A proteomic study of *Methylobacterium extorquens* reveals a response regulator essential for epiphytic growth

Benjamin Gourion*, Michel Rossignol†, and Julia A. Vorholt*‡

*Laboratoire des Interactions Plantes Micro-Organismes (LIPM), Institut National de la Recherche Agronomique/Centre National de la Recherche Scientifique, BP52627, 31326 Castanet-Tolosan, France; and †Unité Mixte de Recherche 5546, Centre National de la Recherche/Université P. Sabatier, F-31326 Castanet-Tolosan, France

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Aerial plant surfaces are colonized by diverse bacteria such as the ubiquitous *Methylobacterium* spp. The specific physiological traits as well as the underlying regulatory mechanisms for bacterial plant colonization are largely unknown. The purpose of this study was to identify proteins produced specifically in the phyllosphere by comparing the proteome of *Methylobacterium extorquens* colonizing the leaves either with that of bacteria colonizing the roots or with that of bacteria growing on synthetic medium. We identified 45 proteins that were more abundant in *M. extorquens* present on plant surfaces as compared with bacteria growing on synthetic medium, including 9 proteins that were more abundant on leaves compared with roots. Among the proteins induced during epiphytic growth, we found enzymes involved in methanol utilization, prominent stress proteins, and proteins of unknown function. In addition, we detected a previously undescribed type of two-domain response regulator, named PhyR, that consists of an N-terminal sigma factor (RpoE)-like domain and a C-terminal receiver domain and is predicted to be present in essentially all *Alphaproteobacteria*. The importance of PhyR was demonstrated through phenotypic tests of a deletion mutant strain shown to be deficient in plant colonization. Among PhyR-regulated gene products, we found a number of general stress proteins and, in particular, proteins known to be involved in the oxidative stress response such as KatE, SodA, AhpC, Ohr, Trx, and Dps. The PhyR-regulated gene products partially overlap with the bacterial *in planta*-induced proteome, suggesting that PhyR is a key regulator for adaptation to epiphytic life of *M. extorquens*.

fitness | sigma factor | stress | two-component system

Molecular microbial ecology is often hampered by the difficulty of unraveling how the environment shapes bacterial physiology and allows microorganisms to multiply. One such habitat is the aerial parts of plants that are colonized by various microorganisms, mostly bacteria, which are often found in numbers averaging 10^6 to 10^7 cells per cm^2. Epiphytes, defined as bacteria that are capable of multiplying on plant surfaces, encounter rather harsh conditions in the phyllosphere environment. This habitat is generally considered to be poor in nutrients. In addition, residing microorganisms are exposed to the atmosphere and radiation and are subjected to rapid changes with respect to their physical environment (1). Many plant-colonizing bacteria do no apparent harm to their hosts and might even be beneficial to the plant, whereas others are plant pathogens and can, after establishment of an epiphytic population, ultimately destroy the tissue on which they are living.

The chemical and physical features of leaf surfaces are not well known, and the same is true for the traits that allow these bacteria to multiply in the leaf habitat. Our current knowledge about bacterial physiology in the phyllosphere stems mainly from targeted approaches. Thus, phenotypes such as flagellar motility, UV-mediated mutagenic repair, and exopolysaccharide production contribute substantially to epiphytic fitness (2–4). In addition, random mutagenesis has been performed to identify novel targets important for phyllosphere colonization (5). Gene expression profiling is a good strategy for providing information about adaptations to specific conditions or environments. One powerful strategy for targeting gene expression in the natural context is through promoter trap analysis [i.e., *in vivo* expression technology (IVET)] (6). Variants of this approach have been successfully applied to identify genes induced during phyllosphere colonization of bacterial pathogens (7–9). However, by definition, the IVET strategy can only give an incomplete picture of the physiology of bacteria. Indeed, it is well known that changes at the protein level are not necessarily predictable from transcript levels because of differences in translation efficiency, proteolysis, and posttranslational modifications. In this study, we have therefore chosen a more direct way to gain insights into the physiology of bacteria in the phyllosphere through the analysis of the proteome of a bacterium in this ecosystem.

For this work, we have used the *Alphaproteobacterium Methylobacterium extorquens* AM1, a well studied model pink-pigmented facultative methylotroph (PPFM) (10), whose draft genome sequence is available (see www.integratedgenomics.com/genomereleases.html#6). *Methylobacterium* spp. are common leaf epiphytes that represent an important bacterial population on leaves (11, 12) and have been found on all analyzed plants (13). *Methylobacterium* spp. on plant surfaces benefit from methanol produced by plants (14) by means of methylotrophy (10, 15). However, methanol is not the only carbon substrate that these bacteria are able to consume in the phyllosphere (14). The presence of *Methylobacterium* may be beneficial to plants through the production of plant hormones (13, 16). The ubiquitous presence of *Methylobacterium* on plant surfaces makes them an interesting model for discovering the particular traits that these bacteria have acquired as successful epipytes. This work provides the identification of previously undescribed candidate proteins of *Methylobacterium* required for phyllosphere colonization and, in particular, the identification of a key regulator controlling adaptation to this habitat.

