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보 문

Temperature dependent 2,3-dihydroxybenzoic acid production in *Acinetobacter* sp. B-W

Kyoung-Ja Kim[1]*, Jae-Hun Lee[1], and Yong-Joon Yang[2]

[1]*Department of life Science and Biotechnology, College of Natural Science, Soonchunhyang University, Asan 336-745, Republic of Korea*
[2]*Department of Plant and Food Science, Sangmyung University, Cheonan 330-720, Republic of Korea*

Acinetobacter sp. B-W의 온도 의존적 2,3-dihydroxybenzoic acid 생산

김경자[1]* · 이재훈[1] · 양용준[2]

[1]순천향대 생명시스템학과, [2]상명대학교 식물식품공학과

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ABSTRACT: A soil microorganism producing iron chelator (siderophore) under low iron stress (up to 2 μM of iron) was identified as *Acinetobacter* sp. B-W by 16S rDNA sequence analysis, biochemical-, physiological tests and morphological analysis using electron microscope. Catechol nature of siderophore was detected by Arnow test. Although optimal cell growth was identified at 36°C in iron-limited media, significant quantities of siderophore were produced only at 28°C. Biosynthesis of siderophore was strongly inhibited by growth at 36°C. Production of siderophore was completely inhibited by 10 μM $FeCl_3$. Iron chelator produced from *Acinetobacter* sp. B-W was purified from supernatant using butanol extraction, Sephadex LH-20 column chromatography and HPLC. Purified sideropore was identified as 2,3-dihydroxybenzoic acid by HPLC, TLC and IR analysis.

Key words: *Acinetobacter* sp., catechol type, 2,3-dihydroxybenzoic acid, siderophore, temperature dependent

Iron is essential for the growth of almost all living organisms. Although iron is an abundant element, its bioavailability in aerobic environments is limited because of the insolubility of ferric iron. To overcome the difficulty of acquiring sufficient iron for growth, many microorganisms synthesize a variety of biomolecules that chelate ferric iron and mobilize it for use (Neilands, 1981; Crosa, 1989; Neilands, 1995). Both bacteria (mainly aerobic) and fungi produce a chelator of ferric ion, siderophores, under iron-limiting conditions (Crosa, 1984; Neilands, 1995). The origin of the word siderophore is from a Greek word meaning “iron bearer”. Siderophores are low molecular weight compounds (400−1000 Da) with high affinity for ferric ion (Raymond and Dertz, 2004; Skaar, 2010). Broadly, siderophores can be classified into three categories depending upon the moiety that donates oxygen ligands for Fe^{+3} coordination: a) catecholates (or phenolates) b) hydroxymates (or carbooxylate) and c) the mixed types (Miethke and Marahiel, 2007; Wencewicz *et al*., 2009). Many bacteria, including *Pseudomonas* sp., *Azotobacter* sp., *Rhizobium* sp., *Bacillus* sp., etc., are able to excrete catechol type siderophores (Hoefte, 1993; Carrillo-Castaneda *et al*., 2000). Members of the genus *Acinetobacter* have been reported to be involved in a variety of nosocomial infection s including bacteraemia, urinary tract infection, and secondary meningitis, with increasing frequency (Bergogne-Bérénin and Towner, 1996). Temperature regulated siderophore production has been identified in several bacteria (Garibaldi, 1972; Cogswell and Weinberg, 1980; Worsham and Konisky, 1984) and one yeast species (Ismail, 1985). But it was reported that growth temperature in *Aeromonas* sp. had little effect on production of siderophore (Naidu and Yadav, 1997).

***For correspondence.** E-mail: kyoungjakim@hotmail.com;
Tel.: +82-41-530-1352; Fax: +82-41-530-1350

In this study temperature dependent production and structure elucidation of catechol type siderophore from *Acinetobacter* sp. B-W are reported.

