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ORIGINAL ARTICLE

Isolation of Auxin- and 1-Aminocyclopropane-1-carboxylic Acid Deaminase-producing Bacterium and Its Effect on Pepper Growth under Saline Stress

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Abstract Plant tissues produce ethylene under the environmental stresses such as drought, salinity, and heavy metals. Ethylene concentration can be reduced by 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase produced by plant growth-promoting rhizobacterium (PGPR), which cleaves the ethylene precursor ACC. The present study focused on alleviation of environmental stress by selected PGPR, which could suppress fungal plant disease. These PGPRs were capable of utilizing ACC as sole source of nitrogen and also produced auxin. Seed germination of red pepper was reduced with increasing salt concentration, and approximately 98.2% of seeds germinated in the absence of salt, whereas only 36.2% seeds germinated in the presence of 175 mM NaCl. Seed germination was also decreased by 62.1 and 19.9% in the presence of 120 mM NaCl and 120 mM NaCl + ACC deaminase-producing PGPR *Pseudomonas fluorescens* 2112, respectively, compared to uninoculated control. The effect of salinity stress with different salt concentration on pepper plants and their alleviation with PGPR was evaluated. Non-inoculated pepper plants died after 5 week when grown in the presence of high salt (120 mM NaCl), whereas 80% of pepper plants inoculated with *P. fluorescens* 2112 survived under the high salt stress. Salt stress also decreased the fresh and dry weights of pepper grown, compared to the negative control, whereas pepper plants inoculated with *P. fluorescens* 2112 retained the biomass similar to control plants. These results indicate that ACC deaminase and auxin producing *P. fluorescens* 2112 is a multi-functional PGPR that can promote the growth and development of pepper plants by alleviating the high-salt stress.

Keywords 1-aminocyclopropane-1-carboxylic acid deaminase · plant growth promoting rhizobacteria · *Pseudomonas fluorescens* · saline stress

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Introduction

Ethylene is an important gaseous phytohormone, involved in the growth and development of plants such as seed germination, root length, and abscission of leaf and petal (Bleecker and Kende, 2000; Zhu, 2001). Plants can synthesize ethylene as a secondary metabolite when exposed to biotic or abiotic stresses including high salt concentration, drought, and soil contamination by heavy metals, and attacks by pathogens (O'Donnell et al., 1996; Glick, 2005). However, ethylene concentrations above threshold can stun plant growth and reduce the efficiency of restoration (Mayak et al., 2004; Cheng et al., 2007).

Salinity is a natural phenomenon commonly found in arid and semiarid regions, but it can be induced by irrigation. In addition, about 20% of arable land and more than 50% of irrigated land worldwide, including Korea, are being influenced by salinity (Siddikee et al., 2010). Crops cultivated under high salinity, are induced to produce ethylene, which can either retard the plant growth or decrease the yield production or constrain the both (Egamberdieva et al., 2008).

The plant growth promoting rhizobacteria (PGPR), known as soil microbes that inhabit the rhizosphere community can stimulate plant growth and suppress diseases directly and/or indirectly (Glick, 1995; Kloepper et al., 2007). PGPR stimulate plant growth through the biosynthesis of plant growth promoting hormones

such as auxin and gibberellins. PGPR can control pest by secreting antibiotics such as 2,4-diacetylphloroglucinol (2,4-DAPG) and phenazine. They can also synthesize the hydrolytic enzymes such as β-glucanase and chintinase, which have antibiotic characteristics. PGPRs are also capable to produce the siderophore (Fe^{3+} specific-binding material) which chelats the ferric ion (Fe^{3+}) and constrains the growth of pathogenic microbes (Lee and Kim, 2001; Kim, 2004; Jang et al., 2009). In addition, PGPR have the nitrogen (N_2) fixation capability and convert the molecular nitrogen (N_2) into nitrate NO_3. Plants can consume nitrogen only in the form of nitrate and they can also promote plant growth by solubilizing the insoluble phosphate (Siddikee et al., 2010). They can also synthesize the plant growth regulation hormones such as cytokinin and abscisic acid which are directly involved in plant growth promotion (Glick, 2004; Jang et al., 2009).

