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Analysis of genes contributing to plant-beneficial functions in plant growth-promoting rhizobacteria and related Proteobacteria

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The positive effects of root-colonizing bacteria cooperating with plants lead to improved growth and/or health of their eukaryotic hosts. Some of these Plant Growth-Promoting Rhizobacteria (PGPR) display several plant-beneficial properties, suggesting that the accumulation of the corresponding genes could have been selected in these bacteria. Here, this issue was targeted using 23 genes contributing directly or indirectly to established PGPR effects, based on genome sequence analysis of 304 contrasted Alpha- Beta- and Gammaproteobacteria. Most of the 23 genes studied were also found in non-PGPR Proteobacteria and none of them were common to all 25 PGPR genomes studied. However, ancestral character reconstruction indicated that gene transfers -predominantly ancient- resulted in characteristic gene combinations according to taxonomic subgroups of PGPR strains. This suggests that the PGPR-plant cooperation could have established separately in various taxa, yielding PGPR strains that use different gene assortments. The number of genes contributing to plant-beneficial functions increased along the continuum -animal pathogens, phytopathogens, saprophytes, endophytes/symbionts, PGPR- indicating that the accumulation of these genes (and possibly of different plant-beneficial traits) might be an intrinsic PGPR feature. This work uncovered preferential associations occurring between certain genes contributing to phyto-beneficial traits and provides new insights into the emergence of PGPR bacteria.

Plant roots host a large variety of bacteria, many of them cooperating with the plant and enhancing plant nutrition, stress tolerance or health¹. Several different modes of action are documented in these Plant Growth-Promoting Rhizobacteria (PGPR). Direct effects on plants may involve enhanced availability of nutrients^{2,3}, stimulation of root system development via production of phytohormones and other signals⁴ or interference with plant's ethylene synthesis^{5,6}, and/or induced systemic resistance⁷. Indirect beneficial effects of PGPR on plants entail competition or antagonism towards phytoparasites^{8,9}.

Despite extensive literature on PGPR's modes of action (especially in the Proteobacteria), the molecular features that define a PGPR remain elusive, because the PGPR status is not always well defined. First, PGPR may occupy different microbial habitats, as they range from saprophytic soil bacteria that colonize the rhizosphere to bacteria that can also colonize internal root tissues. This means that the distinction is not often simple respectively with saprophytes without plant-beneficial effects (especially plant commensals) and with vertically-inherited endophytes or plant endosymbionts. Second, several bacteria display alternate ecological niches, and at times some may function as PGPR. For instance, certain tumor-inducing *Agrobacterium* strains have plant growth stimulation potential on non-susceptible plant hosts¹⁰, a property also found in an *Escherichia coli* gut commensal¹⁰. Third, the genes implicated in plant-beneficial functions range from genes directly conferring plant-beneficial properties, such as *nif* (nitrogen fixation)¹¹ or *phl* (phloroglucinol synthesis)¹², to genes contributing to a variety of cell functions indirectly or secondarily including plant-beneficial ones, such as *pqq* (pyrroloquinoline quinone synthesis)¹³. Fourth, many PGPR strains are not yet recognized as such (as determination of PGPR status requires experimental assessment), and it is very likely that not all plant-beneficial traits and the corresponding genes have already been identified. Fifth, the assessment of genes encoding plant-beneficial properties is commonly restrained to particular bacterial clades¹⁴ if not particular PGPR strains^{9,12}, without a more general analysis of gene distribution across several bacterial clades¹⁵.


Table 1 | Distribution of plant-beneficial function contributing (PBFC) genes according to the primary ecological lifestyle documented for the bacteria studied

Gene function	Gene	PGPR (25 ^a)	Endophytes/symbionts (56)	Saprophytes (29)	Phytopathogens (59)	Animal pathogens (135)
Phosphate solubilization	<i>pqqB</i>	20	36	26	34	13
	<i>pqqC</i>	20	36	26	35	13
	<i>pqqD</i>	20	36	26	35	13
	<i>pqqE</i>	20	36	26	35	13
	<i>pqqF</i>	10	17	8	16	7
	<i>pqqG</i>	7	17	9	13	4
	2,4-Diacetylphloroglucinol synthesis	<i>phlA</i>	3	0	0	0
<i>phlB</i>		3	0	0	0	0
<i>phlC</i>		3	0	0	0	0
<i>phlD</i>		3	0	0	0	4
<i>hcnA</i>		3	9	2	0	4
Hydrogen cyanide synthesis	<i>hcnB</i>	3	9	2	0	4
	<i>hcnC</i>	3	9	2	0	4
	<i>budA</i>	5	2	3	14	5
Acetoin/2,3-butanediol synthesis	<i>budB</i>	5	2	3	14	5
	<i>budC</i>	11	12	4	10	5
	<i>nirK</i>	6	14	1	1	108
Nitric oxide synthesis	<i>ipdC</i>	5	2	3	10	5
Auxin synthesis	<i>ppdC</i>	2	2	0	0	0
	<i>acdS</i>	9	31	16	26	44
ACC deamination	<i>nifD</i>	9	23	3	3	0
Nitrogen fixation	<i>nifH</i>	9	23	3	3	0
	<i>nifK</i>	9	23	3	3	0

^aThe number of bacteria is indicated in parenthesis.