Materials and Methods

Microorganisms, media, and culture conditions

Acinetobacter sp. B-W was isolated from soil in Cebu city of Philippines. Half g of soil was suspended in 50 ml of distilled water and the supernatant was spread on the chrome azurol S (CAS) agar medium (Neilands, 1981). On CAS agar plates, siderophore-producing (Sid^+) bacteria form colonies with an orange halo. This occurs because iron is removed from the original blue CAS-Fe (III) complex during siderophore production. Formation of siderophore halos (Neilands, 1981) was evaluated following 5 days of colony incubation at 30°C. The colony showing the largest orange halo was selected as siderophore producing bacterium for further investigation. *Acinetobacter* sp. B-W was maintained on a semi synthetic minimal medium (Stintzi and Mayer, 1994): glucose 5 g/L, $(NH_4)_2SO_4$ 0.5 g/L, yeast extract 0.05 g/L, KH_2PO_4 0.2 g/L, $MgCl_2$ 0.6 H_2O 0.2 g/L, $FeSO_4$ 0.005 g/L, agar 20 g/L, pH 7.0. The siderophore production medium for *Acinetobacter* sp. B-W was a chemically defined one (modified PGI medium, pH 7.0) with the following compositions (in g/L): glucose (5), sodium glutamate (2.5), $MgSO_4 \cdot 7H_2O$ (0.5), K_2HPO_4 (1.0), $MnSO_4$ (0.002). The siderophore production medium with an iron concentration of 2 μM and 10 μM were referred as low iron medium (LIM) and high iron medium (HIM), respectively. $FeCl_3 \cdot 6H_2O$ was added to the medium for LIM and HIM. The culture for siderophore production was carried out at 28°C for 36 h.

Identification of siderophore producing bacteria

The bacterial strain with the highest siderophore production was identified by Gram staining, physiological and biochemical test using an API kit (bioMérieux) and 16S rDNA sequence analysis. Morphological characteristics of this strain were observed with electron microscope. Thereafter the strain was identified according to *Bergey's Manual of Determinative Bacteriology* based on its morphological, biochemical, and physiological characteristics.

Siderophore detection

The CAS solution assay (Schwyn and Neiland, 1987) was used to quantitate siderophore activity in culture supernatant by measuring the decrease in the absorbance of blue color at 630 nm. Standard curves relating CAS reactivity to the iron-binding ligands were determined using the fungal siderophore desferrioxamine (Desferal; CIBA-GEIGY). Hydroxamate and catechol functionality of 10-fold-concentrated siderophore extracts of *Acinetobacter* sp. B-W were examined by the Csaky test (Csaky, 1948; Gillam *et al*., 1981) and the Arnow reaction (Arnow, 1937), respectively. In these assays, hydroxylamine and 2,3-dihydroxybenzoic acid, respectively, were used as the standards.

Isolation and purification of siderophores

Bacterial cultures grown in 600 ml of modified PGI medium for 36 h at 28°C were harvested by centrifugation at 8,000 rpm for 10 min. Iron-free siderophores were obtained by the following method (Payne, 1994; Milagres *et al*., 1999). Culture supernatants were extracted three times with equal volumes of butanol. The concentrated organic extracts were dissolved in 5 ml of methanol. Partial purification of the siderophores was achieved by the fractionation of the organic extracts on a Sephadex LH-20 (Pharmacia) column in the methanol elution. The eluting solutions were purified with Chelex-100 to remove the iron. The CAS assay-reactive fractions were pooled and concentrated 10-fold by evaporation. The resolution of siderophore was carried out on HPLC Nucleosil ODS (C_{18}) column using methanol (Gills *et al*., 1996) as a mobile phase, at the flow rate of 1.0 ml/min.

Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) was performed on 0.25-mm-thick silica gel 60 F254 in the following solvent systems: Benzene-acetic acid-water (120:70:3), Butanol-acetic acid-water (12:3:5), Butanol-pyridine-water (14:3:3), chloroform-methanol (2:1), Toluene-1,4-dioxane-acetic acid (45:10:2). The plates were examined under UV light or sprayed with 0.12 N $FeCl_3$ in 0.1 N HCl to detect iron-binding compounds (O'Brien *et al*., 1970). To detect catechol-type compounds, they were sprayed either the reagents of the Arnow assay or 1% ferric ammonium citrate and then with potassium ferricyanide (Rogers, 1973).

