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REVIEW

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Insights into plant-beneficial traits of probiotic *Pseudomonas chlororaphis* isolates

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Abstract

Pseudomonas chlororaphis isolates have been studied intensively for their beneficial traits. *P. chlororaphis* species function as probiotics in plants and fish, offering plants protection against microbes, nematodes and insects. In this review, we discuss the classification of *P. chlororaphis* isolates within four subspecies; the shared traits include the production of coloured antimicrobial phenazines, high sequence identity between housekeeping genes and similar cellular fatty acid composition. The direct antimicrobial, insecticidal and nematocidal effects of *P. chlororaphis* isolates are correlated with known metabolites. Other metabolites prime the plants for stress tolerance and participate in microbial cell signalling events and biofilm formation among other things. Formulations of *P. chlororaphis* isolates and their metabolites are currently being commercialized for agricultural use.

INTRODUCTION

Pseudomonas chlororaphis isolates exist worldwide. From the frequency of their detection, many isolates are plant colonizers. Interest in *P. chlororaphis* isolates is driven by the properties of their metabolites, which show potential for use in formulations beneficial to plant health [1]. The *P. chlororaphis* isolates associate with both mono- and dicotyledonous plants growing in commercialized and natural habitats. A recent review of *P. chlororaphis* isolates indicated that these bacteria colonize 11 different plant hosts grown on 4 different continents [1]. *P. chlororaphis* isolates from soils include: UFB2 [2] from soybean field soil; L19 [3] from coal-, heavy metal- and petroleum-contaminated saline soils [4]; and MCC2693, a psychrophile from a mountain ecosystem [5].

The aim of this review is to highlight the currently identified traits of *P. chlororaphis* isolates that would benefit from further study because of their potential agricultural value. The present scientific methods and technologies have provided extensive knowledge of the behaviour of *P. chlororaphis* isolates within the confines of the laboratory; however, studies are currently investigating the process that occur in the field to identify the traits of *P. chlororaphis* isolates that will aid the development of successful commercial products.

This review begins with a background of how microbial isolates are classified as belonging to the four subspecies of the *P. chlororaphis* group. Isolates of this group have protective, 'probiotic-like' effects on their hosts. The arsenal of metabolites produced by *P. chlororaphis* group isolates are discussed to illustrate their protective roles for the plant hosts. Volatile organic compounds (VOCs) are significant because they allow the microbe to influence its environment in three dimensions (including through the atmosphere). Next, microbial survival mechanisms are addressed with a focus on biofilm formation and microbial nutrition. Although *P. chlororaphis* isolates are deemed nonpathogenic [6], isolates have the potential to kill plant-associated organisms such as nematodes and insects. Further, these isolates can be denitrifiers; therefore, their potential effects on ecosystem function should be carefully considered before their widespread use in the field. The final section of this review focuses on the potential commercial use of metabolites of *P. chlororaphis* isolates.

The information reviewed in these sections reveals the potential for *P. chlororaphis* isolates to provide plant protection that extends further than the intact cells. The model shown in Fig. 1 illustrates there are at least three overlapping layers and spheres of protection that arise from colonization of the root surface. Each of these layers offers different mechanisms with the potential to contribute to plant health. The mechanisms and traits of importance in the rhizosphere for the beneficial activities of *P. chlororaphis* isolates summarized in Fig. 1 are discussed in the subsequent sections.

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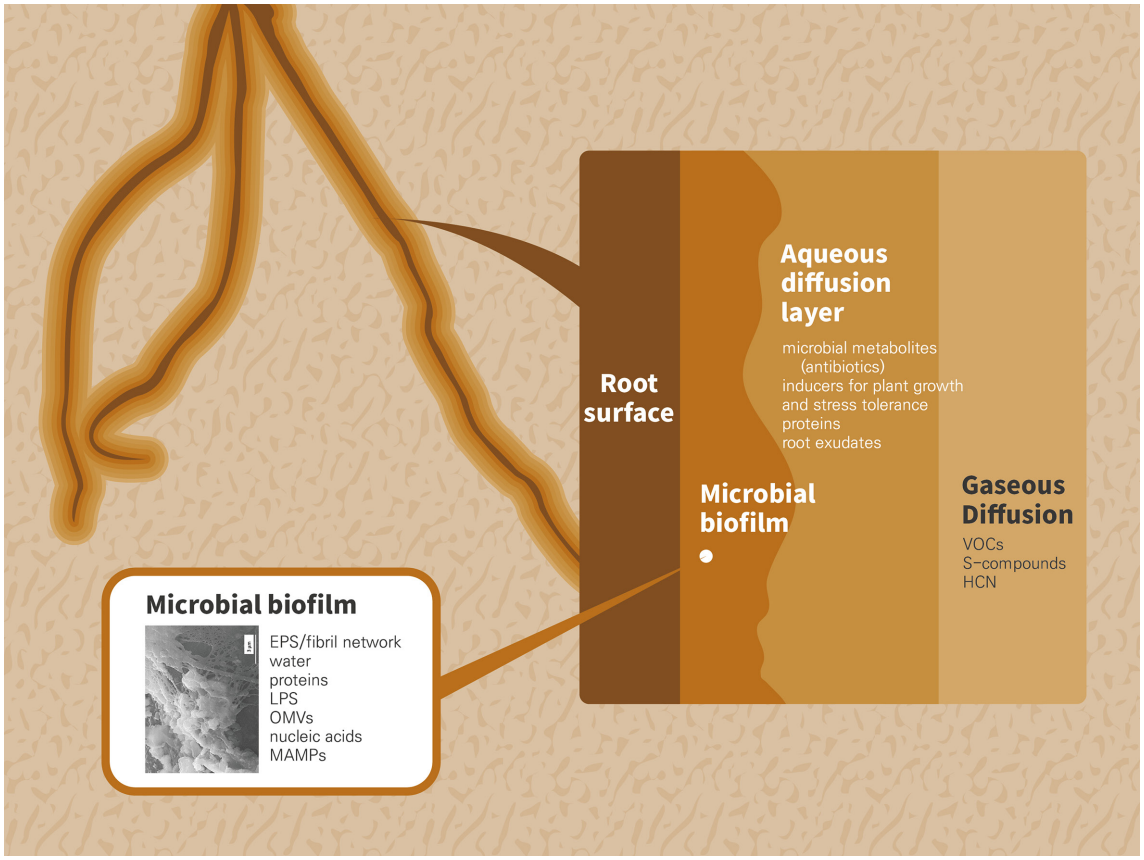

