Role of *Dickeya dadantii* 3937 chemoreceptors in the entry to Arabidopsis leaves through wounds

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**SUMMARY**

Chemotaxis enables bacteria to move towards an optimal environment in response to chemical signals. In the case of plant-pathogenic bacteria, chemotaxis allows pathogens to explore the plant surface for potential entry sites with the ultimate aim to prosper inside plant tissues and to cause disease. Chemoreceptors, which constitute the sensory core of the chemotaxis system, are usually transmembrane proteins which change their conformation when sensing chemicals in the periplasm and transduce the signal through a kinase pathway to the flagellar motor. In the particular case of the soft-rot pathogen *Dickeya dadantii* 3937, jasmonic acid released in a plant wound has been found to be a strong chemoattractant which drives pathogen entry into the plant apoplast. In order to identify candidate chemoreceptors sensing wound-derived plant compounds, we carried out a bioinformatics search of candidate chemoreceptors in the genome of *Dickeya dadantii* 3937. The study of the chemotactic response to several compounds and the analysis of the entry process to Arabidopsis leaves of 10 selected mutants in chemoreceptors allowed us to determine the implications of at least two of them (ABF-0020167 and ABF-0046680) in the chemotaxis-driven entry process through plant wounds. Our data suggest that ABF-0020167 and ABF-0046680 may be candidate receptors of jasmonic acid and xylose, respectively.

**Keywords:** chemoreceptors, *Dickeya dadantii* 3937, entry, jasmonic acid, xylose.

**INTRODUCTION**

*Dickeya dadantii* 3937 (Dd3937) is an enterobacterium included in the Top 10 List of phytopathogenic bacteria. This catalogue is based on the scientific/economic importance of the plant bacterial pathogens included (Mansfield *et al*., 2012). *Dickeya dadantii* causes soft rot in a wide range of plant species, including important crops, such as potato (Charkowski *et al*., 2012). Several mechanisms are needed for the adaptation and virulence of *D. dadantii* inside the plant. The most important virulence factor is the production of a large set of enzymes and isoenzymes (pectate lyases, polygalacturonases, pectin methylesterases, etc.) that disassemble the plant cell wall (Barras *et al*., 1994). Other processes involved in the adaptation of *D. dadantii* to the hostile plant conditions are as follows: (i) iron uptake, for which *D. dadantii* synthesizes two siderophores: chrysobactin and achromobactin (Munzinger *et al*., 2000; Persmark *et al*., 1989); (ii) mechanisms to counteract the presence of plant toxic substances, such as multidrug resistance (MDR) efflux pumps (Barabote *et al*., 2003; Maggiorani Vallecillos *et al*., 2006); (iii) other mechanisms to overcome the presence of antimicrobial compounds (Costechareyre *et al*., 2013; Llama-Palacios *et al*., 2003, 2005; López-Solanilla *et al*., 1998; 2001; Río-Alvarez *et al*., 2012). Moreover, the *hrp*-encoded type III secretion system (T3SS) is required for the full virulence of *D. dadantii* (Bauer *et al*., 1994; Yang *et al*., 2002; Yap *et al*., 2005).

All the above-cited mechanisms are needed for the adaptation and virulence of *D. dadantii* inside the plant. Nevertheless, bacteria spend the majority of their life outside the plant and bacterial entry into the plant is critical. The life cycle of *D. dadantii* during the infection of potatoes requires adhesion to the plant surface and penetration into the plant tissues, either via wound sites or through natural openings, such as stomata (Reverchon and Nasser, 2013). For this, an active bacterial movement towards entry sites is required. Chemotaxis, which enables bacterial cells to move towards certain stimuli and away from others (Porter *et al*., 2011), is crucial for the colonization of the host and for the establishment of a successful infection (Moen and Vanderleyden, 1996).

The bacterial chemotaxis system is the best-studied biological gradient sensor and has been analysed in detail in *Escherichia coli*. ...
Bacterial chemoreceptors are hexagonally packed trimers of receptor dimers (Briegel et al., 2012) that recognize specific chemicals (even redox state) at the periplasm and regulate the flagellar function, assisted by a signal transduction pathway composed of the so-called Che proteins (Hazelbauer and Lai, 2010). The structure of a typical bacterial chemoreceptor is as follows: (i) a sensor domain or ligand-binding region (LBR), which can be either periplasmic or cytoplasmic, and which detects chemical signals and induces a conformational change; (ii) a HAMP-type adaptation domain (cytosolic), which transmits the molecular stimulus created by ligand binding at the LBR to the: (iii) MCP domain or signalling domain (cytosolic), which undergoes reversible methylation at multiple sites and transduces the signal to the downstream signalling cascade of Che proteins (Falke and Hazelbauer, 2001). Despite the fact that the chemotaxis system is conserved among bacteria, chemoreceptors differ largely in their protein topology. A large-scale comparative genomic analysis of the MCP signalling and adaptation domain family (from 312 prokaryotic genomes) allowed the identification of seven major conserved MCP signalling domain. The Dd3937 chemoreceptors were classified into six different topologies (Fig. 1A), as described previously (Lacal et al., 2010a). The amino acid sequences of these 47 chemoreceptors were searched for transmembrane regions (TMs) using the DAS server (Table 1).

In this study, we carried out a bioinformatics analysis followed by an experimental approach to identify putative chemoreceptors involved in the perception of compounds released in plant wounds. Moreover, we analysed the role of these chemoreceptors in the entry to the plant apoplast and the colonization ability in Arabidopsis plants.