Despite these limitations, however, a number of emblematic PGPR model strains have been extensively characterized over the last 20 years, uncovering the molecular basis of at least some of their plant-beneficial effects. These studies have evidenced that many PGPR strains typically harbor more than one plant-beneficial property^{8,16}, and it could be hypothesized that the accumulation of genes contributing (whether directly or indirectly) to plant-beneficial traits has been selected by the interaction of these bacteria with plants. On this basis, it could even be expected that PGPR might be identified by their particular assortment of genes contributing to plant-beneficial functions. So far, a more general description of the occurrence of these genes, including in bacteria not interacting with plants, is still lacking. Such knowledge would bring fundamental insights into the potential associations of phyto-beneficial traits in PGPR bacteria, and this can now be achieved based on genome comparisons and phylogenetic analyses^{17,18}.

Hence, our objective was to assess the distribution of 23 genes contributing to eight key plant-beneficial functions using genomic and phylogenetic analyses, as well as ancestral state character reconstruction to infer possible gene transfers. These plant-beneficial function contributing genes (hereafter referred to as PBFC genes) were investigated using the genomes of 25 emblematic proteobacterial PGPR (i.e. bacteria colonizing root surface and/or tissues and displaying plant growth-promotion effects). These genomes were also compared with those of 279 other Alpha-, Beta- and Gammaproteobacteria representing various taxonomic groups and ecological status, such as (i) endophytes/symbionts (i.e. asymptomatic, endophytic bacteria possibly in symbiotic interaction with the plant but for which plant-beneficial effects are not documented, as well as root-nodulating, diazotrophic bacteria), (ii) saprophytes (i.e. bacteria from various environments including soil; some of them possibly colonizing roots but without established plant-beneficial effects), (iii) plant pathogens and (iv) animal pathogens.

The 23 genes selected included (i) the nitrogenase-encoding genes *nifHDK* responsible for nitrogen fixation in proteobacterial PGPR from *Azospirillum*¹¹, *Burkholderia*¹⁹ and other genera, (ii) the pyrroloquinoline quinone-encoding genes *pqqBCDEFG* contributing to

mineral phosphate solubilization in the PGPR *Pseudomonas fluorescens* F113²⁰, *Erwinia herbicola*²¹ and *Enterobacter intermedium*²², (iii) the indole-3-pyruvate decarboxylase/phenylpyruvate decarboxylase gene *ipdC/ppdC* of the indole-3-pyruvate pathway for synthesis of the auxinic phytohormone indole acetic acid (IAA) in *Azospirillum brasilense* Sp245¹⁵, *Enterobacter cloacae* UW5²³ and other *Enterobacteriaceae* PGPR²⁴, (iv) the copper nitrite reductase gene *nirK* leading to formation of the NO root-branching signal in *Azospirillum brasilense* Sp245²⁵, (v) the 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene *acdS* in *Pseudomonas putida* GR12-2²⁶ and various other *Pseudomonas* PGPR⁶, which enables degradation of the plant's ethylene precursor, (vi) the acetoin genes *budAB* and 2,3-butanediol gene *budC* (induced systemic resistance) in the PGPR *Enterobacter* sp. 638²⁷, and (vii) genes *hcnABC* (hydrogen cyanide) and *phlACBD* (2,4-diacetylphloroglucinol) for synthesis of antimicrobial compounds in *P. fluorescens* F113, *P. protegens* CHA0 and many other PGPR pseudomonads¹².

Results

Contrasted co-occurrence patterns of PBFC genes in proteobacterial PGPR. In the 25 sequenced PGPR strains, which belonged to the genera *Azospirillum*, *Rhizobium/Agrobacterium* (Alphaproteobacteria), *Azoarcus*, *Burkholderia*, (Betaproteobacteria), and *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Serratia*, (Gammaproteobacteria), the PBFC genes were found in 2 (for gene *ppdC*) to 20 (*pqqBCDE*) of the genomes (Table 1). The PGPR strains harbored from 1 (i.e. *acdS* in *Burkholderia* 'cepacia' 383 and *B. phytofirmans* PSJN) to 14 of the 23 PBFC genes studied (in *P. protegens* Pf-5, *P. brassicacearum* NFM421 and *P. fluorescens* F113), which gave 7.5 ± 3.1 PBFC genes per strain (Supplementary Fig. S1a). The exact test of Fisher ($P < 0.05$) evidenced that *phlACBD* and *hcnABC* significantly occurred together in certain PGPR strains (Fig. 1) i.e. pseudomonads. Three other separate groups of co-occurring genes were identified, i.e. *budAB* and *ipdC*, the operon *nifHDK* and the clustered genes *pqqBCDE*. No other significant co-occurrence of PBFC genes was found.



Similar or lower prevalence of PBFC genes in Proteobacteria of other ecological types. The genomes of 279 other sequenced Proteobacteria corresponding to saprophytes or endophytes/symbionts without established PGPR status, as well as pathogens of plants or animals, were studied as well. For the 56 endophytes/symbionts, PBFC genes were found in 0 (for *phlACBD*) to 36 (*pqqBCDE*) of the genomes (Table 1). Whereas two bacteria did not display any of the 23 PBFC genes, they were extensively found in others, with eight strains exhibiting as many as 10 PBFC genes each. Overall, the endophytes/symbionts harbored 6.1 ± 2.6 PBFC genes per strain, but the difference with PGPR was not significant ($P = 0.06$). Exact-Fisher pairwise tests of the co-occurrence of PBFC genes ($P < 0.05$) revealed four groups, i.e. *hcnABC* and *pqqBCDE* linked by *pqqFG* genes, as well as *nifHDK/acdS* and *budAB/ipdC* further apart (Fig. 2a).