Results and Discussion

Proteome Analysis of *M. extorquens* During Phyllosphere Colonization. With the aim to identify proteins that are specifically induced when *M. extorquens* AM1 colonizes the phyllosphere of *Arabidopsis thaliana* ColO plants, we performed a differential analysis of the

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Abbreviations: MM, minimal medium; ROS, reactive oxygen species; 2-DE, 2D gel electrophoresis.

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‡To whom correspondence should be sent at the present address: Institute of Microbiology, Swiss Federal Institute of Technology Zurich, Eidgenössiche Technische Hochschule Höggerberg, CH-8092 Zurich, Switzerland. E-mail: vorholt@micro.biol.ethz.ch.

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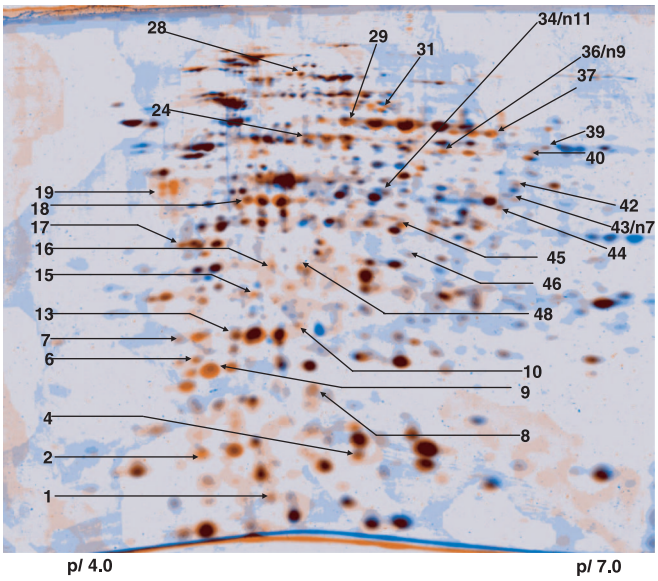

Fig. 1. Dual-channel image analysis of 2-DE protein pattern of *M. extorquens* AM1 to reveal proteins induced during phyllosphere colonization. Proteins from cells harvested from the phyllosphere of *A. thaliana* ColO are colored in orange, and those of cells harvested from the surface of solid MM containing succinate as the only carbon source are colored in blue. Spots of identified proteins are marked (see Table 1).

proteome of *M. extorquens* that had colonized plants under gnotobiotic conditions after seed inoculation by comparison with the proteome of bacteria that were cultivated on the surface of synthetic minimal medium (MM) under the same conditions of light and temperature. We used succinate as a carbon source, because it enters directly into the tricarboxylic acid (TCA) cycle and allows us to observe the induction of methylotrophy markers (17). Proteins were separated by 2D gel electrophoresis (2-DE), and we identified those that were induced at least 3-fold based on image analysis (Fig. 1). In total, 40 proteins were identified as up-regulated during phyllosphere colonization (Table 1). To distinguish between proteins specific for phyllosphere colonization with respect to more general epiphytic adaptation, we compared the proteome of bacteria from the aerial parts with that of rhizosphere-colonizing bacteria. We identified 9 proteins that were >3-fold induced relative to the rhizosphere proteome (Table 1), out of which 5 were not identified in the earlier comparison. The high similarity between the phyllosphere and rhizosphere proteomes suggests a similar adaptation to the epiphytic state in both plant environments. Relatively few down-regulated proteins were identified (see Table 3, which is published as supporting information on the PNAS web site), and these generally corresponded to housekeeping proteins, which could reflect a general down-regulation of metabolism during epiphytic growth compared with *in vitro* conditions.

Among the proteins induced during bacterial growth in the phytosphere (leaf and root surface environments), key markers of methylotrophic metabolism (17) were found to be up-regulated (e.g., MxaF and Fae) with respect to growth on synthetic medium containing succinate as a carbon source (Table 1). The induction of these enzymes upon epiphytic growth is in agreement with a previous study in which an advantage of wild-type (WT) *M. extorquens* cells in competition with methylotrophy-minus mutants was demonstrated, suggesting methanol utilization by the methylotroph (14). Another protein induced during phyllosphere colonization was PhaA, which initiates synthesis of the reserve polyhydroxy butyrate (PHB) (ref. 18; Table 1). It has been shown that PHB formation is stimulated by a deficiency of nutrients such as NH_4^+, SO_4^{2-}, Mg^{2+}, Fe^{2+}, or Mn^{2+} (19). The observed induction of PhaA might thus represent part of a general adaptation to nutrient-limiting conditions as would be expected for phyllospheric growth where the carbon source might not be a growth-limiting factor (14). The nature of this limiting factor is possibly suggested by the phyllosphere-specific induction of two putative periplasmic ABC transporter components predicted to be involved in iron and sulfate uptake (Table 1). Interestingly, iron and sulfate have been suggested to be critical for phyllosphere colonization in other organisms (9).

