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Transcriptional profiling of Gram-positive *Arthrobacter* in the phyllosphere: induction of pollutant degradation genes by natural plant phenolic compounds

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Summary

Arthrobacter chlorophenolicus **A6 is a Gram-positive, 4-chlorophenol-degrading soil bacterium that was recently shown to be an effective colonizer of plant leaf surfaces. The genetic basis for this phyllosphere competency is unknown. In this paper, we describe the genome-wide expression profile of *A. chlorophenolicus* on leaves of common bean (*Phaseolus vulgaris*) compared with growth on agar surfaces. In phyllosphere-grown cells, we found elevated expression of several genes known to contribute to epiphytic fitness, for example those involved in nutrient acquisition, attachment, stress response and horizontal gene transfer. A surprising result was the leaf-induced expression of a subset of the so-called *cph* genes for the degradation of 4-chlorophenol. This subset encodes the conversion of the phenolic compound hydroquinone to 3-oxoadipate, and was shown to be induced not only by 4-chlorophenol but also hydroquinone, its glycosylated derivative arbutin, and phenol. Small amounts of hydroquinone, but not arbutin or phenol, were detected in leaf surface washes of *P. vulgaris* by gas chromatography-mass spectrometry. Our findings illustrate the utility of genomics approaches for exploration and improved understanding of a microbial habitat. Also, they highlight the potential for phyllosphere-based priming of bacteria to stimulate pollutant degradation, which holds promise for the application of phylloremediation.**

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Introduction

Plant leaf surfaces (collectively referred to as the phyllosphere) provide a large and unique habitat for microbial life. Even though the phyllosphere can be a harsh and stressful environment with rapid changes in temperature, relative humidity and harmful ultraviolet radiation, it is typically colonized by large populations and diverse communities of bacteria, fungi and other microorganisms (Leveau, 2006; Meyer and Leveau, 2012; Vorholt, 2012; Rastogi *et al*., 2013). A relatively understudied aspect of phyllosphere microbiology is the ability of several phyllosphere bacteria to degrade aromatic pollutants, such as toluene, phenol and phenanthrene (De Kempeneer *et al*., 2004; Sandhu *et al*., 2007; 2009; Waight *et al*., 2007; Yutthammo *et al*., 2010), as well as various foliar pesticides (Ning *et al*., 2010; Zhou *et al*., 2011). Such bacteria have potential towards phylloremediation (Sandhu *et al*., 2007), i.e. the removal of foliage-associated organic pollutants by members of the phyllosphere community.

Representatives of the genus *Arthrobacter* (high GC Gram-positive, family Micrococcaceae, order Actinomycetales, class Actinobacteria, phylum Actinobacteria) are well known for their exceptional resistance to various stresses and their ability to degrade a wide variety of organic pollutants (Mongodin *et al*., 2006). *Arthrobacter* species are common members of phyllosphere communities (Rastogi *et al*., 2012), and they were recently shown to exhibit a high level of epiphytic fitness (Scheublin and Leveau, 2013). This combination of properties makes *Arthrobacter* a target genus for studies on phylloremediation.

Little is known about the genes underlying phyllosphere competency in *Arthrobacter*. From the few studies that are available for other bacterial genera (Marco *et al*., 2005; Gourion *et al*., 2006; Fink *et al*., 2012; Yu *et al*., 2013), it

has become clear that phyllosphere exposure affects the expression of genes involved in motility, chemotaxis, biofilm formation and attachment, as well as genes related to nutrient starvation, and osmotic, oxidative and desiccation stresses. For the plant pathogen *Pseudomonas syringae*, genes involved in virulence, such as toxin production genes also showed different transcript levels (Yu *et al*., 2013). The proteome of *Methylobacterium extorquens* featured several induced proteins during epiphytic growth, including enzymes involved in methanol utilization, stress proteins and regulatory proteins (Gourion *et al*., 2006). In a metaproteomic study of the total phyllosphere community, proteins related to carbohydrate transport, carbon and nitrogen metabolism, motility, and stress were among the most abundantly expressed (Delmotte *et al*., 2009).

In the study we describe here, we employed whole-genome transcriptome arrays of *Arthrobacter chlorophenolicus* A6 to gain a better understanding of its phyllosphere competency. Strain A6 is a 4-chlorophenol-degrading isolate from soil (Westerberg *et al*., 2000). Recently, strain A6 was demonstrated to be an excellent phyllosphere colonizer (Scheublin and Leveau, 2013). It has been studied extensively with regard to the genes that contribute to 4-chlorophenol degradation, and its complete genome sequence is available (Nordin *et al*., 2005; Unell *et al*., 2009). We designed transcriptome arrays to investigate which genes were induced in the phyllosphere of common bean (*Phaseolus vulgaris*) as compared with growth on agar surfaces. Since water availability is an important factor in phyllosphere survival and activity (Beattie, 2011), we included both high and low relative humidity treatments for the phyllosphere-grown cells. In addition, we compared the expression profiles of strain A6 on agar surfaces with or without 4-chlorophenol. These analyses revealed an unexpected connection between epiphytic growth and 4-chlorophenol exposure, which we followed up on in more detail by quantifying the expression of 4-chlorophenol degradative genes in response to plant phenolic compounds using reverse-transcriptase real-time PCR and identification of naturally occurring phenolic compounds on bean leaf surfaces by gas chromatography-mass spectrometry (GC-MS).

Results

Using custom-made microarrays, we determined and compared the transcriptional profiles of *A. chlorophenolicus* A6 cells that were recovered in quadruplicate from (i) bean leaf surfaces after incubation for 48 h at 97% relative humidity (PhylH, for phyllosphere high humidity), (ii) bean leaf surfaces after incubation for 48 h, of which the first 24 h were at 97% relative humidity and the second 24 h at 50% relative humidity (PhylL, for phyllosphere low humidity), (iii) the surface of a 1/10 strength tryptic soy agar plate supplemented with 1 mM 4-chlorophenol after incubation for 48 h at 97% relative humidity (A+CP, for agar plus 4-chlorophenol), and (iv) the surface of a 1/10 strength tryptic soy agar plate after incubation for 48 h at 97% relative humidity (A-CP, for agar without 4-chlorophenol).

Clustering of the transcriptome microarray data showed a clear separation between phyllosphere samples (PhylH and PhylL) on the one hand and agar samples (A+CP and A-CP) on the other (Fig. 1). Among the agar samples, A+CP replicates also clearly separated from A-CP replicates. Such a separation was less obvious for the PhylH and PhylL samples (Fig. 1). We observed a strongly positive correlation between the expression of individual genes at high and low relative humidity in the phyllosphere (Fig. 2A), which suggests a similar bacterial experience under these two conditions. In fact, for only three genes that were differentially expressed in the phyllosphere compared with growth on agar, the expression was significantly different ($P < 0.05$) between the PhylH and PhylL samples. The first, Achl_4566, is part of the so-called *cph* gene cluster for 4-chlorophenol degradation and will be discussed in more detail below. Like Achl_4566, Achl_0518 was expressed more highly under conditions of low humidity. It encodes a putative substrate transporter belonging to the major facilitator superfamily MFS_1, with high sequence similarity to proline/betaine transporters of other *Arthrobacter* species. The expression of the third gene, Achl_2563, was lower under conditions of low humidity compared with high humidity. Its predicted product is also annotated as an MFS_1 protein. With 41% sequence similarity to a valanimycin resistance gene of *Streptomyces viridifaciens* (accession number AAN10244), Achl_2563 might be involved in antibiotic efflux, but why its expression is suppressed at low relative humidity is not evident.

A weak but significant positive correlation was observed between the PhylH and A+CP treatment (Fig. 2B), and between the PhylL and A+CP treatment (not shown), suggesting that there were more genes that responded in the same way to these two conditions than there were genes that responded in opposite directions. Included in this list of genes are several that contribute to the degradation of 4-chlorophenol, as will be detailed below. A complete list of differential gene expression is given in Supporting Information Table S1. A number of specific differences (Table 1) will be highlighted further below, organized primarily by gene function. Unless otherwise noted, any reference to up- or down-regulation of genes is relative to gene expression on agar surfaces in the absence of 4-chlorophenol (i.e. A-CP).