Fig. 1. The multilayered protective mechanisms pertinent to a root colonized by a *P. chlororaphis* isolate. The layers considered are at the root surface, the patchy biofilm of microbial cells, the sphere generated by aqueous diffusion of secreted metabolites and the extended volume in the rhizosphere impacted by volatile diffusion. At the root surface, metabolites/volatiles, including VOCs, released by root colonists could be transported in the plant upward apoplastically and/or by the plant vasculature (xylem/phloem) to initiate plant responses such as changes in growth and stress tolerance. Microbial products could also function in pathogenicity towards nematodes and insects. Growth of the bacterium as a biofilm offers protection to the bacterial cells and the ability to trap materials through charge–charge interactions (e.g. nucleic acids would provide negative charges to bind metal ions) and physically because of the fibrillar nature of the extracellular matrix. The presence within the EPSs and OMVs of discrete structures that act as microbially associated molecular patterns (MAMPs) would activate plant defences. The insert image is a scanning electron micrograph showing cells of strain O6 *P. chlororaphis* colonizing a 7 day-old wheat root from a seed inoculum; the methods are described by Jacobson *et al*. [72]. The diffusion of secreted metabolites and enzymatic proteins in the pore waters surrounding the root would extend their functions to a wider volume in the rhizosphere than just the root surface. Diffusion of the volatiles released from the colonized root through pore spaces in the soil and into the air would influence surrounding soil organisms and shoot tissues and neighbouring plants.

What is in a name for these colourful bacteria?

P. chlororaphis isolates are Gram-negative, motile, green-/yellow-/orange-coloured gamma-proteobacteria that are now assigned to their own taxonomic group (*Pc*; NCBI Taxonomy Browser) among seven *Pseudomonas* spp. groups. Currently there are four subspecies designated as *P. chlororaphis* isolates: *chlororaphis*, *aurantiaca*, *aureofaciens* and *piscium*. Bacterial isolates were first identified as pseudomonads in the late 1800s by Miguela, and the opportunistic human pathogen, *Pseudomonas aeruginosa,* was designated as the first type culture. This early work on taxonomy is elegantly discussed by Palleroni [7], who comments that the complexity of classification of *P. chlororaphis* isolates is partly due to the extreme metabolic diversity of pseudomonads. Classification based on 16 s rRNA gene sequences, a tool first used in 1965, clarifies the predicted evolutionary relationships among *P. chlororaphis* isolates. In 2007, the *P. chlororaphis* group was split into subspecies (*P. chlororaphis* subsp. *aurantiaca*, *P. chlororaphis* subsp. *chlororaphis* and *P. chlororaphis* subsp. *aureofaciens*) based on genetic and phenotypic properties [8]. Alignment of 16S rRNA gene sequences grouped type cultures of the subspecies *aurantiaca*, *chlororaphis* and *aureofaciens* together and apart from other *Pseudomonas* species [8]. DNA–DNA hybridization values were 76 % for two *aurantiaca* type cultures, 67–81 % for three *chlororaphis* type cultures and 76–88 % for three *aureofaciens* type cultures [8]. These findings, as well as the observed variability in the sequences of the housekeeping genes *atpD*, *recA* and *carA*, while the fatty acid

Table 1. Correlation between colony pigmentation and *P. chlororaphis* isolate classification

Name of *P. chlororaphis* subspecies	Pigmentation colour/ phenazines	Type culture
chlororaphis	Green PCA	ATCC 9446
aureofaciens	Gold PCA, 2-OH PCA, 2-OH PHZ	ATCC 13985
aurantiaca	Orange/yellow PCA, 2 OH PCA, 2 OH PHZ	ATCC 33663
piscium	Yellow/green. PCA and PCN	NCIMB 14478

These descriptions are based on publications by Biessy *et al.* 2018 [11], Burr *et al.* 2010 [9] and Peix *et al.* 2007 [8]. The abbreviations for phenazines are as follows: phenazine-1-carboxylic acid, (PCA); phenazine-1-carboxamide, PCN; 2-hydroxyphenazine-1-carboxylic acid, 2-OH PCA; 2-OH hydroxyphenazine, 2-OH PHZ.

compositions agree, support their designation as subspecies of *P. chlororaphis* [8].