Some PGPR strains have the capability to decrease the ethylene production. Such strain produces ACC deaminase which catalyzes the degradation of ethylene precursor, ACC into α-ketobutyrate and ammonium (Glick, 2004). If ACC deaminase producing PGPR exist in the rhizosphere, they will cleave the ACC into α-ketobutyrate and ammonia which results in the difference in the ACC concentration between the inside and outside the plant roots (Dimkpa et al., 2009). In order to maintain the balanced concentration of ACC, the plants expel more ACC from the inside to the outside. As a consequence, the ACC concentration inside the roots decreases, which can increase plant growth since the concentration of the stress molecule, ethylene, is reduced (Jalili et al., 2009).

Recent studies reported that ACC deaminase producing bacteria can survive well in high salt environment and that they help plants to reduce stress effects (Siddikee et al., 2011). The basic aim of the present study was to characterize the selected indigenous antagonistic bacteria and investigate the effects of plant growth promotion. The selected PGPR strains were analyzed to increase the salt stress tolerance as well as to promote the plant growth.

Materials and Methods

Antagonistic bacterial strains. The six PGPR strains were used in present study e.g. *Bacillus megaterium* KL39 (Jung and Kim, 2003), *Bacillus thuringiensis* BK4 (Jung et al., 2003), *Pseudomonas fluorescens* 2112 (Lee and Kim, 2001), *Pseudomonas maltophilia* AM5 (Kim, 2004), *Serratia proteamaculans* 3095 (Lee and Kim, 1999), and *Chryseomonas luteola* 5042 (Yun et al., 2001). All the selected strains were isolated from eco-friendly or low-chemical pesticide cultivated land with a variety of crops and were developed as antagonistic bacteria for microbial agent. *B. megaterium* KL39, *B. thuringiensis* BK4, *P. fluorescens* 2112, and *C. luteola* 5042 have antifungal activities against *Phyotophthora capsici* and/or *Fusarium oxysporum* by producing antibiotics (Lee and Kim, 2001; Yun et al., 2001; Jung and Kim, 2003; Jung et al., 2003). *P. maltophilia* AM5 and *S. proteamaculans* 3095 also have antagonistic mechanisms against plant fugal pathogens by having β-1,3 glucannase and chitinase activity (Lee and Kim, 1999; Kim, 2004). The selected PGPR strains were cultured in Luria-Bertani (LB) broth.

Determination of ACC deaminase activity. Qualitative analysis of ACC deaminase activity was performed as described previously (Jacobson et al., 1994). Bacteria were grown in the LB broth at 28°C for 24 h, and pellets were collected after centrifugation at 4,670 × g for 15 min. The pellets were thoroughly washed twice with 0.2 M phosphate buffer (pH 7.0) and were re-incubated in the DF salt minimal medium at 28°C for 48 h supplemented with 3 mM ACC (Sigma-Aldrich, USA) as the only N source (Glick, 1995). The ability of bacteria to utilize ACC was verified by maintaining the same cells as controls in the absence of $(NH_4)_2SO_4$ as N source. ACC deaminase activity was determined quantitatively by measuring the amount of α-ketobutyrate produced by the ACC deamination as described previously (Penrose and Glick, 2003). The concentration of α-ketobutyrate was determined by comparing the absorbance of sample to a standard curve of α-ketobutyrate (Sigma-Aldrich) at 540 nm. the enzyme activity was calculated based on the μmol of α-ketobutyrate released per mg of protein per h. Protein concentrations were determined according to Lowry et al. (1951).

Determination of auxin production. Auxin biosynthesis was estimated by a colorimetric technique using Salkowski reagent consisting of 27.6 mM $FeCl_3$ and 6.6 M H_2SO_4 (Lim and Kim, 2009). PGPR strains were incubated in King's B medium at 28°C in shaking incubator at 4,670 × g for 3 days. After centrifugation, the recovered supernatant was mixed with Salkowski reagent and kept in dark for 30 min and then examined by a spectrophotometer at 535 nm. The standard curve was constructed using a range of 1–45 μg/mL indole-3-acetic acid (IAA; Sigma-Aldrich).

