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Some Characteristics of a Plant Growth Promoting *Enterobacter* sp. Isolated from the Roots of Maize

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ABSTRACT

Some properties of an *Enterobacter* sp. isolated from the roots of maize are described. Isolation was carried out using the semisolid enrichment culture technique and subsequent plating, both on nitrogen free biotin medium. Morphological, biochemical and phylogenetic characterization using the MicroSeq™ 16S rDNA technique were employed in identification of isolate, which was revealed to be closest matched at 99.4% with *Enterobacter asburiae.* The isolate possessed properties of plant growth promoting bacteria. Thus, it produced indole-3-acetic, plant polymer hydrolyzing enzymes, pectinase and cellulase as well as ammonia *in vitro*. The isolate grew well in the presence of both 3% NaCl and 10 µg of streptomycin. In plate bioassays, isolate promoted the germination of both maize and rice seeds as well as root and lateral root growth resulting in weight increases of seedlings over their controls. Experiments to quantify ability of isolate to promote plant growth was performed using hydroponics solutions and as appropriate, an inoculum of the isolate. Pot experiments were also employed. Results from these studies showed that isolate enhanced nitrogen accumulation and significantly ($P < 0.05$), improved the growth of maze seedlings over controls. Isolate has potential for utilization as inocula for sustainable production of cereals.

Keywords: Cereals; Plant Growth Promoting *Enterobacter*

1. Introduction

Plant growth promoting rhizobacteria (PGPR) achieve their effect by diverse mechanisms as recently reviewed by Lugtenberg and Kamilova [1]. The supply of nutrients such as nitrogen is improved through various mechanisms of biological nitrogen fixation (BNF), while phosphate and potassium are made available through solubilization of their insoluble forms, often abundant in the soil. PGPR may also provide plants with essential vitamins. Other mechanisms employed to promote plant growth include plant growth regulating effects (phytohormones), both positive and negative, induced systemic resistance to microbial pathogens and siderophore production aiding plant nutrition by chelation. These organisms have also been reported to enhance plant growth through miscellaneous beneficial effects such as osmotic adjustment, stomatal regulation and modification of root morphology, which may lead to enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism [1].

Research on BNF, particularly the Rhizobium-legume symbiosis has made significant progress. Biofertilizer technologies for the increased agricultural production of legumes based on these research findings are today considered essential ingredients for sustainable agriculture. However, cereal crops like rice, maize and wheat remain by far the world's most important sources of food, both for direct human consumption and indirectly, as inputs to livestock production [2]. According to Charpentier and Oldroyd [3], nitrogen fixing cereals would be the breakthrough necessary to underpin sustainable food production for 9 billion people, the projected population of the world by 2050.

Research efforts employing molecular biology and genetic manipulation techniques are ongoing to induct nodular symbiosis between non-legumes on the one hand and the rhizobia and other free-living atmospheric nitrogen fixers on the other. While this procedure appears to be in the future, recommendable BNF technologies for some cereals, such as rice have been achieved using various rhizospheric bacteria, particularly members of the genus *Azospirillum.* Several biofertilizer formulations, for example, Mazospirflo-2™ (Soygro Ltd, South-Africa), containing *Azospirillum brasilense* or combinations of genera such as *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Zoogloes* as in Biopower™ (Nuclear Institute for Biotechnology and Genetic Engineering, Pakistan), are being marketed successfully today. Nonetheless, the pro-

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blem of low efficiency of these inoculants persists, requiring often, supplementation with inorganic fertilizer. As a consequence, the use of this class of microbial inoculants in the field has not been extensive**,** underscoring the need for sustained search for more efficient organisms.

We report here, some characteristics of an *Enterobacter* sp., which promoted the germination and growth of maize and rice seedlings under laboratory and green house conditions. This organism has the potential for direct exploitation or the contribution of genes for the engineering of hybrids suitable for the formulation of biofertilizers for cereals.