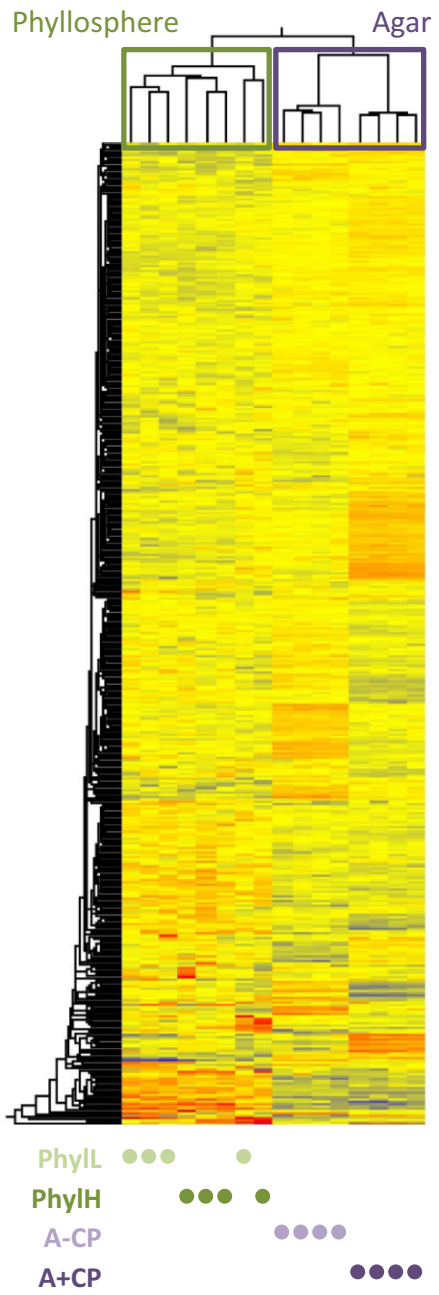

Fig. 1. Cluster diagram of *A. chlorophenolicus* A6 transcriptome array samples. Samples were organized by hierarchical clustering using Euclidian distances and the average linkage rule. PhylH, high-humidity phyllosphere treatment; PhylL, low-humidity phyllosphere treatment; A-CP, agar surface treatment; A+CP, agar plus 4-chlorophenol treatment.

Chlorophenol degradation genes

All but one of the 11 genes in the *cph* gene cluster (Achl_4564–4574) (Nordin *et al*., 2005) were induced in response to 4-chlorophenol (Fig. 3, black bars). The exception was Achl_4571 (*cphR*), which is annotated as a transcriptional activator protein. We found that three genes in the cluster, Achl_4564, Achl_4565 and Achl_4566, were also induced during growth on bean leaf surfaces (Fig. 2; Fig. 3, white and grey bars). These genes form a putative operon coding for the three-step conversion of hydroquinone to 3-oxoadipate as follows: Achl_4564 codes for CphC-II, converting hydroquinone to hydroxyquinol (Nordin *et al*., 2005); Achl_4566 codes for CphA-II, which is a predicted hydroxyquinol 1,2-dioxygenase; and the product of Achl_4565 is CphF-II, which is predicted to catalyse the conversion of maleylacetate to 3-oxoadipate. Not induced in the phyllosphere were Achl_4570 (*cphB*) and Achl_4573 (*cphC-I*), both of which are predicted to code for the production of hydroquinone from 4-chlorophenol, or Achl_4569 and Achl_4574, whose gene products are CphA-I and CphF-I, presumed paralogs of CphA-II and CphF-II respectively.

We confirmed by reverse transcription quantitative PCR (RT-qPCR) that Achl_4564 and Achl_4566, but not Achl_4569, were induced during epiphytic growth (Fig. 4A). In liquid culture, the expression of Achl_4569 was stimulated only by 4-chlorophenol (1 mM), while Achl_4564 and Achl_4566 were induced by 4-chlorophenol (1 mM), phenol (1 mM), hydroquinone (1 mM, 10 μM and 100 nM, but not 10 nM) and arbutin (1 mM and 10 μM, but not 100 nM). No induction was observed upon exposure to 1 mM concentrations of the following (plant) phenolic compounds: 4-hydroxybenzoic acid, protocatechuic acid, coumaric acid, caffeic acid, ferulic acid, quercetin, catechol or resorcinol (Fig. 4B). Arbutin is a glycosylated form of hydroquinone and has been identified in leaf extracts of several plant species (see *Discussion*). Using GC-MS, we were unable to detect arbutin in leaf washes from bean plants that were used in our experiments. However, we consistently found hydroquinone in these leaf washes in the amount of 1.5 ng per leaf averaged (Supporting Information Fig. S1). Other phenolic compounds that we identified in at least one of three replicate samples included caffeic acid, ferulic acid, 4-hydroxybenzoic acid, and protocatechuic acid; we did not detect 4-CP in the leaf washes.

Nutrient acquisition

Besides various phenolics, several other compounds were detected by GC-MS analysis of the bean leaf surface washes. These included compounds that one expects to find in the phyllosphere environment, such as intermediates of the citric acid cycle, tartrate, glycol, pentoses, hexoses, disaccharides, polar amino acids, long-chain alcohols and fatty acids (Supporting Information Table S2). Glucose, along with fructose and sucrose, is known to be the most abundant carbon source on bean leaf surfaces (Leveau and Lindow, 2001), but the