We also found several putative dehydrogenases/oxidoreductases to be induced during phytospheric growth (Table 1). Of these, RMQ03452 is phyllosphere-specific, sharing a high percentage of sequence identity with the AcoD of *Ralstonia eutrophus*, which is involved in the catabolism of acetoin and ethanol (20), and with AldB of *Escherichia coli*, which is thought to have a role in detoxifying alcohols and aldehydes (21). The RMQ03452 protein in *M. extorquens* might contribute to carbon dissimilation or detoxification of alcohols or aldehydes that are produced by plants (22). *Methylobacterium* spp. are specialists in dealing with toxic compounds, as is already clear when considering that formaldehyde is a central intermediate of methylotrophic metabolism (10, 15). This question of detoxification vs. catabolism also arises for a putative lactoylglutathione lyase (GloA) (Table 1). In *E. coli*, GloA is required for detoxification of methylglyoxal, which is known to cause DNA damage (23). Because it has been reported that methylglyoxal is formed during catabolism of certain amino acids and other compounds such as acetone (24), methylglyoxal might therefore also be an intermediate produced upon breakdown of nutrients of the facultative methylotroph.

The analysis of the *in planta* proteome of *M. extorquens* AM1 clearly reflects an adaptation to survival under stress conditions (Table 1). The identified stress proteins fall into two classes: chaperones/proteases and oxidative stress-related proteins. Among the former are two paralogues of the periplasmic DegP/HtrA family (25), which suggests a response to extracytoplasmic stress and/or the need for assistance in the maturation of components of the cell envelope required for epiphytic growth. In addition, there is a predicted protease of the DJ-1/Pfp-1 superfamily that is a homologue of the general stress protein 18 (GSP18) of *Bacillus subtilis* (26) and heat-shock proteins that are well known to be induced by various types of environmental stress (27). The oxidative stress response is suggested by the up-regulation of superoxide dismutase, catalases, and the Dps protein. Dps is a nonspecific DNA-binding protein and a key component of the protection strategy against H_2O_2 (28, 29), UV irradiation (30), and electrophiles such as methylglyoxal (23, 28) (see above). The formation of reactive oxygen species (ROS) is a normal event and a by-product of electron transport under aerobic conditions (31). Because the detoxification of ROS becomes particularly important under starvation conditions (32), the observed induction of ROS-removing enzymes in *M. extorquens* AM1 might thus be a reaction to endogenously formed ROS. On the other hand, it is well known that plant cells challenge bacteria by means of an oxidative burst (33) and superoxide dismutase (SOD), and Kat and Dps have been shown to counteract the toxic effects of ROS produced by plants (34, 35). All of the stress proteins that we identified appeared to be epiphytic-specific rather than phyllosphere-specific. However, another protein, ClpP, was found to be induced in the phyllophere rather than in the rhizosphere. Clp proteases of *E. coli* are known to play an important role in cytoplasmic quality control and participate in numerous regulatory mechanisms that are important in nongrowing or slow-growing cells. ClpP interacts with ClpX or ClpA, which exhibit different substrate specificities (36).

A Response Regulator Common to *Alphaproteobacteria* Essential for Plant Colonization. The analysis of the *in planta* proteome of *M. extorquens* AM1 revealed the induction of a putative two-component system response regulator, RMQ08198, that we named

Table 1. List of proteins from *M. extorquens* AM1 found to be induced during phyllosphere (P) colonization relative to MM and rhizosphere (R) colonization