Within the 29 saprophytes, PBFC genes were found in 0 (for *phlACBD* and *ppdC*) to 26 (*pqqBCDE*) of the genomes (Table 1). Although three bacterial strains showed none of the studied genes, one strain (*Pantoea* sp. At-9b) exhibited as many as 12 genes. Globally, saprophytic strains contained 5.5 ± 1.8 genes per genomes. This is significantly lower than in PGPR ($P < 0.05$) but not different from endophytes/symbionts ($P = 0.44$). Co-occurrence analysis of PBFC genes in saprophytic bacteria evidenced five separate groups, i.e. *hcnABC*, *pqqBCDE*, *pqqFG*, *nifHDK* and *budABC/ipdC* (Fig. 2b).

In the 59 phytopathogenic bacteria, PBFC genes were found in 0 (for the 8 genes *ppdC*, *phlACBD* and *hcnABC*) to 35 (*pqqCDE*) of the genomes (Table 1). Whereas seven phytopathogens (*Xylella* sp. and *Xanthomonas albilineans*) did not contain any of the 23 PBFC genes, as many as 8 PBFC genes occurred in *Erwinia* and *Pantoea* species. This gave overall 4.3 ± 2.2 PBFC genes per strain, which was lower than in PGPR and endophytes/symbionts ($P < 0.05$) but not significantly lower than in saprophytes ($P = 0.06$). Exact-Fisher pairwise tests ($P < 0.05$) of the co-occurrence of PBFC genes revealed two independent groups, i.e. *pqqBCDEFG* linked to *acdS* via *pqqG*, and *budABC/ipdC* with *nifHDK* (Fig. 2c).

Most PBFC genes were not prevalent in the 135 animal pathogens. Except *nirK* present in 109 of them, the other PBFC genes were not often found (ranging from 4 genomes for *pqqG*, *phlD* and *hcnABC* to 44 genomes for *acdS*) or not found at all (*nifHDK*, *ppdC*, *phlACB*). The number of PBFC genes varied from 0 (in 9 animal pathogens) to 9 (in 7 other animal pathogens), i.e. 1.8 ± 1.2 PBFC genes per strain

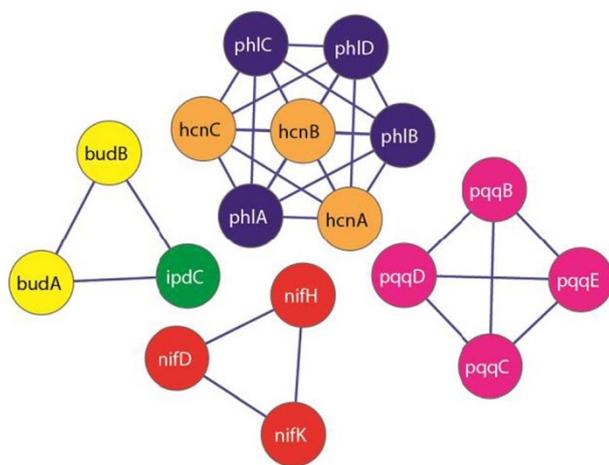


Figure 1 | Co-occurrence network of the PBFC genes for the 25 PGPR genomes. The genes are depicted with a colored circle according to their encoded function. Each co-occurrence is represented by an edge linking the corresponding genes and materialized by a line (based on Fisher exact test; $P < 0.05$). Several PBFC genes found in PGPR (i.e. *pqqF*, *pqqG*, *budC*, *nirK*, *ppdC* and *acdS*) did not display significant co-occurrence with any other(s).

overall, which was lower than for all other ecological types (all $P < 0.05$). Exact-Fisher pairwise tests ($P < 0.05$) evidenced a single group comprised of three subgroups extensively linked with one another, i.e. *budABC/ipdC*, *pqqBCDEF* and *hcnABC/pqqG/phlD* (Fig. 2d).

Distribution of PBFC genes across all 304 proteobacterial genomes reveals taxonomic specificities. Whereas *phlACB* were only retrieved in PGPR (in 3 of 25 genomes), the other PBFC genes were recovered in bacteria of different ecological types. Many occurred in PGPR as well as in endophytes/symbionts, saprophytes and phytopathogens, especially *pqqCDE* (36 of 56, 26 of 29 and 35 of 59 genomes, respectively), and with a lower prevalence *ipdC* (2 of 56, 2 of 29 and 10 of 59 genomes, respectively) and *nifHDK* (23 of 56, 3 of the 29, and 3 of 59 genomes, respectively). In contrast, the *hcnABC* genes were retrieved in PGPR (3 of 25 genomes), saprophytes (2 of 29 genomes), endophytes/symbionts (9 of 59 genomes) and animal pathogens (4 of 135 genomes), but were absent in plant pathogens.

The distribution of certain PBFC genes according to bacterial ecological type could, at least in part, reflect taxonomic properties. This is indicated by the occurrence of PBFC genes in taxa restricted to a given ecological type (Fig. 3). In particular, *ppdC* was only retrieved in certain *Azospirillum* PGPR and *Bradyrhizobium* in the endophyte/symbiont category. For many PBFC genes, however, their occurrence within a taxon was related to species/strain ecology. This was the case for *phlACBD* (*Pseudomonas* PGPR), *hcnABC* (all *Pseudomonas* types except phytopathogens), and *nifHDK* (mainly in PGPR and endophytes/symbionts from various proteobacterial taxa). The relation to ecology, if any, was not as strong for *ipdC* and *budAB* (Enterobacteriaceae), *acdS* (all Burkholderiaceae considered and various Alphaproteobacteria and Gammaproteobacteria), *nirK* and *pqq* genes (various Proteobacteria corresponding to several ecological types).

