difference between treatments. Disease index corresponds to the mean in the percentage of diseased leaves per treatment relative to that of the control plants (100%). One experiment representative of three replicates is shown.

*thaliana* – *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) was used as described by Pieterse and colleagues (1996). Briefly, 2-week-old seedlings were planted in potting soil containing *P. putida* cells at two different densities. After 4 week incubation, the aerial parts of the plants were dipped in a suspension of *Pst* DC3000. The severity of lesions in the leaves was evaluated 4–5 days after infection. Figure 1 shows that KT2440 caused a significant drop in the incidence of disease, with more than a 30% reduction in the disease index, when the higher density of bacteria was present in soil ($10^7$ cfu per gram of substrate). As KT2440 could not be detected on challenged leaves by standard detection techniques (detection limit below 100 cfu per about 15 leaves), we concluded that this effect is mediated by ISR. Although $10^7$ cfu $g^{-1}$ is a standard density used with a substantial number of strains for the establishment of ISR in Arabidopsis against *Pst*, inoculum size has been shown to be crucial for *Pseudomonas fluorescens* WCS374, which provides effective protection, in this case, only at a low rhizobacteria density of $10^3$ cfu $g^{-1}$ (Djavaheri, 2007).

In order to identify plant signalling pathways required for the establishment of ISR by KT2440, different Arabidopsis lines (Table 1) were assayed and compared with the wild-type accession Columbia (Col-0): *jar1*, a JA response mutant (Staswick *et al.*, 1992); *etr1*, an ET-insensitive mutant (Bleecker *et al.*, 1988); transgenic NahG plants (Delaney *et al.*, 1994) expressing the bacterial *nahG* gene encoding salicylate hydroxylase, which is responsible for the removal of SA and is therefore impaired in SA signalling; and *npr1*, a mutant in the NPR1 transcriptional regulator at the convergence of both ISR and SAR pathways (Cao *et al.*, 1994). The finding that *jar1*, *etr1* and *npr1* lines were unable to develop ISR (Figs 1B-D) was not surprising because both the JA and ET signalling pathways have been shown to be essential for ISR induced by *P. fluorescens* WCS417r in Arabidopsis (Pieterse *et al.*, 1998). However, our results show that for KT2440, plant-produced SA seems to be important to ISR in Arabidopsis, because NahG plants did not exhibit

**Table 1.** Bacterial strains and plant lines.

| Bacteria and plants | Relevant characteristics | Reference |
|---|---|---|
| Bacterial strains | | |
| *P. putida* KT2440 | Wild type | Nakazawa (2002) |
| *P. putida* KT2440R (KTR) | Rif$^r$ | Espinosa-Urgel and Ramos (2004) |
| *P. putida* KTRTn*7*-Sm | miniTn*7*-Sm intergenic specific insertion in KTR; Rif$^r$, Sm$^r$ | Matilla *et al.* (2007) |
| KTR PP2561 | miniTn*5* at PP2561, a $Ca^{2+}$ binding haem peroxidase; Rif$^r$, Km$^r$ | Matilla *et al.* (2007) |
| *P. syringae* pv. *tomato* DC3000 | Pathogen of Arabidopsis | Cuppels (1986) |
| Plant lines | | |
| *A. thaliana* | Ecotype Columbia (Col-0) N1092 | L.C. van Loon |
| *A. thaliana etr1* | ET insensitive mutant | Bleecker *et al.* (1988) |
| *A. thaliana jar1* | JA response mutant | Staswick *et al.* (1992) |
| *A. thaliana npr1* | Mutant in defence regulatory protein NPR1 at the convergence of JA and SA signalling pathways | Cao *et al.* (1994) |
| *A. thaliana* NahG | Transgenic plant expressing the bacterial *nahG* gene encoding salicylate hydroxylase | Delaney *et al.* (1994) |

Km$^r$, kanamycin resistant; Rif$^r$, rifampin resistant; Sm$^r$, streptomycin resistance; ET, ethylene; JA, jasmonic acid; SA, salicylic acid.

