Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria

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Specific strains of the *Pseudomonas fluorescens-putida* group have recently been used as seed inoculants on crop plants to promote growth and increase yields. These pseudomonads, termed plant growth-promoting rhizobacteria (PGPR), rapidly colonize plant roots of potato, sugar beet and radish, and cause statistically significant yield increases up to 144% in field tests[1-5]. These results prompted us to investigate the mechanism by which plant growth was enhanced. A previous study indicated that PGPR increase plant growth by antagonism to potentially deleterious rhizoplane fungi and bacteria, but the nature of this antagonism was not determined[6]. We now present evidence that PGPR exert their plant growth-promoting activity by depriving native microflora of iron. PGPR produce extracellular siderophores (microbial iron transport agents)[7] which efficiently complex environmental iron, making it less available to certain native microflora.

Plant growth-promoting fluorescent *Pseudomonas* strains A1, BK1, TL3B1 and B10, isolated from potato periderm or roots, exhibited *in vitro* antibiosis on King's medium B[8] agar plates (KB) against the bacterium *Erwinia carotovora*, which causes potato soft rot and seedpiece decay (Fig. 1). When KB plates were amended with 1 μM $FeCl_3$, the antibiosis against *E. carotovora* did not occur, nor was the yellow-green fluorescence of PGPR produced. The above PGPR also exhibited on KB plates *in vitro* antibiosis against *Escherichia coli* K-12 AN193, which does not produce its native siderophore, enterobactin, but not against its enterobactin-producing parent, *E. coli* K-12 AN194. These results suggested that in iron-deficient conditions, PGPR were producing fluorescent siderophores which were responsible for antibiosis against *E. carotovora* and *E. coli* K-12 AN193. Yellow-green fluorescent siderophores have previously been isolated from iron-limiting cultures of *P. fluorescens*[9-11].

We next investigated the effect of iron on plant growth-promoting activity by PGPR in soils. When potato seedpieces were dipped into PGPR suspensions (10^9 Colony-forming units (CFU) per ml) immediately before planting in field soils in a previously described greenhouse assay[5], statistically significant increases in plant growth (total plant wet weight) occurred 2 weeks after emergence. Average increases (six replications with three plants each) were 47% for strain A1, 63% for strain B10 and 74% for strain BK1. The control was an autoclaved suspension of strain A1. In contrast, when 120 ml of 100 μM ethylenediaminetetra-acetatoferrate (III) (Fe^{III} EDTA$^-$) per 600 g of soil were added on alternate days to soils planted with PGPR-inoculated potato seedpieces, PGPR did not statistically increase growth (±4%). Addition of iron alone to soils did not significantly increase plant growth over the control. The effect of iron (III) on colonization of roots by PGPR was examined with strain A1 as previously described[2,6]. Although the addition of Fe^{III} EDTA$^-$ resulted in no increase in plant growth, the bacteria, nevertheless, colonized roots at populations averaging 3.2×10^3 CFU per cm root as compared with 4.4×10^3 CFU per cm without iron.

These results suggested the intriguing proposition that a fluorescent siderophore from PGPR may mimic the biological action of PGPR. To test this, we isolated a yellow-green fluorescent pigment capable of binding iron (III) from broth cultures of strain B10 grown in an iron-deficient medium containing glycerol and proteose peptone no. 3. The pigment was purified to homogeneity by a procedure similar to that of Meyer and Abdallah[10]. This pigment exhibited properties typical of a siderophore, including complete repression of production in culture media containing micromolar amounts of iron (III). Furthermore, both the pigment and its ferric complex reversed iron starvation of strain B10 on KB plates induced by the synthetic ferric complexing agent, ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA), which is not utilized by the cells[12]. As the chemical and physical properties of this siderophore seem to be different (J.L. and M.T., unpublished results) from those of others isolated earlier[10,11,13-15], we name this new siderophore, pseudobactin. When paper disks containing 20 μl of 200 μM pseudobactin were applied to KB plates previously seeded with *E. carotovora*, 3-mm radial growth inhibition zones about the disks were observed within 12–16 h. Red-brown ferric pseudobactin caused no antibiosis against *E. carotovora* up to millimolar concentrations. These results suggest that the *in vitro* antibiosis of PGPR against *E. carotovora* is caused by iron deprivation induced by the PGPR's siderophore. *E. coli* K-12 AN194 was not susceptible to PGPR because enterobactin may be able to reverse iron starvation precipitated by PGPR's siderophore.