Spot no.*	RMQ no.	Gene product(s)†	CD search	*Mr*	p*I*	Ratio,‡ P/MM	Ratio,‡ P/R
		Metabolism					
29	RMQ05966	MxaF, methanol dehydrogenase, large subunit (M31108)	pfam01011	67.2	5.8	+	
48	RMQ00044	MxaJ protein (M31108)	pfam00497	27.4	6.0	∞	
4	RMQ09682	Fae, formaldehyde activating enzyme (L43136)		20.7	7.0	+	
16	RMQ08765	PqqB, PQQ biosynthesis polypeptide (L25889)		30.6	5.4	∞	
34 n11	RMQ03830	PhaA, β-ketothiolase (AF287907)	pfam00108	44.1	6.7	+	+
42	RMQ09548	Crr, crotonyl-CoA reductase (L48340)	pfam00107	47.5	6.3	∞	
2	RMQ01365	Gap20 (AF442749)		19.0	5.8	∞	
45	RMQ05381	Malyl-CoA lyase-like protein	pfam03328	37.9	5.8	∞	
24 n10	RMQ03452	Aldehyde dehydrogenase	pfam00171	62.8	6.4	+	∞
28	RMQ07560	Xanthine oxidase-related aldehyde oxidoreductase	pfam02738	80.6	5.3	+	
44	RMQ07805	Putative NADP-dependent oxidoreductase	pfam00107	35.9	6.0	+	
P12	RMQ11717	Putative quinoprotein		36.0	6.9	+	
1	RMQ02894	GloA, lactoyglutathione lyase	pfam00903	16.5	5.7	+	
n3	RMQ09259	Adenylate kinase	pfam00406	21.3	5.1		+
		Transport					
n4	RMQ08930	ABC-type Fe+ transport system, periplasmic component		36.6	6.6		+
n6	RMQ06383	ABC-type sulfate transport system, periplasmic component		24.1	7.8		+
15	RMQ02495	Putative amino acid binding protein	pfam00497	28.5	5.2	∞	
31	RMQ05493	Putative oligopeptide binding protein	pfam00496	70.2	6.7	+	
		Stress proteins					
P28	RMQ01248	DegP/HrA, Trypsin-like serine proteases	pfam00089	50.4	6.7	∞	
40	RMQ04833	DegP/HrA, Trypsin-like serine proteases	pfam00089	51.2	8.4	∞	
n5	RMQ08088	ClpP, ATP-dependent Clp protease proteolytic subunit	pfam00574	23.1	5.8		+
6	RMQ05519	Protease I (Serine protease) DJ-1/Pfpl family (GSP18)	pfam01965	20.7	4.9	∞	
P22	RMQ06501	Hsp70, heat-shock protein 70 (DnaK)	pfam00012	51.9	4.9	+	
P35	RMQ06982	Hsp70, heat-shock protein 70 (DnaK)	pfam00012	68.5	5.2	+	
P4	RMQ02206	Hsp20, heat-shock protein 20	pfam00011	18.4	5.2	∞	
P15	RMQ02531	SodA, Superoxide dismutase	pfam02777	22.5	5.8	+	
37a	RMQ09549	KatE, catalase (L48340)	pfam00199	63.5	7.1	∞	
37b	RMQ11789	KatE, catalase	pfam00199	59.9	5.8	∞	
9	RMQ05258	Dps, DNA protection protein	pfam00210	19.9	5.0	+	
		Proteins of unknown function					
7, 12	RMQ09016	NfU-like/thioredoxin-like protein	pfam01106	20.3	4.8	∞	
43 n7	RMQ10082	Major royal jelly protein§	pfam03022	41.4	6.3	∞	+
P26	RMQ06718	Major royal jelly protein§	pfam03022	40.2	5.4	∞	
n12	RMQ07439	Protein of unknown function		68.3	6.4		+
8	RMQ10020	Protein of unknown function	pfam05974	18.5	5.4	∞	
10	RMQ09099	Protein of unknown function		21.1	5.9	∞	
13	RMQ08861	Protein of unknown function		20.3	5.3	∞	
17	RMQ03063	Protein of unknown function		31.7	4.9	∞	
18	RMQ00428	Protein of unknown function§		31.9	5.3	+	
P30	RMQ05730	Protein of unknown function§		28.9	6.8	+	
19	RMQ01102	Protein of unknown function		43.5	5.0	∞	
P25	RMQ00267	Protein of unknown function		39.1	5.2	∞	
39	RMQ03107	Protein of unknown function	pfam00450	55.3	6.4	∞	
46	RMQ03170	Orf88, dioxygenase (AY034474)	pfam00903	36.4	6.2	+	
36 n8	RMQ09688	Putative nucleoside binding protein		54.7	9.0	∞	∞
		Regulator					
P16	RMQ08198	Response regulator (PhyR, this work)	pfam06182	29.1	4.8	∞	

*Spot numbers that are preceded by ''P'' were identified on gels from independent experiments stained with silver nitrate rather than with SYPRO Ruby. Spot numbers preceded by ''n'' were detected to be induced in bacteria that were grown in the phyllosphere with respect to the rhizosphere. All other proteins were identified from the proteome of bacteria grown in the phyllosphere with respect to minimal medium supplemented with succinate.

†Accession nos. are in parentheses.

‡Spots indicated as ''∞'' were only detectable in the proteome from bacteria grown in the phyllosphere and not in the references (MM and rhizosphere, respectively). Spots indicated as ''+'' were found to be at least 3-fold induced. Proteins were identified from 2D gels stained with SYPRO Ruby and found in two biological repetitions whereby the majority of the spots were in addition also found induced on gels that were stained with silver nitrate.

§RMQ10082 and RMQ06718 represent protein paralogues, and RMQ00428 and RMQ05730 represent another pair of protein paralogues.