induction and/or expression of ISR (Fig. 1E), in contrast to the Arabidopsis/WCS417r model system (Pieterse *et al*., 1996). Yet, a similar result as for KT2440 has been reported for the salicylate siderophore-producing bacteria *Pseudomonas aeruginosa* 7NSK2 in tobacco (De Meyer *et al*., 1999) but in this case, bacterial produced SA was the unique determinant of ISR. Nevertheless, 7NSK2 can elicit ISR in NahG Arabidopsis (Ran *et al*., 2005), differently from what was observed with KT2440. One possibility to explain our result is that deprivation of plant-produced SA was detrimental for the achievement of systemic resistance by KT2440 and therefore this strain might elicit both the ISR and the SAR pathway. In view of the observation of van Wees and colleagues (2000), this simultaneous activation of both pathways might lead to increased disease suppression. Alternatively, from our experiments we can not rule out a pleiotropic effect in NahG plants related to the accumulation of catechol (van Wees and Glazebrook, 2003). In this context, bacterial colonization of the NahG Arabidopsis root has been demonstrated to be hampered (Rudrappa *et al*., 2007) and the subsequent lack of the plant–bacteria interaction is expected to be crucial for ISR elicitation. Further studies considering a wider variety of rhizobacteria and plant mutants are required to generalize the requirement of signalling molecules such as SA, ET and JA on the induction of ISR.

### Haem peroxidase mutant fails to elicit ISR

While root colonization by specific bacteria of many different genera can reduce disease in foliar tissue against various pathogens, bacterial ISR determinants that have been identified so far seem to be specific to the pair plant/pathogen (Meziane *et al*., 2005). Bacterial traits that have been shown to be important for ISR are flagella, lipopolysaccharide, certain antibiotics, and iron chelating and iron regulated compounds (Höfte and Bakker, 2007). Bacterial volatiles (Ryu *et al*., 2004) and the quorum-sensing molecules N-acyl-L-homoserine lactones (Schuhegger *et al*., 2006) have also been shown to trigger ISR. We have observed that close physical interaction occurs between plant root and bacteria by using scanning electron microscopy (our unpublished results). On this basis and with the aim of unveiling putative determinants for effective ISR between Arabidopsis and KT2440, we focused on bacterial traits preferentially expressed in the rhizosphere and with a putative extracellular location. For this we took advantage of recently published bacterial *rup* (rhizosphere upregulated) genes, identified as consistently induced in the first genomic analysis of a bacteria in the rhizosphere, which was carried out with KT2440 (Matilla *et al*., 2007). It was reported in this work that a bacterial mutant in PP2561, a large haem peroxidase 3619 amino acids long, was hampered in competitive colonization of the maize rhizosphere and same result has been observed with independent mutants at different positions of the gene (not shown). Flanking the monocistronic gene encoding PP2561 and divergently transcribed was *rup2560*, which had been identified by its induction in the rhizosphere as mentioned above for the *rup* genes. The PP2560 protein is annotated as a type I secretion system (TISS) ATPase. This TISS is encoded in the negative strand by what appears to be an operon consisting of the orfs PP2558 through PP2560. PP2561 is very likely a protein secreted by the flanking TISS. This protein presents a serralysin C-terminal domain, containing calcium-ion binding domains, which is found within most, if not all, gene products secreted by the type I pathway (Delepelaire, 2004). The analysis of available bacterial genomes reveals that PP2561 is *P. putida* species-specific. Our results in Arabidopsis with strain PP2561 showed that this mutant was severely hampered in competitive colonization with the wild type (Fig. 2A), such that after 12 days in the rhizosphere the wild-type strain had displaced the mutant from 50% to 15%. This result corroborated previous findings with maize plants (Matilla *et al*., 2007). We also found that the mutant PP2561 was more sensitive to oxidative stressors such as cumene and tertbutyl hydroperoxides than the wild type (our unpublished results) and that the colony size of this strain was smaller than that of the wild type after recovery from the rhizosphere. It is important to note that this mutant strain did not appear to be compromised in seed adhesion (M. Espinosa-Urgel, pers. comm.), nor in its ability to colonize the rhizosphere of Arabidopsis in the absence of competition (Fig. 2B). Importantly, we found that PP2561 mutant was unable to induce systemic resistance (Fig. 3). As ISR experiments were performed with KT2440 in competition with native bacteria present in the seeds, the compromised efficiency in competitive colonization of mutant PP2561, likely due to its increased sensitiveness to oxidative stress, seems to be responsible for its inability to achieve ISR.

