SCREENING AND IDENTIFICATION OF NEW TYPES OF EXOPOLYSACCHARIDES-PRODUCING LACTIC ACID IN THE INNER MONGOLIA DAIRY PRODUCTS

– Research paper –

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Abstract: Exopolysaccharides (EPS) is a type of polysaccharide produced by lactic acid bacteria (LAB) that can be directly used in foods to make the products more excellent. Therefore, batch studies were performed to explore the effect of different LAB on the production of EPS and antioxidant activity. Five strains with high EPS yield and antioxidant activity were screened out from 66 strains isolated from Tibetan dairy products. The results show that EPS production of the five strains (B55, B62, B30, 7830 and K2) were 110.66, 145.48, 132.78, 122.11 and 111.72 mg·L⁻¹, respectively, and they have a higher DPPH free radical scavenging activity (56.29, 66.43, 62.94, 68.71, 61.87%). Five LAB strains were identified and classified based on screening, purification and 16S rDNA sequences. Molecular characterization based on partial sequence 16S rDNA homology confirmed the initial identification as *Lactobacillus fermentum* (B55, B62), *Lactobacillus plantarum* (7830), *Pediococcus acidilactici* (B30) and *Lactobacillus helveticus* (K2).

Keywords: Exopolysaccharides; lactic acid bacteria; DPPH radical scavenging activity; Phylogenetic tree

INTRODUCTION

Exopolysaccharides (EPS) is a type of carbohydrate compounds by lactic acid bacteria (LAB) secreted into the culture medium during growth and metabolism. It is the product of microbial adaptation to the environment. EPS are water-soluble glue with unique physical properties. EPS production have received extensive attention as they are produced by food-grade microorganisms with GRAS (generally recognized as safe) and alternatives to the polysaccharides of plant and animal origin. It was reported that LABs isolated from Inner Mongolian traditional yoghurt have the ability to produce EPS (Zhang, 2014) and the EPS produced by LAB has the ability to impart beneficial rheological and functional properties to foods such as yogurt, as a natural thickener, the product has a suitable viscosity and reduced synergy.

Mende et al. (2007) isolated EPS from *Streptococcus thermophilus* and applied it to yoghurt. It was found that EPS can increase the

apparent viscosity of yogurt significantly. The EPS with negatively charged would be attracted electrostatically to casein particles with positively charged in milk, enhancing the polysaccharide-protein network structure. Purwandari et al. (2007) showed that EPS could make yoghurt have better rheological properties and the relationship between its content and yoghurt texture was not obvious. Zhang et al. (2011) added EPS to yoghurt at 1% inoculum size which produced from *Streptococcus thermophilus* ST1, that found the viscosity of yoghurt was improved. Girard et al. (2007) thought that the formation of yogurt gel was closely related to the kinetics of strain acidification and the participation of EPS can improve the consistency and viscosity of the final obtain yogurt. The study found the some EPS have the ability to antitumoral activities and cholesterol-lowering activity (Ouweland et al., 2002; Parvez et al., 2006). It is because the EPS produced by LAB would be effected by the probiotic organism and the EPS secreted that the properties of final product would be enhanced obviously.

The important cause of oxidative damage is free radicals which is also important indicators for

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evaluating the antioxidant properties. Wu et al. (Wu *et al.*, 2010) considered that the EPS produced by *Bifidobacterium* could help prevent gastrointestinal infection and inhibit the growth of harmful bacteria in food. Zhang et al. (2012) studied the in vitro antioxidant activity of EPS which isolated from *Lactobacillus plantarum* C88 and found that the EPS has the functions of scavenging oxygen free radicals, increasing antioxidant enzyme activity and reducing lipid peroxidation. Uchida et al. (2010) studied the hypocholesterolemic effect of EPS from *L. lactis* in vivo and found that oral administration of EPS could effectively reduce the incidence of atherosclerosis in hyperlipidemic rabbits and inhibit disease progression. The study on EPS structure of LAB has important significance in theory and application, because the structure and function of LAB EPS are inextricably linked (Yang et al., 2007).