In 2010, a new branch was added to the *P. chlororaphis* group: *P. chlororaphis* subsp. *piscium* [6, 9]. Burr *et al.* [9] characterized two isolates of this subspecies; both from fish intestines. However, a strain from a tomato rhizosphere, known as PCL 1391, which confers strong protection against tomato foot and root rot [10], was also classified as belonging to *P. chlororaphis* subsp. *piscium*. As discussed by Peix *et al.* [8], certain phenotypic traits are variable among the *P. chlororaphis* subspecies, such as the potential for denitrification and the use of arabinose or ketogluconate as C sources [8, 9]. A visually distinguishing feature for cells in the different *P. chlororaphis* subspecies is pigmentation when cultured on a rich medium [Table 1, [8]]. The pigmentation varies with the array of antifungal phenazines produced by the *P. chlororaphis* isolate. An analysis conducted by Biessy *et al.* [1] showed that each isolate in the *P. chlororaphis* group had unique singleton genes within their genomes, which ranged in size from 6.66 to 7.30 kbp. The isolates share a common core of 71 % of their genes with pseudomonads of different species that also produce phenazines. This common core of genes is increased to 87 % within each subspecies of the *P. chlororaphis* group. Despite these studies on classification, most published studies of *P. chlororaphis* isolates do not identify to the subspecies level.

Beneficial traits of *P. chlororaphis* isolates in the rhizosphere: metabolites for direct antagonism of pathogens and systemic activation of plant defences

Phenazines

The intensity of colony pigmentation in *P. chlororaphis* strains, when grown on a rich medium, contributes to their ease of isolation and detection during studies. This pigmentation is due to the production of three-ring nitrogen-containing phenazines, which have antimicrobial properties and affect cell signalling [11]. In a 1962 paper [12], the difficulty of identifying an isolate as *P. chlororaphis* or *P. aeruginosa* based solely on pigmentation is discussed, because cells of both these species can produce a green colouration. However, the correlation between phenazine structure and colour and isolate identity (Table 1) is discussed in more recent publications, and is strengthened by genome analysis and transcriptomics [1, 11].

The metabolic pathways that transform the product of the shikimic acid pathway, chorismate, to one of several phenazines produced by plant-beneficial pseudomonads, including those of the *P. chlororaphis* group [e.g. phenazine 1 carboxylic acid (PCA), phenazine 1-carboxamide (PCN), 2-hydroxyphenazine-1-carboxylic acid (2-OH PCA) and 2-OH hydroxyphenazine (2-OH PHZ)] are discussed by Biessy *et al.* [11]. The genes involved in the synthesis of the basic phenazine rings are clustered (genes *phz*A, *phz*B, *phz*C … *phz*G) and the gene order is highly conserved among beneficial phenazine producers [11]. These clusters are preceded by the regulatory genes (e.g. *phzI* and *phzR*). The findings highlight how the genes in these beneficial phenazine-producing pseudomonads differ from those in other microbes (e.g. *Streptomyces*, *Burkholderia* and *Pectobacterium* spp.) that also synthesize phenazines but with different structures [13]. Comparisons among plant-beneficial phenazine-producing pseudomonads show that all isolates in the *P. chlororaphis* group generate PCA; the *P. chlororaphis* subspecies *aurantiaca* and *aureofaciens* do not produce PCN, whereas this phenazine is synthesized by all *P. chlororaphis* subspecies *piscium* [1]. Genes, such as *phzH* for PCN and *phzO* for 2-OH PCA, account for the variation in the side groups of the final phenazine products [1].

The mechanisms by which microbes regulate phenazine synthesis have been studied intensively in many *P. chlororaphis* isolates, revealing overall similarities and some differences. Synthesis is conditioned by environmental factors, including cell density, which, when above a threshold density, triggers global changes in gene expression through a quorum-sensing system. The quorum-sensing systems of *P. chlororaphis* isolates are governed by cell-signalling molecules called acyl homoserine lactones (AHSLs), which are produced under the control of a 'Gac' sensor kinase mechanism [11]. Both translational and transcriptional control measures condition the synthesis of AHSLs, although there are differences in the regulatory processes among pseudomonad isolates. For example, most *P. chlororaphis* isolates have two regulatory loci for AHSL synthesis; however, three have been reported in *P. chlororaphis* subsp. *aurantiaca* PB-St2 [14]. The knowledge of these pathways is the most detailed for isolates 30–84, due to the efforts of the Pierson group [15, 16], and PA23, from the work of deKievit's research team [17, 18]. Important findings include the fact that phenazines, such as PCA, regulate their own gene expression [19] and the discovery of overlap between Gac regulation and that of other global regulators,

such as RpoS and Anr [17, 20, 21]. Readers are referred to the review by Biessy and Filion [11] for details of the complex regulation of gene expression for phenazine synthesis.

The review by Biessy and Filion [11] also summarizes the properties of the phenazines that are important for plant interactions. The most widely researched is the direct inhibition of microbial growth by phenazines, a finding that suggests the potential for biological control of plant microbial pathogens by *P. chlororaphis* isolates. Fungal and bacterial growth inhibition is readily observed when these microbes are grown in medium adjacent to the *P. chlororaphis* isolates where the phenazines diffuse from the bacterial colonies. The presence of phenazine-producing microbes is correlated with soils that suppress certain pathogens, as in the classic example of take-all decline soils [22], in which the level of crop damage caused by a fungal pathogen declines with continuous cropping of wheat on the same fields [22].