The comparison of the 304 genomes showed that, unexpectedly, PBFC genes previously described as clustered (even forming operons in many cases) were not necessarily found together in a same genome (Fig. 4). For instance, *pqqFG* were close to *pqqBCDE* in *Pseudomonas* (and a few other genera), whereas *pqqBCDE* occurred without *pqqG* (encoding a family-S9 peptidase) and especially *pqqF* (encoding a family-M16 peptidase) in most other Proteobacteria. Similar observations were made for *phlD* and *phlACB*, as well as *budAB* and *budC*. Yet, the groups revealed by exact-Fisher pairwise tests ($P < 0.01$) corresponded mainly to genes involved in a same function (Fig. 3). This analysis showed that *hcnABC* and *phlD* linked the other *phl* genes with the six *pqq* genes, themselves linked to *budABC/ipdC* via *nirK* and to *nifHDK*. *nifHDK* were also linked, separately, to *ppdC* and to *acdS*.

Distribution of PBFC genes is partly related to proteobacterial phylogeny. We assessed whether the distribution of PBFC genes exhibited significant phylogenetic signal, meaning that closely-related species have similar gene content. Fritz and Purvis D index analysis (Table 2) showed that distribution of the PBFC genes was significantly influenced by evolutionary relationships between proteobacterial species, as indicated by D scores significantly less than 1. The genes *phlACBD*, *pqqFG*, *budABC*, *ipdC*, *ppdC* and *hcnABC* showed a strong phylogenetic signal, while *acdS*, *nifHDK* and *pqqBCDE* showed weaker signals.

Horizontal gene transfers had significant effects on PBFC gene distribution in Proteobacteria. When the impact of genome plasticity was assessed, by computing events of acquisitions and losses across proteobacterial species, no loss was detected for *pqqF*, *phlACBD*, *ppdC* and *nirK* (Table 3). On the contrary, a few losses were inferred for the other genes, ranging from 1 loss for *pqqG*, *budABC*, *ipdC* and *hcnABC* to 6 losses for *pqqB*. In comparison,

