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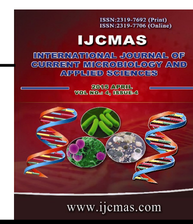

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Original Research Article

Evaluation of the potential bioactivity of an endophytic bacteria isolated from *Magnolia dealbata* Zucc

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A B S T R A C T

Keywords

Magnolia dealbata Zucc., cytotoxicity, antimicrobials, trypanomicidal, bio-pharmaceuticals, Pseudomonads

Plant associated microorganisms have proven to be an important source for discovery of novel bioactive compounds for agricultural, pharmaceutical and food industries. The aim of this research was to disclose untapped bioactivities of endophytic bacteria isolated from the medicinal tree *Magnolia dealbata* Zucc. A total of 55 bacterial strains were isolated from this tree and tested for antimicrobial activity. Eight of them, identified as Pseudomonads strains, displayed a broad antimicrobial activity. Two of these strains also exhibited antitumor and trypanocidal activities. Ribosomal 16S RNA sequences supported this Pseudomonads strains as highly related with the Fluorescent group, recognized as the mayor secondary metabolite producers.

Introduction

The most important use of bioactive compounds has been as anti-infective drugs. Between 2000 and 2007, the market for such antimicrobials increased 11 billion dollars and included approximately 160 antibiotics and their derivatives (Sanchez *et al*., 2012).

Antibiotic multidrug resistance from the main pathogenic bacteria and the increasing demand for treatment of chronic diseases has led to exploration of new sources of bioactive compounds (Demain and Sanchez 2015).

During the past 60 years, the preferred sources for isolation of new bioactive producing microbes have been soil, air and water. Recently, the search has focused on non-conventional sources (geysers, oceans, caves, animals, and plants). Plant associated microorganisms have proven to be important for discovery of novel bioactive compounds

(Strobel 2003). The ecological interaction between microorganisms and vegetal hosts drives to unique symbiotic chemical communication in which microbial metabolites are in a delicate balance with those produced by the plant-host. Around 315,000 plants inhabit the earth, and each one of these could host at least one or more endophytes. Very few of these plants have been partially or completely studied. The use of plants as a source of microorganisms, has regained interest after isolation of the endophytic fungus *Taxomyces andreanae* from the tree *Taxusbrevifolia*. Like its plant host, this microorganism is able to produce the antitumor agent Taxol (Craggand Newman 2007). The higher Taxol production levels reported today are 227 mgL^{-1} from the fungus *Alternaria alternate* var. *monosporus*, isolated from the bark of *Taxusyunanensis* after ultraviolet andnitrosoguanidine mutagenesis (Duan *et al*., 2008).

Magnolia is a genus containing ~120 species belonging to the Magnoliaceae family. Members of this family inhabit rain forest in North America and Asia, were large populations have been reported (Judd *et al*., 2010). They have ornamental trees of more than 20 m tall, greatly appreciated by local communities because their beautiful flowers and medicinal properties. They are recognized by their analgesic, anthelmintic, laxative, and antibiotic activity against pathogenic bacteria to trait different conditions like stomach sickness and typhoid fever as well (Yung-Hsiang *et al*., 2011). Among them, *Magnolia ovobata* (Kotani *et al*., 2005) *Magnolia officinalis* (Yung-Hsiang *et al*. 2011) and *Magnolia dealbata* (Alonso-Castro *et al*., 2011) are reported to produce magnolol honokiol and obovatol, phenolic compounds with antitumoral, antibacterial, antidiabetic, antihypertensive, anxiolytic and antiepileptic activities (Young-Jung *et al*., 2011; Alonso-Castro *et al*., 2011). Our research group is interested in the use of endemic medicinal plants as a source of microbial endophytes with potential to produce compounds of pharmaceutical interest. For this purpose, we have chosen *Magnolia dealbata* Zucc., which in addition to its ornamental use, has been used for many years in the popular Mexican medicine for treatment of circulatory and respiratory affections (Pijoan 2003). Therefore, the aim of this work was to disclose untapped bioactivities of endophytic bacteria isolated from the medicinal tree *M. dealbata* Zucc.