In addition to direct growth antagonism of microbes, the phenazines also induce systemic resistance in plants such that protection against shoot pathogens is triggered by root colonization with *P. chlororaphis* isolates. Both PCN- and PCA/2-OH PHZ-producing microbes possess this ability [23–25]. Duke *et al.* [23] observed that aerial application of isolates induces both localized and systemic responses associated with resistance. Thus, the direct microbial antagonism caused by phenazine production is reinforced by the phenazine-mediated upregulation of plant defence mechanisms.

Pyrrolnitrin and resorcinols

Phenazines are not the only metabolites synthesized by *P. chlororaphis* isolates that offer plants protection against microbial pathogens. As shown by Biessy *et al.* [1], *P. chlororaphis* group isolates synthesize other antimicrobials, including the chlorinated product, pyrrolnitrin, and the alkyl resorcinols. These compounds have different importance in plant pathosystems, depending on the identities of the plant, pathogen and pseudomonad. Mutational analysis of *P. chlororaphis* subsp. *piscium* PCL 1391 revealed that the phenazine PCN was a key metabolite for controlling fusarium root rot in tomato [26]; however, although isolate PCL 1606 produces PCN, the synthesis of 2-hexyl-5-propyl resorcinol, from the *dar* gene cluster, is more important for combatting the white rot fungus in avocado [27]. However, pyrrolnitrin, rather than phenazines, is the key antimicrobial produced by *P. chlororaphis* subsp. *aureofaciens* PA23 that controls white rot on canola [28]. Pyrrolnitrin is also the key metabolite in the inhibition of fusarium head blight by *P. chlororaphis* G05 [29]. Whether these observations are influenced by the nutrients available to the pseudomonad awaits resolution. The synthesis of phenazines predominates over pyrrolnitrin in *P. chlororaphis* isolate O6 in medium with abundant glucose [30]. Thus, the question arises of the role of the composition and concentrations of plant metabolites in the root exudates in regulating the production of antimicrobials by *P. chlororaphis* isolates in the rhizosphere. The phenazine PCA is detected in soils harbouring phenazine-producing pseudomonad isolates [31]; however, the extent to which this phenazine originates from *P. chlororaphis* group isolates or from other bacteria, such as *P. synxantha* detected in the soils, is unknown.

Hydrogen cyanide (HCN)

HCN, a very simple, soluble and volatile compound formed from glycine, is another part of the *P. chlororaphis* group's toxic arsenal because of its ability to bind to structures containing heme groups. The nematocidal activity of *P. chlororaphis* isolate PA23 is correlated with both pyrrolnitrin and HCN [32]. An isolate of *P. putida* also kills nematodes via multiple mechanisms involving volatiles [33]. In addition, Flury *et al.* [34], working with *P. chlororaphis* isolate PCL 1391 and other pseudomonads, show that HCN, rather than phenazines or pyrrolnitrin, is the active component responsible for nematode larval death. The production of HCN by *P. chlororaphis* isolate O6 causes cell death in both nematodes [35] and aphid nymphs [36]. HCN is detected in the air space of tomato plants growing with roots colonized by *P. chlororaphis* O6 (35). The successful control of root-lesion nematodes by the *P. chlororaphis* Sm3 is reported in greenhouse-grown strawberries; however, no studies on the underlying mechanism are discussed in this work [37]. Similarly, under commercial greenhouse conditions, the application of *P. chlororaphis* O6 formulations results in the control of nematodes on pepper plants [38].

Volatile organic compounds (VOCs)

Much interest is currently directed towards microbially produced VOCs [39–43] that protect the plant in three dimensions through gaseous diffusion. This process is independent of water flux and aqueous diffusion, processes that would distribute the metabolites such as the phenazines. Generally, VOC activity is assessed by growth of the organisms (i.e. plants and microbes) separately on media in enclosed vessels where only the air space is in common. These dual but separate culture systems are made possible, for example, by the presence of a solid barrier across an agar plate separating media, but not the air space. When the VOC composition is analysed by sampling this air space from dual cultures with *P. chlororaphis* isolates, an array of metabolites with compositions that change with the growth medium is reported. It is possible that VOCs that are not found in nature are released from the medium used for microbial growth. In addition, medium components not found in nature may be metabolically transformed into VOCs due to the versatility of microbial metabolism, i.e. these VOCs would not be produced on a colonized root. Dual culture assays only containing microbes also negate the detection of any VOCs generated by the plant in response to contact with microbial cells or secreted metabolites. The composition of the VOCs from microbially colonized plants has not yet been comprehensively studied. Another process important in understanding the role of VOCs is that diseased plants alter their VOC patterns and it is suggested that some of these compounds will attract soil microbes with antifungal properties [44].