### Changes in the profile of Arabidopsis root exudates in response to KT2440

To our best knowledge no compounds have been identified in root exudates that are specifically involved in ISR, except for galactinol that has been recently identified as a signal of ISR in leaves (Kim *et al*., 2008). Having found a *P. putida* KT2440 trait, PP2561, that is crucial for ISR in Arabidopsis, we decided to explore whether we could use this mutant in order to characterize ISR-elicited plant-specific responses that occur at the rhizosphere. One such response that can be measured is changes in the composition of compounds present in root exudates. It has been proposed that root exudates

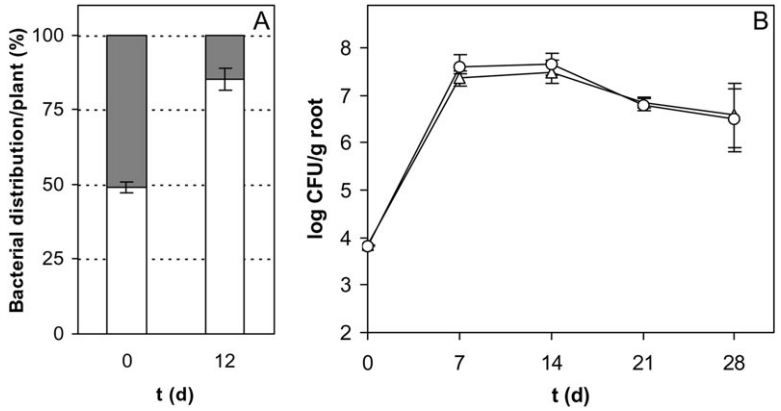


**Fig. 2.** Colonization ability of *P. putida* wild-type and *rup* mutant strains in the rhizosphere of *A. thaliana*. KTRTn*7*-Sm (Matilla *et al.*, 2007) was used as the wild-type strain in the experiments in order to utilize its resistance marker in competition assays with the PP2561 mutant. KTRTn*7*-Sm and KTR strains are equally competitive in root colonization (not shown). Competitive colonization of KTRTn*7*-Sm and PP2561 mutant (A), independent colonization of KTRTn*7*-Sm and PP2561 mutant (B). Seeds were surface sterilized in bleach for 1 min vortexing and rinsed five times in sterile distilled water. Germination took place on Musharige and Skoog (MS)-agar (0.8%). Commercial MS medium (Caisson laboratories, Inc MSP P0509) adjusted to pH 5.8 was supplied with sucrose 1% (w:v). The obtained 7 day seedlings were transplanted, incubated and watered as described in *Supporting information* (Appendix S1). For assessing colonization, roots were vortexed for 1 min in $MgSO_4$ 10 mM and serial dilutions were plated on KB-agar (King *et al.*, 1954) (1.5%) medium supplied with cycloheximide, 40–100 μg $ml^{-1}$, rifampin, 10 μg $ml^{-1}$, and streptomycin, 100 μg $ml^{-1}$, for selecting KTRTn*7*-Sm, or kanamycin, 25 μg $ml^{-1}$ for selecting the *rup* mutant respectively. In A, white and grey corresponds to the KTRTn*7*-Sm and PP2561 mutant strains respectively. In B, cfu attached per g of seed is shown at the initial time. Circles correspond to KTRTn*7*-Sm and triangles to PP2561. Error bars represent the standard deviation for 10 (A) and six plants (B) respectively. Colonization assays were highly reproducible between experiments.