Materials and Methods

Isolation of microorganisms

Microorganisms were isolated from the Mexican endemic tree *Magnolia dealbata* Zucc., in Coyopolan, Ver. Mexico (19° 21´05.9´´ N & 97° 03´30.8´´W, at 1591 m above sea level). A specimen of this tree is present at the Herbarium of the Estación Biológica los Tuxtlas, Ver., Mexico with the catalogue number MEXU 6469. For microbial isolation, cork and roots were disinfected before being dissected from the tree (Zinniel *et al*., 2002). Before analysis, all collected samples were transported in polyethylene bags with distilled water under sterile conditions. Samples were subsequently washed with 50% and 70% ethanol (30 min each) and finally with sterile distilled water. Fragments of these samples were transferred to either Petri dishes with Nutrient Agar (NA) (BD, Sparks, MD, USA) and incubated at 29°C for 6-7 days. Phenotypically different colonies were isolated until axenic cultures were observed and these were tested for antibiotic activity. Light microscopy and Gram staining performed a primary classification of bacteria.

Extracts preparation

For bioactivity, different production media were tested; NB+0.5% of glucose was selected as the production media in further experiments. One liter of 24 h fermentation broth in production media was prepared from strains 1AC and 28A. Organic extracts were prepared for antibiotic MIC and cytotoxic- trypanocidal IC_{50} determinations with the following directions: Biomass was mashed during 3 h in a mixture of dichloromethane (CH_2Cl_2): methanol (MeOH) 1:1 (400 ml). The product was passed through a series of Whatman (number 1), 0.45 and 0.22 μm Millipore filters and the extract concentrated to dryness in a rotary evaporator. The fermentation medium filtrate was extracted three times with CH_2Cl_2 and four times with ethyl acetate (CH_3-COO-CH_2-CH_3), using 500 mL solvent per liter of filtrate. The extracts were concentrated to dryness in a rotary evaporator. A control was prepared by extracting sterilized and incubated culture medium.

Antibiosis assay

Preliminary antibiotic tests were performed in NA plates at 29°C for the reference strains *Bacillus subtilis* ATCC 6633 and *Saccharomyces cerevisiae* ATCC 9763 at 37ºC for strains *Micrococcus luteus* ATCC 9341 and *Escherichia coli* ATCC 11229, previously grown in NB for 24 h. Different volumes from the concentrated bacteria fermentation filtrates were used to evaluate antibiotic production using the Kirby-Bauer method (http://aminj.myweb. uga.edu/ KIRBY-BAUER.html).

The minimal inhibitory concentration (MIC) of the organic extracts from strains 1AC and 28A was determined as a decrease in turbidity and assessed by OD_{600} in 96 well plates according to Wiegand *et al*., (2008). Each well containing 100 μL of nutrient broth medium was inoculated with a McFarland suspension (0.5 value) of the reference strains. Different concentrations of the organic extracts were tested against these strains (Gram-positive bacteria, *E. coli* and *S. cerevisiae*). The plates were incubated at 37ºC for *E. coli* and *M. luteus* and at 29ºC for *S. cerevisiae* and *B. subtilis.* As positive controls, nalidixic acid, erythromycin and cycloheximide, were utilized. Additionally, Nutrient Broth extract was used as negative control.

Cytotoxic activity

Before evaluation of their cytotoxic activity, endotoxins were removed from organic extracts from strains 1AC and 28A through immobilization with Polymixin B (Thermo Scientific™ Detoxi-Gel Endotoxin Removing Column). Concentrations of 1 to 50 $\mu g ml^{-1}$ of the purified extracts were examined, in triplicate, in 96 well plates containing 5000 cells per well of each cell line. Tested cell lines were MCF-7 (human breast cancer; ATCC HTB-22) and4T1 (murine breast epithelial cells; ATCC CRL-2539). MCF-7 was cultured in Dulbecco´s Modified Eagle´s/Ham´s Nutrient Mixture F12 (Sigma-Aldrich, St Louis MO) (1:1) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. 4T1 cell line was cultured in RPM1 medium (Sigma Aldrich, St Louis MO) supplemented with 10% FBS as recommended by supplier. Both cell lines were purchased from the American Type Culture Collection (Rockville, MD). Cell viability was assayed using the colorimetric method reported by Mosmann (1983), which is based on the reduction of tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to chromogenic formazan (2*E*,4*Z*)-(4,5-

dimethylthiazol-2-yl)-3,5-diphenyl-formazan) by a mitochondrial reductase. As positive controls, cisplatin (CDDP) and doxorubicin were utilized. Additionally, DMEM, DMSO and concentrated NB media were used as negative controls.