RESULTS

**Dd3937 possesses an unusually high number of chemoreceptors among Enterobacteria**

We screened the Dd3937 genome (http://asap.ahabs.wisc.edu/asap/home.php) for the presence of proteins containing the highly conserved MCP signalling domain. The Dd3937 genome encodes 47 proteins containing the MCP domain. Despite the fact that Dd3937 belongs to Enterobacteria, this result contrasts with the average number of MCP genes found in other Enterobacteria: 29 MCPs per genome (Lacal et al., 2010a). The amino acid sequences of these 47 chemoreceptors were searched for transmembrane regions (TMs) using the DAS server (Table 1). According to the presence of TMs, Dd3937 chemoreceptors were classified into six different topologies (Fig. 1A), as described previously (Lacal et al., 2010a). In the case of Dd3937, all topologies, including class Ia,
show similar results to those reported by Lacal et al. (2010a). Furthermore, in the most abundant topology Ia, we grouped the LBRs according to their length (Fig. 1B). Dd3937 LBRs (Ia) can be clustered into two groups as described previously by Lacal et al. (2010a). Most LBRs (60%) are characterized by sizes corresponding to cluster I, the majority of the LBRs being those with sizes between 150 and 159 amino acids (Fig. 1B).

LBR sequences were analysed by CLUSTAL W2 (Larkin et al., 2007), searching for diversity among the sequences (Table S1, see Supporting Information). The results showed that the level of sequence conservation was generally low and no duplications were observed. Moreover, LBRs of each of the 47 MCP proteins in Dd3937 were searched for Pfam components (Finn et al., 2014), using a customized BioPerl (Stajich et al., 2002) script. Only hits

**Table 1** Amino acid coordinates of methyl-accepting chemotaxis protein (MCP) domains and transmembrane regions (TMs) determine the topology of the Dd3937 chemoreceptor.

| ID            | UNIPROT† | TM   | MCP       | Topology class‡ |
|---------------|----------|------|-----------|-----------------|
| ABF-0014536   | E0SH54   | 10   | 32        | 181 203         | 334 501 Ia          |
| ABF-0014618   | E0SM30   | 36   | 58        | 214 232         | 367 531 Ia          |
| ABF-0014722   | E0SF47   | 7    | 29        | 190 209         | 344 511 Ia          |
| ABF-0014824   | E0DP4    | 10   | 32        | 183 205         | 340 507 Ia          |
| ABF-0015168   | E0SH41   | 5    | 24        | 177 199         | 335 501 Ia          |
| ABF-0015513   | E0SBF1   | 20   | 42        | 200 222         | 360 523 Ia          |
| ABF-0015600   | E0SE76   | 12   | 34        | 278 295         | 431 598 Ia          |
| ABF-0015603   | E0SE77   | 38   | 60        | 300 317         | 451 618 Ia          |
| ABF-0016115   | E0SN86   | 13   | 35        | 269 285         | 421 588 Ia          |
| ABF-0016585   | E0SA2    | 10   | 32        | 189 211         | 343 510 Ia          |
| ABF-0016979   | E0SW8    | 10   | 32        | 183 205         | 343 510 Ia          |
| ABF-0017097   | E0SC7    | 17   | 39        | 272 290         | 427 593 Ia          |
| ABF-0017662   | E0SF0    | 5    | 27        | 187 204         | 342 508 Ia          |
| ABF-0017665   | E0SF12   | 36   | 58        | 213 235         | 372 538 Ia          |
| ABF-0017668   | E0SF13   | 10   | 32        | 184 206         | 344 511 Ia          |
| ABF-0017672   | E0SF14   | 10   | 32        | 184 206         | 347 514 Ia          |
| ABF-0017674   | E0SF15   | 20   | 39        | 201 223         | 359 526 Ia          |
| ABF-0017863   | E0SI11   | 10   | 32        | 189 211         | 346 510 Ia          |
| ABF-0017896   | E0SH9    | 11   | 33        | 178 200         | 337 503 Ia          |
| ABF-0018502   | E0SF0    | 10   | 32        | 187 209         | 346 512 Ia          |
| ABF-0018541   | E0SF6    | 10   | 32        | 182 204         | 360 505 Ia          |
| ABF-0018585   | E0SX6    | 20   | 42        | 199 221         | 357 524 Ia          |
| ABF-0018754   | E0SM4    | 10   | 32        | 179 201         | 336 503 Ia          |
| ABF-0018765   | E0SN3    | 20   | 42        | 204 226         | 356 518 Ia          |
| ABF-0018892   | E0SJ1    | 20   | 42        | 194 216         | 355 522 Ia          |
| ABF-0019050   | E0AP6    | 15   | 37        | 270 292         | 427 591 Ia          |
| ABF-0019306   | E0SF78   | 13   | 35        | 282 299         | 435 601 Ia          |
| ABF-0019718   | E0SH9    | 7    | 29        | 180 202         | 336 502 Ia          |
| ABF-0019790   | E0SF98   | 10   | 32        | 187 209         | 344 510 Ia          |
| ABF-0019851   | E0SM37   | 7    | 29        | 179 194         | 333 499 Ia          |
| ABF-0019852   | E0SM36   | 20   | 42        | 195 214         | 348 515 Ia          |
| ABF-0019855   | E0SM35   | 13   | 35        | 185 204         | 340 507 Ia          |
| ABF-0019858   | E0SM34   | 31   | 53        | 202 222         | 357 522 Ia          |
| ABF-0020167   | E0SD0    | 10   | 29        | 178 200         | 340 507 Ia          |
| ABF-0020252   | E0SF13   | 25   | 47        | 288 314         | 439 604 Ia          |
| ABF-0020431   | E0SX8    | 5    | 27        | 168 190         | 327 489 Ia          |
| ABF-0046680   | E0MA1    | 58   | 77        | 348 370         | 504 670 Ia          |
| ABF-0017537   | E0SD21   | 10   | 32        | 142 147         | 421 586 Ia          |
| ABF-0017419   | E0SN1    | 138  | 160       |                | 295 462 Ib         |
| ABF-0014726   | E0SF49   | 159  | 178       | 188 200         | 335 497 II          |
| ABF-0019309   | E0SF75   | 164  | 181       | 186 205         | 341 506 II          |
| ABF-0014843   | E0SDM9   | 147  | 151       | 165 180 185 196 | 337 499 III         |
| ABF-0016380   | E0EN9    | 30   | 52        | 183 205 336 350 | 490 656 III         |
| ABF-0018511   | E0SE2    | 13   | 30        | 196 203 315 330 | 468 633 III         |
| ABF-0016436   | E0SM7    | 297  | 417       |                | 297 417 IVa         |
| ABF-0017090   | E0SK2    | 474  | 615       |                | 474 615 IVa         |
| ABF-0017824   | E0SI40   | 17   | 131       |                | 17 131 IVa          |