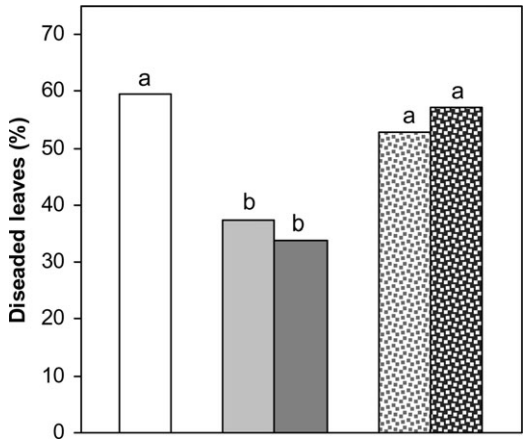


**Fig. 3.** Induced resistance capacity of *rup* mutants against infection of *Arabidopsis thaliana* Col-0 by *Pst* DC3000. Experiments and statistical analysis were carried out as described in the legend for Fig. 1. For the treatment with KTR, substrate was inoculated to a final density of $5 \times 10^3$ cfu $g^{-1}$ (pale grey) and $5 \times 10^7$ cfu $g^{-1}$ (dark grey) respectively. Identical treatments were used for PP2561 strain (pale and dark dotted). Control plants are shown in white. Experiments were repeated three times. At the lower inoculum density of KT2440, less reproducibility among experiments was observed (Figs 1 and 3). The PP2561 strain is a mutant derivative of KTR and exhibits kanamycin resistance due to the miniTn*5* transposon insertion (Matilla *et al.*, 2007). The transposon insertion site, between residues 733 and 734, mapping to the first peroxidase domain, was determined by using arbitrary PCR as reported previously (Espinosa-Urgel *et al.*, 2000).

are important to mediate plant–plant and plant–microbes interactions in the rhizosphere (Bais *et al.*, 2006 and references therein). Furthermore, evidence exists suggesting that the composition of root exudates varies in response to changes in plant–microbe interactions (Phillips *et al.*, 2004; Kamilova *et al.*, 2006). Walker and coworkers demonstrated that chemical and biological (fungal) elicitation induced root secretions containing compounds with antibacterial, antifungal and allelochemical properties (Walker *et al.*, 2003). Modifications in the plant exudates have been reported to not only be linked to elicitation but also to increased susceptibility of the plant to disease. Thus, Bais and colleagues (2005) demonstrated that bacterial pathogens can avoid the presence of antimicrobials in plant exudates, in order to bring about their own infection. This indicates that changes in root exudation represent a key feature through which putative compounds involved in communication between plants and bacteria may be characterized. Furthermore, it appears that changes in exudates might occur during ISR, because the exogenous application of methyl jasmonate to plants, an analogue of JA, which is a signalling molecule for ISR in some bacterial strains, induced important increases of phytochemicals in root exudates (Badri *et al.*, 2008).

In order to gain insight into KT2440-mediated ISR, we compared the root exudates profile of sterile *Arabidopsis thaliana* cultivated '*in vitro*' and gnotobiotic plants incubated with KT2440, by using high-performance liquid