Seed germination assay. Pepper seeds (*Capsicum annum* L., Buchon; Seminis Vegetable Seeds, Korea) were surface sterilized by soaking them in 70% ethanol for 1 min, followed by 2% NaOCl for 30 s. Bacteria grown in LB broth were centrifuged at 4,670 × g for 15 min and pellets were re-suspended in DF salt medium supplemented with 3.0 mM ACC as the sole N source and incubated for 48 h at 28°C to induce ACC deaminase activity. The cells were harvested, washed, and re-suspended in sterile 10 mM $MgSO_4 \cdot 7H_2O$ solution. The surface-sterilized pepper seeds were kept in the bacterial suspension (7.0×10^8 colony forming units (CFU)/mL) for 4 h. control treatment was conducted as same but the seeds were soaked in 10 mM $MgSO_4 \cdot 7H_2O$ solution instead of bacterial suspension. Twenty pepper seeds were used for each treatment. The seeds were transferred on sterilized filter papers, soaked with a solution of 0, 75, 120, and 175 mM NaCl in a Petri dish. The germination test was lasted for 4 days at 28°C and 70% humidity. The number of seeds sprouting was measured every day and the experiment was repeated three times.

***In vivo* pot trial.** Pepper seeds were surface-sterilized as described above and sown in the pot (one seed per hole) having 50 g of sterilized soil. The pots were kept at 28°C and a relative humidity of 50% with 12 h dark/light cycle with illumination of

5,000 lux until the five-leave stage (TKS 2, FloraGard, Germany). The pepper plants of same size and length were selected and repotted in 90.0×90.0×70.0 mm pots on 200 g of sterilized soil. After application of exogenous salt stress for one week, plants were treated every 3 days with 50 mL of NaCl solution (0, 75, 120, and 175 mM). Each bacterial strain was incubated as described above, centrifuged and pellets were collected. The pellets were re-suspended in 10 mL of sterile distilled water and inoculated with 7.0×10^8 CFU/mL per pot by irrigation. The salt-treated plants were the negative control and the de-ionized distilled water-treated seeds were the positive control. The seedlings were irrigated with 50 mL of sterile distilled water every 3 days. All treatments were repeated three times with 20 plants per experiment, and the plants were observed for visible symptoms of stress reaction under the salt stress condition. Symptoms included withering or drying of the leaves and stem. The biomass of pepper plants was measured after 3 weeks from the high-salt stress treatment and dry mass was determined by completely drying the plants at 70°C.

Colonization of *P. fluorescens* 2112 in pepper rhizosphere. The soil in the rhizosphere of the red pepper after treating with *P. fluorescens* 2112 was recovered and colonization of *P. fluorescens* 2112 was monitored for 3 weeks. All experiments were carried out simultaneously and the effects of *P. fluorescens* 2112 on promoting pepper growth and inducing the resistance of pepper under the salt stress of 75, 120, and 175 mM NaCl were measured. Plants were randomly selected and one plant was rooted from each treatment group and the soil that adhered to the plants was carefully dusted off. The viable cell count in 10 g of the soil was measured using pseudomonas isolation agar (PIA, BD, Franklin Lakes, USA) dilution plating method since the colonies of *Pseudomonas* species in PIA media could be easily distinguished visually on green color.

Statistical analysis. The data are shown as the means ± SD. Significant difference was verified Duncan's multiple range test at 95% confidence level. Statistical analyses were performed using SAS software version 8.2 (SAS Institute, Inc., USA).

Results and Discussion

ACC deaminase activity. All the selected strains were capable to utilize and grow in the DF salt medium with ACC as the sole N source. The selected strains showed variation in their ACC deaminase activity (Table 1). Among these strains, *P. fluorescens* 2112 exhibited maximum ACC deaminase activity (6.20 μM). The other five strains showed lower growth rate compared to the growth in the medium containing $(NH_4)_2SO_4$ as the N source. The activities of *B megaterium* KL39, *B. thuringiensis* BK4, *P. maltophilia* AM5, *S. proteamaculans* 3095, and *C. luteola* 5042 were 0.68, 0.73, 0.66, 0.70, and 0.59 μM, respectively. Therefore, *P. fluorescens* 2112 was used in subsequent experiments to assess the effect of high salt concentrations on seed germination and growth of red pepper plant growth under the high salt condition. The ACC of roots is catabolized by ACC deaminase to α-ketobutyrate and ammonia. The bacteria utilize the NH_3 evolved from ACC as a source of N and thereby restrict the accumulation of ethylene within the plant, which otherwise inhibits plant growth (Glick, 2005). Therefore, the selected multi-functional PGPR may have great potential for use in developing microbial agents with potent antagonistic mechanisms and ACC deaminase activity.