* Nomenclature from the ASAP database (https://asap.ahabs.wisc.edu/asap/logon/php).  † Nomenclature from the UNIPROT database (http://www.uniprot.org/).  ‡ Topology class according to Lacal et al. (2010a).
Fig. 1 (A) Classification of Dd3937 chemoreceptors into six different topologies (roman numerals): 47 MCP sequences from Dd3937 were analysed. Classification is based on clustering annotated by Lacal et al. (2010a). The prediction of transmembrane regions (TMs) was performed using the DAS server (Cserzo et al., 1997). Arabic numbers indicate the relative abundance of receptors with a given topology in Dd3937 and in bacteria according to Lacal et al. (2010a). MCP, methyl-accepting chemotaxis protein; LBR, ligand-binding region. (B) Relative abundance of Dd3937 MCP sequences (belonging to class Ia) according to the size of the LBR (amino acids).
with an E-value of less than or equal to 0.005 were retained (Table S2, see Supporting Information). The results showed that 23 of the LBRs fall into the annotated domain 4HB_MCP_1 (four helix bundle sensory module for signal transduction) (Ulrich and Zhulin, 2005), which is a ubiquitous sensory module in prokaryotic signal transduction. Five are homologues to the annotated domain TarH, a member of a family of transmembrane receptors that mediate the chemotactic response in certain enteric bacteria, such as Salmonella typhimurium and E. coli (Kim et al., 1996). Homology with other annotated domains involved in sensory processes can be found.

**Global BLAST of LBRs from Dd3937 chemoreceptors suggests putative receptors for plant compounds**

In order to identify Dd3937 chemoreceptors involved in the sensing of plant-specific compounds, LBRs were further analysed by bioinformatics methods. LBR sequences were extracted from the protein sequence as described in Experimental procedures. LBR sequences were subjected to local BLAST against a bacterial genome database (this database contains complete bacterial genomes available as of 14 February 2014 from GeneBank Ref Seq) using an ad hoc PERL script (for more details, see Experimental procedures). Table S3 (see Supporting Information) shows similar LBR sequences (E-value ≤ 10^-30) to those found in other bacteria. Figure 2A shows the distribution of Dd3937 LBRs among the following groups of bacteria: (i) other Dickeya strains; (ii) plant-pathogenic bacteria; (iii) animal-pathogenic bacteria (considering the occurrence of a high number of similar LBR sequences in animal pathogens); and (iv) non-pathogenic bacteria (including bacteria present in plants, soil and water). Among the 47 LBRs of Dd3937, nine were widely distributed among bacteria regardless of their niche (animals, plants, soils and water). Another seven LBRs, apart from being present in plant-pathogenic bacteria, were also found in environmental (non-pathogenic) bacteria. Moreover, another four LBRs, apart from being present in Dickeya strains, were also found in environmental (non-pathogenic) bacteria. Only for one LBR were similar sequences found exclusively in animal and environmental bacteria. The largest group, 13 LBRs, was found in other plant-pathogenic bacteria besides the Dickeya genus, and another large group containing 12 members included LBRs exclusively present in the Dickeya genus. This means that 56% of Dd3937 LBRs show similarity only with other LBRs from phytopathogenic bacteria (Fig. 2B). Interestingly, one Dd3937 LBR

![Fig. 2](A) Distribution of Dd3937 ligand-binding regions (LBRs) among bacteria. *Nomenclature from the ASAP database (https://asap.ahabs.wisc.edu/asap/logon/phhp). In bold, genes selected for further analyses. Grey boxes indicate that similar sequences to a given Dd3937 LBR were found in the global BLAST analysis. (B) Schematic representation of the distribution of Dd3937 LBRs among bacteria according to their ecological niche: path., pathogenic; environ., environmental.
was not found in other *Dickeya* strains, but was found in other plant-pathogenic bacteria.