Trypanocidal activity

Organic extracts from strains 1AC and 28A were tested against *Trypanosomacruzi* CL-Brener (Zingales et al., 1997) and their IC_{50} values determined. For this purpose, *T. cruzi* was grown in Liver Infusion Tryptose (LIT) supplemented with 25 mg L^{-1}haemin and 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) (Camargo 1964). *T. cruzi* CL-Brenner was donated by Dr. B. Zingales from Sao Paulo University, Brazil.

The extract concentrations tested ranged from 100 to 400 μg ml^{-1}. For this purpose, 96 well plates containing 100 μl NB, $3x10^6$epimastigotes and the corresponding extract, were incubated for 24 and 48 h at 28ºC. After incubation, morphological changes and epimastigotes were counted in a microscope using a Neubauer chamber. Morphological changes of epimastigote cells were observed through HEMA staining of paraformaldehyde fixed cells. Benznidazole, Nifurtimox and G418 were employedas positive controls.

Molecular and phenotypic identification of bacteria

For this purpose, eight bacterial strains showing antibiotic activity were grown and their gDNA extracted according to the Miniprep protocol reported by Wilson (1994). For 16S rDNA amplification, the primers tested were: fwd5'-CCGAATTCGTCGACAACAGAGTTTGATCATGGCTCAG and rev 3'-CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT (Weisburg *et al.* 1991). PCR amplification conditions were: initial activation of the Taq-DNA-Polymerase for 5 min at 94°C, followed by 30 cycles of 30 s denaturation at 94°C, annealing for 1 min at 56.5°C and extension for 2 min at 72°C, with a final extension step at 72°C for 4 min.

Amplicons of the expected sizes were purified from unincorporated nucleotides using the QIAEXII kit (Qiagen). Purified amplicons were ligated into the pGEM-T Easy vector (Promega) according to supplier instructions. The resulting constructions were transformed by electroporation into *E. coli* DH5α (1250 MeV, 5 ms). Plasmids were purified and sequenced, and the resulting nucleotide sequences were compared against the NCBI Genebank database.

Bacterial phenotypic identification and antibiotic susceptibility were performed with VITEK® -2 GN ID card- 21341 and antibiotic susceptibility tests with VITEK® -2 Gram-negative susceptibility card AST GN-25 (BioMérieux).

Siderophores production

For this purposes, strains were grown in Erlenmeyer flasks (250-mL) with 50 mL King's (KB) medium (King *et al.* 1954) after 24 h at 29ºC fluorescence was observed with UV light. When necessary, siderophores were eliminated by passing the concentrated media through a sepharose column charged with $CuSO_4$ (Xiao and Kisaalita 1995). Siderophores were eluted with a volume of HEPES buffer 120 mM plus 100 mM NaCl, and nine volumes of acetate buffer 20 mM plus 100 mM NaCl. The decrease in fluorescence was monitored by UV light and spectrophotometry at 400 nm. Eluates were tested for bioactivity

against *M. luteus* using the Kirby Bauer assay (http://aminj.myweb.uga.edu/KIRBY-BAUER.html).

Result and Discussion

A total of 55 endophytic bacteria were isolated from the tropical tree *Magnolia dealbata* Zucc, 17 were Gram-positive and 38 Gram-negative, including cocci and bacilli. Screening tests of the bacteria isolates revealed 14 different inhibition patterns against bacteria, yeast or both. Among them, four Gram-negative bacteria (1AC, 1AD, 1AE, 16A) isolated from cork and four obtained from root (14A, 21A, 24A, 28A) were able to inhibit at least three assay microorganisms. The most sensitive bioassay microorganism was *M. luteus*, whereas *E. coli* was inhibited to a lesser extent.