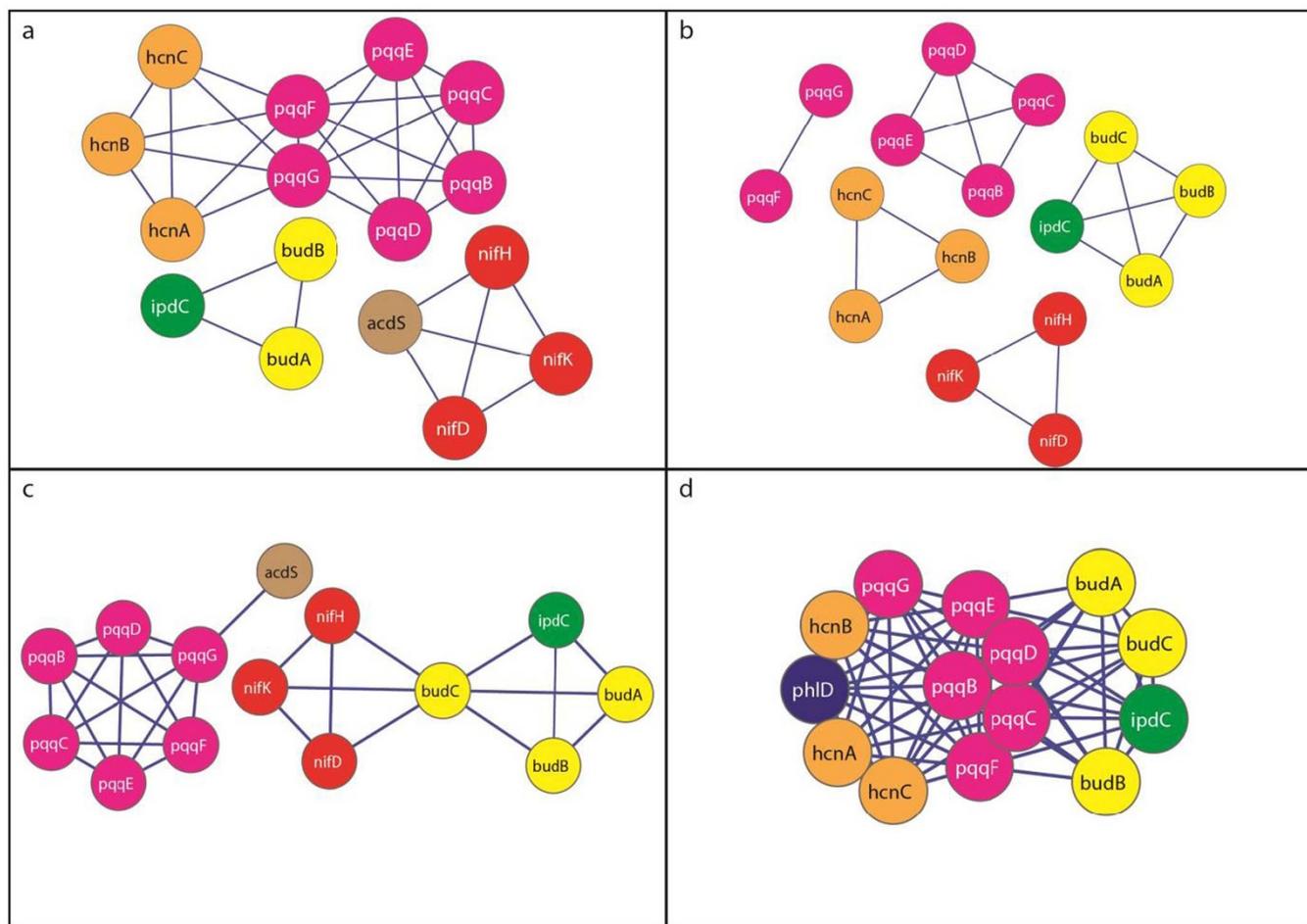


Figure 2 | Co-occurrence network of PBFC genes according to primary ecological classification of bacteria. The genes are depicted with a colored circle according to their encoded function. Each co-occurrence is represented by an edge linking the corresponding genes and materialized by a line. Computations were made for (a) endophytes/symbionts, (b) saprophytes, (c) phytoparasites, (d) animal pathogens.

the number of acquisitions was of a larger scale, from 1 for *ipdC* and *budAB* to 21 for *acdS* (Table 3).

All 23 genes appeared at least once in a distant ancestor of the species studied (Fig. 5). *ipdC*, *ppdC* and *phlACBD* are clade specific; *ipdC* appeared in the last common ancestor (LCA) of *Pantoea* and *Erwinia* genera, *ppdC* in the LCA of *Azospirillum brasilense* and the LCA of *Bradyrhizobium* strains ORS78 and BTai1, and *phlACBD* in the LCA of *Pseudomonas fluorescens* F113 and *Pseudomonas brassicacearum* NFM421 and the LCA of *Pseudomonas protegens* Pf-5. *budABC* appeared in the LCA of Enterobacteriaceae; *budAB* are clade specific but *budC* was acquired at least 7 times in other clades. The *pqqBCDEFG* genes appeared in the LCA of the *Pseudomonas*; *pqqG*, *pqqF* and *pqqBCDE* were acquired respectively 4, 5 and 15 times by other taxa. At the extreme, *nifHDK* underwent at least 18 acquisitions and *acdS* (which appeared in the Burkholderiaceae LCA) 21 acquisitions, in both cases across the three phyla considered.

Discussion

In this study, plant-beneficial properties of PGPR were for the first time assessed on a broad scale, by considering (i) a large range of PBFC genes corresponding to various types of plant-beneficial properties, (ii) PGPR strains of contrasted taxonomic status (from the Alpha- Beta- and Gammaproteobacteria), and (iii) a selection of non-PGPR Proteobacteria with primarily other biotic relations with plants (i.e. endophytes/symbionts and phytopathogens) or other types of ecology (i.e. saprophytes and animal pathogens).

It could have been thought that the PGPR status entailed the presence of a core collection of PBFC genes shared by all PGPR strains, but the current results based on 25 emblematic PGPR strains indicate that none of the 23 key PBFC genes of the study were common to all strains, even though as many as 20 PGPR genomes displayed *pqqBCDE*. PQQ is a co-factor potentially implicated in several cellular processes (and incidentally contributing also to phosphate solubilization), which may explain its wide occurrence in PGPR^{13,28}. In comparison with Proteobacteria of other lifestyle, PBFC genes restricted to PGPR were not found, except for *phlACB* but these genes were present in only 3 *Pseudomonas* PGPR. However, the number of PBFC genes increased along the continuum animal pathogens (only 1.8 PBFC genes/strain), phytopathogens, saprophytes, endophytes/symbionts, PGPR (as many as 7.5 PBFC genes/strain). The same findings were made when assessing the number of functions expected from these PBFC genes, except that the difference between animal and plant pathogens was not significant (Supplementary Fig. S1b).

Our gene distribution data suggest that PBFC genes might be selected in plant-associated habitats and counter-selected elsewhere, as exemplified by the very low number of these genes in animal pathogens (where only *nirK* was prevalent). This is in accordance with the expectations that most of the corresponding functions would not be relevant for animal physiology and plant is not the primary habitat of these bacteria. For instance, nitrogen fixation is counter-selected in pathogenic bacteria^{29,30}. In addition, results suggest that amongst all the plant-associated bacteria, specific lifestyle is

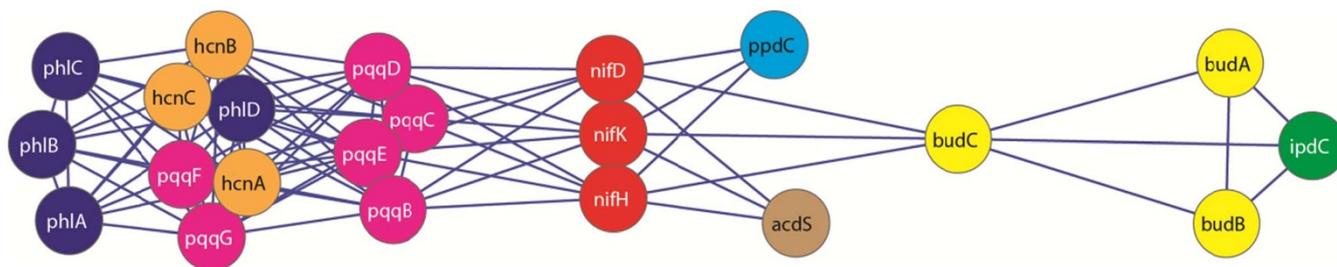


Figure 4 | Co-occurrence network of the PBFC genes for the 304 genomes. The genes are depicted with a colored circle according to their encoded function. Each co-occurrence is represented by an edge linking the corresponding genes and materialized by a line. *nirK* does not appear in the figure because this gene did not show any significant co-occurrence with other PBFC gene(s).

asymptotically, probably because these alternative hosts promote bacterial survival before recolonizing the next animal primary host³⁵. This could explain why certain animal-associated strains displayed PBFC genes. Furthermore, opportunistic human pathogens such as *Pseudomonas aeruginosa* PAO1 and PA14 can also infect roots and lead to plant death³⁶.