For *P. chlororaphis* O6, VOC production with growth on two different media is reduced by mutations in the Gac system,

which eliminates regulation by the acyl homoserine lactones involved in quorum sensing. Similarly, the reduction in VOC emissions observed in *gac*S mutants of *P. fluorescens* isolates [45] suggests that Gac dependence is a common trait among the plant-associated pseudomonads. One of these Gac-regulated metabolites with marginal volatility, 2R,3R-butanediol, is produced by the microbial fermentation of pyruvate by the *P. chlororaphis* strains O6 and M71 [46, 47]. The plant responds to the butanediol with narrowed stomatal openings and the induction of systemic responses, changes that are involved in drought and pathogen tolerance [24, 48]. Butanediol also promotes plant growth, a response that is partly attributed to the activation of genes encoding plant cell wall-modifying enzymes and enhanced C metabolism [49]. In addition, the composition of plant root exudates is altered in plants exposed to this VOC, a process that modifies microbial root colonization [50]. Thus, 2R,3R-butanediol induces an array of responses in the plant that benefit plant health.

Another class of plant-active compounds with low volatility are the polyamines. Polyamine production in *P. chlororaphis* O6 has implications for biocontrol [51]. In plants, polyamine oxidases generate reactive oxygen species (ROS), which are involved in defence system signalling [51]. sConsequently, the production of polyamines by bacterial cells may stimulate signalling in the host plant cells.

Many additional simple volatiles are detected from *P. chlororaphis* group isolates. For the *P.* subsp. *aureofaciens* SPS-41 [52], the production of 3-methyl butanol, 2-methyl butanol and phenylethyl alcohol is associated with direct growth antagonism towards the fungus causing black rot in sweet potato tubers. The hydrocarbon, undecane, is detected from *Pc*. subsp. *aurantiaca* KNU17Pc1 [53] and *P. chlororaphis* O6 (unpublished) when isolates are grown on rich medium. Other VOCs detected in both O6 (unpublished) and M71 [46] isolates include S-containing compounds, such as hydrogen sulfide, dimethyl sulfide and methanethiol. These metabolites may be correlated with induced plant defence responses. There are a wide variety of VOCs for which the production by *P. chlororaphis* isolates needs to be clarified under field conditions where the microbe is colonizing a plant or other host. Moreover, their modes of action as a single compound or within a 'VOC cocktail' should be established.

SURVIVAL STRATEGIES OF BACTERIAL ISOLATES

Sources of nutrients

The isolation of *P. chlororaphis* group strains from many soils, rhizospheres and plants indicates that they have strong survival mechanisms in nature. Their association with plant roots provides them with diverse C and N sources from the metabolites in root exudates. The catabolism of these metabolites is indicated by alterations in root exudate composition for colonized plants [54]. For example, citrate is one of the organic acids in wheat root exudates that is preferentially utilized by a *P. chlororaphis* isolate [54]. The various catabolic pathways of pseudomonads enhance their abilities to process an array of organic molecules into biomass. The *P. chlororaphis* isolate O3 degrades lignin-like structures [55] and genes conferring tolerance to toluene are present in other *P. chlororaphis* isolates [56]. These traits raise the possibility that some *P. chlororaphis* isolates may be of commercial use in recycling and environmental remediation.

The ability of *P. chlororaphis* isolates to kill other soil organisms, such as nematodes, insects, algae [57] and other microbes would provide copious and varied nutrients for microbes to augment those available from the plant. The role of volatile HCN and organic metabolites produced by *P. chlororaphis* isolates in killing insects and nematodes has already been discussed. However, members of the *Pc* group also produce insecticidal peptides. One gene cluster involved is the set of '*fit*' genes, most thoroughly studied in *P. fluorescens* isolates [58]. Loci for the '*fit* ABCDEFGH' cluster are found in *P. chlororaphis* isolates O6 and 30–84 [59] and these microbes cause death in tobacco hornworm when injected or ingested [60]. However, the '*fit*' system may be augmented by other insecticidal peptides, for example the peptide IPD072Aa, produced by *P. chlororaphis* isolates. The expression of the *P. chlororaphis* gene encoding this peptide in maize results in plants that are toxic to the rootworm pest [61]. Inspection of the *P. chlororaphis* O6 genome reveals an analogue to this gene; thus, this trait may be widespread among *P. chlororaphis* isolates. These examples show the array of traits within the *P. chlororaphis* group of pseudomonads that can achieve the same goals, such as insecticidal activity.

The aggressiveness of the *P. chlororaphis* isolates as root colonizers [62] may also be enhanced by a completely different mechanism, that is, the killing of competing bacteria by the production of bacteriocins, called tailocins, as demonstrated for *P. chlororaphis* isolate 30–84 [63, 64]. Tailocins are related to the pyocins of *P. aeruginosa* [65] and were first implicated in isolate 30–84 in connection with phenazine promotion in the biofilm structure [19]. The tailocins promote colonization of the root surface by isolate 30–84 by killing other potential colonizers that are sensitive, i.e. other pseudomonads and bacterial genera. This mechanism is effective for colonization of the isolate 30–84 at the root surface but not for bulk soil colonization [64].