Due to their spectrum of microbial inhibition, as well as differences in colony morphology, organic extracts from supernatant (SOE) and organic extracts from biomass prepared from 24 h fermentations (in NB + glucose), of two bacteria (1AC and 28A) were selected for MIC evaluation. As seen in Table 1, 6.25µg/mL SOE from the 28A strain fermentation filtrates, were required to reach the Gram-positive microorganisms MIC value. However, SOE from 1AC strain filtrates exhibited dissimilar activities against *M. luteus* and *B subtilis*, requiring 12.5µg/mL in the first case and half this concentration for the second. These MIC values were almost 10 times weaker than those observed for erythromycin

In addition, the required concentrations of SOE filtrates to reach the MIC dosage against *S. cerevisiae* and *E. coli* were more than 100-fold higher than those required for the positive controls nalidixic acid and cycloheximide, respectively. Furthermore, activities of SOE from fermentation filtrates were stronger than those obtained from biomass. Furthermore, when compared to erythromycin, the SOE filtrates from the 28A strain displayed an 8-fold weaker activity against *E. coli*.

Molecular characterization through 16S rDNA, rpoD and gyrB analysis of 1AC and 28A bacteria showed more than 94% similarity to the *Pseudomonas* genus. These findings were corroborated by carbon source utilization assays and antibiotic sensitivity tests using the VITEK® 2 (Biomérieux) system for microbial identification (Table 2). The VITEK® 2 assay strongly suggested that both strains belong to the fluorescent Pseudomonads group. Distance and Maximum Likelihood methods were tested with 16S rDNA in order to obtain the best suitable and supported phylogenetic tree (Fig. 1). Both tests corroborate our last results and additionally disclose that1AC and 28A strains are strongly related with *Pseudomonas protegens* Pf5 and *Pseudomonas fluorescens* CHA0, two of the mayor secondary metabolite producers in the Pseudomonads genus (Gross and Loper 2009). According to the ribosomal coding sequence analysis, *Pseudomonas* sp. strain 28A remained as an unidentified member of the fluorescentgroup. This finding, lead us to think on 28A strain as a strong novel candidate for future bioactivity endeavors.

Additionally, cytotoxic activities of SOE obtained from *Pseudomonas* sp. 1AC and 28A were established against breast cancer cell lines MCF-7, from human, and 4T1from mice, which very closely mimics stage IV human breast cancer. As shown in Table 3, the stronger inhibition was observed with SOE than those from biomass, requiring less than 0.1 µg ml^{-1}, to reach the IC_{50} against both cell lines. Furthermore, this effect was

even better than the anticancer drug controls doxorubicin and CDDP, which served as positive controls.

Activity against cell lines was seen before and after passing the filtrates through an endotoxin-removing column. These fractions were cleaned up to ensure that cytotoxic effect on cell lines was not due to an endotoxin activity, but owed to the extract itself. Cell treatment with a range from 1 to 50 µg mL^{-1} of the SOE filtrates, showed that even at a concentration of 1 µg mL^{-1}, the cell viability value could not reach the 100% seen on the negative control, meaning that even the lowest concentration tested possess a strong cytotoxic effect.

Highest cytotoxic effect was observed against 4T1 cells using SOE of the strain 28A, requiring less than 1 µg mL^{-1} to achieve about 80% inhibition; on the other hand, at the same concentration, 60% inhibition was achieved against MCF7 cells. These values are comparable with those exerted by doxorubicin, about 80% and 50%, respectively.

In view that inhibition of the filtrate extracts was similar to that of the antitumor agent doxorubicin, suggested that could be acting against cell lines with high proliferative capacity. This can be supported on the basis of similar morphological changes observed on cell lines treated with both, filtrate extracts or doxorubicin (not shown). Nevertheless, the mechanism for the inhibitory activity of cancer cell growth of the extract needs to be investigated in detail.