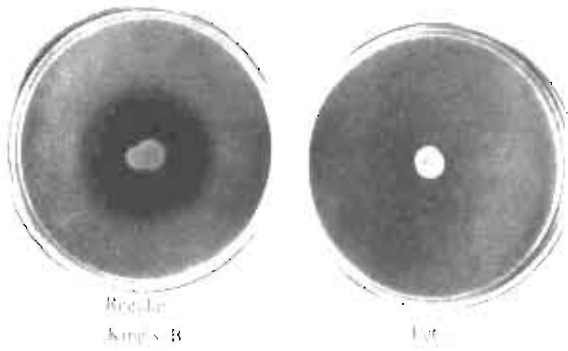

Fig. 1 Plant growth-promoting rhizobacterial antibiosis against *Erwinia carotovora* eliminated by iron (III). Both King's B agar plates were inoculated in the centre with 10 μl of a suspension of fluorescent *Pseudomonas* strain B10 ($\sim 10^6$ CFU ml^{-1}), incubated for 24 h at 28 °C, and sprayed with a suspension of *E. carotovora* ($\sim 10^6$ CFU ml^{-1}). The plate on the right contained 10 μM $FeCl_3$, whereas the plate on the left contained no added iron (III). The zone of growth inhibition of *E. carotovora* and the fluorescence of strain B10 were examined at 24 h. Similar results were obtained with fluorescent *Pseudomonas* strains A1, BK1 and TL3B1.

The effect of pseudobactin on plant growth and rhizoplane fungal colonization was determined in the greenhouse assay (Table 1). Both B10 suspensions and pseudobactin at 10 μM caused significant plant growth increases compared with water-treated controls. Ferric pseudobactin at 50 μM and B10 with 50 μM Fe^{III} EDTA$^-$ did not increase plant growth. In addition to increasing plant growth, pseudobactin and B10 treatments resulted in statistically significant reductions of fungal populations on the rhizoplane relative to the water-treated control. Fungal colonization of the rhizoplane, measured by the method of Huisman *et al.*[16], averaged 5.5 CFU per 10 cm root on water-treated plants compared with 2.3 CFU per 10 cm (−59%) on B10-treated plants and 1.4 CFU per 10 cm (−74%) on pseudobactin-treated plants.

Based on these results, we propose the following scenario to account for the enhancement of plant growth by PGPR.

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Following inoculation and planting of crop seeds, PGPR rapidly colonize roots of the developing plant. As a result of a limited supply of iron in the rhizoplane, PGPR produce siderophores which sequester iron in the root zone, making it unavailable to certain rhizoplane microorganisms. These microorganisms are unable to obtain essential quantities of iron for growth either because they do not produce siderophores, produce comparatively less siderophores than those of PGPR, and/or produce siderophores which have less affinity for iron than those of PGPR. The populations of these microorganisms, some of which are quasi-pathogens (T. V. Suslow and M.N.S., unpublished results), are reduced, and a more favourable environment for root growth is created. Our results with mutants of PGPR support this hypothesis. Certain mutants obtained by treatment with *N*-methyl-*N'*-nitro-nitrosoguanidine or UV light at 254 nm were non-fluorescent, did not exhibit antibiosis against *E. carotovora* and did not promote growth increase of potato in the greenhouse assay even though they were able to colonize plant roots[6]. At least five mutants derived from strains BK1, TL3B1 and TL3B2 were examined. These mutants did not produce siderophores, in contrast to their parents. An alternative hypothesis that plant growth-promoting rhizobacterial siderophores bind iron and make it directly and perhaps more readily available to the plant seems unlikely because ferric pseudobactin did not promote plant growth (Table 1).