2. Materials and Methods

2.1. Isolation of Rhizosphere Bacteria

The roots of maize (*Zea mays*) plants in their vegetative stage of growth (about two month-old), were harvested from a farm on our campus at Awka, Nigeria (6.22N; 7.07E). Roots were returned to the laboratory in sterile polyethylene bags and adherent soil removed by rinsing in serial baths of tap water. The isolation of *Azospirillum* sp. was the original purpose of this study and thus the semisolid enrichment culture technique as compiled by Bashan *et al*. [4], was employed with minor modifications and performed as follows. Washed roots were cut into 3 cm segments and disinfected by soaking in 70 % ethanol for 5 min, in 6.25 % sodium hypochlorite for 10 min, followed by several rinses in sterile distilled water. Intact root pieces (0.5 to 1.0 cm) were then placed into tubes of semisolid (0.05%) nitrogen free biotin medium (NFb) and incubated without shaking for 5 days at 30˚C. This medium was composed (g/l) of: DL-malic acid 5; KOH 4; K_2 HPO_4 0.5; $MgSO_4 \cdot 7H_2O$ 0.2; $CaCl_2$ 0.02; NaCl 0.1; $FeSO_4 \cdot 7H_2O$ 0.5; Agar 5 g and (mg/l) of: $NaMoO_4 \cdot 2H_2O$ 2; $MnSO_4 \cdot H_2O$ 10. The medium also contained 2 ml of 0.5 % solution of bromothymol blue in 95% ethanol and had a final pH of 6.8.

White pellicles from tubes, which showed this characteristic feature, were subcultured onto solid semi selective, Congo red-NFb medium for *Azospirillum* [5]. This medium was similarly composed as the enrichment medium, above but contained 2% agar and additionally, 0.05 g/l yeast extract and 15 ml/1 of 1:400 aqueous solution of Congo-red. The later was autoclaved separately and added just before plates were poured.

Red to scarlet colonies suggestive of *Azospirillum* was purified by repeated streaking on same medium. One isolate, MR5, was selected for further studies on the basis of its exceptional luxuriant growth on Congo red-NFb medium when compared with the other isolates. This isolate was stored on nutrient agar slopes at 4˚C during the period of the study.

2.2. Morphological and Biochemical Characterization of Selected Isolate

The shape, dimensions and motility of PGPR, in addition to the range of carbohydrate sugars they utilize are important determinants of their ability to colonize plant roots. Therefore, the colony and then, the cellular morphology of pure cultures of the isolate were determined by light microscopy. Some standard biochemical tests for Gram negative bacterial genera including catalase, O/F of D-glucose and hydrolysis of gelatin were performed. The ability of the isolate to utilize various carbohydrates such as the disaccharide, sucrose, the trisaccharide, raffinose, the pentose, xylose, the alcohol, mannitol and the polysaccharide starch, were also determined by growing isolate in minimal salts medium comprised, $NH_4H_2PO_4$ 1 g; KCl 0.2 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; Agar 10 g, Distilled water 1 L and 4 ml 0.2% solution of bromothymol blue. The medium was supplemented with separately sterilized, 1% (wt/vol) of the appropriate carbon substrate.

2.3. Phylogenetic Characterization and Identification of Isolate

The isolate was identified by the Bacterial 500bp Standard technique using the MicroSeq™ 16S rDNA kit at the National Collections of Industrial and Marine Bacteria Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen. AB21 9YA (NCIMB). The 16SrDNA sequence analysis was carried out using the NCIMB Ltd. internal work instructions; WI-NC-58 rev 6, 138 rev 4, 147 rev 13, 149 rev 8, 191 rev 2, 198 rev 0, 214 rev 1, 215 rev 3, 226 rev 0 and 253 rev 1. The resulting sequence data were aligned and compared with the 16S rRNA sequence database of the NCIMB Microseq Database and the public data base European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany.

2.4. Determination of the Ability of Isolate to Promote Plant Growth

There are no efficient high-throughput assays systems for the selection of superior strains of plant growth promoting bacteria. A combination of bioassay methods were employed for this purpose during this work. The modified plate bioassay [6] for the ability to promote the germination and growth of seedlings was done as follows. Maize seeds, *Zea mays* (T2BR Comp 2-W), kindly provided by the International Institute for Tropical Agriculture, Ibadan, Nigeria (IITA) and rice (FARO 44), purchased locally were employed for this test. Seeds were selected on the basis of their uniformity in weight and surface sterilized with 95% ethanol and 2.5% sodium hypochlorite, then washed several times with sterile distilled water before each test. Growth of the seed-

lings were quantified by measurement of root and shoot weights.