Although biomass and SOE from strains 1AC and 28A exhibited growth inhibition against *T. Cruzi* (Table 4), the biological activity obtained from SOE was superior to that from biomass. As observed before, SOE from *Pseudomonas* sp. 28A displayed higher effect against the parasite than those from *Pseudomonas* sp. 1AC. Remarkably, the 28A SOE IC_{50} value was in the same range of that shown by commercial Benznidazole and 10-fold better than G418. Morphological changes and decreased motility were observed as fast as 24h after adding the supernatant treatments, i.e., cells loosed the flagellum and turned from elongated to spherical shapes with a wrinkled cell membrane. Similar morphological changes were observed with the positive controls Benznidazole and Nifurtimox. In addition, no morphological changes were observed with G418 and biomass extracts (data not shown). Except from viscosin, a surfactant produced by *Pseudomonas fluorescens* SBW25 (Abdullah et al., 2014), no other trypanocidal agents have been obtained from Pseudomonads species. This result supports the SOE obtained from *Pseudomonas* sp. 28A, as a strong candidate to untapped new candidates to fight this important parasite.

One characteristic of the *Pseudomonas* genus is its ability to produce siderophores (Meyer and Abdallah 1978). Under iron depletion, siderophores (iron chelating molecules) can be monitored in the concentrated media by their blue-green fluorescence under UV light.As iron chelating agents, these compounds are able to produce Fe^{2+} stress and cause cytotoxic or bactericidal effects. Thus, in order to discard that siderophores were responsible for the inhibitory effects previously observed, they were eliminated. After siderophores elimination, all biological activity from fermentation filtrates extracts remained intact (not shown).

In this work, the value of the medicinal tree *M. dealbata* Zuc., as a source of microorganisms producing different pharmacological activities has been

demonstrated. Some of these isolates, identified as *Pseudomonas* sp., showed strong potential as a source of antiinfective, anticancer and trypanocidal drugs. The chemical characteristics of the produced compounds remain to be elucidated.

The medicinal tree, *Magnolia dealbata* Zucc., harbors a wide diversity of microorganisms. Around 10% of the bacterial strains isolated displayed at least antimicrobial activity. *Pseudomonas* was the must common isolate among these bioactive bacterial strains. Remarkably two Pseudomonads strains(1AC and 28A)exhibits not only antimicrobial but cytotoxic and trypanocidal activity as well. *Pseudomonas* sp. 28A evince as a better candidate for further studies since organic extracts obtained from supernatant presents strong cytotoxic and trypanocidal activity, similar or even better than the observed for the positive controls. Studies regarding to trypanocidal compound characterization are currently being carried out. This work supports endophytic bacteria as chemical factories and excellent candidates for isolation of bioactive compounds looking forward for most potent and less toxic molecules with potential pharmacological applications.

Table.1 Supernatant and biomass MIC values from strains 28A and 1AC toward test microorganisms

Organic extracts obtained from:	**MIC values (µg/mL)**			
	E. coli	*S. cerevisiae*	*M. luteus*	*B. subtilis*
28A Supernatant[a]	400.00	100.00	6.25	6.25
28A Biomass[a]	>3200.00	>3200.00	12.50	6.25
1AC Supernatant	>3200.00	>3200.00	25.00	12.50
1AC Biomass	>3200.00	>3200.00	25.00	12.50
Controls				
Nutrient Broth[b]	>3200.00	>3200.00	3200.00	3200.00
Nalidixicacid[c]	3.12	—[d]	200.00	3.12
Erythromycin[c]	50.00	>200.00	0.78	0.78
Cycloheximide[c]	—	0.78	—	—

aObtained from 24 h NB fermentations.
bNegative control: Nutrient Broth.
cPositive controls: Nalidixic acid, erythromycin and cycloheximide.
d(—):It means not determined.

Table.2 Molecular and biochemical identification of selected bacteria

	NCBI*				VITEK® 2	
Strain	Genus/species	Identity (%)	Covering (%)	Maximum score	Genus/species	Identity (%)
1AD	*Ps. putida*	95	98	2183	*Ps. fluorescens*	93.3
	Pseudomonas sp.	96	89	2024		
1AE	*Gamma proteobacterium*	97	79	1094	*Ps. fluorescens*	33.6
	Ps. fluorescens	97	78	1092	*Ps. aeruginosa*	33.2
14A	*Ps. putida*	95	98	2183	*Ps. fluorescens*	98.3
	Pseudomonas sp.	96	89	2024		
16A	Uncultured *Pseudomonas*	99	93	1567	*Aeromonas salmonicida*	95.0
	Endophyte Bacterium SS14	99	93	1567		
21A	*Pseudomonas* sp.	98	96	1230	*Ps. fluorescens*	99.0
24A	Endophyte Bacterium SS14	99	99	1768	*Ps. fluorescens*	99.0
	Ps. tolaasii	99	99	1765		
28A	*Pseudomonas* sp.	98	94	1687	*Ps. fluorescens*	93.3
	Ps. fluorescens	98	98	1681		