**Mutants in individual chemoreceptors of *Dd*3937 are affected in the perception of JA and xylose**

In order to unveil the role of the perception of plant molecules during the interaction of *Dd*3937 with plants, chemoreceptors from the different groups shown in Fig. 1 were selected. Thus, ABF-0018754 was selected as a control for chemotaxis assays as it is the candidate for serine perception in *Dd*3937. ABF-0046680 was chosen from the group of chemoreceptors whose LBRs showed similarity with those of plant-pathogenic bacteria, but also environmental bacteria. ABF-0014536 and ABF-0017668 were selected from the group of chemoreceptors whose LBRs showed similarity only with those of plant-pathogenic bacteria; ABF-0018541 and ABF-0017824 were selected from the group of chemoreceptors whose LBRs showed similarity with those of *Dickeya* strains (not with other plant-pathogenic bacteria) and environmental bacteria. ABF-0017662, ABF-0017665, ABF-0017863 and ABF-0020252 were selected from the group of chemoreceptors whose LBRs showed similarity only with those of *Dickeya* strains. Finally, ABF-0020167 was chosen because of its particularity of not showing similarity with other *Dickeya* strains, but with other plant-pathogenic bacteria. These chemoreceptors were mutagenized by different approaches (for more details, see Experimental procedures). ABF-0018754, chosen as a control for chemotaxis assays, restored serine perception when heterologously expressed in the non-chemotactic *E. coli* UU1250 strain (Bibikov et al., 2004) (Fig. S1, see Supporting Information).

In order to elucidate how the mutations in the chemoreceptors cited above affected the movement of *Dd*3937 towards putative entry sites, such as wounds, the chemotactic response of wild-type (WT) and mutant strains towards JA and xylose was analysed. As mentioned previously, JA secreted in plant wounds is a strong chemoattractant for *Dd*3937 (Antunez-Lamas et al., 2009) and xylose is released when the plant cell wall is altered.

The analysis of the chemotaxis towards these plant compounds was performed using the classical ’in-plug’ assay (Tso and Adler, 1974), which is based on the ability of bacteria to swim in soft agar and move towards/away from a given chemical. The results were recorded from soft agar–bacteria plates based on either the observation of attraction haloes or of the absence of a chemotactic response (Fig. 3). Independent of the intensity of the haloes, these were considered as a positive chemotraction when an apparent ring of accumulated cells was found at a certain distance from the plug.

From the results of the ’in-plug’ assays, we selected mutants 14536, 17824, 18541, 20167 and 46680 for quantitative chemotaxis assays towards JA, and mutants 18541, 20167, 20252 and 46680 for quantitative chemotaxis assays towards xylose. For

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**Fig. 3** (A) Typical attraction haloes of *Dd*3937 wild-type (WT) towards jasmonic acid (JA) and xylose in the ’in-plug’ assay. A drop containing a given chemical, either JA (10 mM) or xylose (10 mM), or their corresponding controls, ethanol (0.04% v/v) and water, was deposited on the (0.25%) soft-agar bacteria, and haloes were recorded after 20 min. (B) Chemotactic response of *Dd*3937 strains towards JA and xylose. Chemotactic response of *Dd*3937 WT and mutant strains was evaluated in the presence of a concentration gradient of JA (10 mM) and xylose (10 mM). The chemotactic response was considered as attraction (+) or non-chemotactic response (Ø) in comparison with that of the WT strain for each chemical. Similar results were obtained in three independent experiments.

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For this purpose, a Palleroni-like assay was performed using 5-μL tips instead of glass capillaries, as handling allows a high-throughput screening in comparison with the classic Palleroni assay (Pallaroni, 1976). This assay allows the calculation of the chemotaxis index...
As bacterial cells can move actively towards/away from a given chemical contained in the capillary, values of CI greater than 1.0 are considered as chemoattraction and values less than 1.0 as chemorepulsion (Fig. 4). The results in Fig. 4 indicate that, in the case of JA, only 18541 and 20167 mutants showed a reduced chemoattraction towards this plant hormone. In the case of xylose, 20252 and 46680 mutants were affected in the perception of this sugar. As a control for the quantitative (capillary) assays, chemotaxis towards serine of the WT and mutant 18754 was assayed in each experiment (data not shown). Strains overexpressing the WT chemoreceptors restored the ability to develop a chemoattraction response in the presence of the respective compound (Fig. 4B, D).

To check that these results were not the product of an altered chemotactic ability, *Dd*3937 mutants were tested for chemotaxis towards serine. All mutants, except 18754 (candidate serine receptor of *Dd*3937), showed the same chemotaxis towards serine as did the WT strain (Fig. S2, see Supporting Information). Moreover, to demonstrate that these mutants do not have a general defect in motility, we carried out a swimming assay in soft agar-rich medium. This assay, which reflects the sum of taxis towards compounds present in the medium, showed that the spreading of these strains was not altered with respect to that of the WT strain (Fig. S3, see Supporting Information).

**Dd*3937 chemoreceptors contribute in a different manner to entry into Arabidopsis leaves**

As stated above, how pathogens enter plant tissues is critical for pathogenesis. To ascertain the particular relevance of the selected
Dd3937 chemoreceptors in this process, and how this could affect the subsequent development of symptoms, WT and mutant strains were challenged to entry and colonization assays in Arabidopsis leaves. Arabidopsis thaliana has been described and characterized previously as a susceptible host of Dd3937 (formerly Erwinia chrysanthemi 3937) (Dellagi et al., 2005; Fagard et al., 2007).