Results and Discussion

Identification of siderophore producing bacteria

Siderophore producing bacterial strains were isolated from soil in Cebu city of Philippines by using chrome azurol S (CAS) agar plate. A bacterium showing the largest orange halo on the CAS agar plate was selected as siderophore producing bacterium for further investigation. 16S rDNA gene was amplified by PCR with bacterial universal primers and determination of the nucleotide sequence of PCR product (Fig. 1) was performed by Macrogen Inc. (Korea). Phylogenetic analysis was performed using BLAST program of NCBI. Morphological characteristics were observed by an electron microscope (Fig. 2). On the basis of 16S rDNA sequence analysis, morphological characteristics, physiological-, biochemical test using commericial kit, this strain was identified as *Acinetobacter* sp. *Acinetobacter* species are Gram-negative bacteria belonging to the wider class of Gamma-proteobacteria, and non motile, oxidase-negative, catalase positive and occur in pairs under magnification. Members of the genus *Acinetobacter* have been reported to be mostly found in soil, different water sources, and many healthcare environments (Perez *et al*., 2007; Peleg *et al*., 2008).

Bacterial growth and production of siderophore

To determine the relationship of cell growth and siderophore production, *Acinetobacter* sp. B-W was cultured in a modified PGI medium at 28°C for 36 h with shaking (130 rpm). As depicted in Fig. 3, siderophore production in modified PGI medium started during exponential phase of growth following

1	ACCGGACCTC ATGTCACTAG CCGTTGGGGC CTTTGAGGCT TTAGTGGCGC AGCTAACGCG	60
61	ATAAGTAGAC CGCCTGGGGA GTACGGTCGC AAGACTAAAA CTCAAATGAA TTGACGGGGG	120
121	CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGATGCAA CGCGAAGAAC CTTACCTGGC	180
181	CTTGACATAG TAAGAACTTT CCAGAGATGG ATTGGTGCCT TCGGGAACTT ACATACAGGT	240
241	GCTGCATGGC TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC	300
301	AACCCTTTTC CTTATTTGCC AGCGAGTAAT GTCGGGAACT TTAAGGATAC TGCCAGTGAC	360
361	AAACTGGAGG AAGGCGGGGA CGACGTCAAG TCATCATGGC CCTTACGGCC AGGGCTACAC	420
421	ACGTGCTACA ATGGTCGGTA CAAAGGGTTG CTACCTAGCG ATAGGATGCT AATCTCAAAA	480
481	AGCCGATCGT AGTCCGGATT GGAGTCTGCA ACTCGACTCC ATGAAGTCGG AATCGCTAGT	540
541	AATCGCGGAT CAGAATGCCG CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA	600
601	CACCATGGGA GTTTGTTGCA CCAGAAGTAG CTAGCCTAAC TGCAAAGAGG GCGGTTACCA	660
661	CGGTGTGGCC GATGACTGGG GTGAAGTCGT AAGGGAAAAC CCCCAAAAAG GGGG	720

Fig. 1. Analyzed nucleotide sequence data of amplified 16S rDNA of strain B-W by PCR (forward direction).

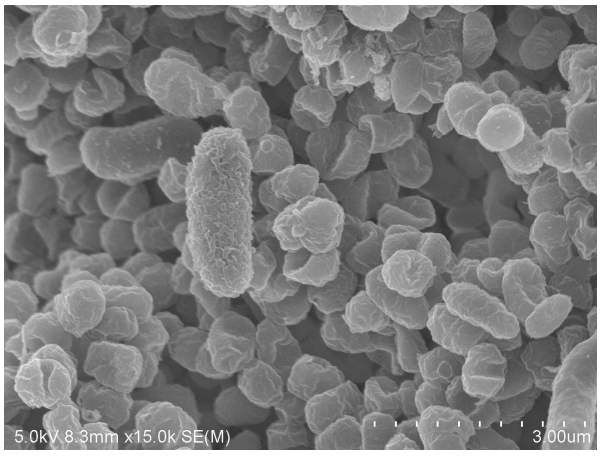

Fig. 2. SEM micrograph of *Acinetobacter* sp. B-W.