Plant polymer hydrolyzing activities using cellulase and pectinase were qualitatively determined using plate assays. For the cellulase assay, NFb plates supplemented with 0.25% carboxymethyl cellulose (CMC) were inoculated with isolate. After incubating for 48 h at 30˚C, the plates were overlaid with 1 mg ml^{-1} Congo red solution for 30 min [7]. Congo red solution was then poured off followed by washing the surface of the plate with 1 M NaCl solution. Pectinase activity was determined by growing isolates on yeast extract pectin medium, composed g/L^{-1} distilled water of; Pectin 5.0; KH_2PO_4 4.0; Na_2HPO_4 6.0; yeast extract 1.0 and agar 18.0. After incubation at 30˚C for seven days, iodine-potassium iodide solution (1.0 g iodine, 5.0 g potassium iodide and 330 ml H_2O) was added to detect clearance zones [8].

Indole-3-acetic acid (IAA) production was determined by the method of Loper and Scroth [9], while ammonia and hydrogen cyanide production were determined respectively by methods described by Cappucino and Sherman [10] and Lock [11].

Isolate was screened for tolerance for 3% NaCl by streaking two lines in a cross fashion across the surface of nutrient agar plates in which NaCl concentration was adjusted to 3%. Controls were set up on plates without added salt. Streptomycin resistance was determined by routine disk (10 µg) diffusion assay on nutrient agar.

To determine the ability of the isolate to enhance accumulation of nitrogen *in planta*, and to compare its efficiency to promote plant growth against the NPK fertilizer, the following experiment was set up using the completely randomized design. Maize seeds were grown in hydroponics solutions, which lacked or contained the mineral nutrients nitrogen (N), phosphorus (P) and potassium (K) and as appropriate an inoculum of the test organism composed of 10^7 cfu/ml washed cells. Treatments were as follows: T_0. Distilled water (DW), T_1. DW + Inoculum, T_2. DW + P + K, T_3. DW + N + P + K, and T_4. DW + Inoculum + P + K. All treatments were replicated five times. Solutions containing P + K were composed of 125 mg/L of K_2HPO_4 (250 ppm, K and 100 ppm, P), while the N + P + K solutions contained additionally, 391 mg/L NH_4NO_3 (230 ppm, N).

Maize seeds, selected and sterilized as has been described earlier, were allowed to germinate and grow on the hydroponics solutions for a period of 14 days. The plants were carefully harvested and roots and shoots were separated. These were weighed separately, and results obtained were subjected to Analyses of Variance (ANOVA). Shoots from the DW and the DW + Inoculum treatments were subsequently dried to constant weight at 50˚C - 60˚C in an air-circulating oven and their nitrogen contents per 100 mg of plant material determined by Kjeldahl digestion [12].

2.5. Effect of Inoculation of Isolate on Growth of Maize Seedlings in Pot Experiments

Soil for this experiment was sandy loam in nature with a pH of 4.5 and contained 1.112 g/Kg total N. About 10 kg of soil was thoroughly mixed and distributed in 1 Kg portions into polyethylene bags. The variety of maize already described was used as test plant. The experimental design was the completely randomized design with five replicates. Treatments were: T_0 control (No inoculation) and T_1 (Seed inoculated by soaking in culture of isolate containing about 10^7 cfu/ml washed cells for one hour. The plants were placed in a green house and watered regularly for 45 days. Plants were carefully up rooted at the end of this period, washed clean of sand particles and dried at 50˚C for 72 h. The weights of their shoots and roots were recorded. The results were subjected to analysis of variance (ANOVA) and the Tukey HSD (0.05) test.

3. Results

3.1. Morphological and Biochemical Characteristics of Isolate

Colonies on Congo red-NFb medium were luxuriantly growing, 3 - 5 mm diameter, pink colonies with a raised elevation after 72 h incubation at 30˚C. Edges of the colonies usually became irregular with time. On nutrient agar, 30˚C/48 h, the isolate grew as 3 - 4 mm mucoid colonies, with a convex elevation and entire edges. On prolonged incubation the organism swarmed actively, covering the surface of the agar plate. Microscopy revealed isolate as Gram negative motile short rods. Biochemical characteristics of the isolate are given in **Table 1**.