To compare the ability to detect plant compounds released in a wound, we first performed entry assays in A. thaliana Col-0 leaves, as described previously (Antunez-Lamas et al., 2009). For each particular strain, the cells which entered the plant apoplast were measured relative to the initial inoculum of cells (cells recovered from the leaf/cells put on the wound; expressed as a percentage), and these ratios were used for comparison with the WT strain ratio. Figure 5A shows the differences in entry ability of the 10 MCP mutants tested in comparison with the WT strain. Two mutants, 20167 and 46680, were significantly affected in their ability to enter the plant apoplast in contrast with the WT strain.

Entry assays in A. thaliana leaves were performed as described previously. 20167-C and 46680-C complemented strains showed entry percentages close to those of the WT levels (Fig. 5B).

To ascertain whether the delay in entry into the plant apoplast exhibited by 20167 and 46680 mutants could affect the subsequent establishment of infection, the same assay described above was performed, changing the incubation time from 2 to 24 h (Fig. 5C). Figure 5C shows that the delay in entry of 20167 observed at 2 h affected the population inside the leaves at 24 h.
whereas 46680 reached WT population levels at 24 h. To check that the decrease in the 20167 population inside the leaves at 24 h was not a result of impairment in colonization ability, but a direct consequence of reduced entry, a non-entry-dependent colonization assay was performed. For this, WT and 20167 cell suspensions were infiltrated into the abaxial side of A. thaliana leaves and bacterial populations were estimated at 24 h. Figure 5D shows that, when infiltrated, the population of the 20167 mutant is at the same level as that of the WT strain.

**DISCUSSION**

The *E. coli* chemotactic pathway has provided a paradigm for sensory systems in general. However, the increasing number of sequenced bacterial genomes shows that, although the central sensory mechanism seems to be common to all bacteria, there is added complexity in a wide range of species (Krell et al., 2011; Wadhams and Armitage, 2004). Indeed, the analysis of bacterial genomes reveals that motile bacteria differ enormously in the number of chemoreceptors. Chemoreceptor abundance in a bacterial genome ranges from 64 in *Magnetospirillum magnetotacticum* (Alexandre et al., 2004) to a single chemoreceptor in other bacteria (Lacal et al., 2010a). Forty-seven chemoreceptors have been identified in *Dd3937* through bioinformatics analyses. In comparison with its close Enterobacteria relatives (*Shigella, Salmonella, Yersinia, Escherichia* spp.), which show a small number of chemoreceptors, for instance five in *E. coli* (Lacal et al., 2010a; Wadhams and Armitage, 2004), *Dd3937* possesses a large number of chemoreceptors. This is congruent with the hypothesis that microorganisms living in complex and variable environments must be able to detect a greater variability of changing conditions. Indeed, genome sequencing has revealed that many microorganisms from aquatic and soil environments possess large numbers of chemoreceptors (Alexandre et al., 2004). Moreover, to assess the relationship between the abundance of chemoreceptors and lifestyle, regardless of taxonomic classification, Lacal et al. (2010a) analysed 264 bacterial genomes for the presence of chemoreceptors, and similar results were obtained, showing that the number of chemoreceptors per genome depends mostly on bacterial lifestyle and metabolic diversity, but weakly on genome size. Among the 264 bacterial genomes analysed, only three phytopathogenic bacteria, *Xylella fastidiosa*, *Xanthomonas oryzae pv. oryzae* and *Agrobacterium tumefaciens* C58, were included. These authors also analysed the topology of the chemoreceptors defining several classes. In the case of *Dd3937*, the most abundant topology is that of class Ia, as it mainly occurs in bacteria (Lacal et al., 2010a). For the rest of the classes, similar results to those shown for bacteria were obtained in *Dd3937*. Moreover, the analysis of the LBR length in the most abundant topology class (Ia) also showed the same clustering into two defined groups described by Lacal et al. (2010a).

The detailed analysis of the distribution of *Dd3937* LBRs among known bacterial genomes allowed us to determine that more than 50% of the LBRs show high similarity only with sequences from other plant-pathogenic bacteria. These in silico-based data suggest that these chemoreceptors would be implicated in the perception of plant-derived compounds. The large number of putative chemoreceptors involved in this process demonstrates the relevance of the perception of plant compounds during the interaction. Indeed, the identification of host–microbe interaction factors in the genome of *Dd3937* with supervised machine learning shows that methyl-accepting chemotaxis genes are highly enriched among the predicted host–microbe interaction factors in this bacterium (Ma et al., 2014).