to be maximal at 24 h incubation. During stationary phase there was gradual decrease in the pH of medium towards acidic range and this may be attributed to the synthesis of siderophores and along with other secondary metabolites (Budzikiewicz, 1993). When 20 µl of cell free supernatant of *Acinetobacter* sp. B-W was added to CAS agar plate, an instant color change from blue to orange red was noticed indicating the presence of siderophores in broth (Fig. 4). The effect of iron concentration on the siderophore production by *Acinetobacter* sp. B-W was studied in modified PGI medium supplemented with varying amounts of ferric ion. Siderophore production was sensitive to the increase of ferric ion concentration in the medium (Fig. 5); a concentration of ferric ion less than 2 µM was needed for siderophore production. Higher concentration of ferric ion considerably decreased the siderophore production. At a concentration of 10 µM in the medium, the siderophore

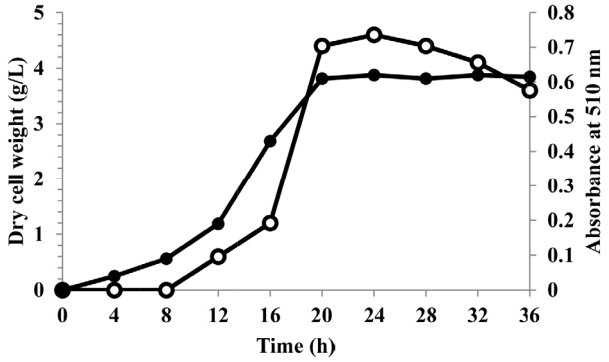

Fig. 3. Production of siderophore by *Acinetobacter* sp. B-W during growth. Cells were grown in modified PGI medium at 28°C for the determination of dry weight (●) and siderophore content by Arnow test (○).

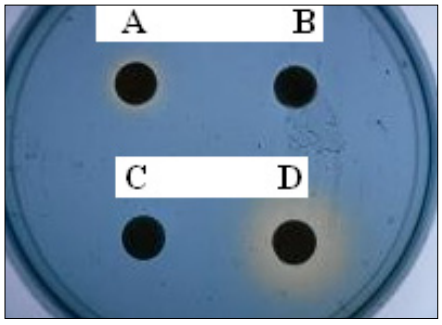

Fig. 4. CAS agar assay. The CAS agar assay was performed on CAS plate with paper discs those were coated with 20 µl of followings; (A) Supernatant of *Acinetobacter* sp. B-W at 6 h growth, (B) Modified PGI medium, (C) Supernatant of *Acinetobacter* sp. B-W at 24 h growth with 10 µM $FeCl_3$, (D) Supernatant of *Acinetobacter* sp. B-W at 24 h growth.

production of strain B-W was completely inhibited. As shown in Fig. 6, although iron supplementation in liquid cultures accelerated growth of strain B-W, differential levels of iron availability did not greatly influence maximum biomass production, which may be explained by successful bacterial adaptation to different culture conditions regarding iron availability. In general, the growth curves were not much different depending on the iron concentration in the medium (Fig. 6). Up to 20 h, the growth rate was fast for all three culture conditions (modified PGI medium with 0, 2, and 10 µM $FeCl_3$). After this period, slight increase in growth and then no significant increase in the growth were observed. Our experimental data suggest that more siderophores were produced when the B-W grew slowly (under ferric ion deficiency) than when it grew rapidly (under ferric ion supplementation). This fact agrees to the statement made by Neilands (1984) that high iron concentration in the medium generally results in excellent microorganism growth but only modest yields of siderophore.

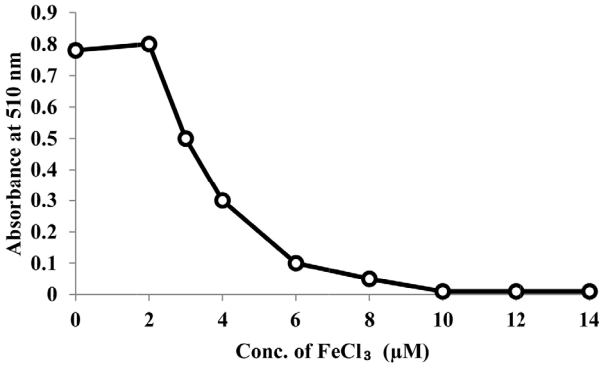

Fig. 5. Effect of iron concentrations on the production of siderophore by *Acinetobacter* sp. B-W grown in modified PGI medium at 28°C. The siderophore concentration was estimated by Arnow test.