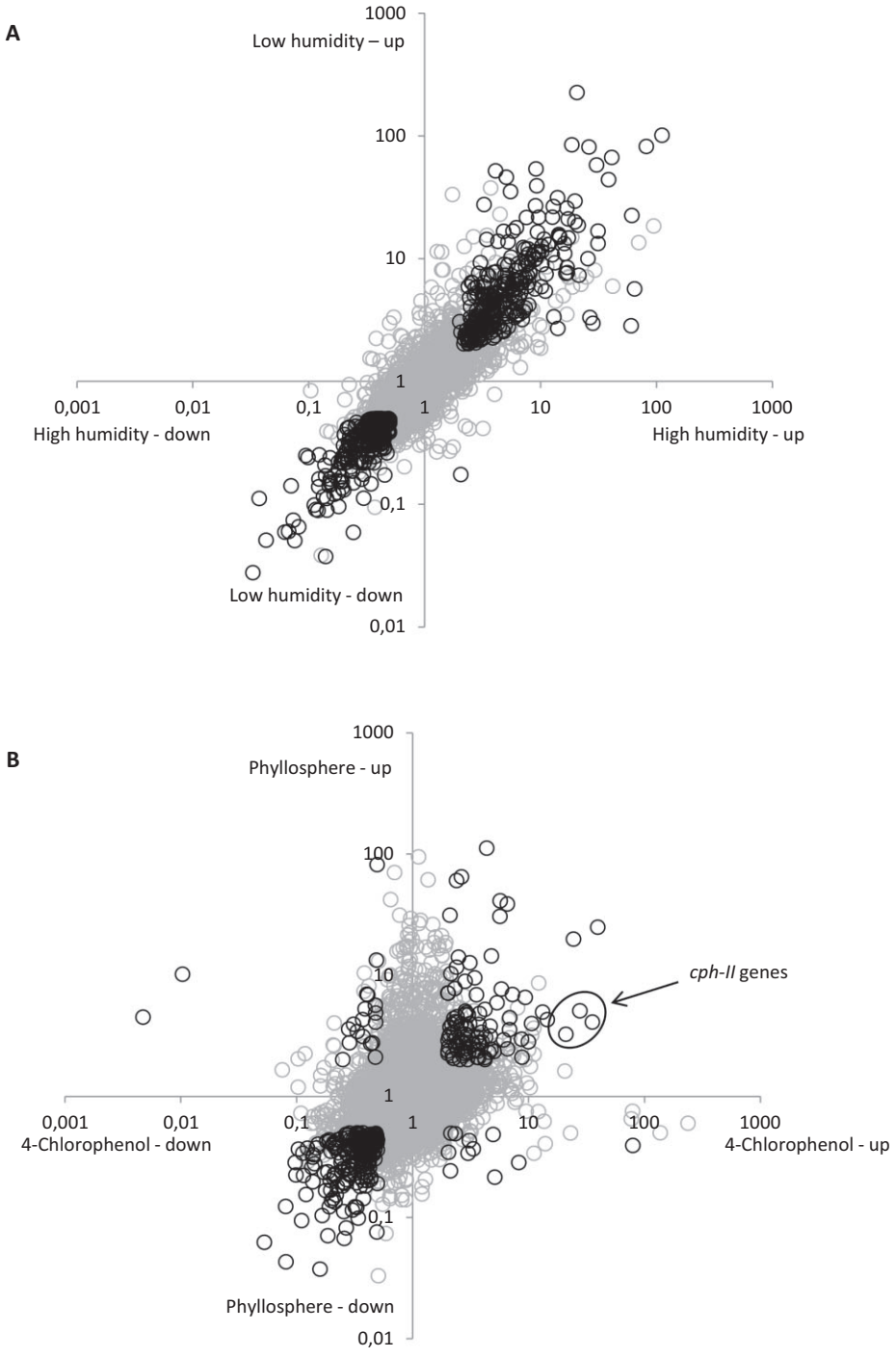

Fig. 2. Correlations between the fold change in gene expression under high- and low-humidity conditions in the phyllosphere (A), and between high humidity in the phyllosphere and 4-chlorophenol exposure (B). All changes in gene expression are relative to agar surface without chlorophenol. Black symbols represent genes that were significantly ($P < 0.05$) more than twofold differentially expressed in both treatments. The other genes are indicated in grey symbols. Indicated by a circle and arrow are the *cph-II* genes, i.e. *cphC-II*, *cphA-II* and *cphF-II*, which form a putative operon and have been implicated in 4-CP degradation, coding for the conversion of hydroquinone to oxoadipate.

Table 1. Differential expression of selected genes in *A. chlorophenolicus* A6.

Locus Tag[a]	PhylH[b]	PhylL[b]	A+4CP[b]	Predicted function
Achl_0049	**7.6**	**21.6**	1.6	Hypothetical protein
Achl_0050	**16.8**	**25.7**	–1.0	Hypothetical protein
Achl_0051	**26.2**	**81.1**	1.5	Protein of unknown function DUF1469
Achl_0052	**18.6**	**84.5**	1.9	Hypothetical protein
Achl_0159	**4.1**	**2.4**	**–2.1**	Beta-Ig-H3/fasciclin
Achl_0362	**111.8**	**101.3**	**4.4**	Phosphate ABC transporter, periplasmic phosphate-binding protein
Achl_0363	**21.1**	**18.7**	1.8	Phosphate ABC transporter, inner membrane subunit PstC
Achl_0364	**14.6**	**14.9**	1.8	Phosphate ABC transporter, inner membrane subunit PstA
Achl_0365	2.4	2.9	**–2.5**	Phosphate ABC transporter, ATPase subunit(EC:3.6.3.27)
Achl_0518	1.4	**11.3**	–1.5	General substrate transporter
Achl_0710	**10.2**	**5.9**	–96	Malate synthase A (EC:2.3.3.9)
Achl_0711	**4.5**	**4.3**	–211	Isocitrate lyase (EC4.1.3.1)
Achl_0848	**7.4**	**8.8**	1.5	Phosphate uptake regulator, PhoU
Achl_1321	**81.6**	**81.9**	**–2.0**	Hypothetical protein
Achl_1726	3.6	**11.0**	1.6	2-hydroxypropyl-CoM lyase (EC:2.1.1.14)
Achl_1744	**6.2**	**6.7**	1.8	Daunorubicin resistance ABC transporter ATPase subunit
Achl_1844	**3.2**	**3.1**	**3.0**	Putative transcriptional regulator
Achl_1845	**2.6**	**2.2**	**2.3**	FeS assembly protein SufB
Achl_1846	**2.7**	2.2	**2.7**	FeS assembly protein SufD
Achl_1847	**2.1**	1.9	**3.6**	Rieske (2Fe-2S) domain protein
Achl_1848	1.8	1.6	**2.3**	FeS assembly ATPase SufC
Achl_1849	2.1	2.1	**2.5**	Protein of unknown function DUF59
Achl_2231	**22.3**	6.2	–1.1	Nitrogen regulatory protein P-II
Achl_2232	**70.3**	13.5	–1.4	Ammonium transporter
Achl_2284	**2.5**	**2.3**	–1.7	Beta-Ig-H3/fasciclin
Achl_2389	2.0	**7.3**	1.6	Cysteine desulfurase
Achl_2563	**–4.1**	**–17.0**	**2.1**	Major facilitator superfamily MFS_1
Achl_2643	**20.6**	**225.1**	1.6	Flp/Fap pilin component
Achl_2644	**9.1**	**53.9**	–1.6	TadE family protein
Achl_2645	**5.5**	**35.2**	–1.8	Hypothetical protein
Achl_2646	4.5	**22.9**	–1.3	SAF domain protein
Achl_2647	**2.7**	**5.7**	–1.1	Flp pilus assembly protein ATPase CpaE-like protein
Achl_2648	2.2	**12.6**	1.2	Type II secretion system protein E
Achl_2649	1.0	**3.5**	–1.6	Type II secretion system protein
Achl_2650	1.7	**33.3**	1.2	Type II secretion system protein
Achl_2712	**13.0**	**3.4**	–2.0	Phage shock protein C, PspC
Achl_2713	**6.8**	1.9	**–2.4**	Hypothetical protein
Achl_2714	**3.2**	1.6	–1.7	Phage shock protein C, PspC
Achl_2817	4.3	**8.0**	**6.7**	Sulfate adenylyltransferase, large subunit(EC:2.7.7.4)
Achl_2818	5.4	**9.5**	**8.0**	Sulfate adenylyltransferase, small subunit(EC:2.7.7.4)
Achl_2819	**3.3**	**5.1**	**2.8**	Phosphoadenosine phosphosulfate reductase (EC:1.8.4.8)
Achl_2820	8.6	**12.9**	**12.2**	Sulfite reductase (ferredoxin) (EC:1.7.7.1)
Achl_2971	1.8	1.7	**2.6**	Hypothetical protein
Achl_2972	1.1	–1.1	**7.2**	Flagellar biosynthesis protein FlhA
Achl_2973	–2.3	**–2.3**	–1.2	Type III secretion exporter
Achl_2974	1.8	1.8	**3.4**	Flagellar biosynthetic protein FliR
Achl_2975	1.1	–1.2	**4.4**	Flagellar biosynthetic protein FliQ
Achl_2976	1.5	–1.0	1.9	Flagellar biosynthetic protein FliP
Achl_2977	1.3	1.1	**2.8**	Flagellar biosynthesis protein FliO
Achl_2978	1.7	2.0	**2.8**	Flagellar motor switch protein FliN
Achl_2979	1.1	–1.1	**7.2**	Surface presentation of antigens (SPOA) protein
Achl_2980	2.4	1.9	**2.9**	OmpA/MotB domain protein
Achl_2981	1.4	–1.1	**4.5**	MotA/TolQ/ExbB proton channel
Achl_2982	1.4	1.3	**3.2**	Flagellar FlbD family protein
Achl_2983	1.2	1.2	**5.4**	Protein of unknown function DUF1078 domain protein
Achl_2984	–1.1	–1.2	**5.3**	Flagellar hook capping protein
Achl_2985	2.3	2.5	**2.2**	Hypothetical protein
Achl_2986	1.3	–1.1	**4.3**	NLP/P60 protein
Achl_2987	2.2	1.8	1.8	Flagellar export protein FliJ
Achl_2988	2.7	1.9	**6.8**	ATPase, FliI/YscN family(EC:3.6.3.14)

