PERGAMON

Soil Biology and Biochemistry 31 (1999) 1847–1852

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Cytokinin production by *Paenibacillus polymyxa*

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Accepted 23 June 1999

Abstract

The production of hormones has been suggested to be one of the mechanisms by which plant growth-promoting rhizobacteria (PGPR) stimulate plant growth. To evaluate whether the free-living soil bacterium, *Paenibacillus polymyxa*, releases the hormone group cytokinins and, if so, their identity, the content of cytokinins in the growth media, before and after cultivation of this bacterium, was determined by immunoaffinity chromatography (IAC). This method allows the isolation of almost all known cytokinins and their metabolites. Separation and characterization were done by high performance liquid chromatography (HPLC) with on-line ultraviolet (UV) detection, and final identification was by gas chromatography-mass spectrometry. *Iso*-pentenyladenine (iP) was identified in the two defined media used for the cultivation of *P. polymyxa*, but not earlier than at its late stationary growth. A third medium, supplemented with yeast extract, contained *iso*-pentenyladenine riboside (iPR) and some additional cytokinin-like substances before inoculation. When the same medium was sampled after the cultivation of *P. polymyxa* up to its logarithmic growth phase, the cytokinin concentration had decreased. After prolonged cultivation of *P. polymyxa*, small amounts of iP appeared in all three media, and iPR had disappeared from the yeast-containing medium, which indicates that the bacterium can metabolize cytokinins. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Paenibacillus polymyxa; PGPR; Cytokinin production; IAC; HPLC

1. Introduction

The role of the rhizosphere microbiota in the promotion of plant growth has received increasing attention (Frankenberger and Arshad, 1995). One of the most often reported plant growth promoting rhizobacteria (PGPR) is *Bacillus polymyxa*, now named *P. polymyxa* (Ash et al., 1993). It has a range of reported properties, including nitrogen fixation (Lindberg et al., 1985; Heulin et al., 1994); soil phosphorus solubilization (Duff et al., 1963; Singh and Singh, 1993); production of antibiotics (Davis et al., 1968; Rosado and Seldin, 1993), chitinase (Mavingui and Heulin, 1994), and other hydrolytic enzymes (Nielsen and Sorensen, 1997), and a enhancement of soil porosity (Gouzou et al., 1993).

The reason for stimulation of plant growth by *P. polymyxa* is not primarily its nitrogen-fixing ability, as in several strains, isolated by Lindberg and Granhall (1984), the nitrogen-fixing ability did not correlate with the growth promoting effect (Lindberg et al., 1985). Similar results have been observed in several studies with other diazotrophic PGPR bacteria (Kapulnik et al., 1983; Lin et al., 1983; Smith et al., 1984). As the effect of inoculation with these PGPR bacteria resembled a hormonal effect, Lindberg et al. (1985) suggested that bacterial plant hormone production might explain at least part of the overall effect on plant growth.

Some associative nitrogen-fixing bacteria produce auxins, gibberellins and cytokinins (Vancura and Macura, 1961; Tien et al., 1979). The production of growth promoting compounds similar in activity to indole-3-acetic acid by *P. polymyxa* has been suggested

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PII: S0038-0717(99)00113-3

to be a stimulus to growth in crested wheatgrass (Holl et al., 1988). In their studies, cytokinin production was also studied in culture filtrates of *P. polymyxa* using a soybean callus test. Although yeast extract, which has been demonstrated to contain cytokinins, was used, no cytokinin activity was detected in the filtrate.

Cytokinins have key regulatory roles in plant growth and development. They promote seed germination, de novo bud formation, release of buds from apical dominance, stimulation of leaf expansion and of reproductive development, and retardation of senescence (Mok, 1994). Some of these effects have been observed in wheat when inoculated with *P. polymyxa* strains B1 and B2 (Lindberg et al., 1985; Lindberg and Granhall 1986). One of these strains (B2) was, therefore, chosen for further investigation.