Table 1. Some biochemical characteristics of isolate.

Test	Result
Catalase	+
Oxidase	-
Citrate utilization	+
Fermentation/oxidation of glucose	+
Sucrose utilization	+
Raffinose	+
Xylose	+
Mannitol	+
Starch hydrolysis	+
Gelatinase	-

3.2. Phylogenetic Characteristics and Identification

Contrary to expectation, phylogenetic characterization of isolate MR5 revealed that it was not an *Azospirillum*. The "10 top hits" from the comparison of its 16S rRNA sequence data using the NCIMB Microseq Database is shown in **Table 2**. This revealed that isolate MR5 or NCOO1927-MR5 as designated by the NCIMB was most closely matched with *Enterobacter asburiae* (99.4%). This isolate also matched *Enterobacter* sp. and *Enterobacter cloacae*, ATTC 13047 at 98.97 %, respectively on this database. However, comparison of this sequence with the EMBL database produced a 100 % match with both *Enterobacter* sp. and the type strain *Enterobacter cloacae*, ATTC 13047.

Figure 1 shows the phylogenetic relatedness of *Enterobacter* sp.-NCOO1927-MR5 to some type strains and other similar bacteria constructed using the neighbor joining technique and the NCIMB database. This figure suggests a closer relationship between this isolate and *Enterobacter asburiae* than with *Enterobacter cloacae.*

3.3. Results of Tests for Ability of Isolate to Promote Plant Growth

The isolate promoted the germination of both maize and rice seeds in the plate bioassays. Inoculated maize showed 100% germination, compared to 80% in the controls, while inoculation improved germination of rice seeds from 70% in the controls to 90% in the tests. Inoculation with our isolate promoted root growth, including lateral root development of tested seedlings, resulting in weight increases over their controls at the average rates of 43% for maize and 37% for rice. Similar increases were observed for shoot weights, 35% in maize and 40% in rice.

The isolate produced the plant polymer hydrolyzing enzymes, cellulase and pectinase and 20 µg/ml of indole acetic acid. It also produced ammonia, but failed to produce hydrogen cyanide. Isolate also grew well in nutrient agar containing 3% NaCl and showed resistance to 10 µg streptomycin.

Shoots of maize seedlings grown in the presence of inoculum showed a higher N accumulation than shoots grown without it (**Table 3**). Furthermore, growth of maize seedlings, measured as means of weights of shoots and roots, were comparable in the DW + N + P + K and the DW + Inoculum + P + K treatments but differed signifycantly from seedlings grown in the DW and the DW + P + K treatments at $P < 0.05$.

3.4. Effect of Inoculation of Isolate on Growth of Maize Seedlings in Pot Experiments

Means of shoot weights for maize seedlings, 0.56 ± 0.10 for the control and 0.818 ± 0.08 for test were significantly different at $P < 0.05$. Even though mean root

Table 2. Ten closest matches with *Enterobacter* sp.-NCOO1927-MR5 (MicroSeq™ 500).

Sequence Name	% Match	Sequence Name	% Match
Enterobacter asburiae	99.4	*Enterobacter cancerogenus*	98.92
Enterobacter hormaechei	99.27	*Enterobacter kobei*	98.92
Enterobacter pyrinus	99.03	*Leclercia adecarboxylata*	98.83
Enterobacter cloacae cloacae	98.97	*Klebsiella pneumoniae pneumoniae*	98.63
Enterobacter cloacae dissolvens	98.97	*Citrobacter youngae*	98.6