Table 1. *cont.*

Locus Tag[a]	PhylH[b]	PhylL[b]	A+4CP[b]	Predicted function gene product
Achl_2989	1.2	1.3	**3.2**	Hypothetical protein
Achl_2990	**2.2**	1.9	**2.7**	Flagellar motor switch protein FliG
Achl_2991	**4.4**	**4.0**	**6.9**	Flagellar M-ring protein FliF
Achl_2992	2.4	2.1	**2.3**	Flagellar hook-basal body complex subunit FliE
Achl_2993	1.8	1.3	**3.6**	Flagellar basal-body rod protein FlgC
Achl_2994	1.8	1.3	**5.3**	Flagellar basal-body rod protein FlgB
Achl_2995	1.4	–1.1	**4.6**	Hypothetical protein
Achl_2996	–1.0	–1.2	**3.8**	Flagellar protein FliS
Achl_2997	–1.6	–2.0	**3.6**	Flagellar hook-associated 2 domain protein
Achl_2998	–1.1	–1.6	**7.6**	Flagellin domain protein
Achl_2999	1.2	–1.1	**5.7**	FlgN family protein
Achl_3000	2.0	1.3	**6.3**	Flagellar hook-associated protein FlgK
Achl_3001	1.3	1.3	**5.6**	Flagellar hook-associated protein 3
Achl_3258	**28.2**	**3.0**	1.2	Protein of unknown function DUF322
Achl_3259	**10.0**	1.9	–1.6	Hypothetical protein
Achl_3260	**64.7**	**5.7**	**2.6**	Hypothetical protein
Achl_3261	**26.6**	**3.3**	1.1	Hypothetical protein
Achl_3262	**60.3**	**2.8**	**2.4**	Hypothetical protein
Achl_3263	**14.1**	**2.7**	–1.2	CsbD family protein
Achl_3264	**9.4**	1.9	–1.3	RNA polymerase, sigma-24 subunit, ECF subfamily
Achl_3265	**6.7**	1.3	–1.6	Hypothetical protein
Achl_3266	**4.4**	1.5	–1.4	Hypothetical protein
Achl_3518	**17.4**	7.0	1.7	Ammonium transporter
Achl_3525	**7.6**	5.4	1.5	Glutamine synthetase, type III(EC:6.3.1.2)
Achl_3724	**31.2**	**16.7**	–1.3	Ferric reductase domain protein transmembrane component domain protein
Achl_3725	**25.7**	**10.0**	–1.1	FMN-binding domain protein
Achl_3726	**17.0**	**7.7**	1.3	ApbE family lipoprotein
Achl_3731	**3.8**	**4.3**	**2.6**	Siderophore-interacting protein
Achl_3732	**3.1**	**3.0**	**2.4**	ABC transporter related(EC:3.6.3.34)
Achl_3733	**4.0**	**4.5**	**3.1**	Transport system permease protein
Achl_3734	**5.9**	**6.7**	**5.3**	Transport system permease protein
Achl_3735	**6.9**	**7.0**	**7.3**	Periplasmic binding protein
Achl_3864	**94.6**	18.4	1.1	Hypothetical protein
Achl_4158	**3.7**	2.8	–1.1	Peptidase A24A prepilin type IV(EC:2.1.1.-,EC:3.4.23.43)
Achl_4451	**–30.3**	**–36.1**	–2.0	Hypothetical protein
Achl_4564	**5.1**	**46.0**	**27.7**	Monooxygenase FAD-binding(EC:1.14.13.20)
Achl_4565	**3.3**	**27.5**	**21.0**	Iron-containing alcohol dehydrogenase (EC:1.3.1.32)
Achl_4566	**4.1**	**51.9**	**35.5**	Intradiol ring-cleavage dioxygenase (EC:1.13.11.1)
Achl_4567	1.3	**5.2**	**3.1**	Hypothetical protein
Achl_4568	–1.3	–1.3	**79.0**	Hypothetical protein
Achl_4569	–1.5	–2.0	**77.1**	Intradiol ring-cleavage dioxygenase (EC:1.13.11.1)
Achl_4570	**–2.5**	**–2.7**	**79.5**	Flavin reductase domain protein FMN-binding
Achl_4571	–1.9	–1.9	–1.8	Transcriptional activator domain protein
Achl_4572	–1.7	–1.1	**237.6**	Protein of unknown function DUF1486
Achl_4573	–2.0	–1.7	**137.0**	4-hydroxyphenylacetate 3-hydroxylase (EC:1.14.13.3)
Achl_4574	1.6	1.9	**20.6**	Iron-containing alcohol dehydrogenase
Achl_4629	**3.7**	**2.7**	**2.1**	Relaxase/mobilization nuclease family protein
Achl_4630	**2.1**	1.8	**3.7**	Mobilization protein

a. Only those genes referred to explicitly in the text are listed here. For a complete list, see Supporting Information Table S1.
b. The values shown are fold changes in the expression of *A. chlorophenolicus* A6 genes on leaves at high relative humidity (PhylH), on leaves at low relative humidity (PhylL) or on agar plates supplemented with 4-chlorophenol (A+4CP), compared with agar plates without 4-CP. Genes with a more than twofold change in gene expression and a corrected P value < 0.05 are indicated in bold.

microarray data did not support the notion that *A. chlorophenolicus* A6 utilizes these sugars during epiphytic growth. Perhaps the expression of genes for catabolism of glucose went undetected due to the fact that growth on leaf surfaces was compared with growth on tryptic soy agar, which features glucose as the main carbon source. However, the microarray data did show phyllosphere-induced expression of genes involved in the acquisition of other nutrients, specifically phosphate, nitrogen, iron and sulphur, as explained below.

Cluster Achl_0362–0365 codes for subunits of a phosphate ABC transporter system and was highly expressed in the phyllosphere. The fold-change in transcript level for Achl_0362, which encodes a periplasmic phosphate-binding protein, was highest of all differentially expressed phyllosphere genes (Supporting Information Table S1).

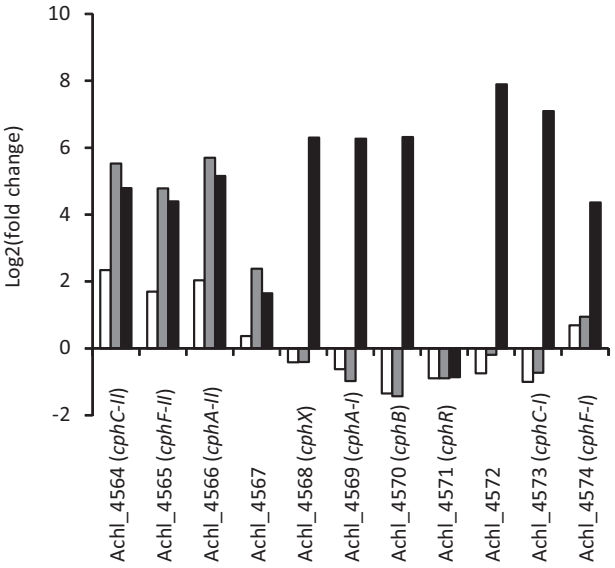

Fig. 3. Fold change in the expression of *A. chlorophenolicus* 4-chlorophenol degradation genes on the transcriptome arrays. White bars represent the comparison between the high-humidity phyllosphere and agar surface treatment, grey bars between the low-humidity phyllosphere and agar surface treatment, and black bars between the A+CP and A-CP agar surface treatment.

