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Plant Growth-Promoting *Pseudomonas putida* WCS358 Produces and Secretes Four Cyclic Dipeptides: Cross-Talk with Quorum Sensing Bacterial Sensors

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Abstract. The most universal cell-cell signaling mechanism in Gram-negative bacteria occurs via the production and response to a class of small diffusible molecules called *N*-acylhomoserine lactones (AHLs). This communication is called quorum sensing and is responsible for the regulation of several physiological processes and many virulence factors in pathogenic bacteria. The detection of these molecules has been rendered possible by the utilization of genetically engineered bacterial biosensors which respond to the presence of exogenously supplied AHLs. In this study, using diverse bacterial biosensors, several biosensor activating fractions were purified by organic extraction, HPLC and TLC of cell-free culture supernatants of plant growth-promoting *Pseudomonas putida* WCS358. Surprisingly, it was observed that the most abundant compounds in these fractions were cyclic dipeptides (diketopiperazines, DKPs), a rather novel finding in Gram-negative bacteria. The purification, characterization, chemical synthesis of four DKPs are reported and their possible role in cell-cell signaling is discussed.

Gram-negative bacteria use small diffusible signal molecules to regulate gene expression in response to bacterial population density; this process is now called "quorum sensing" (reviewed by Fuqua et al. [3]). Most commonly, the signal molecules characterized from Gram-negative bacteria so far are *N*-acylhomoserine lactones (AHLs) differing in their length and degree of saturation of the acyl chain [3]. Various reports have shown that quorum sensing is associated with the regulation of antibiotic production in *Erwinia* and *Pseudomonas*, bioluminescence in marine vibrios, virulence factors in *Pseudomonas aeruginosa*, swarming motility in *Serratia* and conjugal transfer in *Agrobacterium* [15]. If methods could be developed to interfere with quorum sensing systems of gram-negative pathogens, a novel means of controlling their pathogenicity might be possible [5]. The biosynthesis of the AHL signal molecule occurs through the activity of a signal synthase protein, a member of the LuxI protein family, and accumulation of the AHL molecule above a threshold level will result in its interaction with a member of the LuxR family of activators allowing transcription of target genes [3, 15].

It is becoming evident that AHL signaling is the most common "language" among Gram-negative bacteria, the study of quorum sensing in Gram-negative bacteria was facilitated by the use of bacterial AHL biosensors. These biosensors respond in an easily detectable way to the exogenous presence of AHL signaling molecules [10, 16]. An example is the *Escherichia coli* AHL biosensor containing a plasmid having a *luxR* family gene and promoter region coupled to the *lux* structural operon (eg. *E. coli* (pSB401), [16]) responding by the emission of light in the presence of exogenous AHL. Similarly, *Agrobacterium tumefaciens* NT1 (pDCI4E33) responds to exogenous AHL by activation of transcription of the *lacZ* gene [2] and *Chromobacterium violaceum* CVO26 by purple pigment formation [10].

Other types of signaling molecules have been identified in Gram-negative bacteria, for example, in *Ralsto-*

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nia solanacearum [1] and in *Xanthomonas campestris* [11] cell-density-dependent mechanism employ rather different signaling molecules. In Gram-positive bacteria, several systems are regulated by cell density using modified peptides as the signal molecules [8]. Recently, one study has reported that several Gram-negative bacteria produce and secrete cyclic dipeptides and that these molecules potentially cross-talk with the AHL quorum sensing system [6]. The precise role of these molecules is currently unclear. The study of Holden et al. [6] to our knowledge is the first report of cyclic dipeptides in bacteria and they have shown a possible role in stimulating and antagonizing AHL bacterial sensors. In this study we report that plant growth-promoting *Pseudomonas putida* WCS358 produces at least four different cyclic dipeptides and that some of them potentially cross-talk with the quorum sensing LuxI and LuxR homologs.