Mutational inactivation of a particular PBFC gene may reduce (without necessarily abolishing) plant-beneficial effects in PGPR strains^{1,37}, and genetic acquisition of an additional PBFC gene has the potential to enhance PGPR performance^{8,39}. This indicates that possessing multiple PBFC genes should confer a better efficiency at enhancing plant growth. In this context, the analysis of co-occurrence patterns (exact Fisher tests) can be useful to identify selection of multiple PBFC genes and their potential synergistic effects. However, gene co-occurrence may also take place because species that share a recent evolutionary history also share similar gene contents, a phenomenon known as phylogenetic signal. Indeed, the Fritz and Purvis index clearly pointed to gene associations related to phylogenetic signal, i.e. PBFC genes were more likely to be conserved in closely-related species. This also raises the possibility that the

potential to become a PGPR may rely (at least in part) on ancestral features in the corresponding bacterial taxa, which is in phase with previous findings on particular PGPR populations⁴⁰ and more generally on function distributions in Gammaproteobacteria⁴¹.

The distribution pattern of PBFC genes amongst Proteobacteria of various lifestyles and the relation to bacterial taxonomy prompted us to assess in more details the evolutionary history of these genes. Ancestral character reconstructions showed few losses of PBFC genes, even in animal-associated bacteria, but many more gene acquisitions. Indeed, the role of horizontal gene transfer has been substantiated with various types of PGPR^{42,43} and suggests that cooperation interactions between Proteobacteria and plant roots might have established separately in various taxa, yielding PGPR strains whose effect(s) on the plant may rely on different and taxa-specific combinations of modes of action. Further genome sequencing efforts targeting close relatives of these PGPR would be needed to confirm this possibility. Despite conservation of PBFC genes across different ecological lifestyles, a differential use/regulation of these genes depending on environmental and host conditions is likely⁴⁴, as can take place during exaptation⁴⁵. Indeed, expression patterns of PBFC

Table 2 | Phylogenetic patterns of gene distribution in selected Proteobacteria. Values were calculated for the 1000 partitions of the species phylogenetic tree

Gene ^a	D ^b	P(D > 0) ^c	P(D < 1) ^c	Phylogenetic signal strength
<i>pqqB</i>	0.05 (0.01/0.07)	0.16/0.41	0	Strong
<i>pqqC</i>	0.04 (0.00/0.07)	0.16/0.45	0	Strong
<i>pqqD</i>	0.04 (0.00/0.07)	0.16/0.45	0	Strong
<i>pqqE</i>	0.04 (0.01/0.07)	0.17/0.45	0	Strong
<i>pqqF</i>	-0.17 (-0.20/-0.15)	0.95/0.99	0	Very strong
<i>pqqG</i>	-0.18 (-0.21/-0.17)	0.95/0.98	0	Very strong
<i>phlA</i>	-0.40 (-0.76/-0.06)	0.48/0.88	0.00/0.04	Very strong
<i>phlB</i>	-0.40 (-0.75/-0.05)	0.49/0.89	0.00/0.04	Very strong
<i>phlC</i>	-0.40 (-0.75/-0.06)	0.48/0.88	0.00/0.03	Very strong
<i>phlD</i>	-0.24 (-0.36/-0.12)	0.63/0.86	0	Very strong
<i>hcnA</i>	-0.18 (-0.30/-0.06)	0.62/0.95	0	Strong
<i>hcnB</i>	-0.18 (-0.30/-0.05)	0.61/0.95	0	Strong
<i>hcnC</i>	-0.18 (-0.29/-0.06)	0.61/0.94	0	Strong
<i>budA</i>	-0.34 (-0.37/-0.30)	0.98/0.99	0	Very strong
<i>budB</i>	-0.34 (-0.37/-0.30)	0.98/0.99	0	Very strong
<i>budC</i>	-0.02 (-0.08/-0.03)	0.40/0.70	0	Strong
<i>nirK</i>	-0.05 (-0.09/-0.02)	0.60/0.89	0	Very strong
<i>ipdC</i>	-0.33 (-0.41/0.28)	0.96/0.99	0	Very strong
<i>ppdC</i>	-0.34 (-0.27/-0.47)	0.65/0.80	0	Very strong
<i>acdS</i>	0.10 (0.07/0.13)	0.05/0.19	0	Moderate
<i>nifD</i>	0.28 (0.23/0.33)	0.01/0.05	0	Weak
<i>nifH</i>	0.28 (0.24/0.33)	0.00/0.04	0	Weak
<i>nifK</i>	0.28 (0.23/0.33)	0.00/0.04	0	Weak

^aThe genes studied are involved in phosphate solubilization (pyrroloquinoline quinone; *pqqBCDEFG*), 2,4-diacetylphloroglucinol synthesis (*phlACBD*), hydrogen cyanide synthesis (*hcnABC*), induced systemic resistance (acetoin and 2,3-butanediol; *budAB* and *budC*, respectively), NO synthesis (copper nitrite reductase; *nirK*), IAA synthesis (indole-3-pyruvate decarboxylase/phenylpyruvate decarboxylase; *ipdC/ppdC*), plant ethylene regulation (ACC deamination; *acdS*), and nitrogen fixation (nitrogenase; *nifHDK*).