In recent decades, The LAB-EPS development have been vigorously carried out due to the special advantages in product structure, performance and production. The development of new microbial EPS has become one of the hot spots in industrial microbiology research. Quantity production of polysaccharides was authorized in 1947, the yield of dextran from *Leuconostoc mesenteroides* were 2000 metric tonnes, and FDA approval of food grade xanthan extracted from *Xanthomonas campestris* in 1969 which was consumed more than 20000 tonnes

per year, mainly used as thickener (Sutherland, 2002). Since LAB are food-grade industrial producing bacteria which have a higher safety compared with other bacteria. The research on LAB-EPS has gradually increased in just the past few years. However, the low yield and poor stability of strains are still the dominant factors that restrict their large-scale production. In-depth study of probiotic media to increase extracellular polysaccharide production will provide greater possibilities for the development of the probiotic industry.

The use of traditional polysaccharides is strictly limited, it is because they lack reproducibility in terms of nature, purity, supply and cost. Furthermore, their rheological properties may not fully meeting the requirements. It is finds that novel polysaccharides not only meets most needs of food industry, but also has the ability to be an efficient substitute for the traditional polysaccharides. Therefore, the development of LAB-EPS as a new type of polysaccharide is promising, and LAB that produce EPS have also become potential strains. The purpose of this experiment is to screen out strains which can be used as a starter in milk that have both EPS-producing and anti-oxidant properties. In this experiment, 66 strains of the laboratory-preserved strains were screened to obtain 5 strains that produced antioxidant EPS and the strains were identified.

MATERIALS AND METHODS

Microorganism and culture conditions

The LAB were named previously and isolated from different sources like Kefir grains, commercial dairy product and fermented bovine milk from Inner Mongolia (designated as K1, K2, K3, K4, K5, K6, K7, K8, K9, K10, K11, K12, K13 K14, K15, K16, L49, 7469, 7830, 8014, B25, B26, B30, B43, B55, B56, B62, B82, B90, BB01, GC, GD, L13, L20, L21, L22, L24, L28, L35, L4, L41, L48, L5, L54, L57, L60, L61, L64, L86, La5, LB6, LC, LD, Lp621, Lp69, Lpp, P36, P71, S46, S47, S72, S73, S75, S76, S79 and S91).

The above 66 LAB isolates were maintained in the collection of the School of Food and Biological Engineering, Shaanxi University of Science and Technology. The different strains were each incubated for 24 hours in the MRS (De Man, Rogosa and Sharpe) at an inoculum size of 5% to obtain the activity. All strains were sub-cultured in their appropriate medium at 37 °C.

Preparation of crude EPS

The 66 strains of laboratory freeze-dried bacteria powder were activated for three generations in the basic sugar-producing medium. The EPS was prepared in MRS medium by incubating third generation inoculum (10^9 CFU·mL⁻¹) in static condition for 48 hours at 37°C. Then to remove the cell pellet, the bacterial suspension was centrifuged at 8000 r/min for 15 minutes with the teperature of 4 °C and the supernatant was addedd with 10% TCA (Trichloroacetic acid solution) to remove protein, then double volume-chilled ethanol was added and the mixture was stored overnight at 4 °C. The mixture was centrifuged at 8000 r·min⁻¹ for 15 minutes and the pellet (crude EPS) collected was dissolved in deionized water (Savadogo *et al.*, 2004). The total amount of carbohydrates in the pellet was estimated using the method of phenol-sulphuric acid method as described below.

Rescreening strains producing EPS

Some strains with the potential to produce more EPS were screened out from 66 strains. The

operation of rescreening was same as the previous screening. The crude polysaccharide particles dissolved in distilled water and put in a dialysis bag for 24 hours, which operation would remove small molecules such as monosaccharides, pigments, and peptides. The absorbance of polysaccharides was measured by phenol-sulfuric acid method meanwhile the DPPH radical scavenging activity and the ferrous ion chelation rate were measured.