Materials and Methods

Bacterial strains and culture conditions. The *Pseudomonas putida* used was strain WCS358, isolated from the rhizosphere of potato roots [4]. For the purification of AHLs, culture of *P. putida* WCS358 was grown to late exponential phase either in M9 medium [12] in a Biostat M 2 liters (B. Braun GmbH, Melsungen, Germany) or Tecbio 10 (Tecninox, Parma, Italy) fermenters at 30°C. *Agrobacterium tumefaciens* strain NT1 (pDCI4E33) was grown in AB minimal mannitol (ABM) liquid medium at 30°C [7]. *Chromobacterium violaceum* CV026 is a double mini-Tn5 mutant derived from ATCC31532 and was grown in LB medium at 30°C. *E. coli* strain JM109 (pSB401) with the fusion of *luxRI′*::*luxCDABE* was grown in LB medium plus tetracycline 10 μg/ml at 37°C.

Purification and characterization of autoinducers and DKPs. The supernatant from 10 l of an overnight M9 culture of *P. putida* WCS358 was concentrated to 500 ml by rotary evaporation and extracted twice with the same volume of ethyl acetate plus 0.1 ml/l of acetic acid. The extract was dried at room temperature and resuspended in pure methanol. This extract was fractionated by HPLC with a semipreparative C_{18} reverse-phase column (1 × 25 cm), using a methanol:water gradient from 20:80 to 80:20 eluent composition in 35 min at a flow rate of 2.5 ml/min. Fractions of 5 ml each were collected and aliquots of 0.5 ml from each fraction were dried and resuspended in 10 μl of ethyl acetate. They were applied to C_{18} reversed-phase TLC plates with fluorescent detector F_{254} (20 × 20 cm, Merck, Darmstadt, Germany) and developed using methanol/water (60:40 vol/vol) as eluent, according to Shaw et al. [13]. After chromatography, the solvent was evaporated and the dried plates were overlaid with either a culture of *C. violaceum* CV026 mixed with LB top agar, as described by Shaw et al. [13], or *A. tumefaciens* NT1 (pDCI4E33) mixed with ABM medium plus 40 μg/ml of X-Gal. Positive fractions containing the autoinducers were detected by the appearance of either a purple or blue spot on the TLC plate (see below), respectively. Reverse phase C18 TLC was used for both analytical and preparative purposes. In the latter, after having loaded the positive fractions onto the TLC plates and developed as described above, a 2-cm strip on the left side of the plate was overlaid with either the *C. violaceum* CV026 culture in LB top agar or *A. tumefaciens* in ABM top agar plus X-Gal, to detect the position of the autoinducer on the TLC plate. The C18 matrix in the regions of the preparative TLC plate corresponding to the compound(s) to be analyzed were scraped off and extracted three times with 3 ml of acetone. The combined extracts from the same spot were then clarified by centrifugation and dried. The residue was redissolved in an appropriate solvent for NMR and mass spectroscopy analysis.

Nuclear magnetic Resonance Spectroscopy (NMR). ^{1}H NMR measurements were performed on a Bruker AC 200 spectrometer equipped with a 5-mm ^{1}H selective probe. The experiments were performed at 300K and the signal chemical shifts (in ppm) were referred to tetramethylsilane. All the samples were dissolved in [^{2}H]-chloroform with a typical concentration of 5 mg/ml. The spectral width was 4000 Hz, the digital resolution was 0.2 Hz/pt and the acquisition time was 2.3 s.

The assignment of ^{1}H signals was made by means of a two-dimensional correlation spectroscopy (2D COSY) experiment. The two-dimensional spectra were acquired using 128 scans/series with 1 K and 0.5 K data points in F2 and F1 dimensions respectively with zero-filling in F1.

Chemical synthesis of cyclo dipeptides (Diketopiperazines, DKPs). Synthesis in solution: PyBOP assisted coupling of Boc-AA1-OH (AA1 = Phe, Leu, Tyr) and HCl.H-AA2-OEt (AA2 = Pro, Val) in MeCN (5–10 mmol scale) gave crude ethyl ester of Boc protected dipeptide as an oil or crystalline product with yields >90% and purity >95%. Protected dipeptides without purification were Boc-deprotected with formic acid for 3.5 h at room temperature. The formic acid was evaporated and the dipeptide esters were boiled in mixture of toluene and 2-butanol according to the modified procedure of Sukuki et al. [14]. The crude DKPs (portions from 50–100 mg) were separated by RP-HPLC to purity >98% at the following conditions: column Waters RCM, 100×25 mm I.D., gradient 0–30% over 60 min at 6 ml/min, detection at 214 nm.