As even a limited enhancement of cytokinin production in transgenic plants may increase plant biomass and longevity (Gan and Amasino, 1995), there is a renewed interest in the possibility that bacteria supply cytokinins to soil. Because cytokinins are a diverse group of labile compounds and present in small amounts in biological samples, they have often been difficult to identify and quantify. We have used immunoaffinity chromatography (IAC) to isolate, high performance liquid chromatography (HPLC) with online ultra violet (UV) spectrum detection to separate and characterize, and gas chromatography-mass spectrometry to identify cytokinins in culture media with or without yeast extract, before and after the growth of *P. polymyxa*.

2. Materials and methods

2.1. Organism and growth conditions

P. polymyxa strain B2, isolated from the rhizosphere of wheat (Lindberg and Granhall, 1984), was used. The cells were kept at −70°C until use.

The basal medium for cultivation of the bacterium was that of Okon et al. (1977), modified by Lindberg and Granhall (1984), and further modified in three ways:

A. (g l^{-1}) $MgSO_4 \cdot 7H_2O$ (0.2), NaCl (0.1), $CaCl_2$ (0.02), $Na_2MoO_4 \cdot 2H_2O$ (0.02), $MnSO_4 \cdot H_2O$ (0.01), FeEDTA (0.125 Fe), glucose (10), yeast extract (0.6) (Oxoid Ltd., Basingstoke, Hampshire, England), biotin (0.1 mg), pyridoxin (0.2 mg), in 0.1 M potassium phosphate, pH 7.0.

B. as medium A, but 1 g of casamino acid (Bacto) was used as an organic N-source instead of yeast extract.

C. as medium A, but $(NH_4)_2SO_4$ (60 mg N l^{-1}) was used as an inorganic N-source.

The three media were autoclaved without glucose for 20 min at 120°C. Glucose was autoclaved separately for 10 min before addition to the medium.

The bacterium was cultivated at 30°C on a rotary shaker (130 rpm), and growth was monitored by absorbance ($A_{600\ nm}$). The cells were harvested at the late logarithmic phase of growth (OD 0.45 to 0.5) and 9 to 10 h after the onset of the stationary phase of growth (OD 0.8 to 0.85). The latter time was chosen because cytokinins were then detected in *P. polymyxa* culture filtrates using a biotest (Pantzar C. and Granhall U., unpublished data). At harvest, the cultures were centrifuged (7700*g*, 4°C, 30 min), and 20 ml of the supernatant (containing less than 1000 cells ml^{-1}) was decanted.

2.2. Cytokinin extraction and prepurification

Methanol (30 ml; −20°C) was added to 20-ml aliquots of the decanted supernatant. Kinetin riboside (KR, 100 pmol) (Sigma) was added as an internal standard and sonicated for 20 s with a 475 W sonicator XL2020 (Heat Systems, Farmingdale, NY), fitted with an 1.25 cm tapered horn set at half maximum output. The sonication was repeated as the extract reached −10, −5°C and 0°C (ca 30 min). Insoluble material was removed by centrifugation (13500*g*, 4°C, 20 min). The methanol was removed by rotary evaporation.

The aqueous phase of the extract was passed through a C_{18} column (Bond Elute, Analytichem Int., Harbor City, CA). Each column was eluted with 2 ml of 60% methanol. The eluates were evaporated under vacuum and dissolved in phosphate buffered saline (PBS, 50 mM sodium phosphate, 140 mM sodium chloride, 1 mM EDTA Triplex 3, pH 7). The pH was adjusted with NaOH to approximately 7. Each extract was passed through a column (13 × 60 mm) containing 1 ml of Sepharose 6B (Pharmacia, Uppsala, Sweden).

2.3. Immunoaffinity chromatography (IAC)

Cytokinins were isolated from the purified samples by IAC using polyclonal antibodies raised against zeatin riboside and iPR (Sigma) as described by Nicander et al. (1993), except that the column temperature was 30 to 35°C. The eluate was evaporated under vacuum to less than 300 μl and diluted by the addition of PBS. Every sample was passed through the column a second time, and the new eluate was evaporated under vacuum.