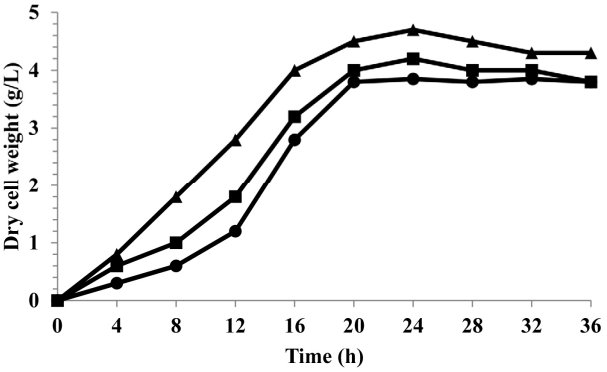

Fig. 6. Growth curve of *Acinetobacter* sp. B-W grown in different iron concentrations. (●) 0, (■) 2 µM, (▲)10 µM.

Effect of temperature on the growth and siderophore production

The influence of growth temperature on siderophore production was studied by growing cultures at 20, 24, 28, 32, 36, or 40°C (Fig. 7). The optimal growth temperature of *Acinetobacter* sp. B-W is around 36°C. The growth rate steadily increases with temperature from 20 to 36°C. Above 40°C, the growth rate decreases drastically. Siderophore production was increased with temperature from 20 to 28°C and then decreased from 30 to 40°C. Siderophore production of *Acinetobacter* sp. B-W was strongly inhibited at 36°C and it could indicate that siderophore synthesis of strain B-W is temperature sensitive. A bacterial cell must adapt to its environment that could be important for its

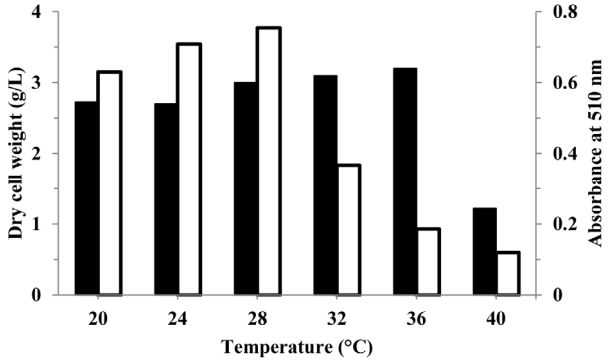

Fig. 7. Siderophore production by *Acinetobacter* sp. B-W grown in modified PGI medium at different temperatures for 24 h. (■) dry weight, (□) siderophore production.

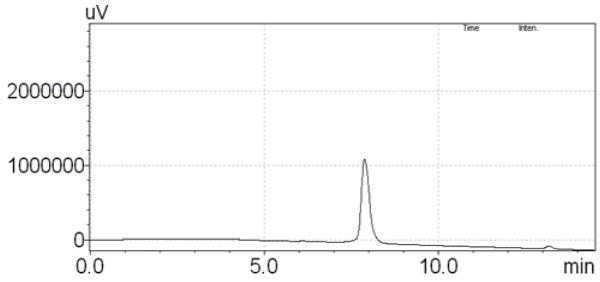

Fig. 8. HPLC of the purified siderophore from *Acinetobacter* sp. B-W.

success (Winkelmann, 2004). Temperature regulated siderophore production has been identified in several bacterial genera (Garibaldi, 1972; Cogswell and Weinberg, 1980; Worsham and Konisky, 1984) and this type regulation in which siderophore production is reduced at elevated temperatures has been directly associated with virulence in *Pseudomonas aeruginosa* (Meyer *et al*., 1996). High growth levels in the absence of siderophore in strain B-W at 36°C indicate that strain B-W may possess an alternative non-siderophore based iron assimilation system. It has been identified up to five iron assimilation mechanisms in *E. coli* (Earhart, 1996).