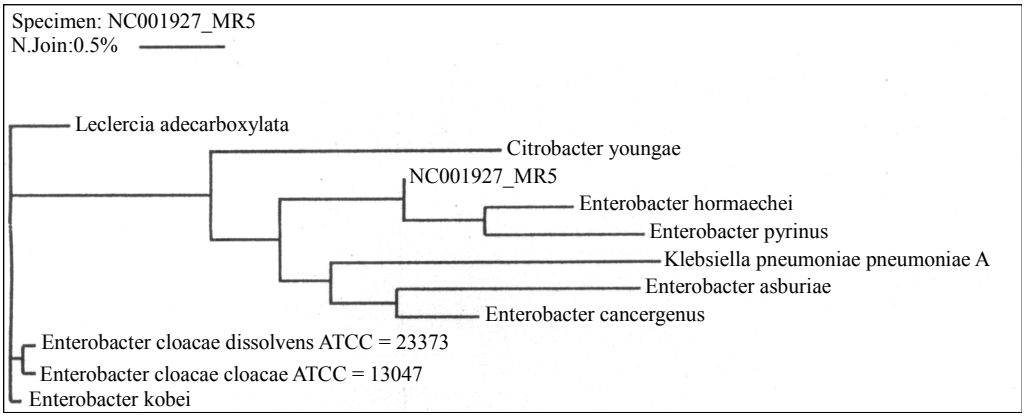

Figure 1. Phylogenetic tree showing relatedness of *Enterobacter* sp.-NCOO1927-MR5 to similar bacteria.

Table 3. Nitrogen composition and mean weights of shoots and roots of maize seedlings grown in hydroponics solutions containing different combinations of mineral elements and inoculum of isolate.

Treatments	DW	DW + Inoculum	DW + P + K	DW + N + P + K	DW + Inoculum + P + K
N Composition of shoot	0.49%	0.55%	-	-	-
Mean root weight	$0.068^{a} \pm 0.05$	$0.208^{b} \pm 0.07$	$0.144^{a} \pm 0.06$	$0.28^{b} \pm 0.07$	$0.248^{b} \pm 0.09$
Mean shoot weight	$0.23^{a} \pm 0.05$	$0.454^{a} \pm 0.04$	$0.342^{a} \pm 0.12$	$0.668^{b} \pm 0.34$	$0.608^{b} \pm 0.08$

weight of control seedlings, 1.03 ± 0.09 was not statistically different from that of test roots, 1.34 ± 0.27, there was nevertheless, an increase of about 30.1% in growth of root material.

4. Discussion

The definitive identification of *Enterobacter* sp.-NCOO-1927- MR5 reported by the NCIMB was *Enterobacter* sp. This is because the 16SrDNA sequence of this isolate matched different species on the different databases, NCIMB and the public EMBL consulted. In terms of DNA-relatedness, Grimont and Grimont [13] have described *E. cloacae* as heterogeneous with many groups. These authors in fact stated that *E. asburiae* belongs to one of the *E. cloacae* groups. Further studies are needed to elucidate the identity of this isolate and indeed, the ambiguities in the taxonomy of the *Enterobacter*. For the rest of this report, this isolate is simply designated as *Enterobacter* sp.-NCOO1927-MR5.

Enterobacter sp.-NCOO1927-MR5, shared many morphological and biochemical characteristics with earlier isolates of *E. asburiae* [14,15]. Notable among these characteristics are their motility, production of, cellulase and pectinase enzymes as well as the ability to utilize a wide range of sugars. Flagellar motility in bacteria is an important factor in the colonization of plant roots, especially their interiors [16]. Cellulase and pectinase enzymes act as virulence factors for pathogenic bacteria of plants and are believed to be involved in the invasion of host plants by PGPR, as reported for *E. asburiae* JM22 [17]. The possession of these characteristics may have contributed to the ability of this isolate in colonizing roots of tested seedlings.

Plants are known to exude a wide range of substances [18]. The ability of *Enterobacter* sp.-NCOO1927-MR5 to utilize a wide range of sugars, may contribute to the success of this species in adaptation to roots of different plants. Generally, the *Enterobacter* genus is one of the most common genera of bacteria isolated as plant endorhizosphere bacteria. They have been found as endorhizosphere bacteria in maize, rice, cotton, cucumber, common bean, broccoli and sweet potato, to list but a few [19-22].

The efficiency of PGPR is critically dependent, among others, on their ability to tolerate saline conditions prevalent in many soils, as well as resist numerous antibiotics produced by competing flora. As has been variously reported [21,22], *Enterobacter* sp.-NCOO1927-MR5 was shown in this study to tolerate 3% NaCl as well as resist inhibition by the antibiotic, streptomycin. The production of ammonia is frequently reported for PGPR, a process most probably resulting from the deamination (ammonification) of the amino acids present in the peptone used for this assay. It has been suggested that ammonia may have a role in antagonism against competing flora, particularly the fungi [22,23].