PhyR (for "phyllosphere-induced regulator"). This protein is interesting in several respects as follows. (*i*) A National Center for Biotechnology Information (NCBI) CD search revealed that PhyR carries a RpoE (σ^E)-like domain at the N terminus of the protein (Fig. 2*A*). A sigma-factor-like domain has not yet been described as part of a response regulator and might suggest that PhyR could possibly initiate transcription by itself. (*ii*) The sigma factor RpoE plays a major role in maintaining the integrity and function of the envelope and provides resistance to environmental stresses including desiccation and oxidative stress in *Pseudomonas* spp. (4, 37, 38). (*iii*) The domain structure of PhyR shows that the predicted phosphorable receiver domain is located at the C terminus (Fig. 2*A*), although this domain is usually located at the N terminus in described response regulators (39).

The identification of a previously undescribed type of response regulator in the proteome of *in planta*-grown *M. extorquens* AM1

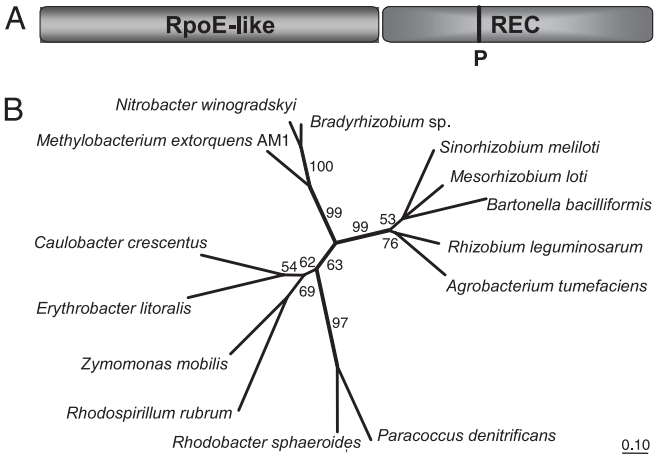

Fig. 2. Structure and phylogeny of PhyR. (*A*) Predicted two-domain structure of PhyR. A strictly conserved aspartate residue (corresponding to position 190 of PhyR) is predicted to be the phosphorylation site (P) of the receiver domain according to Prosite (www.expasy.ch). (*B*) Phylogenetic tree of PhyR homologues in various *Alphaproteobacteria* using the treepuzzle algorithm and the Jones–Taylor–Thornton evolutionary model and based on the amino acid sequences aligned with ClustalW (see Fig. 4, which is published as supporting information on the PNAS web site). Branches that were recovered in <50% of 1,000 reconstructed treepuzzle trees are shown as multifurcations; percentage values for branches with ≥50% recovery are given in the tree. Original tree construction included all available PhyR homologue sequences currently available in the GenBank database. A selection was made of 13 representative sequences plus PhyR of *M. extorquens* AM1.

prompted us to evaluate the importance of PhyR for plant colonization by constructing a deletion strain. Growth rates of the mutant were found to be unaltered with respect to WT when plant colonization was mimicked *in vitro* under mixed growth conditions (i.e., in the presence of succinate and methanol) (14). However, *in planta* colonization experiments revealed a severe growth defect of the PhyR deletion mutant (Fig. 3). Cell numbers of the mutant were below the detection limit for 65% of 3-week-old plants. When we then cloned the PhyR gene in trans, we were able to restore the colonization capacity to the WT level (data not shown).

Interestingly, a BLAST search revealed that PhyR homologues are present in essentially all free-living *Alphaproteobacteria* for which a genome sequence is available, but not in any other bacteria. This finding clearly indicates a more general function for PhyR homologues than adaptation to the phyllosphere and an ancient origin within this proteobacterial subgroup based on phylogenetic analysis (Fig. 2*B*).

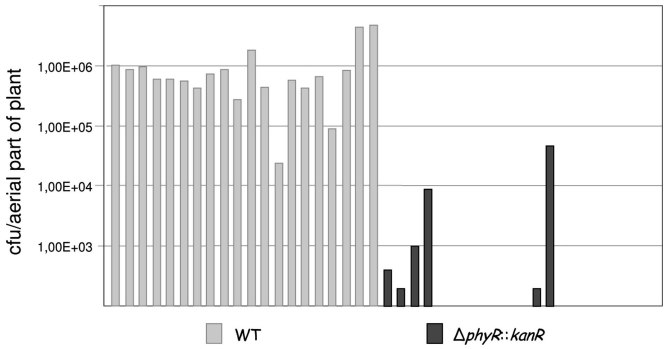

Fig. 3. Plant colonization of *M. extorquens* WT and the *phyR* deletion strain. The detection limit was at 10^2 cfu per plant. Three other independent experiments showed congruent results (data not shown).