Solid-Phase Synthesis: Boc-protected dipeptides (0.5–1 mmol scale) were assembled on Wang resin using Fmoc/But chemistry. Optically pure diketopiperazines were cleaved and deprotected from the resin in one step according to Kowalski et al. [9]. The yield/purity of the crude products were >97%.

Detection using bacterial AHL biosensors. Biological activity was tested using three biosensors. One was *E. coli* (pSB401) where plasmid pSB401 is a *luxRI′*, *PluxI-lucCDABE* which responds to exogenous AHL by the emission of light [16]. *Chromobacterium violaceum* CVO26 is a double Tn5 mutant having one transposon in a *luxI* homologue and the other in a repressor gene; this mutant is unable to produce AHL and is defective in violacein production, a purple pigment produced by *C. vialoaceum* [10]. *A. tumefaciens* strain NT1 (pDCI4E33) were plasmid pDCI4E33 contains a *traG::LacZ* fusion and *traR* are cloned into pDSK519 [2]. Providing exogenous AHLs results in the production of β-galactosidase which is easily detectable. Mutant CVO26, *E. coli* (pSB401) and *A. tumefaciens* (pDCI4E33) were used in plate and TLC overlays to detect presence of exogenous AHLs by production of purple pigment formation, light emission, and blue color in presence of X-gal, respectively.

Effect of DKPs on quorum-sensing bacterial sensors. One milliliter of 10 mM solution in ethyl acetate of each of the four DKPs synthesized was dried by rotary evaporation and resuspended into 20 μl of chloroform. Aliquots were spotted onto the RPC18-TLC plate which was developed as described above. The plate was then air dried and overlaid with a thin film of top agar containing either the *E. coli* (pSB401) or *Agrobacterium tumefaciens* NT1(pDCI41E33) or *C. violaceum* CV026 biosensors. The medium used in the soft agar was LB for *E. coli* and *C. violaceum*, and AB minimal mannitol medium containing 40 μg/ml of X-Gal for *A. tumefaciens*. TLC plates were