2.4. High performance liquid chromatography (HPLC)

The HPLC system consisted of a pump (Merck-Hitachi 655A-12) with a low-pressure gradient mixer

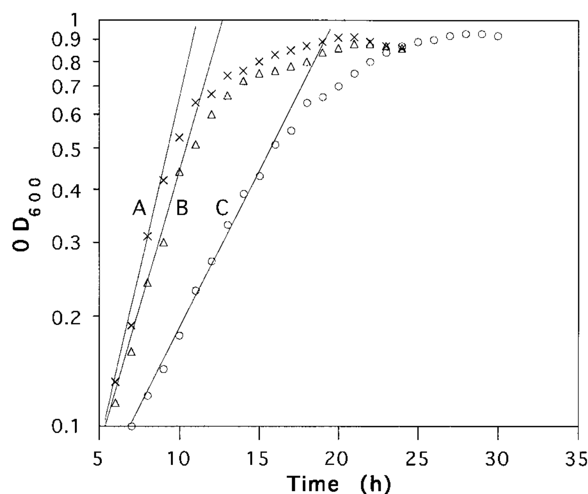

Fig. 1. Growth of *P. polymyxa* in medium A (×), medium B (△) and medium C (○). Harvests were made at the late logarithmic phase of growth (OD 0.45 to 0.5) and at the late stationary phase of growth (OD 0.8 to 0.85).

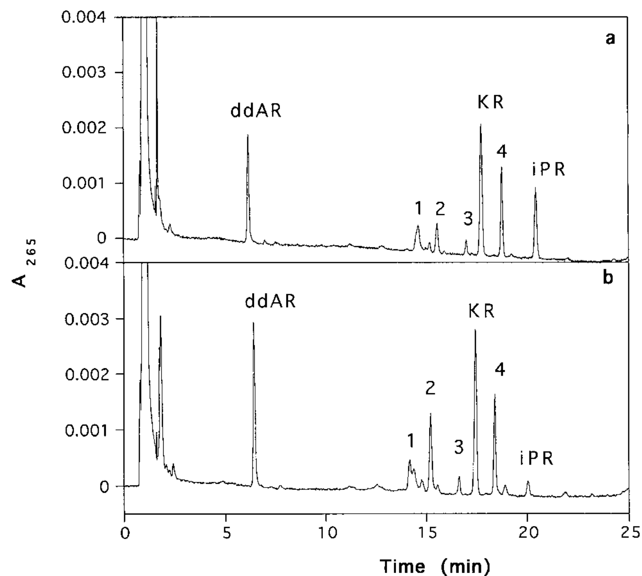

Fig. 2. HPLC chromatograms of IAC-purified medium A. Kinetin riboside (KR, 100 pmol) was added at the extraction and dideoxyadenosine (ddAR) was added before the separation by HPLC. (a) Uninoculated medium, (b) medium in which *P. polymyxa* had been grown until the late logarithmic phase.

(a 6-ml mixing volume between the pump and the sample injector), an on-line scanning spectrophotometric UV detector (Spectra-Focus, Spectra-Physics, San Jose, CA), and a 125 × 4 mm and a 75 × 4 mm Supersphere RP-select B column connected in series. The gradient profile was: 0 min, 1.0%; 8 min, 3.0%; 21 min, 16.6%; and 25 min, 40.0% acetonitrile in 2% acetic acid. The flow rate was 2 ml min^{-1}. A standard run included a mixture of the authentic cytokinins, zeatin, dihydrozeatin and iP; their nucleosides, nucleotides and 9-glucosides; and KR (Sigma, St. Louis, MO). Dideoxyadenosine (ddAR, 100 pmol) (Amersham Pharmacia Biotech) was added before injection as a means to check the retention time. The fractions with putative cytokinins were stored at −20°C.