Identification of Proteins That Are Positively Regulated by PhyR. The phenotype of *phyR* mutants indicate that it is an important regulator and that one or more important physiological traits of phyllospheric growth are under PhyR control. To identify genes that are induced by PhyR, we performed proteome analysis with 2-DE so that we could readily recognize proteins that had been identified during the *in planta* proteome analysis. To this end, we cloned *phyR* in the expression vector pCM80 (40) and introduced the plasmid into *M. extorquens* AM1 Δ*phyR*. The proteome of this *phyR*-overexpressing strain was compared with the *phyR*-deficient strain containing the empty vector as control. The 42 proteins that we identified as PhyR-regulated are in Table 2.

These results show that PhyR is partly responsible for the induction of some of the proteins that we had found to be induced during phyllospheric growth of the bacterium. However, we are unable to distinguish whether they are directly or indirectly regulated by PhyR. Among these proteins are KatE, SodA, Hsp20, Dps, GloA, and several uncharacterized proteins. As mentioned earlier, these proteins are known to be involved in coping with stress caused by electrophiles (GloA, Dps) and ROS (KatE, SodA, Dps). The latter group comprises proteins protective not only against superoxide anions and hydrogen peroxide but also alkyl hydroperoxides (see induction of Ohr and AhpC; Table 2) that are all important components of the plant defense response against microbial infection (33, 41) and by-products of aerobic metabolism (see above).

Several dehydrogenases (pfam00107 and -00106) were found to be induced by PhyR. The substrate spectrum and role of these dehydrogenases is unknown. They might be involved in substrate utilization during starvation to furnish the additional energy supply associated with processes such as repair of oxidized proteins and lipids. Nevertheless, a possible role for one of the putative dehydrogenases (RMQ06018) can be proposed. This protein represents a putative glutathione-dependent formaldehyde dehydrogenase (42) that might fulfill an auxiliary role coping with excess formaldehyde alongside the well described H_4MPT- and H_4F-dependent pathways for formaldehyde oxidation in *Methylobacterium* (15).

In several model bacteria, stress responses have been well studied. Whereas many regulators are specifically involved in one type of stress, other regulators control diverse functions. σ^S is a master regulator of the general stress response in bacteria that belong to *Gammaproteobacteria* (43). Mutants deficient in σ^S are less able to survive upon starvation and are more sensitive to oxidative and osmotic stress as well as UV and desiccation stress in both logarithmic- and stationary-phase cells in Enterobacteriaceae and pseudomonads (44–46). In *Bacillus*, σ^B has been postulated to be the functional homologue of σ^S. The general stress regulon of σ^B provides the cells with a nonspecific, multiple, and preventive stress resistance in which the protection against oxidative stress is an essential part of the response (47).

PhyR has a central role in the adaptation of *Methylobacterium* to the plant environment. Our proteome analysis points to a rather large PhyR-dependent regulon within which the oxidative stress response is an important part (Table 2), reminiscent of the role of σ^S/σ^B in adaptation for surviving stress and starvation in nature (43, 47). So far, the regulatory elements representing the functional homologues of σ^S/σ^B with their corresponding activation mechanisms in *Alphaproteobacteria* are unknown. It is therefore tempting to speculate that PhyR is involved in a σ^S/σ^B-like response. Biochemical analysis will be important to clarify whether PhyR represents a chimeric protein with a functional output domain that acts as a bona fide sigma factor, suggested by the σ^E-like domain.

Conclusions

Little is known about traits important for phyllosphere colonization, and even less is known about the regulatory mechanisms that determine the adaptation of plant epiphytes in general and *Methylobacterium* spp. in particular. Our proteome profiling approach for bacteria that have colonized the phyllosphere is clearly advanta-