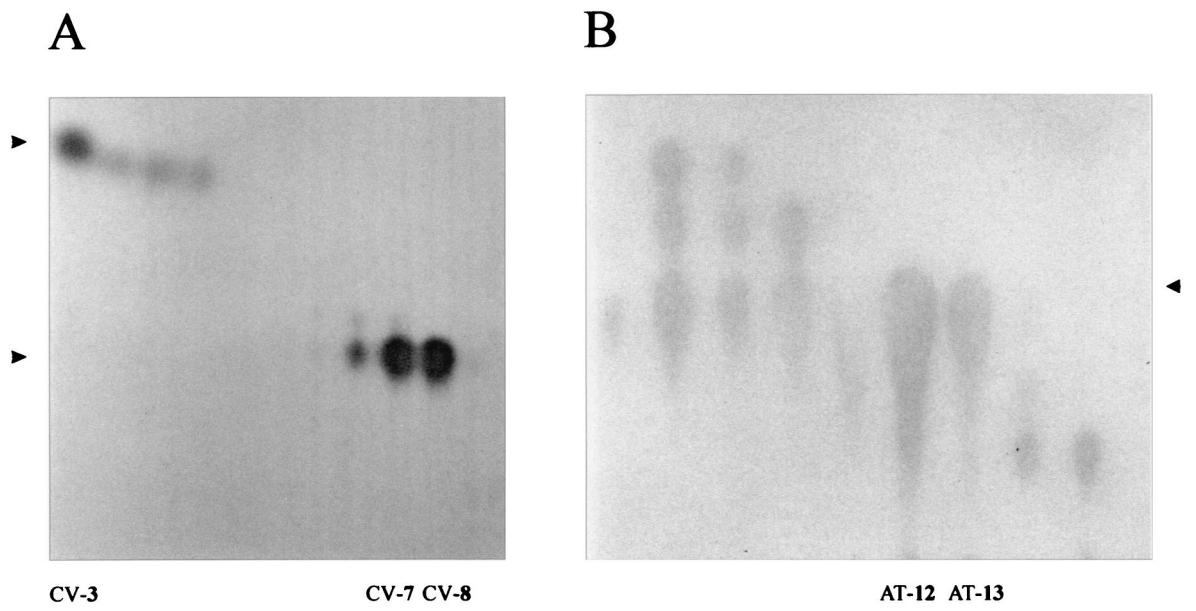

Fig. 1. Induction of violacein synthesis and β-galactoside production in (**A**) *C. violaceum* CV026 and (**B**) *A. tumefaciens* NT1 (pDCI4E33). Biosensor activating fractions were purified by organic extraction and HPLC (see text). HPLC fractions were then subjected to TLC and these plates were then overlaid with *C. violaceum* CV026 or *A. tumefaciens* NT1 (pDCI4E33). Arrows indicate the spots activating the biosensor from which the cyclic dipeptides (DKP) were purified. Also indicated as numbers are the HPLC fractions utilized for the preparative TLC (see text).

incubated over night at 30°C. Detection of the activation spots was according to McClean et al. [10] for *C. violaceum*, by autoradiography for *E. coli*, and by detection of blue spots for *A. tumefaciens*.

Results and Discussion

Identification and characterization of cyclic dipeptides (DKPs) from *P. putida* WCS358 culture supernatant. The ethyl acetate extract of *P. putida* WCS358 cell-free culture supernatant was fractionated by semi-preparative HPLC on a C18 reverse phase column. Fractions were then assayed for the presence of molecules able to activate the quorum sensing biosensors, either *A. tumefaciens* NT1(pDCI41E33) or *C. violaceum* CV026 (see Materials and Methods and Fig. 1). Four positive fractions responding to the AHL biosensors (Fig. 1) were further purified by preparative TLC and the chemical structure of the most abundant molecule present not necessarily the "active" molecule was analyzed by ^{1}H-NMR and by mass spectroscopy. The structure of these four compounds corresponded to four different cyclic dipeptides (DKPs), corresponding to compounds with different retention time present in the fractions able to activate the biosensors used in this study. Three of these molecules were found in fractions able to activate the *C. violaceum* biosensor (CV-3, CV-7, CV-8) and one in a fraction active on the *A. tumefaciens* biosensor (AT-13), as shown in Fig. 1. The diketopiperazines identified are cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Leu), cyclo(L-Phe-L-Pro), and cyclo(L-Val-L-Leu), respectively (Fig. 2 and see below).

Elucidation of structures. ^{1}H NMR spectra of CV-3, CV-7, CV-8, and AT-13 samples were measured in [^{2}H]-chloroform at 200 MHz and the signal assignment was obtained by means of 2D COSY experiments. From the analysis of these spectra, the samples were assigned to four different cyclo-dipeptides and precisely CV-3 = cyclo(L-Pro-L-Tyr), CV-7 = cyclo(L-Pro-L-Leu), CV-8 = cyclo(L-Phe-L-Pro), and AT-13 = cyclo(L-Val-L-Leu). Mass spectroscopy data correlated with the chemical structures determined by NMR (data not shown).

In order to confirm the chemical structures of the purified samples, the corresponding cyclo-dipeptides were prepared by means of chemical synthesis. The synthetic standards were analysed by NMR spectroscopy and their ^{1}H spectra matched perfectly with the data obtained from the natural samples. The spectral data are reported as follows. The non-equivalent protons of the CH_2 groups are labelled as Ha and Hb, respectively.

Cyclo(L-Pro-L-Phe): δ: 1.8-2.15 (2H, m, Pro 4-Ha,b); 2.16 (1H, m, Pro 3-Ha); 2.35 (1H, m, Pro 3Hb); 2.79 (1H, dd, J = 10.6 Hz/-14.6 Hz Pha 3-Ha); 3.50-3.75 (2H, m, Pro 5-Ha,b); 3.63 (1H, dd J = −14.6 Hz/3.7 Hz Phe 3Hb); 4.09 (1H, dt J = 1.0 Hz/≈8.5 Hz Pro α-H); 4.29 (1H, m J = 10.6 Hz/3.7 Hz/≈1 Hz Phe α-H); 5.62 (1H, bs, NH); 7.15-7.50 (5H, m, Phe aromatic H).