Achl_2231 and Achl_2232, which encode a nitrogen regulatory protein P-II and an ammonium uptake transporter, respectively, were also induced in the phyllosphere. Under nitrogen-limiting conditions, the P-II protein is involved in deadenylation of glutamine synthetase type I, which activates the enzyme. Glutamine synthetase type III, encoded by Achl_3525, also had an increased expression in the phyllosphere, as did the gene for a second ammonium transporter (Achl_3518), located several genes upstream. Ammonium transporters, the P-II protein and glutamine synthetases are all key enzymes in the acquisition of nitrogen at low ammonium concentrations (Javelle *et al*., 2004).

Cluster Achl_3731–3735 includes genes involved in iron uptake, and showed increased expression in the phyllosphere as well as in response to 4-chlorophenol. The cluster features a periplasmic binding protein, two transport system permease proteins and an ABC transporter, which are all part of an iron complex transport system. In addition, the cluster codes for a siderophore-interacting protein. In close proximity, three genes predicted to encode for a membrane-associated ferric iron reductase (Achl_3724–3726) also showed increased levels of expression in the phyllosphere.

Several sulphur assimilation genes were induced in the phyllosphere, as well as on agar with 4-chlorophenol. These genes included Achl_1844–1849 which code for components of the sulphur assimilation (SUF) system iron-sulphur (FeS) cluster. The SUF system operates under iron starvation and oxidative stress (Outten *et al*., 2004). Genes Achl_2817–2820 code for assimilatory sulphate reduction via 3′-phosphoadenylylsulfate (PAPS) and were expressed more highly in the phyllosphere, as were genes coding for incorporation of sulphide, the product of the PAPS assimilation pathway, i.e. 5-methyltetrahydropteroyltriglutamate/homocysteine S-methyltransferase (Achl_1726) and cysteine desulfurase (Achl_2389).

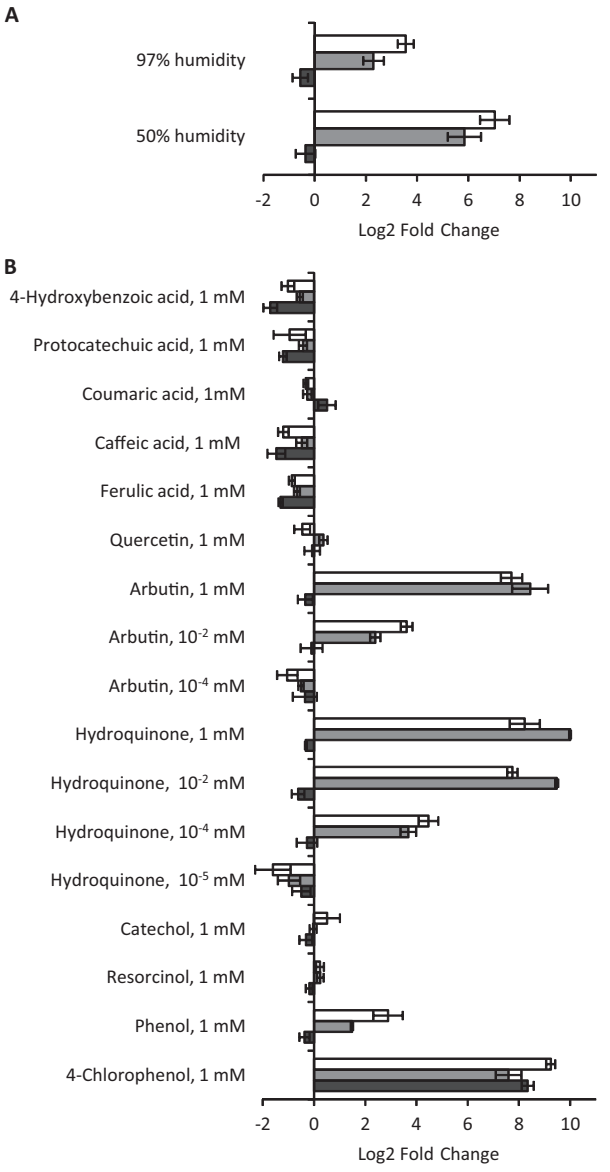

Fig. 4. Fold change in expression of *cph* genes in *A. chlorophenolicus* A6 as determined by RT-qPCR. Gene expression in the phyllosphere at high and low air humidity as compared with an agar surface (A), and gene expression in mineral medium in presence versus absence of several phenolic compounds (B). White bars, Achl_4564 (*cphC-II*); grey bars, Achl_4566 (*cphA-II*); black bars, Achl_4569 (*cphA-I*).

Attachment and motility

The Achl_2643–2650 gene cluster is predicted to be involved in surface attachment. The genes encode Flp pilus assembly proteins. Flp pili belong to a subfamily of the type IV pilin family, which mediates unspecific attachment to surfaces and the formation of microcolonies (Kachlany *et al*., 2001; Pelicic, 2008). Most genes of this cluster were highly expressed in the phyllosphere. Interestingly, this gene cluster is flanked upstream by another Flp pili cluster with a paralogous set of genes, but those genes were not induced in the phyllosphere. Achl_4158, coding for a prepilin peptidase, was expressed fourfold higher in the phyllosphere than on agar surfaces, while other pilin-associated genes were not differentially expressed. There is evidence that the presence of type IV pili increases the phyllosphere fitness of bacteria (Suoniemi *et al*., 1995; Roine *et al*., 1998).

Achl_0159 and Achl_2284 are two other genes with elevated expression in the phyllosphere and with involvement in attachment. Their predicted gene products contain a fasciclin-like (FAS1) domain, which is found in proteins from bacteria to mammals and considered an ancient cell adhesion domain (Ulstrup *et al*., 1995). This finding is in agreement with a metaproteomic study where bacterial proteins with a fasciclin domain were consistently recovered from the phyllosphere of soybean, clover and *Arabidopsis* (Delmotte *et al*., 2009).

The *A. chlorophenolicus* genome contains a cluster of 31 genes involved in flagellar synthesis (Achl_2971–3001). These genes were collectively induced in response to 4-chlorophenol, while only two of them were significantly higher expressed in the phyllosphere, namely Achl_2990 and Achl_2991, encoding a flagellar motor switch protein and a flagellar M-ring protein respectively.

Stress

Gene cluster Achl_3258–3266 was one of the most highly expressed in the phyllosphere. This cluster consists mainly of genes coding for hypothetical proteins, two of which (Achl_3258 and Achl_3265) contain an Asp23 domain, which is an alkaline shock protein family (Kuroda *et al*., 1995). The same cluster also codes for a CsbD family protein (Achl_3263) and a sigma-24 factor (Achl_3264). CsbD is a bacterial general stress response protein, but its role in stress response is unclear (Pragai and Harwood, 2002). The A6 genome contains three *csbD* homologs, all of which showed increased expression in the phyllosphere. The RNA polymerase sigma-24 subunit belongs to the extracytoplasmic function (ECF) subfamily of sigma factors. These sigma factors are involved in responses to extracytoplasmic stresses, such as oxidative stress and desiccation (Testerman *et al*., 2002; Cytryn *et al*., 2007). Nine such sigma-24 genes are present in the *A. chlorophenolicus* genome, and four of them were expressed significantly higher in the phyllosphere than on agar.

Another stress-related gene is *phoU* (Achl_0848), which showed elevated expression in the phyllosphere. PhoU acts as a global negative regulator that increases resistance against multiple antibiotics and stresses by a decrease in cellular metabolism (Li and Zhang, 2007). PhoU, as well as CsbD and Asp23, are expressed in a sigma B-dependent manner. The alternative sigma factor SigB is a master regulator in general stress response in *Bacillus subtilis* and related gram-positive bacteria (Hecker *et al*., 2007). However, the *A. chlorophenolicus* A6 genome does not appear to contain genes annotated as sigma B factors.

Also induced under stress is the phage shock protein C (Darwin, 2005). Cluster Achl_2712–2714 encodes two such phage shock C proteins and one hypothetical protein, all three of which were higher expressed on leaf surfaces than on agar surfaces. Like the ECF sigma factors, the phage–shock–protein system reacts to extracytoplasmic stress (Darwin, 2005).