Table 1 Enhanced plant growth of potato by pseudobactin in greenhouse assay

Treatment	Average plant weight (g)
H_2O control	1.0
50 μM Fe^{III} $EDTA^-$ control	0.9
B10	2.3*
B10 + 50 μM $Fe^{III}EDTA^-$	0.9
10 μM pseudobactin	2.5*
50 μM ferric pseudobactin control	1.1

Potato seedpieces were planted in field soil (sandy loam) from Moss Landing, California. In treatments with fluorescent *Pseudomonas* strain B10, seedpieces were dipped in approximately 10^9 CFU ml^{-1} suspension before planting. Pots were watered with 120 ml of the appropriate solution on alternate days, and a total of nine applications were made. Plants were harvested 10 days after emergence. An average of six replications with three plants per replication were obtained.

* Statistically significant increase ($P = 0.01$) compared with controls.

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Antiquity of the vertebrate pattern of activity metabolism and its possible relation to vertebrate origins

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Vertebrates generally possess well developed capacities for anaerobic metabolism, resulting in formation of lactic acid. Those capacities have traditionally been interpreted in terms of adaptation to hypoxic environments or to special situations such as diving. However, anaerobic metabolism in striated muscle tissue is frequently a major source of ATP utilized during periods of intense activity. The evolutionary significance of anaerobically supported activity has not been discussed, although the interrelationships of capacities for aerobiosis and activity have received considerable attention[1]. We present here evidence that the pattern of activity metabolism utilized by extant species probably dates back to the earliest vertebrates. It is also postulated that the evolution of extensive capacity for anaerobically supported burst activity may have been closely related to the evolution of vertebrates from invertebrate chordates.

Many animals use glycolysis, the anaerobic process by which glucose is degraded to lactic acid. Most invertebrate species resort to significant utilization of glycolysis only in the absence of sufficient environmental oxygen to maintain adequate rates of ATP generation via aerobiosis, or oxidative phosphorylation[2]. However, many vertebrate species rely on prodigious rates of glycolysis for ATP generation during periods of maximal activity[3–6]. Such a pattern of activity metabolism enables many vertebrates to reach 'burst' levels of activity otherwise unattainable if they were solely dependent on aerobic metabolism. Heavy reliance on anaerobic metabolism also has its drawbacks: it is inefficient in utilizing substrate, and is invariably associated with muscle fatigue[7]. In addition, resultant intramuscular lactic acid accumulation and subsequent diffusion of lactate into the cardiovascular system[4] disrupts maintenance of blood and tissue *p*H which may well affect enzymatic activity, protein configuration and so on[8]. This exercise-related *p*H depression may persist in lower vertebrates for several hours, or longer, following cessation of strenuous activity[9].

Although some crustaceans generate moderate quantities of lactate during exercise[10,11], most invertebrates produce little, if any, lactate during maximal activity. For example, during periods of intense activity, the insects are highly dependent on aerobiosis[12] and molluscs anaerobically generate octapene and succinate to supply themselves with sufficient ATP[13]. Hence, the vertebrate capacity for lactate generation during activity should not be considered an adaptation for anaerobiosis *per se*: it is not merely a primitive relict of the occupation of oxygen-depleted environments, but is a specific and relatively unique exploitation of glycolysis to support intense activity.

Lactic acid formation during activity has been demonstrated in many extant gnathostomes (sharks[14], teleost fish[6], amphibians[3], reptiles, mammals[5]). Those data indicate that heavy reliance on lactate formation during activity is widespread among vertebrates. However, the antiquity of this pattern of activity support is still unclear; is it a primitive characteristic dating back to, or even previous to, the origin of vertebrates? To

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