Table 1. Rf values of $FeCl_3$ reacting components produced from *Acinetobacter* sp. B-W

Solvent system	Rf value	
	Sample	2,3-DHBA[a]
Benzene-acetic acid-water (120:70:3)	0.64	0.64
Butanol-acetic acid-water (12:3:5)	0.82	0.82
Butanol-pyridine-water (14:3:3)	0.52	0.52
Chloroform-methanol (2:1)	0.31	0.31
Toluene-1,4-dioxane-acetic acid (45:10:2)	0.35	0.35

Thin-layer chromatography (TLC) was performed on 0.25-mm-thick silica gel 60 F254
[a] synthetic 2,3-dihydroxybenzoic acid

Purification and elucidation of siderophore structure

Siderophore was purified from the supernatant of *Acinetobacter* sp. B-W by extraction with butanol and Sephadex LH-20 column chromatography with methanol as elution solvent. The active fractions were resolved by HPLC. Functionality of siderophore was determined to catechol type by Arrow assay (Arnow, 1937). Csaky test (Csaky, 1948) useful for the detection of hydroxamate type siderophore gave negative results. The purified siderophore was then analyzed by HPLC and IR. Retention time (7.8 min) in HPLC (Fig. 8) and Rf values in TLC (Table 1) were also identical to those of authentic 2,3-dihydroxybenzoic acid (2,3-DHBA). The IR spectrum of this compound exactly matched that of standard 2,3-DHBA (Fig. 9). Apparently, these results showed that B-W produced 2, 3-DHBA. Many high-affinity ferric ion chelating molecules contain catecholate groups that are part of the iron-binding site. Enterobactin, the prototype catechol siderophore produced by *Escherichia coli*, is a cyclic trimer of 2,3-dihydroxybenzoyl-L-serine (O'Brien and Gibson, 1970; Walsh *et al*., 1990). In contrast, siderophores such as vibriobactin and anguibactin produced by *Vibrio cholerae* (Griffiths *et al*., 1984) and *Vibrio anguillarum* (Loehr, 1986; Jalal *et al*., 1989), respectively, are non-cyclic derivatives of 2,3-DHBA. In vibriobactin, the 2,3-DHBA moiety is linked to L-threonine and norspermidine, while in anguibactin DHBA is linked to L-cysteine and hydroxyhistamine.

적 요

철 스트레스(2 μM 이하 농도) 하에서 시데로포어를 생산하는 균주를 토양에서 분리하여 16S rDNA 염기 서열 분석과 생

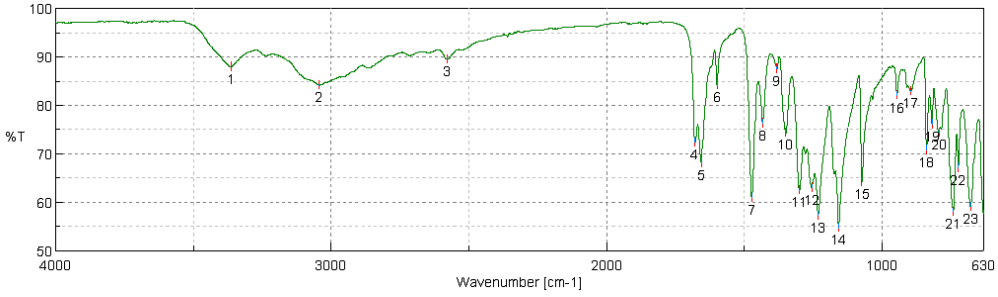

Fig. 9. Infrared (IR) spectrum of the purified siderophore from *Acinetobacter* sp. B-W in KBr disk.

화학적, 생리학적 분석 및 전자 현미경 관찰 등으로 동정한 결과, *Acinetobacter* sp.로 밝혀졌다. 시데로포어의 카테콜 특성은 Arnow법으로 조사되었다. 철을 제한한 배지에서 균주를 배양한 결과, 36°C에서도 잘 자랐지만 시데로포어 생산은 28°C에서 높았다. 36°C에서는 시데로포어 생산이 강하게 억제되었다. 10 μM $FeCl_3$를 첨가한 배지에서는 시데로포어 생산이 완전히 억제되었다. 균주 상등액을 부탄올 추출 후, Sephadex LH-20 컬럼 크로마토그래피와 HPLC를 이용하여 시데로포어를 분리, 정제하였다. 분리, 정제된 시데로포어의 구조는 HPLC, TLC와 IR 분석 결과로부터 2,3-dihydroxybenzoic acid로 확인되었다.

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