The *A. chlorophenolicus* genome contains six drug resistance transporters of the EmrB/QacA subfamily. Four of these were significantly higher expressed in the phyllosphere (14-, 7- and two times 3-fold). In addition, Achl_1744, which is annotated to encode for a daunorubicin resistance ABC transporter, showed sixfold higher expression in the phyllosphere. These data suggest that *A. chlorophenolicus* A6 cells encountered adverse compounds in the phyllosphere that needed to be transported out of the cell.

Horizontal gene transfer

It has been shown previously for *Pseudomonas syringae* and for *Pseudomonas putida* that the phyllosphere stimulates horizontal gene transfer (Normander *et al*., 1998; Bjorklof *et al*., 2000). We observed phyllosphere-induced expression of genes encoding relaxase and mobilization proteins (Achl_4629 and Achl_4630). Both genes were also expressed to a higher level in the presence of 4-chlorophenol. They are located on plasmid pACHL02, which is the same plasmid that harbours the *cph* gene cluster for 4-chlorophenol degradation.

Glyoxylate bypass

The Achl_0710 and Achl_0711 genes coding for key enzymes (malate synthase and isocitrate lyase, respectively) in the glyoxylate bypass were induced in the

phyllosphere. Elevated levels of the same two enzymes were found during the growth of *A. chlorophenolicus* A6 on phenol (Unell *et al*., 2009), which the authors took as an indication of 'insufficient energy'. Activation of the glyoxylate bypass on plant leaf surfaces suggests that strain A6 is assimilating carbon from C2 compounds, possibly acetyl-CoA acquired through catabolism of plant-derived compounds.

Hypothetical proteins

The genes with the second and third most highly increased expression levels in the phyllosphere, Achl_3864 (95-fold) and Achl_1321 (82-fold), are annotated to encode for hypothetical proteins conserved in *Arthrobacter* species but with unknown function. They are not part of an operon with known genes. Achl_1321 has a transmembrane helix and a signal peptide cleavage site outside the cell, suggesting that this protein is secreted by the cell. Similarly, the gene with the highest degree of repression in the phyllosphere, Achl_4451, is a hypothetical protein. Investigation of the function of these three genes would be particularly interesting in the context of phyllosphere colonization.

Another gene cluster of interest is Achl_0049 to Achl_0052. These genes were induced in the phyllosphere compared with agar and expressed higher at high relative humidity. Again, these genes are all hypothetical proteins with unknown functions. Upstream of this cluster is a MarR regulatory gene, which was also higher expressed in the phyllosphere. MarR regulators can control a variety of functions, such as resistance to antibiotics, organic solvents and oxidative stress (Alekshun and Levy, 1999).

Discussion

Where previous studies of the phyllosphere transcriptome have focused on Gram-negative plant and human pathogens (Fink *et al*., 2012; Yu *et al*., 2013), we here present to the best of our knowledge the first transcriptional profile of a Gram-positive phyllosphere-competent strain. Our data set highlights many similarities and differences to the transcriptional profiles of leaf-associated bacteria published to date. We found an increased expression of genes related to nutrient acquisition, attachment, stress response and horizontal gene transfer in the phyllosphere, which is to a large extent in accordance with previous studies that investigated gene and protein expression in the phyllosphere (Marco *et al*., 2005; Delmotte *et al*., 2009; Fink *et al*., 2012; Yu *et al*., 2013). A surprising finding of the present study was the leaf-induced expression of part of the chlorophenol degradation pathway. This will be discussed in greater detail below. Another unique finding was the induction of Flp pili genes. Although attachment is considered to be an important factor in phyllosphere colonization, and a role of pili has been suggested (Leveau, 2006), this is the first time that Flp pili genes have been identified as phyllosphere-inducible. In contrast, leaf exposure of *P. syringae* increased the expression of a large number of genes involved in flagellar synthesis and chemotaxis (Yu *et al*., 2013). Therefore, attachment could be an important phyllosphere survival strategy for *Arthrobacter*, as opposed to motility for *P. syringae*. The expression profile of *A. chlorophenolicus* A6 in the phyllosphere was minimally affected by humidity levels. Moreover, we did not find evidence for (increased) production of osmoprotectants, such as trehalose in the phyllosphere, which is in contrast with the findings for *P. syringae* (Yu *et al*., 2013). However, one of the two significantly higher expressed genes under low versus high relative humidity is annotated to code for a transporter that may aid in osmoadaptation by allowing uptake of compatible solutes, including betaine and proline (Axtell and Beattie, 2002). Many epiphytes can produce these types of compounds, which would suggest that survival of A6 on leaf surfaces may be hardwired to depend in part on the presence and activity of other microbes on the leaf surfaces.

We observed substantial similarities between the transcriptional response of *A. chlorophenolicus* to the phyllosphere and upon exposure to 4-chlorophenol. Genes involved in phosphate and iron uptake, sulphur assimilation, plasmid mobilization genes and several 4-chlorophenol degradation genes were significantly higher expressed under both conditions. Out of 337 genes with a significantly altered expression under both conditions, 91% changed in the same direction, i.e. either up or down, compared with growth on agar in the absence of chlorophenol. Those similarities suggest a priming effect, where exposure to the phyllosphere could lead to a pre-adaptation of bacteria for growth on organic pollutants, such as 4-chlorophenol. Interestingly, a higher expression of genes involved in xenobiotic degradation in the phyllosphere compared with liquid growth medium was also found for the *P. syringae* transcriptome, although this species is not known as a pollutant degrader (Yu *et al*., 2013). In *A. chlorophenolicus* A6, stress-related genes that were induced in the phyllosphere were not responsive to the presence of 4-chlorophenol. This indicates that the stress response to 4-chlorophenol was different from phyllosphere-induced stresses. Under water stress induced by sodium chloride or polyethylene glycol, none of the genes for 4-chlorophenol degradation were induced (S.K.. Moreno and J.R. van der Meer, unpubl. data), suggesting that osmotic stress is not a trigger for their expression. A more likely explanation is that these genes react to

phenolic compounds that are naturally present in the phyllosphere.

Our data demonstrated the phyllosphere-induced expression of three genes within the *cph* gene cluster for 4-chlorophenol degradation. These three genes are thought to constitute a subcluster (cluster II) that evolved independently from the rest of the *cph* gene cluster and were combined by a more recent horizontal gene transfer event (Nordin *et al*., 2005). Based on our qPCR results (Fig. 4), we hypothesize that leaf surface-induced expression of the cluster II genes was triggered by the presence of hydroquinone or derivatives thereof on leaf surfaces. Indeed, we could detect hydroquinone in bean leaf washes, and our data suggest that hydroquinone is available to bacteria such as *A. chlorophenolicus* A6 on the leaf surface in concentrations sufficiently high to stimulate the expression of genes that code for the degradation of hydroquinone. Hydroquinone and its glycosylated form, arbutin, have been identified in the leaves of a broad range of plant species, such as pear, bearberry, *Polygonella myriophylla* and several species in the family Lamiaceae (Pedersen, 2000; Parejo *et al*., 2001; Jin and Sato, 2003; Weidenhamer and Romeo, 2004). In the resurrection fern, hydroquinone and arbutin are important for the plant to deal with desiccation stress (Suau *et al*., 1991). Hydroquinone has also been listed as a compound with antimicrobial (Jin and Sato, 2003) and surface-wetting (Wieckowska *et al*., 2007) properties.

Our findings are of potential interest for phyllosphere-based bioremediation studies. The ability of phyllosphere bacteria to degrade airborne aromatic pollutants, such as phenol, has been previously established (Sandhu *et al*., 2007; 2009). Although the exact relationship between genes involved in (or induced by) hydroquinone and phenol remains to be investigated, our observation that exposure to the phyllosphere induces many of the same degradation genes as exposure to phenol indicates that the phyllosphere might prime bacteria for pollutant degradation. Such priming, for example through a process of 'phyllo-augmentation', could potentially result in increased degradation of and/or a faster response to aromatic pollutants in bio-based environmental clean-up operations.