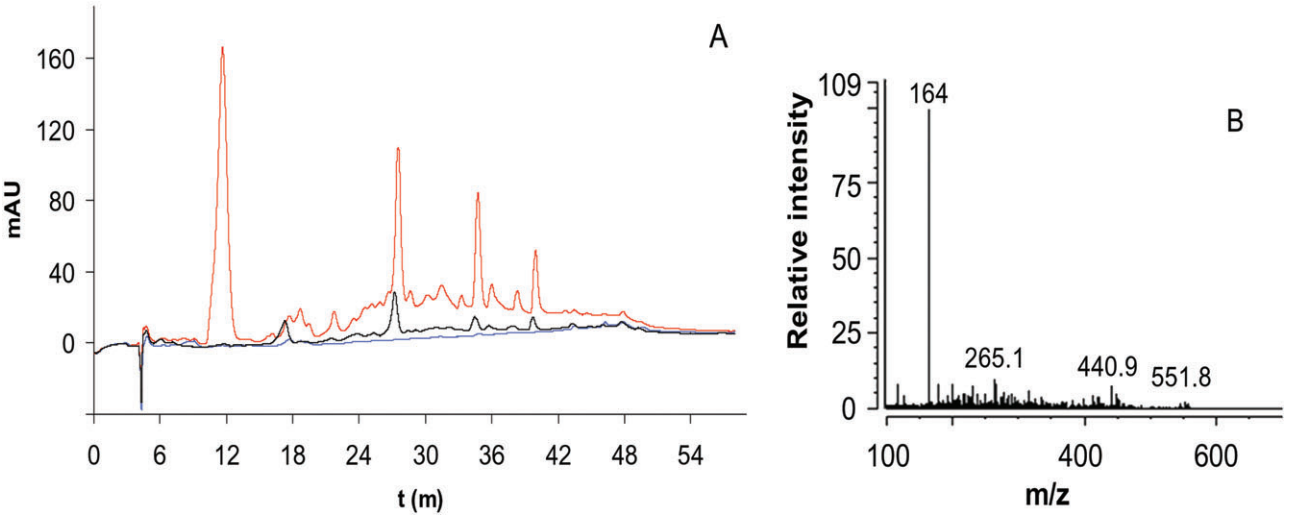


**Fig. 4.** Effect of the KT2440 bacteria upon the exudates profile of Arabidopsis.
A. HPLC chromatograms of plant secondary metabolites profile (black, plants control; red, bacteria-treated plants; blue, bacterial supernatant) at 254 nm. Treatment experiments were carried out in triplicate and each replicate contained either the pooling of 12 plants exudates (4 ml/plant) or 48 ml of bacterial suspension. Plant exudates and bacterial supernatant were centrifuged, filter sterilized, freeze-dried and dissolved in water acidified to pH 2.8. Samples were partitioned with ethyl acetate (1:1; v : v) two times and the organic phase was collected and dried under a nitrogen atmosphere. The dried samples were dissolved in methanol and submitted to HPLC on an Ascentis C18 reverse phase column (5 μM particle size 25 cm × 4.6 mm, Sigma 581340-U), which was coupled to a quadrupole mass spectrometer (MSQ-MS, Thermo Electron Co., Waltham, MA). Samples were loaded onto the column equilibrated with an aqueous solution containing 10% methanol and 0.1% acetic acid. Compounds were eluted by a linear gradient increasing the methanol concentration to 90% during 40 min followed by an isocratic step for additional 15 min. At all times a flow rate of 0.7 ml $min^{-1}$ was applied.
B. Mass spectrum of the principal HPLC peak (at 11 min retention time) recorded in the positive ionization mode. In the negative ionization mode the major peak of this sample was at a m/z ratio of 162. No major differences were observed among replicates. Experiments were repeated three times with high reproducibility.