Phenol-Sulphuric Acid Method for EPS detection

EPS were estimated as total carbohydrates by phenol-sulphuric acid method (Dubois et al., 1956). Glucosidic bond could be hydrolyzed Under the action of concentrated sulfuric acid, these hydrolysed neutral sugars are then partially dehydrated with the elimination of three molecules of water to form furfural or furfural derivatives which could be measured at 490 nm. 1 ml 5 % ($\text{w} \cdot \text{v}^{-1}$) phenol was added to 1 ml sample and 5 ml concentrated sulphuric acid was followed was an ice condition. Then keep the mixture at room temperature for 20 min and the absorbance value was determined at 490nm.

Get the standard curve shown in Equation:

$$y=0.0034x+0.0134(R^2=0.9934).$$

DPPH radical scavenging activity

DPPH radical scavenging capacity was determined by the previous method (Wang et al., 2013). 3.0 mL sample solutions was added in DPPH solution ($0.1 \text{ mmol} \cdot \text{L}^{-1}$ in 95% ethanol), then keep the mixture at room temperature in the dark for 30 min and the absorbance of the mixture was measured at 517 nm. The control group added 95% ethanol solution without DPPH to the sample, and the blank group used ethanol instead of the sample.

The scavenging activity was calculated using Equation:

$$\text{Scavenging activity (\%)} = [A_0 - (A_i - A_j)] \times 100\% / A_0,$$

where A_0 was the absorbance of the DPPH solution without sample solution, the sample part was replaced by 95% ethanol, A_i was the absorbance of the sample with DPPH solution and A_j was the absorbance of the sample with the same condition as A_i , but with 95% ethanol added to replace DPPH solution.

The ferrous ion chelation rate

The ferrous ion chelating rate was assessed according to the method reported by Ma et al., 2013, with minor modifications. One millilitre of samples at different concentrations was mixed with 0.1 mL FeCl_2 ($2 \text{ mmol} \cdot \text{L}^{-1}$), 0.2 mL ferrozine ($5 \text{ mmol} \cdot \text{L}^{-1}$) and 3.7 mL distilled water were added to the

mixture, shaken well, then incubated for 20 min at room temperature. The absorbance of the supernatant was determined at 562 nm. Replace the sample with water was used as the positive standard. The ferrous ion chelating activity was calculated by the following Equation:

$$\text{Ferrous ion chelating rate (\%)} = (A_2 - A_1) \times 100\% / A_2$$

where A_1 was the absorbance of the sample solution and A_2 was the absorbance of the positive standard same condition as A_1 .

DNA extraction and amplification of 16S rDNA sequences

Molecular methods are powerful and efficient tools to classify bacteria taxonomically even between closely related species. These methods are mostly based on polymerase chain reaction (PCR). Genotypic characterization of microorganisms according to their 16S ribosomal RNA sequencing is the best and most reliable approach to identify bacteria on a phylogenetic basis.

The use of molecular tools has broken through the limitations of traditional methods in the identification of bacteria. These techniques are based on PCR using oligonucleotide primers to amplify targeted DNA fragments. These PCR primers are designed at different levels of classification, from genus-specific (Schillinger et al., 2008), to the species-specific level (Coton et al., 2008), to sub-species level (Pu et al., 2002), and also further to the strain-level (Coudeyras et al., 2008). This method is suitable for the initial identification of bacterial species by other methods (usually phenotypic methods).

A pure colony which was picked from each probiotic strain was picked was lysed in 50 mL TaKaRa Lysis Buffer (Cat. No. 9164) (Goranov et al., 2015). After 15 min denaturation at 80°C , then via centrifugation ($14,000g$) the supernatant was used as a template. A TaKaRa 16S rDNA Bacterial Identification PCR Kit (Cat. No. RR176) was using to perform the amplification of the target fragment. The target fragment was amplified by polymerase chain reaction (PCR) using 'Forward primer/Reverse primer2' and could be used for amplification of full sequence DNA fragments. The reaction system and reaction conditions are described as Table 1 shows.

Purification of PCR products

According to the instruction, a Takara MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (Cat. No. 9762) was used to purified the amplified 16S rDNA fragments and the electrophoresis used DL 2,000 DNA Maker which including 2000, 1000, 750, 500, 250 and 100 bp fragments.