The results from the ‘in-plug’ chemotaxis assays indicated that more than one mutant strain showed an altered chemotactic response towards JA or xylose with respect to the WT strain. A possible explanation of these findings would be that a given compound can be detected by more than one chemoreceptor. Indeed, there are several examples in the literature showing this (Kato et al., 2008). The best studied example is the recognition of amino acids by the paralogous receptors PctA, PctB and PctC of *Pseudomonas aeruginosa* (Rico-Jiménez et al., 2013). However, the opposite phenomenon has also been described: one chemoreceptor is able to sense more than one compound. For example, McpS, a *Pseudomonas putida* KT2440 chemoreceptor for tricarboxylic acid (TCA) cycle intermediates, is involved in the chemotactic response towards different ligands, such as malate, succinate and fumarate (Lacal et al., 2010b). Other examples of multi-ligand chemoreceptors are as follows: TcP receptor in *S. typhimurium*, which mediates positive chemotaxis to citrate and negative chemotaxis to phenol (Yamamoto and Imae, 1993), PA2652 of *P. aeruginosa*, which mediates chemotaxis to malate, but not to any of the remaining TCA cycle intermediates (Alvarez-Ortega and Harwood, 2007), and McpX of *Vibrio cholerae*, which functions as a chemoreceptor for multiple amino acids (Nishiyama et al., 2012). Another hypothesis which cannot be ruled out is that a mutation in a given chemoreceptor alters the chemotactic response of the remaining receptors, through gene expression reprogramming. There is much information missing about the gene regulation of chemoreceptors. For example, it is unknown whether all receptors are constitutively expressed, whether they are inducible under certain stimuli and whether there is fine regulation depending on the metabolic needs. It has been shown that the responses of *E. coli* to two opposing chemoattractant gradients are dependent on the chemoreceptor ratio (Kalnin et al., 2010). Therefore, if *Dd3937* chemoreceptor ratios are being altered through individual mutations, one can expect several changing chemotactic behaviours, as found in this study.

The analysis of the chemotactic response to xylose and JA using a quantitative methodology based on capillary assays showed...
that, of the five mutants found to be altered in chemotaxis towards JA in the in-plug assay, only two were affected in the capillary assays. In the case of xylose, of the four mutants altered, only two consistently showed a reduced chemotaxis in the capillary assays. This discrepancy could be a result of the nature of the medium employed, i.e. semi-solid agar in the ‘in-plug’ assay and liquid medium in the capillary assay. Both the availability of the chemoattractant to be perceived and the effects of the conditions during the assay on the motility ability could be responsible for the different results obtained. An important element to take into account is that chemotaxis is a flagellum-dependent phenomenon and the number of flagella is intimately linked to the characteristics of the medium (Jarrell and McBride, 2008).

Although other chemoreceptors could be involved in JA perception, we focused our studies on the role of ABF-0018541 and ABF-0020167 in the active entry process through wounds as the corresponding mutant strains consistently showed a decreased chemotaxis towards this hormone in the two assays performed. Our experiments showed that only the 20167 mutant displayed impaired entry ability to the plant apoplast, which suggested a pivotal role for this receptor during this process. In the case of xylose, although 20252 and 46680 mutants showed reduced chemoattraction towards this sugar in both ‘in-plug’ and quantitative assays, only 46680 was impaired in the entry process, which also suggested a key role for this receptor during the entry to the plant apoplast. However, when analysing the colonization ability, although 20167 and 46680 mutants displayed impaired entry ability, only the 20167 strain was altered in the establishment of a sufficiently large population to prosper in the hostile plant conditions. When 20167 was infiltrated, the bacterial population reached the same level as that of the WT strain. Therefore, the altered perception of compounds produced in wounds, such as JA, is the main contributor to the impaired colonization ability of this strain, which does not seem to be related to the colonization ability or fitness inside the plant tissue. In the case of 46680, although under laboratory conditions the mutant is not affected in its colonization ability, a role in this process under natural conditions cannot be ruled out.

It is noteworthy that, in our in silico analysis, similar sequences to the LBR from ABF-0020167 were only found in plant-pathogenic bacterial genomes. It is also noteworthy that no matches with other Dickeya strains were found. Moreover, global BLAST analyses did not show any match of this LBR with Pectobacterium carotovorum, a phylogenetically related bacterium which does not sense JA (Antunez-Lamas et al., 2009). Therefore, ABF-0020167 could be considered as a putative receptor for JA, although further analyses are required to demonstrate the interaction of its LBR with this plant hormone. It is noteworthy that ABF-0018541 has also been identified as a putative JA chemoreceptor in our capillary assays. The sequence similarity between the LBR of these two chemoreceptors is very low, but it cannot be ruled out that a similar tertiary structure exists, allowing the binding of the same type of molecules. Indeed, both LBR sequences show homology with the annotated Pfam domain 4HB_MCP_1 (four helix bundle sensory module for signal transduction) (Ulrich and Zhulin, 2005), which is a ubiquitous sensory module in prokaryotic signal transduction. A detailed structural analysis of these regions will shed light on their implications in JA binding.

With regard to the ABF-0046680 chemoreceptor, the 46680 mutant was impaired in the ability to sense xylose, but further analyses are required to demonstrate whether: (i) the LBR of ABF-0046680 binds directly to xylose; and (ii) xylose is being released at wound sites.

Moreover, it is expected that the contribution of these chemoreceptors to the efficiency of infection by Dd3937 will depend on the host. As demonstrated previously, the chemotaxis process depends on the presence of a concentration gradient of the compound being sensed (Kalnin et al., 2010), and the gradient created in a wound for a given compound may be different for different hosts.

In summary, the study of Dd3937 chemoreceptors by bioinformatics analyses and experimental approaches has allowed us to identify the ABF-0020167 and ABF-0046680 chemoreceptors as putative receptors for biomolecules of great importance in plant defence: JA and xylose, respectively. Moreover, entry assays through wounds performed in A. thaliana leaves suggest that the perception of plant compounds released from a wound by the chemoreceptors ABF-0020167 and ABF-0046680 is pivotal to the entry into the plant apoplast and to the achievement of a population density sufficient to prosper in the plant apoplast. Further research is needed to demonstrate the interaction between the LBRs and these molecules.