^bMedian value (with the minimum and maximum values in parenthesis).

^cMinimum and maximum values when different.



Table 3 | Number of acquisitions and losses for each PBFC gene^a according to proteobacterial species tree (ancestral character reconstruction)

Acquisitions and losses	pqqB	pqqC	pqqD	pqqE	pqqF	pqqG	phIA	phIB	phIC	phID	hcnA	hcnB	hcnC	budA	budB	budC	nirK	ipdC	ppdC	acdS	nifD	nifH	nifK
Acquisitions	16	16	16	16	4	3	2	2	2	2	4	4	4	1	1	8	11	1	2	21	18	18	18
Losses	6	5	5	5	0	1	0	0	0	0	1	1	1	1	1	1	0	1	0	4	2	2	2
Total occurrences	129	129	129	129	58	50	3	3	3	7	18	18	18	29	29	42	131	25	4	125	38	38	38

^aThe genes studied are involved in phosphate solubilization (pyrroloquinoline quinone; pqqCDEF), 2,4-diacetylphloroglucinol synthesis (phIACBD), hydrogen cyanide synthesis (hcnABC), induced systemic resistance (acetoin and 2,3-butanediol; budAB and budC, respectively), NO synthesis (copper nitrite reductase; nirK), IAA synthesis (indole-3-pyruvate decarboxylase; ipdC/ppdC), plant ethylene regulation (ACC deamination; acdS), and nitrogen fixation (nitrogenase; nifHDK).

genes according to taxonomic and/or lifestyle properties is an important ecological issue, which will deserve further research attention. Bacterial adaptation to new niches is mainly dependent on genetic novelty^{46,47}, which may entail gene acquisitions⁴⁶ or differential regulation⁴⁸. Many examples of traits conferring environmental adaptation that were further co-opted as virulence factor are documented in human pathogens⁴⁹. Similar processes are likely to have taken place in PGPR as well⁵⁰.

In conclusion, the comparison of taxonomically-contrasted proteobacterial PGPR with a wide range of related, non-PGPR bacteria suggested that the emergence of the PGPR status could have paralleled accumulation of PBFC genes in root-adapted bacteria. It is likely that this process took place separately in taxonomically-contrasted Proteobacteria and involved ancient gene acquisitions, which explains why subsequent diversification produced taxonomic subgroups of PGPR strains differing from one another in the range of PBFC genes accumulated.

Methods

Selection of genomes. The genomes used were selected among those available in October 2012. They corresponded to 25 PGPR, 56 endophytes/symbionts (35 endophytes and 21 root-nodulating bacteria), 29 saprophytes (3 from water environments, 6 from bulk soil, 16 from the rhizosphere, and 4 from healthy animal samples), 59 plant pathogens and 135 animal pathogens (124 of them infecting humans). Since distribution can be influenced by phylogenetic relatedness, also called phylogenetic signal¹⁸, genomes were chosen so as to balance the prevalence of the various Alpha-, Beta- and Gammaproteobacterial groups for which PGPR genomes were available, following two principles. First, the primary lifestyle of the selected bacteria had to be documented sufficiently clearly and their genomes were fully sequenced (except in a few cases for orders of particular interest). Second, bacterial orders in which PGPR representatives were available were assessed for genome availability of bacteria corresponding to other lifestyles (especially within the same or closely-related families/genera), and if unsuccessful the phylogenetically-closest order was then targeted.

Homologs retrieval. Homologs of genes contributing to a phyto-beneficial function in PGPR were retrieved using a BLAST-based method. A protein to protein search was done using Blastp⁵¹ with a subset of genes documented to contribute to a given phyto-beneficial function (Supplementary Table S1). As annotations in public databases may contain errors or sometimes fail to accurately predict gene identity, we then did a tblastn⁵¹ search on genomic sequences to overcome these limitations. An E-value threshold of 1e-15 was set to filter blast searches.

Protein family assignment. Assignment of homologous proteins to families having the same putative function was done using a combination of significant sequence identity (see above) and protein domain assignment. Protein domain assignment was done using rps-blast⁵² and the Conserved Domain Database (CDD)⁵³. We separated the NCBI-curated domains (which are considered more accurate) and external sources domains in two distinct databases. The NCBI-curated database was preferentially used for protein domain assignments while external source database was used when the NCBI-curated one could not retrieve results. Proteins were considered of the same family if they (i) had at least 30% of identity on at least 70% of their respective protein sequence length and (ii) shared the same domains with a reference phyto-beneficial protein. Phylogenetic profiles (corresponding to a binary vector with gene's presence and absence respectively indicated as 1 and 0 for each genome) were used to represent the presence/absence of a particular gene in the different organisms for analysis of phylogenetic signal and ancestral state reconstruction.

Gene distribution. For statistical analysis of the number of PBFC genes and number of corresponding functions per genome, according to primary bacterial ecology, the Wilcoxon test was used with the R command *wilcox.test* ($P < 0.05$).

Proteobacterial phylogenetic tree. The proteobacterial phylogenetic tree was based on 31 housekeeping markers identified, aligned and trimmed with Amphora2, as done previously⁵⁴. Trees were inferred by ExaML⁵⁵ with the concatenated alignment, 1000 replicates and the PSR model of rate heterogeneity.