Table 2. List of proteins found to be positively regulated by PhyR

Spot no.	RMQ no.	Gene product*	CD search	*M*r	p*I*	Ratio† phy R^+ vs. phyR^-
		Found in phyllosphere proteome (see Table 1)				
20, 21	RMQ08198	(PhyR, response regulator)	pfam06182	29.1	4.8	∞
1, 2	RMQ09549	KatE, catalase (L48340)	pfam00199	63.5	7.1	+
12	RMQ02531	SodA, superoxide dismutase	pfam02777	22.5	5.8	+
26, 28	RMQ02206	Hsp20, heat-shock protein 20	pfam00011	18.4	5.2	++
29	RMQ05258	Dps, DNA protection protein	pfam00210	19.9	5.0	∞
4	RMQ11717	Putative quinoprotein (glucose dehydrogenase)		36.0	6.9	+
38	RMQ02894	Putative lactoylglutathione lyase	pfam00903	16.5	5.7	∞
8	RMQ03170	Orf88, dioxygenase (glyoxalase family protein) (AY034474)	pfam00903	36.4	6.2	∞
43	RMQ01365	Gap20 (AF442748)		19.0	5.8	+
25	RMQ09016	NifU-like/thioredoxin-like protein	pfam01106	20.3	4.8	∞
24	RMQ08861	Protein of unknown function		20.3	5.3	∞
15	RMQ00428	Protein of unknown function		31.9	5.3	+
		Additional proteins under PhyR control				
3a	RMQ06018	Glutathione-dependent formaldehyde dehydrogenase	pfam00107	42.2	5.7	∞
5	RMQ01240	ADH, alcohol dehydrogenase	pfam00107	39.1	6.5	∞
6	RMQ00842	ADH, alcohol dehydrogenase	pfam00107	35.2	6.3	∞
7a	RMQ07799	Short-chain dehydrogenases/reductases (GSP39)	pfam00106	30.8	5.9	∞
23	RMQ00500	Short-chain dehydrogenases/reductases	pfam00106	27.6	5.1	∞
51	RMQ11711	PccA, propionyl-CoA carboxylase (AY181038)	pfam02786	75.6	4.9	+
14	RMQ06958	MclA, malyl-CoA lyase (U72662)		38.0	5.6	+
18	RMQ06488	MDH, malate dehydrogenase (L33465)	pfam02866	39.1	6.6	∞
30, 31	RMQ02884	Phosphoglycerate mutase	pfam00300	23.9	5.5	+
48	RMQ06654	NAD(P) transhydrogenase α-subunit	pfam01262	39.6	5.6	∞
19	RMQ01528	FixB, Electron transfer flavoprotein, α-subunit	pfam00766	32.5	4.9	∞
11	RMQ02643	WrbA, flavoprotein	pfam00258	21.1	6.2	∞
32	RMQ00895	Carbonic anhydrase	pfam00484	28.4	9.1	+
36	RMQ06760	Ohr, organic hydroperoxide resistance protein-like protein (GSP17o)	pfam02566	15.0	5.7	∞
40	RMQ06032	AhpC (alkyl hydroperoxide reductase)/TSA (thiol specific antioxidant) family protein	pfam00578	18.0	5.9	+
45	RMQ07144	Trx, thioredoxin	pfam00085	17.4	9.7	+
7b	RMQ06181	Putative haloacetate dehalogenase/non-heme chloroperoxidase	pfam00561	30.6	5.8	∞
33	RMQ07321	Putative phospholipid-binding proteins	pfam01161	19.7	5.5	∞
10	RMQ11238	Putative 3-hydroxyisobutyrate dehydrogenase		20.8	5.7	+
22	RMQ12169	CinA-like protein	pfam00994	25.0	4.8	+
27	RMQ09145	Hbd, D-β-hydroxybutyrate dehydrogenase (AY391854)	pfam00106	31.9	8.7	+
42	RMQ02665	GreA, transcription elongation factor	pfam01272	22.1	6.9	+
13	RMQ06933	EF-Ts, elongation factor	pfam00889	35.1	5.3	+
50	RMQ07718	CzcB, Cobalt-zinc-cadmium resistance protein	pfam00529	46.3	6.2	+
3b	RMQ03224	Hypothetical signaling protein		42.4	8.2	∞
34	RMQ04652	Protein of unknown function (GSP26)	pfam01243	19.0	5.7	∞
9	RMQ05442	Protein of unknown function	pfam01442	28.4	5.8	+
41	RMQ01392	Protein of unknown function	pfam03928	14.8	5.0	+
35	RMQ12283	Protein of unknown function		14.9	5.6	∞
37	RMQ12368	Protein of unknown function		12.7	6.6	∞
44	RMQ07641	Protein of unknown function		16.8	5.4	∞

*Accession nos. are in parentheses.

†Spots indicated as ''∞'' were only detectable in the proteome from *M. extorquens* AM1 Δ*phyR* pBG11 (*phyR* overexpression) and not in the strain containing pCM80 (*phyR* minus). Spots indicated as ''+'' were found to be at least 3-fold induced.

geous in detecting the up-regulation of proteins that might have partially overlapping functions, as suggested by the identification of protein paralogues. This work provides a list of candidate proteins that need to be analyzed in more detail with respect to their importance for bacterial fitness. In addition, we have identified a previously undescribed type of two-domain regulator termed PhyR, which plays a key role in the adaptation of bacteria for plant colonization. The PhyR regulon suggests a role in dealing with the various stresses that the bacteria are likely to encounter in the phyllosphere. We assume that this regulator is also of importance in other *Alphaproteobacteria*.