*Results of nucleotide blast using the NCBI Genebank

Table.3 IC_{50} values of supernatant and biomass organic extracts from strains 28A and 1AC for cytotoxic activities

Organic extract prepared from:	**Cell line**	**28A strain IC_{50} (µg/mL)**	**1AC strain IC_{50} (µg/mL)**	**Controls**	**IC_{50}[a] (µg/mL)**
Supernatant	MCF 7	0.057±00.05	34.30±4.76	CDDP	4.90±0.61
Biomass		59.123±7.87	16.10±8.75	Doxorubicin	0.11±0.03
Supernatant	4T1	0.014±00.00	18.64±2.31	CDDP	12.85±0.01
Biomass		29.85±04.14	11.487±3.38	Doxorubicin	0.53±0.05

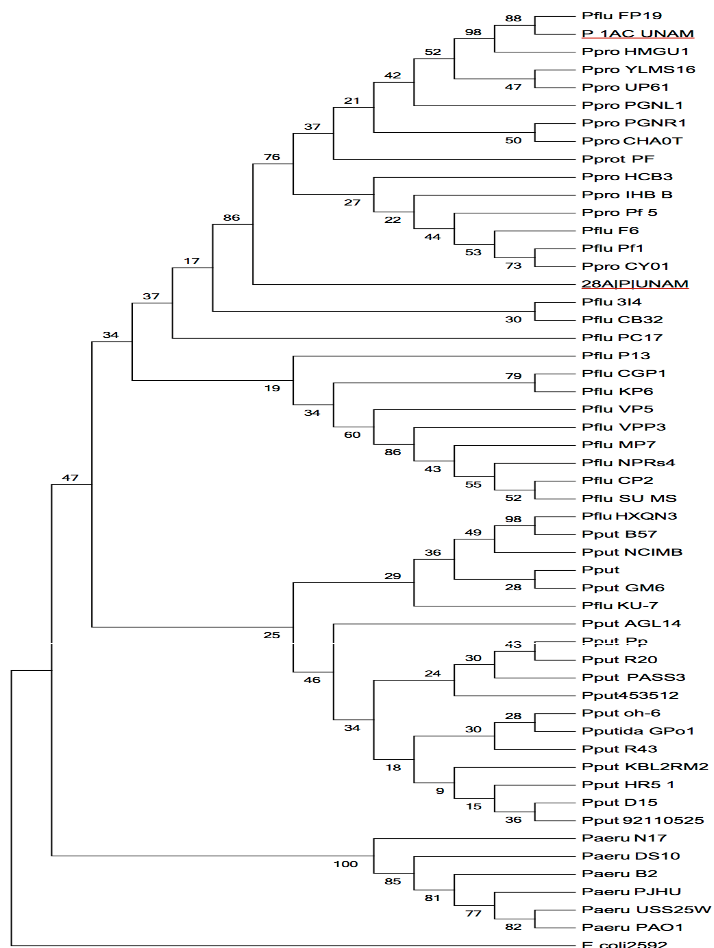
[a]p values <0.05

Table.4 IC_{50} values of supernatant and biomass organic extracts from strains 28A and 1AC with trypanocidal activity

Organic extract prepared from:	**Trypanocidal activity (IC_{50})^{a} µg/mL**
28A supernatant	14.17±8.27
28A Biomass	36.25±5.49
1AC supernatant	97.00±13.00
1AC biomass	280.00±32.00
Controls	
Benznidazole	1.16±0.10
Nifurtimox	0.11±0.01
G418	142.00±92

[a] *p* values <0.05
[b] SD: Standard deviations

Fig.1 Phylogenetic tree based on 16S sequences. The tree was rooted with *E. coli* 2592 and constructed by using the maximum likelihood algorithm and a bootstrap value of 1000. The number at each branch point represents percentage bootstrap support



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