In addition to obtaining nutrition from plants and prey organisms, *P. chlororaphis* cells can also glean essential elements from the soil. Isolates of the *P. chlororaphis* group are termed 'fluorescent' pseudomonads because, under iron-deficient growth conditions, they synthesize effective pyoverdine (PVD)-like siderophores that mine Fe in the environment to be taken up by the bacterial cell. Fe is an essential element for microbes. PVD siderophores exhibit characteristic blue–green fluorescence when non-chelated. The transfer of Fe from loaded PVDs to the plant shows yet another way in which the plants benefit from their associations with these pseudomonads. These siderophores, which extract Fe from soil minerals, may also scavenge other metals, such as Cu, because these ions also bind in the active pocket of the PVD

structure [66]. Indeed, PVDs from the *P. chlororaphis* isolate ATCC 9446 are implicated in the breakdown of organotin antifouling paints, presumably by binding the tin ion and dissolution of the paint solution [56]. Similarly, the release of vanadium from Fe-containing soil minerals observed for *P. chlororaphis* isolate L19 in coal-contaminated soil [3] could be due to selective ion binding by the microbial siderophores. Such observations expand the potential usefulness of the *P. chlororaphis* isolates in commercial remediation ventures.

PVD is not the only type of siderophore produced by *P. chlororaphis* isolates. Genetic loci that allow the synthesis of citrate-based siderophores, called achromobactins [1], are detected in *P. chlororaphis* genomes. Currently, little is known about the ecological significance of these iron-chelators. In *P. chlororaphis* subsp. *chlororaphis*, mutants that overproduce PCN show upregulation of genes for both PVD and achromobactin production [67]. Consequently, there is potential for the genes of both siderophores to be expressed simultaneously by a *P. chlororaphis* isolate. An isolate of *P. chlororaphis* subsp. *piscium* also expands its potential sources of Fe using heme, a trait possibly associated with the isolation of these strains from fish that possess heme in the blood supply. The heme becomes associated with secreted hematophores before being loaded into the cell by special receptors [1]. Finally, phenazines may play yet another role in Fe bioavailability. Dissolution of the Fe-containing minerals, ferrite and hematite, occurs under low-oxygen conditions when exposed to phenazines; 2-OH PHZ is found to be more effective than PCA in mediating Fe release from these minerals [68].

In addition to the chelation of Fe, *P. chlororaphis* isolates subsp. *chlororaphis* and *aurantiaca* solubilize insoluble Zn minerals, such as ZnO and Zn carbonate [69]. The underlying mechanisms are not yet known, and the question of whether the released Zn is bioavailable to plants has not yet been answered . The microbial secretion of organic acids, such as gluconic acid, may be involved in metal solubilization, because these acids also chelate several metals [54]. Gluconate production by other microbes is linked to phosphate solubilization from rock phosphate [48]. Phosphate solubilization by *P. chlororaphis* isolates is a variable trait, occurring in the high-mountain *P. chlororaphis* isolate MCC2693 [5] but not in several other *P. chlororaphis* isolates, as studied by Shahid *et al.* [69]. Genetic analyses reveal that all *P. chlororaphis* isolates have genes for synthesizing PQQ, the cofactor for glucose dehydrogenase. This enzyme oxidizes glucose to gluconate as an alternative mechanism for glucose catabolism [1, 48]. This finding suggests that gluconate is a common metabolite for all *P. chlororaphis* isolates.

Nitrogen metabolism by *P. chlororaphis* isolates can involve denitrification. *P. chlororaphis* isolates use inorganic nitrate as an external electron acceptor when oxygen is limited, causing the production of nitrite. This process may be very important for the maintenance of metabolic flux when there is insufficient oxygen to act as a terminal electron acceptor, such as for the bacterial cells encased within a biofilm matrix. Nitrate flux in the denitrifying pathway is connected to phosphate metabolism through the common regulator protein, Dnr, in *P. aeruginosa* [70]. The regulator, Anr, is involved in sensing low oxygen and activating Dnr, which stimulates the expression of genes involved in the denitrification pathway. However, high phosphate content in pseudomonad cells inhibits the activation potential of Dnr, and thus limits the conversion of nitrate to nitrogen [70]. The extent to which such pathways occur in all *P. chlororaphis* group isolates has not yet been clarified, but is of importance because of the fate of soil nitrate, such as that added purposely as a fertilizer.

The nitrite generated from nitrate can be further metabolized to gaseous nitric oxide (NO). NO is a significant signalling component in plants that is associated with several functions, including the activation of plant defences to biotic and abiotic stressors [71]. The extent to which microbial NO production from *P. chlororaphis* cells within biofilms on plant roots is involved in plant defence activation and remodelling of root morphology remains unclear. The accumulation of NO on *P. chlororaphis* O6-colonized wheat roots is visualized by using fluorescence staining [72]; however, H_2O_2 accumulation is not detected [73]. Further reduction to nitrous oxide (N_2O) followed by its conversion to nitrogen gas completes the denitrification pathway in some pseudomonads. Laboratory studies of *P. chlororaphis* and *P. fluorescens* isolates find that certain microbes only produce N_2O, whereas others also generate N_2 [74]. Two isolates designated as *P. chlororaphis* subsp. *aureofaciens* and *chlororaphis* lack nitrous oxide reductase genes, and the pathway ends with N_2O generation. These isolates possess different nitrite reductases (NIRs): the *aureofaciens* isolate possesses a Cu-NIR and the *chlororaphis* strain possesses a cd_1-NIR. In addition, the two strains in these studies differ in the effectiveness of succinate versus citrate as the reductant substrate [75]. The overall process of denitrification is viewed negatively because it destroys nitrate added to the soil as a plant fertilizer by converting it to nonnutritive gaseous products. It generates a more toxic anion, nitrite, and the released N_2O, which may exacerbate global warming. However, the pathway offers an alternative for *P. chlororaphis* isolates under conditions of hypoxia to maintain cellular function without generating acidic fermentation products.