chromatography-mass spectrometry (HPLC-MS) analyses. To obtain the exudates, 7-day-old axenic seedlings were transferred to 4 ml MS medium supplied with sucrose 1% in 6 well plates and incubated under low light intensity (70 μE $ms^{-2}$, 15/9 h light/dark, 24°C and 20°C respectively). Eighteen-day-old plants were gently washed with sterile distilled water, transferred to either fresh MS medium, or MS inoculated with *P. putida* KT2440 at $10^5$ cfu $ml^{-1}$. Plants were further incubated for 24 h. To obtain bacterial supernatant as a control to compare with, an overnight Luria–Bertani culture was washed in MS media and a bacterial suspension in same media containing $10^5$ cfu $ml^{-1}$ was incubated for 24 h. An interesting finding in our study was that KT2440 increased the organic phase of the root exudation (Fig. 4A). Similar observations were made by Meharg and Killham (1995) who reported that perennial ryegrass increased the secretion of root exudates by 12% in the presence of metabolites derived from *P. aeruginosa*. We do not think that the enhancement of exudation in our experiments was caused only by the lowered exudates concentration in the vicinity of the root, as a consequence of the presence of exudates-utilizing bacteria, given that axenic plants exudates were replaced with fresh medium and so the exudates concentration also reduced. A similar finding to ours has been reported for *P. putida* in wheat (Přikryl and Vančura, 1980). Upon subjecting the organic phase of the exudates to HPLC, we observed a principal peak with a retention time of ~11 min in the exudates of KT2440-incubated plants, unnoticeable both in the exudates of plant alone and in the supernatant of 24 h MS-cultivated bacteria (Fig. 4A). The area of the peak increased with the bacterial inoculum size (between $10^5$ and $10^7$ cfu $ml^{-1}$) and with the incubation time up to 72 h (not shown). Using mass spectrometry in the positive- and negative-ionization mode, mass to charge ratios of 164.0 and 162.0, respectively, were assigned to this principal peak under acidic conditions (Fig. 4B). The maximum of UV spectrum for this peak was 272 nm, which is consistent with an aromatic nature. Analyses of other peaks in the higher molecular weight range indicated that these mass traces correspond to the monocharged state of the compound, consistent with a mass of 163.0 Da. This compound was not found in bacteria-free supernatants of MS-cultivated bacteria. However, trace quantities of a compound with same retention time and mass were detected in the samples of axenic Col-0 plant exudates as an indication that the compound is derived from the plant (not shown).

In order to determine whether this 163 Da compound is involved in ISR, we compared the levels of this compound present in root exudates between Arabidopsis incubated

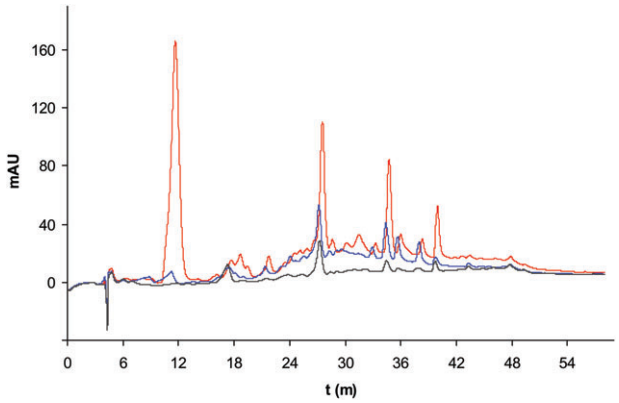


**Fig. 5.** Exudates profiles of Arabidopsis plants in the presence of the wild-type KT2440 and the mutant PP2561 negative in ISR. Methods used were similar to those described in Fig. 4A, except that plants were incubated with KT2440 (red) or with mutant PP2561 (blue); grey chromatogram corresponds to plants control.

with wild-type KT2440 versus the mutant PP2561, which we have shown is unable to stimulate ISR. Bacterial densities of wild-type and mutant strains growing in Arabidopsis root exudates were similar, although because of a smaller size of the PP2561 mutant cells, the turbidity reached in the latter case was diminished. The profile of secondary metabolites in these exudates is shown in Fig. 5. Along with a reduction in several peaks within the exudates of plants colonized by the mutant PP2561, the peak corresponding to the 163 Da compound showed a dramatic reduction versus the wild-type strain (more than 30-fold). As an extra measure, we also subjected aliquots of the exudates 'ex-planta' from anexic plants to *P. putida* KT2440 versus the mutant PP2561 and a very similar profile of metabolites was obtained in both cases, thus excluding the 163 Da compound being the result of any bacterial modification that the mutant was unable to perform (data not shown). It seems therefore that the presence of these compounds in plant exudates is important for ISR, and that they may provide an important readout of the plant–bacteria signalling that is required for successful ISR. Whether the extracellular haem peroxidase encoded by locus PP2561 might have a role in inducing systemic resistance by other means that affecting competitive colonization when added '*in vitro*' is subject of current work. In addition, further biochemical characterization of this protein will help to understand the possible role that a haem peroxidase could have in inducing systemic resistance.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Experimental details.

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