**EXPERIMENTAL PROCEDURES**

**Identification of MCP domains**

For sequence analyses, the Dd3937 genome sequence was downloaded from the ASAP database (http://asap.ahabs.wisc.edu/asap/home.php). The Dd3937 genome was searched for genes encoding chemoreceptors. The search for the MCP domain was performed using an internal pipeline. The MCP domain was downloaded from the Pfam website of the Sanger Centre (http://pfam.sanger.ac.uk) using the Pfam identifier PF00015. This alignment was used to build hidden Markov models (HMMs) by means of HMMer v2.3.2 (Eddy, 1998). Finally, a customized BioPerl (Stajich et al., 2002) script combined with HMMer searched for HMM hits using an E-value of 0.005 to search for MCP-containing proteins.

**Identification of TMs and determination of LBR sequence**

TM domains were identified one by one using the DAS algorithm at the DAS server (Cserzo et al., 1997, 2002). The complete sequences of
chemoreceptors were searched for TMs considering a cut-off value of –2.5. Then, the LBR was extracted from the protein sequence as: (i) the sequence in between two TM domains when present; (ii) the sequence in between the amino terminus and the MCP domain when no TM was present; or (iv) the sequence in between the carboxyl terminus and the TM when only one TM was present; (iii) the sequence in between two TM domains when present; (ii) the sequence in between the amino terminus and the MCP domain when no TM was present. LBRs were identified in all strains, except the 5S pr derivative of Dd3937, where no TM was present.

**Identification of Pfam domains in the LBR sequence**

HMMs based on protein families were downloaded from the Pfam (Finn et al., 2014) website (ftp://ftp.ebi.ac.uk/pub/databases/Pfam/current_release). We focused on the Pfam component, so-called Pfam-A, corresponding to a number of high-quality, manually curated families. LBRs of each of the 47 MCP-associated proteins in Dd3937 were searched for the corresponding Pfam domains using a customized BioPerl (Stajich et al., 2002) script. Only hits with an E-value of less than or equal to 0.005 were retained.

**Global BLAST analysis of LBR sequences**

Amino acid sequences of LBRs were searched against a database of bacterial genome sequences. For this, the complete bacterial sequences of 2997 chromosomes and 2164 plasmids, available as of 14 February 2014, were downloaded from GenBank Refseq. Local BLAST (E-value ≤ 10−30) was performed using an *ad hoc* PERL script. The output file was processed manually.

**Microbiological methods**

The bacterial strains and plasmids used in this study are listed in Table 2. Unless indicated, strains of *E. coli* were grown at 37 °C in Luria–Bertani (LB) medium (Sambrook et al., 1989). Unless indicated, strains of *D. dadantii* 3937 were cultivated at 28 °C in nutrient broth (NB) (per litre: yeast extract, 1 g; peptone, 3 g; NaCl, 2 g; NaH2PO4, 0.5 g; MgSO4·7H2O, 0.5 g; K2HPO4, 0.5 g; glucose, 20 g; pH 7.0) using a Jenway 6300 spectrophotometer (Jenway, Stone/Staffordshire/United Kingdom). For *D. dadantii* strains used in this study, OD600 = 1.0 corresponds approximately to 5 × 108 colony-forming units (cfu).

**DNA manipulation**

The plasmids used in this study are described in Table 2. To analyse the functions of the genes of interest in *D. dadantii* 3937, mutants in these genes were tracked by genomic polymerase chain reaction (PCR) from a mutant grid.
available in our laboratory using two gene-specific primers and a transposon-specific primer. Those mutants not found in the grid were made by two different approaches: In short, the 17662 and 17668 mutants were constructed by Tn7 in vitro mutagenesis using a Genome Priming System (GPS-1) kit (New England Biolabs, Ipswich/Massachusetts/USA), and marker exchange was performed as described previously by Roeder and Collmer (1985). Construction of the 20167 and 46680 mutants was carried out by amplifying with Pfu DNA polymerase (Biolo, Madrid/Spain) an internal sequence between 700 and 800 bp from the Dd3937 genome and cloning into pKNG101 (Kaniga et al., 1991). The resulting plasmid was transferred into Dd3937 via triparental mating (de Lorenzo and Timmis, 1994). As pKNG101 cannot replicate in E. coli, single crossover integrants were selected by resistance to streptomycin. In all cases, the mutations were verified by DNA sequencing.

For gain-of-function assays, ABF-0018754 and AIH-0004387 genes were amplified using a Pfu DNA polymerase from Dd3937 and E. coli K12 genomic DNA, respectively. The primers (5’→3’) used were as follows: 185754F, GCCCATGTCATTGATAC; 185754R, AGCGATTAAAAGGTTTCCC; 0438F7, ATTACCTCGGGCTAATTCTCC; 04383R7, CTATCCTGGCAATTGCTTGC. PCR products were cloned into pGEM-Teasy® vector and the resulting plasmids were transferred into E. coli UU1250 via electroporation (Sambrook et al., 1989).