Cyclo(L-Pro-L-Leu): δ: 0.96 (3H, d J = 6.5 Hz Leu

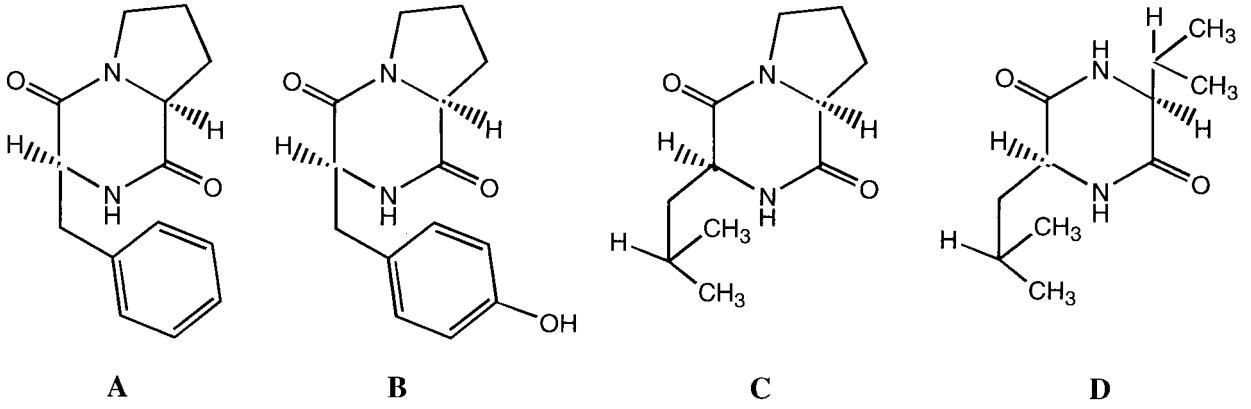

Fig. 2. Structures of: (**A**) cyclo(L-Phe-L-Pro); (**B**) cyclo(L-Tyr-L-Pro); (**C**) cyclo(L-Leu-L-Pro); (**D**) cyclo(L-Leu-L-Val).

CH_3); 1.01 (3H, d J = 6.4 Hz Leu CH_3); 1.55 (1H, m J = 4.9 Hz/ 9.3 Hz/ −14.2 Hz Leu 3-Ha); 1.75 (1H, m, Leu 4-H CH); 1.92 (1H, m, Pro 4-Ha); 2.05 (1H, m, Pro 4-Hb); 2.07 (1H, m, Leu 3-Hb); 2.15 (1H, m, Pro 3-Ha); 2.37 (1H, m, Pro 3-Hb); 3.5-3.7 (2H, m, Pro 5-Ha,b); 4.02 (1H, dd J = 9.2 Hz/ 4.4 Hz Leu α-H); 4.12 (1H, dt J≈8 Hz/ ≈ 1 Hz, Pro α-H); 5.80 (1H, bs, NH).

Cyclo(L-Pro-L-Tyr): δ: 1.8-2.15 (2H, m, Pro 4-Ha,b); 2.16 (1H, m, Pro 3-Ha); 2.35 (1H, m, Pro 3Hb); 2.75 (1H, dd, J = 10.6 Hz/−14.8 Hz Tyr 3-Ha); 3.50-3.75 (2H, m, Pro 5-Ha,b); 3.60 (1H, dd J = −14.6 Hz/3.7 Hz Tyr 3Hb); 4.09 (1H, dt J = 1.0 Hz/≈8.5 Hz Pro α-H); 4.22 (1H, m J = 10.6 Hz/3.7 Hz/≈1 Hz Tyr α-H); 5.15 (1H, bs, OH); 5.62 (1H, bs, NH); 6.82 (2H, d J = 8.5 Hz Tyr aromatic H); 7.10 (2H, d J = 8.5 Hz Tyr aromatic H).

Cyclo(L-Val-L-Leu): δ:1.00 (3H, d J = 6.6 Hz Leu CH_3); 1.00 (3H, d J = 6.6 Hz Val CH_3); 1.03 (3H, d J = 6.3 Hz Leu CH_3); 1.10 (3H, d J = 7.1 Hz Val CH_3); 1.67 (1H, m Leu 3-Ha); 1.79 (1H, m, Leu 4-H CH); 1.87(1H, m Leu 3-Hb); 2.38 (1H, m Val 3-H CH); 3.90 (1H, dd J = 1.35 Hz/ 3.40 Hz Val α-H); 4.02 (1H, m J = 1.35 Hz/ 3.70Hz/ 9.8 Hz Leu α-H).