In summary, we demonstrated that the phyllosphere competency of *A. chlorophenolicus* A6 is linked to the expression of a number of specific gene functions that support epiphytic survival. Most unforeseen and exciting was the discovery that the best studied genes of this bacterium so far, namely the *cph* genes for the degradation of the pollutant 4-chlorophenol, were induced during leaf colonization, and that their elevated expression *in planta* concurs with our demonstration that hydroquinone, an inducer of these genes, is present in the leaf environment.

Experimental procedures

Experimental set-up and sample preparation for transcriptome microarrays

Arthrobacter chlorophenolicus strain A6 was grown at 28°C and 250 r.p.m. in lysogeny broth (LB). At mid-exponential phase, the bacterial culture was centrifuged for 10 min at 3838 *g*. The pellet was resuspended in sterile demineralized water to obtain a bacterial suspension with an optical density at 600 nm (OD_{600}) of 0.15, which corresponded to approximately 8×10^7 colony-forming units (CFU) per millilitre. The above-ground portion of 2-week old bean plants (*Phaseolus vulgaris*, green snap bean, variety Blue Lake Bush 274) with the first two leaves fully expanded were dipped into this bacterial suspension. Five plants were incubated at 97% air humidity for 2 days in a growth chamber; we refer to this as the high-humidity phyllosphere or PhylH treatment. Five other plants were incubated for 1 day at 97% air humidity, followed by a second day at 50% air humidity; we refer to this as the reduced- or low-humidity phyllosphere or PhylL treatment. In addition, 500 µl of bacterial suspension was spread on 1/10 strength Tryptone Soy Agar (TSA; Oxoid, Cambridge, UK) with 15 g agar per litre and supplemented with 1 mM 4-chorophenol (4-CP); we refer to this as the agar plus 4-CP (or A+CP) treatment. Another 500 µl was spread on 1/10 strength TSA without the 4-chlorophenol; we refer to this as the A-CP treatment. Both agar treatments were incubated at 97% humidity for 2 days. We chose agar surfaces as a control (rather than liquid cultures) so as to avoid picking up genes that are surface-induced but not phyllosphere-specific. All plants and plates were incubated in a growth chamber that was maintained a day–night cycle of 16 h and 8 h at 21°C and 16°C respectively. Bacterial population sizes on leaves were estimated at 0, 24 and 48 h post-inoculation to show that they increased during the first 24 h but stabilized during the second 24 h following inoculation (Supporting Information Fig. S2). Similarly, the bacteria on plate established a lawn during the first 24 h, without apparent growth after that and until time of harvest. For each treatment, we prepared four independent replicate experiments. For each replicated experiment, bacteria were recovered from leaves by sequentially putting five leaves from five different plants in 20 ml RNA protection solution [two parts RNAprotect bacteria reagent (Qiagen, Venlo, The Netherlands) and one part phosphate-buffered saline] with 5 s vortexing, 7 min sonication and 5 s vortexing for each leaf. The solution was centrifuged for 20 min at 3838 *g*, and bacterial pellets were frozen at −80°C until RNA extraction. Bacteria from agar plates were washed from the surface with 1 ml RNAprotect solution according to the manufacturer's instructions (Qiagen) and frozen at −80°C until RNA extraction.

Microarray design

YODA software (Nordberg, 2005) was used to design 50-mer probes that target genes from the chromosome and both plasmids of *A. chlorophenolicus* A6. The microarray design has been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE48198 (platform GPL17332). The majority of probes (99.5%) were designed with the following parameters: 1–3

non-overlapping probes per gene, a maximum of 70% identity to non-target sequences, a maximum of 15 consecutive matches to non-target sequences, a melting temperature range of 8°C and a GC content range of 12%. The remaining 0.5% of probes were designed with the following less stringent parameters: a maximum of 80% identity to non-target sequences, a melting temperature range of 15°C and a GC content range of 30%. In total, 13 589 probes were designed that target 99.8% of the predicted protein-coding A6 genes (4581 out of 4590). An additional seven positive control probes were included in the design. Probes were synthesized on microarrays by Agilent Technologies (Santa Clara, CA, USA) using the 8 × 15 000 format.

RNA extraction and microarray procedures

Bacterial pellets were thawed at room temperature and resuspended in 200 μl TE buffer (30 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 15 mg of lysozyme and 2 mg of proteinase K per millilitre. The suspension was incubated at room temperature for 30 min with regular vortexing. Then, 700 μl lysis solution of the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Veenendaal, The Netherlands) was added, and the total volume was transferred to a 2 ml screw cap tube containing 100 mg of 0.1:0.5 mm beads (1:1) (Merlin Bioproducts, Breda, The Netherlands). Tubes were shaken in a Mini-Beadbeater (Biospec Products, Breukelen, The Netherlands) twice for 1 min at 5000 r.p.m. with a 1 min interval on ice. Tubes were centrifuged for 10 s, and the solution without beads was transferred to a fresh tube. After the addition of 500 μl 70% isopropanol, samples were further treated according to the Aurum Total RNA Mini Kit protocol. Columns were eluted with 80 μl elution buffer. An additional 30 min DNase treatment was performed with Ambion TURBO DNA-*free* (Applied Biosystems, Nieuwerkerk a/d ijssel, The Netherlands). After the DNase treatment, the RNA was precipitated with 1/10 volume of 7.5 M ammonium acetate (Sigma-Aldrich, Zwijndrecht, The Netherlands), 1/50 volume glycogen (5 mg ml^{-1}) (Fermentas, St. Leon-Rot, Germany) and 2.5 volumes of ethanol. RNA pellets were washed with 80% ethanol and resuspended in 12 μl nuclease-free water. RNA quality was verified with Experion RNA StdSens (Bio-Rad Laboratories).

The procedures for cDNA synthesis and labelling, and for array hybridization, were based on a protocol described elsewhere, with slight modifications (Johnson *et al*., 2011). An amount of 2–5 μg of RNA was mixed with 1.25 μl random primers (500 μg ml^{-1}; Promega, Madison, WI, USA) in a total volume of 12 μl and incubated at 70°C for 10 min, followed by 4°C for 5 min. Each tube received 13 μl of mastermix containing 0.6 μl Cyanine 3-dCTP (1 mM; Perkin-Elmer, Waltham, MA, USA), 0.6 μl Superase-In (20 U μl^{-1}; Ambion), 1 μl Superscript II (200 U μl^{-1}; Life Technologies, Carlsbad, CA, USA), 5 μl 5× 1st strand buffer, 2.5 μl DTT (100 mM), 0.25 μl dATP-dGTP-dTTP mixture (10 mM each), 0.1 μl dCTP (5 mM) and 2.9 μl nuclease-free water. Labelled cDNA was produced by incubation at 42°C for 120 min, followed by 70°C for 10 min and 4°C for 5 min. RNA was hydrolysed under alkaline conditions as follows: After the addition of 2.5 μl 1 M NaOH, samples were heated at 65°C for 20 min, allowed to cool to room temperature for 10 min and neutralized by the addition of 2.5 μl 3 M Na-acetate (pH 5.2) and 2.5 μl 1 M HCl. The labelled cDNA was then purified using the MinElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The product was eluted in 20 μl EB buffer, and quantity and incorporation efficiency were determined using the MICROARRAY function on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Sixty nanogram of labelled cDNA with incorporation efficiencies between 2% and 3% was loaded onto each microarray, hybridized for 17 h at 65°C, and washed and scanned as described for labelled cRNA in the One-Color Microarray-Based Gene Expression Analysis Manual (Agilent Technologies). The fragmentation step (heating to 60°C for 30 min) was omitted. Hybridization signal intensities were extracted from scanned images using the Agilent Feature Extraction software package (version 10.7.1.1; Agilent Technologies).

To corroborate the array results, three replicate RNA samples each were obtained following the procedures as described above for the agar surface treatments, the high-humidity phyllosphere treatment and the low-humidity phyllosphere treatment. These RNA samples were subjected to RT-qPCR, as described below.