Computation of phylogenetic signal. The phylogenetic signal for each gene was calculated using Fritz and Purvis's D index⁵⁶ implemented in the R package "caper". Computation of random and Brownian motion of evolution probabilities was based on 10,000 permutations. Briefly, a given trait (a gene in our case) displays a highly clustered distribution if $D < 0$, is as clustered as if it evolved under Brownian motion

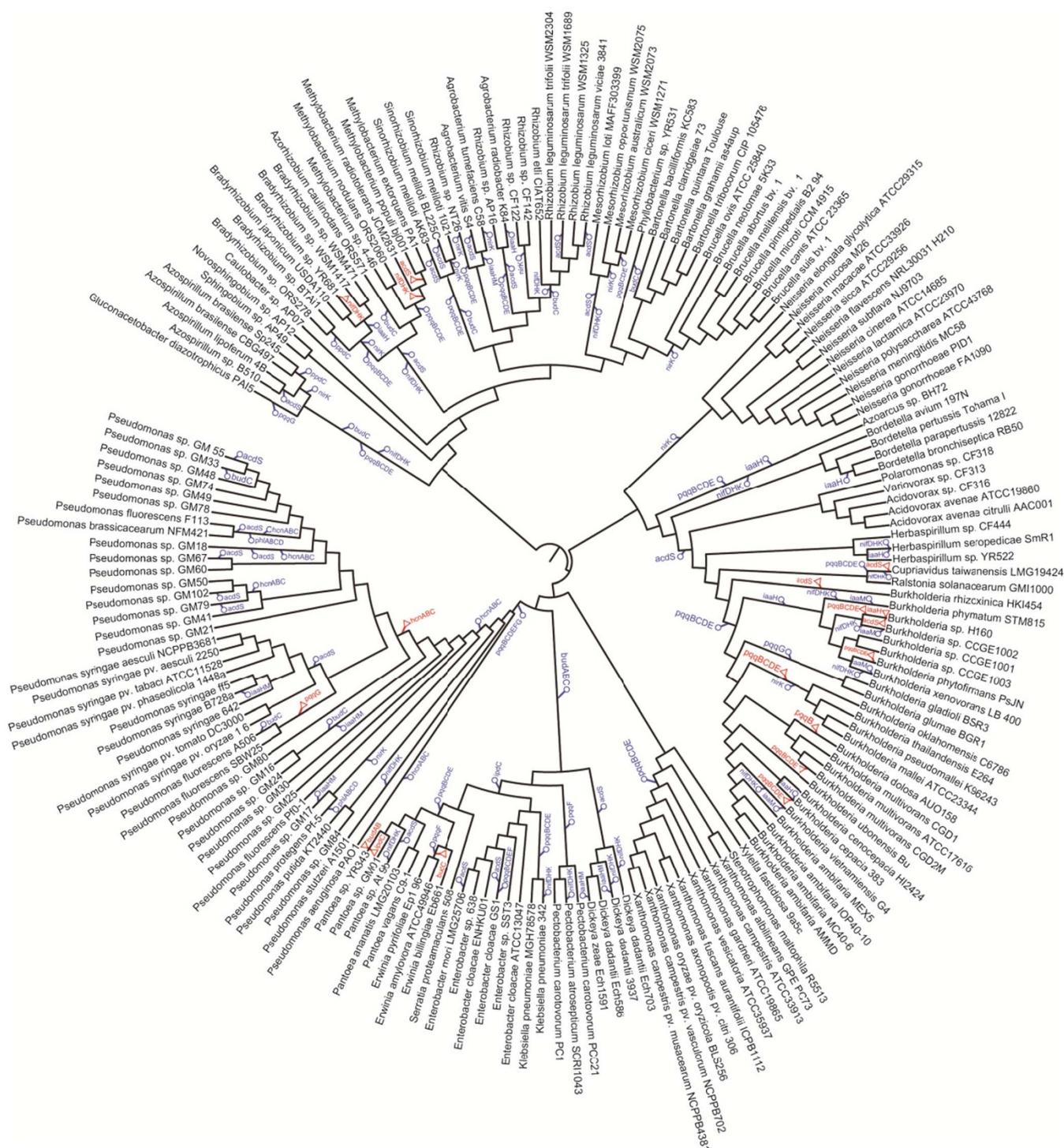


Figure 5 | Reconstruction of acquisitions and losses of PBFC genes in relation to evolutionary history of sequenced bacteria. When different strains of the same species had the same PBFC gene profile, only one representative strain was kept in the Maximum-Likelihood tree to avoid redundant information. Acquisitions are indicated by a blue arrow with a circle and losses by a red arrow with a triangle.

if $D = 0$, displays random distribution if $D = 1$, and is overdispersed if $D > 1$. Comparison of D scores was used to arbitrarily infer the strength of the phylogenetic signal for each gene.

Ancestral state character reconstruction. The GLOOME algorithm was used to infer the presence or absence of each gene on each node of a phylogenetic tree based on their distributions in terminal taxa. The phylogenetic tree used was computed as previously but was based on a filtered alignment. When many bacteria of the same species had the same content in genes of interest, only the reference species indicated in the NCBI database was conserved. This simplified the reconstruction model by removing redundant information. Reconstructions were made with the Maximum

Parsimony method²⁷, which allows to reconstruct ancestral states by minimizing character change events along a phylogenetic tree.

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Author contributions

C.P.C., D.M. and Y.M.L. conceived and designed the project. M.B. performed sequence queries and bioinformatics analyses. M.B., C.P.C., D.M. and Y.M.L. analyzed the findings and wrote the manuscript. All of the authors approved the final manuscript.

Additional information

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