The last sample was dissolved in a deuterated chloroform/methanol 9:1 solvent system to increase the solubility and to improve the signal resolution. In pure deuterated chloroform two broad signals at 5.85 ppm and 6.01 ppm respectively were assigned to the NH protons. Upon addition of deuterated methanol those signals disappeared due to chemical exchange with deuterium atoms.

DKPs and activity on quorum sensing biosensors. Identification of DKPs was possibly due to their presence in HPLC fractions able to activate the quorum sensing biosensors of *C. violaceum* and *A. tumefaciens*. The purification of these molecules was obtained following the activation spots on both the analytical TLC of the HPLC fractions and the preparative TLC, using the two biosensors previously mentioned. In order to confirm their ability to cross-talk to these sensors, an activation assay was performed using the synthetic molecules, and the results are shown in Table 1. Surprisingly, none of the four synthetic cyclic dipeptides identified and characterized from *P. putida* WCS358 showed a positive response with *C. violaceum* CVO26, however three of the four DKPs (Leu-Pro, Tyr-Pro, and Phe-Pro) were purified following activation of violacein production of CVO26 (Fig. 1). Similarly, for the DKP Val-Leu, purified using the *Agrobacterium* biosensor, the synthetic equivalent did not induce this biosensor. The results reported in this study raises several questions regarding signaling molecules in *Pseudomonas putida*. Firstly, the purification of all four DKPs from *P. putida* WCS358 was obtained following AHL-dependent biosensor induction being the most abundant molecule purified from HPLC and TLC fractions activating the sensors. It is therefore postulated that in these fractions four AHLs produced by *P. putida* WCS358 are present. In fact in one of the activating fractions, an AHL was also purified and characterized (G. Degrassi and V. Venturi, unpublished data) thus an AHL will most likely also be present in the other three fractions containing DKPs. The reason for this co-purification of DKPs and AHLs is currently unknown; it could be coincidental or it could have a biological significance with respect to signaling molecules. Some of the synthetic DKPs activate *A. tumefaciens* (pDCI44E33) while others activate *E. coli* (pSB401) (Table 1), and these results are in accordance with those previously reported by Holden et al. [6] for three of the four DKPs identified in this study, cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Leu), and cyclo(L-Phe-L-Pro), except that we found cyclo(L-Pro-L-Tyr) unable to activate *E. coli* JM109(pSB401). In our study we report

Table 1. Influence of DKPs on quorum sensing dependent phenotypes

DKP	*A. tumefaciens* NT1 (pDCI41E33) Activation	*C. violaceum* CV026 Activation	*E. coli* JM 109 (pSB401) Activation
Cyclo (L-Leu-L-Pro)	+	−	−
Cyclo (L-Tyr-L-Pro)	+	−	−
Cyclo (L-Phe-L-Pro)	−	−	+
Cyclo (L-Val-L-Leu)	−	−	+

+ indicates activation of the biosensor, − indicates no activation. The assays used are described in the Material and Methods section.

a new cyclo dipeptide not reported previously, cyclo(L-Val-L-Leu).

This study highlights the production of DKPs by Gram-negative bacteria being to our knowledge only the second report demonstrating this. We observed that these molecules co-purify with AHL as the *P. putida* WCS358 purified fractions activate certain AHL-sensors whereas synthetic DKPs do not activate the biosensor utilized for its identification. However, it was observed that some of the DKPs activate AHL biosensors, thus, as postulated by Holden et al. [6], they could interact with AHL-based quorum sensing. Production of these molecules is a novel finding in Gram-negative bacteria. The role of the molecules in signaling still remains largely unknown, this study demonstrates that DKPs are also present in *P. putida* and highlights that these molecules are more widely produced by Gram-negative bacteria and future work will reveal whether these molecules act as signal molecules, as anti-bacterial agents, as plant growth promoters, or have another biological function. Elucidating all the molecules which are produced and secreted by Gram-negative bacteria could prove very important for understanding gene expression of virulence factors of gram-negative pathogens [5].

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