For complementation of the mutants, ABF-0021067 and AB-0046680 genes were amplified using a Pfu DNA polymerase from Dd3937 genomic DNA employing the following primers (5’→3’): 18541F_PstI, CTGCA GTAGCAATTTGCTCAATTTG; 18541R_XmaI, CCCCCGTTGTAAGCCCATCA TC; 20167F_PstI, CTGCAACTTCTGCTAAGAGGCC; 20167R_XmaI, CCCC GTATCAAGGTCGAGGCT; 20252F_PstI, CTGCACTACATGGAATT; 20252R_PstI, CCCC GGGCGAAAGAGTGTTCTG; 46680F_PstI, TACATGGAATGACGT; 46680R_PstI, TATCAAGGTGCAGTGC; 20252F_PstI, CTGCAG TCATTAATTCTCATGTTG; 46680R_PstI, AGCGATTAAAAGGTTTCCC; 20252R_PstI, AGCGATTAAAAGGTTTCCC; 46680F_PstI, AGCGATTAAAAGGTTTCCC; 46680R_PstI, AGCGATTAAAAGGTTTCCC.

The chemical ‘in-plug’ bacterial chemotaxis assay

Dd3937 cells were scraped from NB plates incubated overnight and suspended in 10 mM MgCl2 at the desired final concentration. Then the bacterial suspension was mixed with Minimal Medium A (MMA) agar medium (Antúnez-Lamas et al., 2009) to a final concentration of 0.25% agar and 2.5 × 10^6 cfu/mL. Once polymerized, a 5-μL drop containing a given chemical was placed on the agar surface (Tso and Adler, 1974). Xylose, serine and JA were used at 10 mM (final concentration). Plates were incubated for 20 min at room temperature. After that, bacterial haloes were recorded. Five replicates were analysed for each experiment. Water was used as a solvent for xylose and serine, and JA was dissolved in ethanol.

**Capillary assays**

The chemotaxis of Dd3937 towards several compounds was measured using a modified Palleroni assay (Palleroni, 1976). CI was defined as the number of cells that accumulated after 20 min within a 5-μL tip containing 2 μL of a given compound divided by the number of cells accumulated within a 5-μL tip containing 2 μL of the solvent in which the compound was dissolved. Values of CI significantly greater than 1.0 were identified as a positive response of the cells to the chemical compound. Values of CI significantly less than 1.0 were identified as a negative response of the cells to the chemical compound. Three replicates (capillaries) were analysed in each experiment. Xylose, serine and JA were tested at 1 mM. Significant differences between the CIs of WT and mutant strains were determined and statistically compared according to Student’s t-test (P < 0.05).

**Plant bioassays**

Entry and entry-dependent colonization assays of Dd3937 strains in A. thaliana leaves were carried out as described previously by Antúnez-Lamas et al. (2009) using 4-week-old Col-0 ecotype plants. A 10-μL drop containing 10^6 cfu/mL was placed on a hole made with a needle on the adaxial side of leaves. Ten leaves were inoculated with each strain. Three leaves were mock infiltrated with 10 mM MgCl2. After 2 or 24 h at 28 °C, leaves were cut and washed with sterile water, and viable cell counts in leaves were determined by serial dilution and plating. For non-entry-dependent colonization assays, 10 leaves were infiltrated into the abaxial side with a suspension containing 2 × 10^6 cfu/mL by syringe infiltration. Three leaves were mock infiltrated with 10 mM MgCl2. After 24 h at 28 °C, leaves were processed as described above.

In both kinds of experiment, significant differences between WT and mutant cells inside the leaves were determined and statistically compared according to Student’s t-test (P < 0.05).

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SUPPLEMENTARY INFORMATION

Additional Supporting information may be found in the online version of this article at the publisher's website:

Fig. S1 Complementation of non-chemotactic strain Escherichia coli UI1250 with putative serine chemoreceptors. Tryptone broth (TB) 0.35% agar plates (A) and TB 0.35% agar plates supplemented with 1 mm serine (B) were inoculated with wild-type (WT), UI1250 or transformed E. coli UI1250 strains expressing the E. coli serine chemoreceptor (AIH-0004387) or the putative serine chemoreceptor of Dd3937 (ABF-0018754). Results were recorded at 48 h. Similar results were obtained in three independent experiments.

Fig. S2 ‘In-plug’ chemotactic response of Dd3937 strains towards serine. A drop containing water or serine (10 mM) was deposited on the (0.25%) soft-agar bacteria. All the Dd3937 strains tested [wild-type (WT) and mutants] showed no chemotactic response towards water; a representative image is shown. Haloes were recorded after 20 min. Similar results were obtained in three independent experiments.

Fig. S3 Swimming ability of Dd3937 strains in rich medium. King’s B (KB) 0.25% agar plates were inoculated with Dd3937 wild-type (WT) and mutant strains at the exponential phase. Diameters of haloes were recorded at 24 h. The means and standard errors are shown. Error bars represent standard error of the mean (SEM). Similar results were obtained in three independent experiments.

Table S1 Percentage identity matrix of the Dd3937 ligand-binding region (LBR).

Table S2 Pafm matches for Dd3937 ligand-binding regions (LBRs). (a) Nomenclature from the ASAP database (https://asap.ahabs.wisc.edu/asap/logon.php). In grey, methyl-accepting chemotaxis proteins (MCPs) under study in this work.

Table S3 Global BLAST of Dd3937 ligand-binding regions (LBRs). Amino acid sequences of LBRs were searched against a database of bacterial genome sequences using BLAST (E-value ≤ 10−30) * indicates that the periplasmic LBR was selected when two LBRs were present. (a) Nomenclature from the ASAP database (https://asap.ahabs.wisc.edu/asap/logon.php).