Biofilm formation

The growth of microbial cells on plant roots is seen as patchy biofilms. Work with *P. chlororaphis* O6 (72) shows that the biofilm cells on plant roots are embedded in a complex extracellular matrix (Fig. 1). This biofilm habitat likely contributes to the survival of isolates in the rhizosphere and soil, as well as their potency in plant protection. The potential beneficial effects of biofilm formation in the rhizosphere, summarized in Fig. 1, are discussed in this section.

Biofilm formation is associated with cell resistance to doses of external antibiotics and hydrogen peroxide that would be lethal to planktonic cells [76]. However, the matrix that embeds the bacterial cells also limits the diffusion of metabolites through the fibrous mass. The concentration of any

antimicrobial products synthesized by the biofilm cells, such as phenazines, pyrrolnitrin and HCN, would better restrict the growth of other microbes sensitive to these chemicals. In addition, biofilm cells would outcompete other microbes, insects and nematodes for the metabolites released as plant root exudates; thus, the potential colonization of a plant by pathogens would be reduced. Biofilm formation by *P. chlororaphis* isolates PA23 and 30–84 is differentially enhanced by phenazines [19, 28]. Phenazines, such as 2-OH PCA, promote the release of extracellular (e-) DNA [19, 77], which can bind cations and modify the biofilm structure, as discussed by Yu *et al*. [77].

Biofilms are observed on the roots of soil-grown plants. Greater biofilm production is observed on wheat roots in dry soil than on those in wet soil for the PCA-producer, *P. synxantha* 2–79 [78]. This greater microbial biomass in dry soil correlates with higher stability and enhanced association of N with the plant roots. Thus, biofilms contribute to the health of both the plant and the soil. The PCA-producing pseudomonads are more dominant in non-irrigated soils than in irrigated soils [79, 80]. The production of water-holding extracellular polysaccharide matrices by microbes promotes healthier soils by mediating soil particle aggregation [81]. The aggregation limits soil run off and loss from wind and rain. The higher level of hydration of the gel-like biofilms on root surfaces is proposed to contribute to drought protection of the plant [82, 83].

In a related study, performed in autoclaved soil, wheat survival under conditions of drought is promoted by colonization with the phenazine-producing *P. chlororaphis* 30–84 [84]. Improved root formation by the colonized wheat is observed and is proposed to enhance water uptake. Induced drought tolerance by the biofilm-former *P. chlororaphis* O6 also occurs in wheat seedlings [72] due to several potential factors: priming by the bacterium to stimulate transcription of a large array of genes associated with drought tolerance [85], alteration of root morphology through the proliferation of root hairs connected to bacterial IAA production [72], butanediol-stimulated stomatal closure [47], NO production elicited by microbial colonization and water-withholding in the bacterial biofilm due to its polysaccharide components [72]. Analysis of priming for drought tolerance in tomato by *P. chlororaphis* M71 [46] also revealed that the induction of antioxidant mechanisms in the plant and the accumulation of the protectant osmolyte proline are important processes in resistance to drought.

Priming of the protective responses in the plant could, in part, involve induction by components of the biofilm matrix. Isolated extracellular polymeric substances (EPSs) protect plants from drought [86]. The EPSs from both planktonic and biofilm cells contain nano-sized outer-membrane vesicles (OMVs), which are released in greater quantities from bacterial cell surfaces under conditions of stress [87]. Atomic force microscope imaging of live *P. chlororaphis* O6 cells subjected to oxidative stress show released OMVs [88]. Studies on other Gram-negative pathogenic bacteria find that OMVs harbour toxins that interact with the host and thereby become part of the pathogen virulence mechanism [89]. However, OMVs also contain lipopolysaccharides and flagellar proteins perceived as 'microbe-associated molecular patterns' (MAMPs) to activate immune responses in plants, a process akin to that observed in animals [90, 91].

Biofilm formation protects microbial cells from water-deficit stress due to its high moisture content and ability to reduce diffusion of toxic materials. Other underlying mechanisms for drought resistance include osmolyte accumulation within microbial cells and their potential secretion. Likely metabolites include proline, glycine, betaine and trehalose, because the genes required for synthesis of these osmolytes are present in *P. chlororaphis* genomes. Trehalose formation is reported for the *P. chlororaphis*. subsp. *aurantiaca* G5 and, like many other traits of the *P. chlororaphis* group isolates, this activity is under Gac sensor kinase global control [92]. Thus, the cells in the biofilm contribute to an extended protectant hydrated layer on the root surface to improve plant and microbial survival to drought and salinity.

The importance of the biofilm habitat to bacterial cells in the *P. chlororaphis* group is supported by the finding that another antifungal metabolite, 2-hexyl-5-propyl resorcinol, stimulates biofilm formation, and that the *dar* genes required for resorcinol synthesis are common to all *P. chlororaphis* isolates [1]. 2-Hexyl-5-propyl resorcinol is essential for the biocontrol action of *P. chlororaphis* isolate PCL 1606 [93] and the production of several additional resorcinol family members is found for *P. chlororaphis* isolate O6 (unpublished). Mutants of *P. chlororaphis* PCL 1606 lacking resorcinol formation are impaired in adhesion, which would limit microbial cell-to-cell interactions involved in biofilm formation [94]. For another bacterium, *Photorhabdus asymbiotica*, the diakyl resorcinols act as cell-signalling molecules [95], suggesting similar roles to the AHSLs for *P. chlororaphis* isolates. Thus, we again observe that, for the *P. chlororaphis* isolates, a single metabolite may have multiple roles.