2.5. Gas chromatography-mass spectrometry with selected ion monitoring (GC-SIM)

The fractions with putative cytokinins were evaporated to dryness and dissolved in 0.5 ml of methanol. Permethylation of standards and samples was performed according to Kovac (1993), except that chloroform was used instead of dichloromethane and the organic phase was backwashed twice with water. After evaporation to dryness in a stream of N_2 at 60°C, the samples were dissolved in 3 µl of chloroform and a 1-µl aliquot was injected into a g.c. (model 8000, Carlo Erba) coupled to a Fison MD 800 EI 60 mass spectrometer (Fison Instrument, UK). The mass spectrometer conditions were: EI ionizing voltage, 70 eV; source temperature, 300°C, and interface temperature, 290°C. Chromatograms were obtained by Selected Ion Monitoring (SIM) with a dwell time of 0.05 s and a mass range span of 0.3 amu.

The gas chromatography system had a splitless injection port at 300°C and a 15 m × 0.25 mm × 0.1 µm DB-5ms (J&W Scientific, USA) column with He as the carrier gas at 27.6 kPa. The temperature program was 80°C for 4 min, increased at 10°C min^{-1} to 290°C, where it was maintained for 10 min.

3. Results

3.1. Bacterial growth

P. polymyxa strain B2 showed different growth patterns in the three media (Fig. 1). The pattern and rate (μ) of growth of the bacterium in the yeast extract medium A ($\mu = 0.34$ h^{-1}) were similar to those in medium B ($\mu = 0.31$ h^{-1}). In medium B, casamino acids were used instead of yeast extract, as the latter has been shown to contain cytokinins (Jameson and Morris, 1989). Medium C, containing only inorganic substances, gave a lower growth rate ($\mu = 0.20$ h^{-1}) than the other two media.

3.2. Cytokinin content in medium A before and after cultivation of *P. polymyxa*

HPLC separation, with UV detection, of the IAC-purified uninoculated medium A revealed UV-absorb-

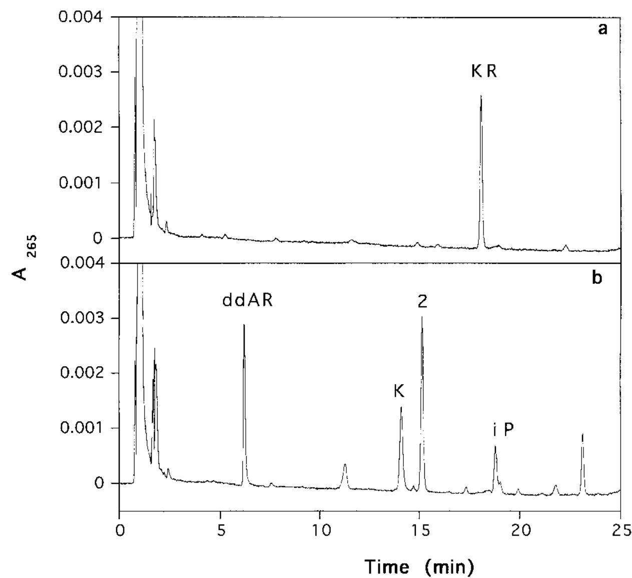

Fig. 3. HPLC chromatograms of IAC-purified media (see Fig. 2 for details). (a) Uninoculated defined media (typical for B and C), (b) typical results for all media (A, B and C) in which *P. polymyxa* had been grown until the late stationary phase.