Microarray data analyses

The expression data were analysed using the Genespring GX software version 11 (Agilent Technologies) as described elsewhere (Johnson *et al*., 2011). In short, data were log2-transformed, normalized by quantile and scaled with the baseline to the median of all samples. All genes were filtered by expression level and were retained when the signal intensity was above the 20th percentile in at least one of the samples. Samples were clustered by hierarchical clustering using Euclidian distances and the average linkage rule; they were clustered on both entities and conditions. All treatments were compared pairwise with the agar surface control treatment using a Welch's *t* test with asymptotic *P* value computation and Benjamini–Hochberg false discovery rate for multiple testing correction. In addition, the high- and low-humidity phyllosphere treatments were compared with each other in a Welch's *t* test. Genes that were at least twofold differentially expressed between treatments with a corrected *P* value lower than 0.05 were considered statistically different. Gene annotations were retrieved from the IMG database (http://img.jgi.doe.gov/).

Array information is available in the NCBI Gene Expression Omnibus database under accession number GSE48198.

Gene expression in the presence of plant phenolic compounds

A. chlorophenolicus A6 cells were harvested from an LB overnight culture by centrifugation for 10 min at 3838 *g*. Cells were resuspended in Brunner mineral medium (MM; DSMZ medium no. 457, Braunschweig, Germany) with 5 mM fructose, adjusted to an OD_{600} of 0.15 and incubated at 28°C and 150 r.p.m. When the OD_{600} reached 0.35, 1 mM of phenolic compound (see below) was added, and the flasks were incubated for another 2 h at 28°C and 150 r.p.m. There were triplicates for each of the 18 treatments. We tested 12 phenolic compounds: phenol, 4-chlorophenol, 4-hydroxybenzoic

Table 2. Primer specifications.

Primer name	Target gene	Sequence	Annealing temperature
1611-f	Achl_1611	GTGGAAGTCATCAACAAG	54
1611-r	Achl_1611	GTCATGTCCAGTTCTAGTG	54
4564-f	Achl_4564	ATATCCCTCAGACGTACC	59
4564-r	Achl_4564	AGACATACTCAGTGGAGAAC	59
4566-f	Achl_4566	GCTTCTATGACGTCCAAT	59
4566-r	Achl_4566	AGTCCCCAGAAGGAGTAT	59
4569-f	Achl_4569	CCAGCACCTACACAACTTCG	59
4569-r	Achl_4569	AAGGCCTAGAACGTCAGAAAG	59

acid, protocatechuic acid, coumaric acid, caffeic acid, ferulic acid, quercetin, arbutin, hydroquinone, catechol and resorcinol. In the controls, no phenolic compound was added. Arbutin was also tested at concentrations of 10^{-2} and 10^{-4} mM, and hydroquinone at 10^{-2}, 10^{-4} and 10^{-5} mM. After 2 h, 3 ml culture was treated with RNAprotect (Qiagen) according to the manufacturer's instructions, and cells were frozen at –80°C until RNA extraction. In addition, the OD_{600} and the number of CFUs per millilitre (CFU ml^{-1}) of the cultures were determined. RNA was extracted according to the protocol described above and resuspended in 35 μl nuclease-free water.

RT-qPCR

Table 2 summarizes the qPCR primers that were designed for the *A. chlorophenolicus* A6 genes Achl_4564, Achl_4566, Achl_4569 and Achl_1611, using the primer3 software (http://primer3.sourceforge.net/) or SciTools of Integrated DNA Technologies (http://eu.idtdna.com/scitools/Applications/RealTimePCR/). Plasmid-encoded genes Achl_4564, Achl_4566 and Achl_4569 are part of the *A. chlorophenolicus* gene cluster for 4-chlorophenol degradation, while Achl_1611 serves as a reference gene; it is chromosomally located and encodes the RNA polymerase sigma factor RpoD. Appropriate annealing temperatures (Table 2) were optimized by testing a range of temperatures.

Two-step RT-qPCR was performed on RNA samples from phyllosphere bacteria (see *RNA extraction and microarray procedures*) and RNA samples from bacteria that were exposed to different phenolic compounds (see *Gene expression in the presence of plant phenolic compounds*). An amount of 420 ng RNA was converted to cDNA with random hexamer primers in a reaction volume of 20 μl (RevertAid First Strand cDNA Synthesis Kit, Fermentas). The cDNA product was diluted 50 times. qPCR mixtures contained 12.5 μl ABsolute™ QPCR SYBR® green mix (ABgene, Fisher Scientific, Landsmeer, The Netherlands), 10 μg BSA, 6.25 pmol of each primer and 5 μl of diluted cDNA template in a total volume of 25 μl. The qPCRs were performed on a Corbett Research Rotor-Gene 3000 thermal cycler (Westburg, Leusden, The Netherlands) with a regime of one step of 15 min at 95°C, and 40 cycles of 60 s at 95°C, 40 s at the respective annealing temperature (Table 2) and 60 s at 72°C. Gene expression was calculated relative to the Achl_1611 *rpoD* gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Analysis of leaf surface washes by GC-MS

For each of the three independent samples, 32 primary leaves of 2-week-old *P. vulgaris* plants were sequentially dipped into 100% methanol for 30 s. The methanol extract was then divided in two parts, both parts received 50 ng of 3,5-dihydroxybenzoic acid as an internal standard and one part was spiked with 50 ng of hydroquinone. The methanol extract was filtered over Whatman filter paper, and the solvent evaporated under a flow of nitrogen. Dried samples were dissolved in 50 μl acetonitrile and subsequently derivatized with 100 μl BSTFA [N,O-bis(trimethylsilyl) trifluoroacetamide] and 10 μl TMCS (trimethylchlorosilane) overnight at room temperature. Samples were diluted with 500 μl acetonitrile and 1–10 μl were injected in an Agilent GC 7890 (Agilent) with BPX-5 column (30 m × 0.32 m × 0.25 μm; SGE, Darmstadt, Germany) in either split or split-less mode. The temperature programme from 50°C to 300°C was as follows: 50°C (5 min) to 100°C (30 min) at 30°C min^{-1}, to 175°C (5 min) at 10°C min^{-1}, to 250°C (5 min) at 10°C min^{-1}, and to 300°C (15 min) at 30°C min^{-1}. The injector was set at 280°C and the He flow was 1.7 ml min^{-1}. Mass spectra were recorded using an Agilent MS 5975C inert XL MSD and analysed with an MSD ChemStation G1701 EA.E.02.00.493. External standards were run for arbutin, caffeic acid, ferulic acid, 4-hydroxybenzoic acid, protocatechuic acid, hydroquinone, catechol, resorcinol, and 4-CP.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. GC-MS analysis of *Phaseolus vulgaris* leaf surface wash extracts. A peak with the retention time (19.6 min) of silated hydroquinone is indicated in a chromatogram section by an arrow (A). The corresponding mass spectrum (B) matched that of silated hydroquinone from the NIST Mass Spectrometry Data Center (C) and of an authentic standard (not shown).

Fig. S2. Population sizes of *A. chlorophenolicus* A6 on bean leaves that were sampled for RNA extraction and subsequent microarray analysis. 'Wet' refers to the PhylH treatment, while 'dry' refers to the PhylL treatment.

Table S1. Fold change in gene expression and corrected *P* values of *A. chlorophenolicus* genes on transcriptome arrays. Genes with a more than twofold change in gene expression and a corrected *P* value lower than 0.05 are indicated in bold. Comparisons were made between (i) high-humidity phyllosphere and agar surface treatment, (ii) low-humidity phyllosphere and agar surface treatment, and (iii) chlorophenol and agar surface treatment. Genes with a significantly different expression between high and low phyllosphere humidity treatments are indicated with asterisks (** = $P < 0.05$, * = $P < 0.1$).

Table S2. Compounds identified in three independent methanol extracts from the surface of bean leaves.