The above studies indicate that biofilm formation is a dynamic part of the association between *P. chlororaphis* isolates and plants and plays vital roles in both microbe and plant protection. Currently, the nature of the embedding matrix and how its composition may vary with time and between plants and isolates require further examination to identify the chemistry of the matrix and the array of genes, both structural and regulatory, that are involved.

Use of *P. chlororaphis* isolates in commercial agricultural products

The commercial use of the *P. chlororaphis* group of biocontrol agents has two branches: the application of specialized metabolites and the formulation of live cell preparations. Genomics, transcriptomics and metabolomics enable the genomic modification of bacteria to target the production of defined metabolites. Phenazines are antibiotics that may have potential as anticancer drugs [96]. The transfer of a *phz*

cluster to a nodulation nitrogen-fixing strain of *Rhizobium etli* correlated a novel antifungal trait with phenazine production, but this modification also destroyed functional nodule formation [97]. A different approach to genetic modification is to transfer the beneficial properties of the microbe to the host. This has been successful in maize, in which the expression of an insecticidal protein reduces insect pest damage [61]. Another example is the expression of a gene from the *P. chlororaphis* isolate G65 to control ethylene production in transformed plants, as summarized in the discussion by Anderson *et al*. [98]. Carlson *et al*. [99] have covered the extensive testing required to demonstrate that genetic modifications are not likely to adversely affect human health. For example, the 'safety' criteria for plant expression of the insecticidal protein, IPD072Aa, included the findings that this protein neither had any similarities to known allergens nor caused morbidity or weight loss in tested animals [99].

Shen *et al*. [100] used genome reduction as a tool to boost 2-OH PHZ production in *P. chlororaphis* isolate GP72, a sequenced biocontrol microbe isolated from green pepper [101]. Overproduction is greatest in a mutant defective in the production of pyoverdine-like siderophores, because the synthesis of this Fe-binding metabolite is an energy- and N-expensive process. Mutants with altered ratios of phenazines exhibit different colony growth patterns to the parental strain, suggesting cell-surface modifications [100]. These observations align with the findings that phenazines modify biofilm structures [19, 28]. The characterization of another mutant with enhanced PCN production also provides information on the traits associated with phenazine formation. Studies of the *P. chlororaphis* isolate HT66 demonstrate that a mutant with increased PCN production had alterations in over 400 proteins [67] and showed increased N metabolism and Fe uptake. An additional finding is that PCN production is influenced by the phosphate supply: a 50 % reduction in phosphate in the growth medium enhances phenazine production. This observation illustrates the importance of the growth medium in generating effective formulations of commercial value.

Studies performed to EPA standards indicate that *P. chlororaphis* isolates pose limited risks to humans and the environment [98]. The supporting evidence includes the absence of pathogenicity islands and type III/IV transfer systems in bacterial pathogens and that no genes required for the production of toxins characterized from pseudomonad plant pathogens are found in *P. chlororaphis* isolates. Indeed the literature has few reports of *P. chlororaphis* isolates from diseased human tissues [102, 103]. Moreover, no disadvantageous effects of *P. chlororaphis* isolate application to field-grown plants are known.

Currently, as reviewed by Anderson *et al*. [98], there are several commercial formulations of different *P. chlororaphis* isolates for agriculture: AtEze for greenhouse use, Cedemon and Cerall for use in cereals, and Howler formulations sold as fungicides. An additional *P. chlororaphis* formulation based on *P. chlororaphis* subsp. *aurantiaca* SR1 is available for use in Argentina to promote the growth of a variety of crops [104]. It is likely that, as the need for 'green', regenerative and sustainable agriculture increases, there will be greater demand for products employing live cells and those based on bioactive metabolites from such microbes, such as *P. chlororaphis* isolates.

SUMMARY OF BENEFICIAL TRAITS

The *P. chlororaphis* group of isolates is a topic of interest because of the biocontrol potential of these bacteria for commercially significant plant pests, such as microbes, insects and nematodes. A model showing the overlapping layers of protection resulting from microbial colonization of a plant root surface is presented in Fig. 1. The multifunctional effects of the interactions between *P. chlororaphis* isolates and the host plant are consistent with their functions as plant probiotics. Indeed, as discussed earlier, *P. chlororaphis* subsp. *piscium* isolates are termed fish probiotics because their presence in the intestine improves fish health.

Several metabolites of isolates from the *P. chlororaphis* group, first identified because of their antifungal activities, are multifunctional. These metabolites participate in metal chelation, insecticidal and nematocidal activities, biofilm formation, induction of priming for plant tolerance to stress and microbial cell signalling. Evidence has accumulated to show that the formation of a biofilm on a plant's surface plays a more important role than just securing an environment for bacterial cells. Instead, the biofilm presence on the root surface aids plant performance and resilience to stress. The beneficial effects of the *P. chlororaphis* group isolates on plants suggests that the use of these traits in commercial formulations for green and regenerative agriculture will be expanded.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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