Some of the *Enterobacter asburiae* isolated earlier were characterized as human pathogens [24] and there had been expression of concerns for safety of human health. However, the majority of strains reported recently has been isolated from environmental sources and have been studied for their various beneficial activities. Such activities include the promotion of plant growth [21,25,26], biocontrol of plant diseases [22,23] and even the conversion of carbohydrate compounds in acid hydrolysates of hemicellulose into ethanol and other fermentation products [15]. These reports have raised the profile of *E. asburiae* as an industrial microorganism.

Enterobacter sp.-NCOO1927-MR5 demonstrated very good potentials for the promotion of plant growth. Infection and promotion of the growth of both maize and rice seeds in the plate bioassays indicate cross-fection abilities. Lack of host specificity is a good attribute for microbial inocula, which may thus be used for the cultivation of a wide range of crops. Zakria *et al*. [21] earlier reported that *Enterobacter* sp. strain 35 isolated from sugarcane successfully colonized and promoted growth of *Brassica oleracea* (broccoli), a dicot. The production of high levels of the plant growth hormone (auxin), IAA *in vitro* is noteworthy. Recent findings have revealed that auxin biosynthesis plays essential roles in many developmental processes in plants including gametogenesis, embryogenesis, seedling growth, vascular patterning, and flower development [27]. IAA production is a major tool employed by PGPR.

Nitrogen is the most critical of the three major elements required for plant growth. The lack of a significant difference in growth of maize seedlings in the hydroponics solutions containing DW and DW + P + K (**Table 3**)

is in agreement with this claim. The relatively higher concentration (per 100 mg of dry plant material) of nitrogen in maize seedlings grown with inoculum compared with those grown without it indicate that *Enterobacter* sp.-NCOO1927-MR5 enhanced nitrogen accumulation in maize as been reported earlier for *E. asburiae* [26]. Lack of facilities did not permit the demonstration of nitrogenase activity or the presence of *nif* genes in this isolate. It is noteworthy however, that *E. asburiae*, is reported to lack the *nif* genes [20,26] but may contribute nitrogen to its host plant by enhancing its uptake. *E. cloacae* on the other hand, is reported to possess these genes [28,29]. Enhancement of nitrogen uptake or its fixation, as well as other mechanisms discussed earlier would probably account for the significant difference, at $P < 0.05$ between weights of inoculated and uninoculated maize seedlings during this study (**Table 3**). Furthermore, the lack of a significant difference in growth of maize seedlings in hydroponics solutions containing DW + N + P + K and DW + Inoculum + P + K suggest comparable efficiency in plant-growth-promoting activity of our isolate with nitrogen chemical fertilizers.

Improvement in growth rate of maize in pot experiments following inoculation with our isolate is comparable with earlier reports. Morales-García *et al.* [22] reported that maize plants inoculated with *Enterobacter* sp. UAPS03001 showed statistically significant greater biomass than the controls under environmental chamber conditions. In field experiments, this species was reported to have also, significantly improved total kernel biomass. Zakria *et al*. [21], have shown that under glasshouse conditions, *Brassica oleracea* (broccoli), a dicot plant inoculated with *Enterobacter* sp. strain 35 had a significantly greater fresh weight than uninoculated plants. Rogers *et al*. [26] obtained similar results under field conditions with hybrid poplar (a short rotation energy crop), after plants were inoculated with *Enterobacter*.

5. Conclusion

Results obtained during this study show that *Enterobacter* sp.-NCOO1927-MR5 has abilities to promote growth of maize. Mechanisms employed for this purpose included enhancement of accumulation of nitrogen and the production of IAA among others. This organism therefore has the potential to promote growth of cereals. It is noteworthy that members of the genus *Enterobacter* are currently in use for biocontrol purposes. Isolation or engineering of species combining these two characteristics will enhance the sustainable production of cereals. The taxonomy of the *Enterobacter* desires further attention to enable a more precise evaluation of the industrial and agricultural potentials of this genus.

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