Table 1 Production of ACC deaminase and auxin by the selected strains

Strains	ACC deaminase activity (μM α-ketobutyrate mg^{-1} protein h^{-1})	Auxin production (μg/mL)
B. megaterium KL39	0.68±0.05	1.89±0.21
B. thuringiensis BK4	0.73±0.21	0.66±0.14
P. fluorescens 2112	6.20±1.26	13.3±0.52
P. maltophilia AM5	0.66±0.05	2.56±0.60
S. proteamaculans 3095	0.70±0.06	1.55±0.48
C. luteola 5042	0.59±0.06	0.75±0.11

Values are means ± SD of three replications.

Auxin production. Auxin is group of phytohormones and IAA is major phytohormone, which stimulates root elongation and cellular division (Kloepper et al., 2004). All the tested multi-functional PGPRs showed a great variation in the synthesis of auxin indole-3-acetic acid (IAA), ranging from 0.75–13.3 μg/mL (Table 1). Among the six PGPRs, *P. fluorescens* 2112 produced the high amount of IAA (13.3 μg/mL). IAA produced by PGPRs affects many plant growth and development activities. IAA is widely used in agricultural field for plant growth and development as well as for high productivity (Mayak et al., 1999; Li et al., 2000). In the present study, multi-functional PGPR *P. fluorescens* 2112 displayed exuberant auxin productivity. The resulting antagonistic and plant growth promotion mechanisms might be beneficially exploited as mutually synergistic agents in crop cultivation.

Seed germination assay. High salt concentrations have adverse effect on seed germination and seedling vigor. The rate of seed germination decreased with increasing concentrations of salt. Almost all pepper seeds of the positive control (without salt stress) germinated in 48 h (98.2%), whereas the rate of seed germination was reduced up to 18.3% by 75 mM NaCl (Table 2). At 120 and 175 mM NaCl, seed germination was also inhibited up to 62.1 and 79.7%, respectively (Table 2). But, plants inoculated with *P. fluorescens* 2112 displayed increased seed germination (19.9 and 69.0% at 120 and 175 mM NaCl, respectively; Table 2).

Seed germination is inhibited under high salt concentration (Glick, 2005). In this study, the ACC deaminase producing PGPR *P. fluorescens* 2112 reduced the repressive effect of salt stress on seed germination. *P. fluorescens* 2112 is capable to produce IAA (13.3 μg/mL). IAA produced by PGPR may stimulate root elongation and cellular division and enhance seed germination under salt stress (Mayak et al., 2001; Patten and Glick, 2002).

Table 2 Effect of *P. fluorescens* 2112 on germination of pepper seed under different salt levels

Treatments	Salt concentration (mM)	Germination rate (%)*			
		24 h	48 h	72 h	96 h
No treatments	0	98.2±0.6^{a}	98.2±0.6^{a}	98.2±0.6^{a}	98.2±0.6^{a}
	75	72.5±2.8^{b}	81.7±3.1^{a}	94.5±3.3^{a}	94.5±3.3^{a}
	120	36.1±2.6^{c}	36.1±5.2^{c}	51.0±3.8^{bc}	51.0±3.8^{bc}
	175	8.9±0.9^{d}	18.5±1.5^{c}	36.2±4.1^{c}	36.2±4.1^{c}
P2112 treatments	0	98.7±0.4^{a}	98.7±0.4^{a}	98.7±0.4^{a}	98.7±0.4^{a}
	75	80.1±3.1^{a}	96.8±1.9^{a}	96.8±1.9^{a}	96.8±1.9^{a}
	120	56.2±1.8^{bc}	78.3±2.0^{a}	96.3±1.2^{a}	96.3±1.2^{a}
	175	12.2±1.8^{cd}	29.2±1.7^{c}	53.6±2.8^{bc}	53.6±2.8^{b}

*Values are means ± SD of three replications. Different lowercase superscripts in the same column represent significant differences verified by Duncan's multiple range test at 95% confidence level.