Experimental Procedures

Bacterial Growth Under *in Vitro* and *in Planta* Conditions. For plant inoculation experiments, *M. extorquens* AM1 was grown in liquid MM (48) containing succinate (20 mM) to midexponential growth phase (OD_{600} = 1.2), centrifuged, washed, and resuspended in 10 mM $MgCl_2$. The bacteria were adjusted to an OD_{600} of 1.0 (10^8 cfu per ml) and used for seed inoculation (4-h shaking at room temperature with slight moving) after sterilization of *A. thaliana* (ecotype Columbia) seeds. Sterilization of seeds was achieved through incubation in 2.4% hypochlorite for 5 min followed by eight washing steps. The plants were allowed to develop under controlled conditions on Murashige and Skoog medium in growth chambers under sterile conditions in Magenta boxes (1 week at 20°C, 16 h light/8 h darkness; 2 weeks at 22°C, 9 h light/15 h darkness). Preliminary experiments were performed to determine a suitable time point of harvest of *M. extorquens* AM1 from plants. It was chosen at 3 weeks to ensure that the overall bacterial population showed logarithmic development at this time point (average cell population: 10^6 cfu per aerial part of each plant). Sterility of uninoculated plants was verified by sonication of leaves in phosphate buffer and plating on KingB medium. For each experiment, ≈150 plants were grown. In addition, bacterial precultures (10^5

cfu/ml) were spread on the surface of agar-solidified MM complemented with 20 mM succinate for 5 days at 22°C, 9 h light/15 h darkness.

Harvest of Bacteria and Preparation of Cell Extracts. The aerial parts of the plants were separated from the roots by cutting with a razor blade, and bacteria were harvested in aliquots of 15 plants in 50-ml plastic tubes filled with cooled TE buffer (10 mM Tris, pH 7.5/1 mM EDTA) supplemented with PMSF (0.3 mg/ml) and Percoll (GE Healthcare, Uppsala, Sweden; 20% final concentration) through alternating sonication and vortexing (45 s/30 s, 3 times). The suspension was centrifuged (12,000 × *g*, 4°C for 10 min) whereby the addition of Percoll facilitated sedimentation of the bacteria, leaving small plant debris in the supernatant. Cells from one experiment were washed, pooled, and frozen until further use. Bacteria from roots and *in vitro* conditions were treated in parallel in a similar way. Total proteins were extracted by using a French pressure cell at 10^8 Pa (two times, 4°C), and the cell extract was recovered after centrifugation (13,000 × *g*, 4°C for 30 min).

Proteome Analysis. 2-DE was performed with 18-cm immobilized pH gradient strips (4.0–7.0; GE Healthcare) as described (17). Five independent experiments were performed, whereby the material (aerial parts, roots, *in vitro*) from two experiments was subjected to SYPRO Ruby staining (Molecular Probes, Leiden, The Netherlands; using 350 μg of protein) and from three experiments to silver nitrate staining (using 120 μg of protein). To identify proteins associated with epiphytic growth of *M. extorquens*, images were analyzed by using the Delta 2D software package (Decodon, Greifswald, Germany). Only proteins that were at least 3-fold induced in the independent experiments were identified. Protein identification was performed by peptide mass fingerprinting as described (17) and liquid chromotography/tandem mass spectrometry (49). Identification of differentially expressed proteins was performed independently from the different gels that represent the different biological repetitions and that were stained with SYPRO Ruby and silver nitrate, respectively, and had to give congruent results.

Mutant Generation and Construction of Complementation Strains. A *phyR* mutant was generated by using the suicide vector pCM184 (50). Complementation of the *phyR* deletion mutant was achieved through cloning of *phyR* with its presumed promoter region by using the forward primer Prom-Phy-f-BamHI tggatcctgccgcgactacgacaaacgag (located 454 nt upstream of the predicted start codon) and the reverse primer Phy-r-KpnI catcggccggtacctttcacgg into the XbaI and HindIII sites of the broad host range cloning vector pCM62 (40) resulting in pBG17. The plasmid was subsequently introduced in the Δ*phyR*::*kanR* mutant. In addition, *phyR* was cloned downstream of the *mxaF* promoter into the PstI/KpnI site of the expression vector pCM80 (40) resulting in pBG11 by using the primer Phy-PstI-f: catggctgcagcagcaacg and Phy-CM80-r-KpnI mentioned above.

Phenotypic Analysis of *M. extorquens* AM1 Constructs and Analysis of the PhyR Regulon. Colonization of the *phyR* deletion strain was compared with *M. extorquens* AM1 WT and the complemented strain Δ*phyR*::*kanR* pBG17. For this purpose, plant inoculation experiments were performed. For sampling, the aerial parts of the plants were placed individually in 1 ml of MM and sonicated for 5 min in an ultrasonication bath. Cell suspensions were then serially diluted and plated onto MM. To identify PhyR-regulated genes, we performed a differential proteome analysis by using *M. extorquens* AM1 Δ*phyR*::*kanR* containing pBG11 and pCM80, respectively, grown to midexponential growth phase in the presence of succinate. Cells were harvested, washed in ice-cold TE buffer supplemented with PMSF, and cell extracts were prepared, and proteome analysis was performed as described above.

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