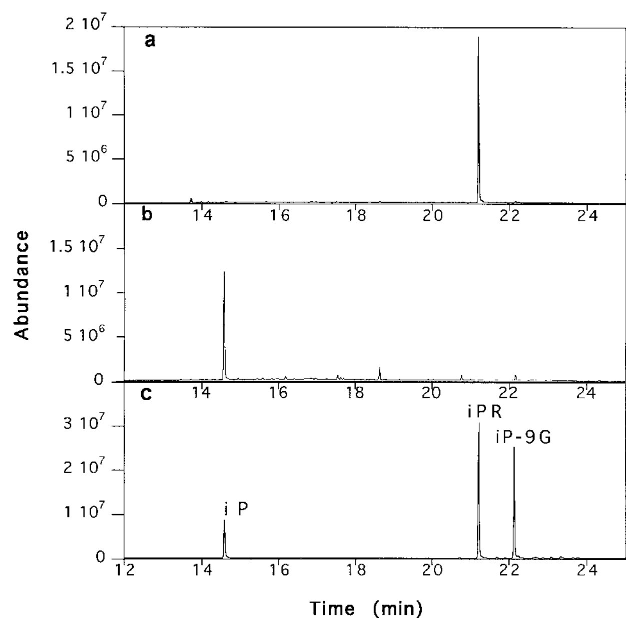

Fig. 4. GC-SIM chromatograms of HPLC-fractions with putative cytokinins from extracts (a,b) and authentic cytokinins (c). (a) Compounds at the retention time for iPR from uninoculated medium A (cf. Fig. 2a), (b) compounds at the retention time for iP from medium C in which *P. polymyxa* had been grown until the late stationary phase (cf. Fig. 3b), (c) the cytokinin standards iP, iPR, and iP-9-glucoside (iP-9-G).

ing substances (Fig. 2a). One peak coeluted with authentic iPR, and its UV spectrum closely matched authentic iPR (data not shown). The identity of iPR was confirmed by GC-SIM (Fig. 4a). Relative abundance ratios were (fragment masses in parenthesis) 100 (174), 80 (202), 44 (216), 42 (217), 19 (348) and 16 (391), and for the standard the corresponding ratios were 100, 80, 45, 43, 19 and 15. Additional peaks of compounds with antibody-binding capacity were also found in the uninoculated medium containing yeast extract (Fig. 2a, peaks No. 1, 2, 3 and 4). These substances had UV spectra typical of adenine modified at positions 6 and 9 (data not shown), but they did not coelute with any known cytokinin.

IAC-purified medium A, after the growth of *P. polymyxa* to the late logarithmic phase, had nearly the same pattern of HPLC peaks (Fig. 2b) as the uninoculated medium (Fig. 2a). However, the amount of iPR per volume of medium was 5- to 6-fold lower, and the unidentified peak No. 2 was 2- to 3-fold higher than in the uninoculated medium.

When *P. polymyxa* was grown until the late stationary phase, the pattern of antibody-binding compounds in medium A changed further. The peak of iPR was replaced by a peak, identified as iP, and most peaks of unknown compounds (No. 1, 3, and 4 in Fig. 2a,b) disappeared. Peak No. 2 remained but had increased 2- to 3-fold compared to that in the medium harvested at the late logarithmic phase (Figs. 2b and 3b).

3.3. Cytokinin content in media B and C before and after cultivation of P. polymyxa

In another set of experiments, media B and C, which did not contain yeast extract, were analyzed. The HPLC of IAC-purified samples from media B and C was the same. The uninoculated media showed no UV-absorbing substances, suggesting that no antibody-binding cytokinins were present initially (Fig. 3a). HPLC of IAC-purified media after growth of *P. polymyxa* until its late logarithmic phase of growth, also did not reveal any cytokinins (data not shown). However, when *P. polymyxa* was grown in media B and C until the late stationary phase, two distinct peaks were detected by HPLC of IAC-purified samples (Fig. 3b). The identity of one of these peaks as iP was confirmed with GC-SIM (Fig. 4b). Relative abundance ratios were (fragment masses in parenthesis) 22 (139), 23 (162), 100 (188), 33 (216) and 16 (231). For the standard the corresponding ratios were 21, 22, 100, 32 and 16.

3.4. Effect of bacterial growth on the internal cytokinin standard

The synthetic cytokinin KR was used as an internal standard. Fig. 3b shows that this compound was not recovered in samples of media from the late stationary

phase. Instead a peak coeluting with kinetin (K), was found, indicating that some factor in the media had deribosylated KR.