Table 3 Effect of *P. fluorescens* 2112 on growth of pepper plants under high salt levels

Treatments	Salt concentration (mM)	Length (cm)		Fresh weight (g/plant)	Dry weight (g/plant)	Average growth reduction (%)*
		Shoot	Root			
No treatments	0	17.20±0.61^{b}	15.32±0.37^{ab}	28.43±0.21^{b}	1.27±0.06^{b}	
	75	11.54±0.53^{d}	8.21±0.42^{d}	21.27±0.14^{c}	0.85±0.11^{d}	34.39
	120	8.75±0.61^{e}	6.02±0.08^{e}	16.73±0.52^{d}	0.78±0.02^{d}	47.39
	175	7.30±0.28^{e}	5.93±0.11^{e}	12.26±0.41^{e}	0.42±0.02^{e}	60.06
P2112 treatments	0	18.78±0.61^{a}	16.21±0.37^{a}	32.12±0.13^{a}	1.57±0.06^{a}	12.8 increased
	75	15.87±1.22^{c}	14.91±0.20^{c}	25.81±1.21^{b}	1.16±0.02^{b}	7.07
	120	13.21±1.75^{cd}	11.52±0.57^{d}	23.78±3.07^{bc}	0.97±0.03^{c}	21.99
	175	7.64±0.05^{e}	5.92±0.15^{e}	12.92±1.28^{d}	0.47±0.01^{e}	58.62

*Average overall growth reduction of red pepper at each level of salt stress.
*Values are means ± SD of three replications. Different lowercase superscripts in the same column represent significant differences verified by Duncan's multiple range test at 95% confidence level.

***In vivo* pot trial.** The growth of pepper plants under high concentration of salt with or without inoculation of *P. fluorescens* 2112 was compared with the positive control (only water treated). The results are summarized in Table 3. Average growth of the red pepper plants was deceased by 34.39 and 60.06% at 75 and 175 mM NaCl, respectively (Table 3). Since the overall reduction of pepper growth was about 47.39% at 120 mM NaCl, the respective concentration of salt was used in subsequent experiments. Inoculated shoots and roots treated with 120 mM NaCl grew up to a height of 13.21 and 11.52 cm, respectively, whereas uninoculated shoots and roots grew up to a height of 8.75 and 16.02 cm, respectively after 3 weeks of time duration. (Table 3 and Fig. 1). The survival rate of the pepper plants was approximately 80% in the presence of high salt concentration and inoculated with *P. fluorescens* 2112 in the course of 5 weeks duration in the experiment. In contrast, none of the plants survived in the absence of *P. fluorescens* 2112. These results showed that ACC deaminase producing *P. fluorescens* 2112 have a great capability to alleviate the negative effect of high salt stress on the growth and development of red pepper plants.

In addition, *P. fluorescens* 2112 had a positive effect on red peppers plants vigor as compared to control. Without stress condition (0 mM NaCl), the red peppers treated with *P. fluorescens* 2112 had a 12% grew higher in term of shoot and root height, and plant weight compared to the water treated pepper.

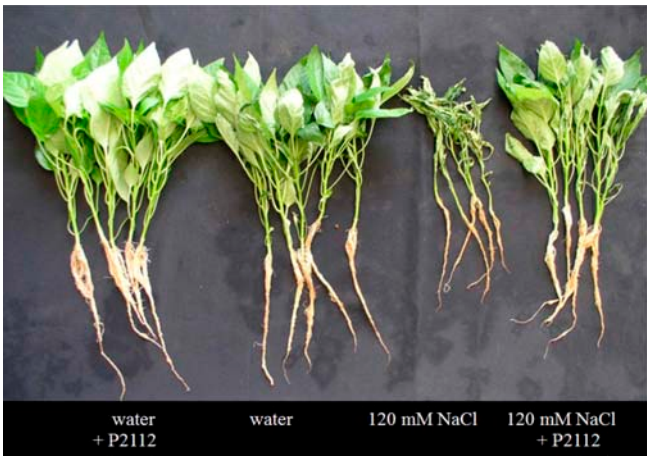

Fig. 1 Effect of *P. fluorescens* 2112 inoculation on pepper growth at high salt concentration. Fifty milliliters of 120 mM NaCl was applied at 3-day intervals, and *P. fluorescens* 2112 was treated once with 7.0×10^{8} CFU/mL per pot. Positive control was watered every 3 days with 50 mL of sterile water. The picture was taken at the age of 3 weeks.