4. Discussion

Plants and plant-associated micro-organisms have been found to contain over 30 growth-promoting compounds of the cytokinin group. These highly active hormones are usually present in very low concentrations. To be able to analyze them in bacterial exudates they must first be separated from the vastly larger amounts of other exudates. This was achieved here by IAC, using a mixture of antibodies that bind virtually all known biologically-active cytokinins, with the exception of cytokinin O-glucosides (Nicander et al., 1993).

Various organisms are reported to produce cytokinins, although it is only in higher plants that cytokinins are unequivocally proven to have a hormonal role. Using the defined media B and C, we show here that the cytokinin iP was released by *P. polymyxa* during its stationary phase of growth. The concentration, as estimated from UV absorbance data in Fig. 3b, was about 1.5 nM. Already as little as 1 nM of cytokinins has been shown to influence plant growth in in vitro experiments (Bennici and Cionini, 1979; Palni et al., 1984). The amount of iP in our in vitro culture does not, of course, accurately reflect the situation in the rhizosphere. The microenvironments into which bacterial cytokinins are excreted could have very small volumes, resulting in high local concentrations. Another factor that could affect microbial cytokinin production in the rhizosphere is the complex chemical environment; it has been shown that cytokinin production in *Azotobacter* sp. is stimulated by various naturally-occurring compounds (Nieto and Frankenberger, 1989). It is well known also that other plant hormones, such as auxines, or other growth regulating substances occur in the rhizosphere. In the vicinity of a plant root such substances could modify the cytokinin effect to the plant in a synergistic way (Stenlid, 1982).

As the release of iP appeared during the stationary phase of growth, the origin could be tRNA in sporulating cells. Cytokinin molecules contain adenine modified at position N6 with an isopentenoid sidechain as a common feature. The same type of compounds are also found as modified bases in tRNA in plants, animals and micro-organisms (Persson et al., 1994).

Bacteria have been shown to release cytokinin types not found in the plant itself (MacDonald et al., 1986). It is possible that peak No. 2 in Fig. 3b is such a cytokinin. The compound showed affinity to the antibodies, and a UV spectra typical of cytokinins, but did not coelute with any known standard.

Autolyzed yeast extract, an ingredient of many bacterial culture media, has been shown to contain large amounts of the cytokinins iP and iPR (Jameson and Morris, 1989). In medium A containing the commercial preparation of Oxoid yeast extract, iPR was identified. Four additional compounds were isolated from the Oxoid extract by the antibody columns (peaks No.1, 2, 3 and 4, Fig. 2a). They moved at positions different from any of the known cytokinins, but they showed the UV spectra of adenine compounds with moieties at positions N6 or N9 (data not shown). Most plant cytokinins have such structures and such UV spectra. The unknown compounds could, thus, be structurally related to cytokinins. Growing yeast cells do not contain these compounds (Timmusk and Nicander, unpublished), and they are, therefore, probably products formed during the autolysis of the yeast. The presence of iP/iPR and the four cytokinin-like compounds, makes yeast extract an unsuitable choice as a medium supplement when studying cytokinin production by bacteria.

Extracts from late stationary phase cultures in all three media modified the internal standard, KR. While recovered intact in the late log phase cultures, the compound was here replaced by the deribosylated form, kinetin (Fig. 3b). It is an experimental observation for which we do not have a clear explanation. It is known that some degradative enzymes are not denatured in 60% methanol used for deproteinization (Bieleski, 1964). We speculate that also deribosylating enzymes might have been present during the handling of the sample.

In summary we can say that iP and one unknown cytokinin-like compound are released by *P. polymyxa* during its stationary phase of growth.

Acknowledgements

This work was performed with financial support from the Knut and Alice Wallenberg Foundation, the Swedish University of Agricultural Sciences, the Swedish Institute and Carl Trygger's Foundation. P.-O. Björkman is acknowledged for the GC-MS analyses and R. D. Hammerton for valuable technical discussions. Torben Haslund is thanked for his efforts on behalf of the project.

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