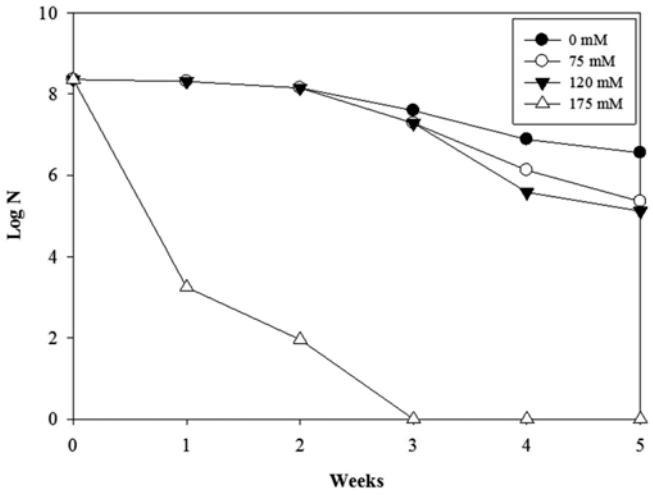

Fig. 2 Root colonization by *P. fluorescens* 2112 inoculation in the red-pepper pots. Fifty milliliters of 120 mM NaCl was applied at 3-day intervals, and *P. fluorescens* 2112 was treated once with 7.0×10^8 CFU/mL per pot.

PGPR have been reported to promote plant growth through either direct or indirect mechanism. Indirect mechanisms include prevention of phytopathogens through the production of antibiotics, extracellular hydrolytic enzymes and siderophores (Mayak et al., 1999). Direct mechanisms include providing plants with phytohormones, fixed nitrogen, soluble phosphate, or iron through bacterial siderophores, or ACC deaminases that can reduce the levels of stress induced ethylene in plants (Glick, 2004). Plants as well as plant associated microbes including PGPRs can produce plant growth phytohormones, e.g., production of indole-3-acetic acid (IAA), cytokinins, and gibberellins, which induce the plant growth. Besides these hormones, improved nutrient acquisition is involved in direct growth promotion. Plant-associated microorganisms can supply macronutrients and micronutrients (Kloepper et al., 2004).

Plant tissues increase the ethylene concentration under environmental stresses such as drought, heavy metals and salinity (Jang et al., 2009). A high concentration of ethylene in plants can inhibit plant growth and the ACC deaminase produced by PGPR can reduce the plant's ethylene concentration by cleaving the ethylene precursor ACC. Consequently, plant growth is stimulated. ACC deaminase producing PGPR induce plant tolerance under environmental stresses such as soil contamination by heavy metals, drought, high salinity and osmotic stress (Nadeem et al., 2007; Saravanakumar and Samiyappan, 2007). In this study, multi-functional PGPR *P. fluorescens* 2112 producing ACC deaminase and auxin could induce the resistance of pepper.

P. fluorescens 2112 suppresses *Phytophthora* blight disease caused by *Phytophthora capsici* by producing antibiotics 2,4-DAPG and siderophore (Lee and Kim, 2001). The present results could prelude the development of microbial agents for organic farming of pepper plants in saline environments, specifically multi-functional PGPR *P. fluorescens* 2112, and their use in the efficient induction of resistance to both biotic and abiotic stresses that can occur during the cultivation of pepper plants.

Colonization of *P. fluorescens* 2112 in the pepper rhizospheres. After treating the red pepper plants with the *P. fluorescens* 2112, stressed with 0, 75, 120, and 175 mM NaCl, the root of the red pepper rhizosphere was recovered to confirm colonization of *P. fluorescens* 2112. At 0–120 mM salt stress condition, the initial cell concentration was maintained at 7.0×10^8 CFU/g for 2 weeks. However, the cells concentration decreased to 1.54×10^2 CFU/g after 2 weeks, and the treated cells were not detected at 175 mM salt (Fig. 2). These results indicate that *P. fluorescens* 2112 successfully colonized in the rhizosphere of red peppers and the PGPR strain induced resistance.

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