DNA sequencing and construction of phylogenetic tree

The 16S rDNA gene fragments sequencing was performed by ABI PRISMTM 33730XL DNA Sequence (Applied Biosystem) by using Primer of Seq forward, Seq reverse and Seq internal primers (Table 2). In order to construct phylogenetic trees, the sequences were analyzed by the NCBI (National Center of Biotechnology Information) BLAST

(basic local alignment search tool) and the neighbor-joining method was used to infer evolutionary history with the unweighted pair group method with arithmetic-mean (UPGMA) (Sokal, 1958). The evolutionary history of the taxa analyzed was represented by taking bootstrap consensus tree inferred from 1000 replicates and the maximum composite likelihood method was used to compute the evolutionary distances.

Table 1. PCR reaction conditions

Reaction system	Reaction conditions			
Supernatant (after denaturation)	1μL	94 °C	5min	1 cycle
PCR premix	25μL	94 °C	1min	
Forward primer (20 pmol/mL)	0.5μL	55 °C	1min	30 cycles
Reverse primer2 (20 pmol/mL)	0.5μL	72 °C	1.5min	
16S-free H ₂ O	23μL	72 °C	5min	1 cycle
Total volume	50μL			

Table 2. Sequencing primer

Primer	Primer sequence
Seq forward	5'-GAGCGGATAACAATTTTCACACAGG-3'
Seq reverse	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'
Seq internal	5'-CAGCAGCCGCGGTAATAC-3'

RESULTS AND DISCUSSIONS

Potential strains were screened out by measuring EPS production

Table 3 shows that 11 strains (Lp69, LB6, L13, L64, 8014, 7830, K2, B62, B55, B30, S72) were screened out with potential for EPS production compared with other strains, their EPS yield were 134.94, 172.82, 163.88, 133.51, 132.44, 131.72, 149.94, 237.15, 187.88, 175.97, 130.24 mg·L⁻¹, respectively. These 11 strains were re-screened to screen out the strains with high-yield EPS with antioxidant activity. The higher content of crude EPS at this time was due to the total carbohydrate content measured by the phenol-sulfuric acid method, while the monosaccharide in the EPS sample was removed during the initial screening.

Antioxidative EPS-producing strains obtained by rescreening

The EPS yield, DPPH free radical scavenging rate and ferrous ion chelation rate of 11 strains were determined as shown in Figure 1. All tested probiotic strains showed the potential to produce EPS and antioxidants. The polysaccharide production of the five strains (B55, B62, B30, 7830 and K2) exceeded 100 mg·L⁻¹ among all the strains these yield of EPS were 110.66, 145.48, 132.78,

122.11 and 111.72 mg·L⁻¹, respectively. This is a new discovery that K2 was isolated from natural kefir grains among them and the research and analysis of this strain could continue. Their EPS yields were lower than that of the initial screening that because residual monosaccharides in many culture media were separated out after the dialysis. At the same time, they have relatively high DPPH radical scavenging activity (56.29, 66.43, 62.94, 68.71, 61.87%) and ferrous ion chelating rate. The different performance of various strains when producing EPS were closely related to their inherent factors.

These strains can all be used as potential strains for the production of EPS. Many studies have used LAB-EPS in products to obtain better product. Yang et al. (Yang *et al.*, 2013) extracted the EPS of a *Streptococcus thermophilus* S1 and added it to buffalo yoghurt in different proportions. The results showed that both the hardness and the viscosity of the yoghurt reached a maximum value when the added amount was 0.01% and the above two indicators showed a downward trend with the continued increase of EPS added, but they were all greater than the yogurt samples without EPS. The analysis indicates that this phenomenon may be related to the network structure of EPS.

Table 3. Screening for EPS-producing strains of LAB

No.	LAB	EPS (mg·L ⁻¹)	No.	LAB	EPS (mg·L ⁻¹)	No.	LAB	EPS (mg·L ⁻¹)
1	L49	74.54±1.1	23	LD	21.19±1.58	45	Lpp	19.5±0.98
2	Lp69	134.93±1.2	24	7469	23.87±1.52	46	S76	27.44±1.17
3	L28	56.67±1.34	25	L22	17.16±0.83	47	L86	20.08±0.66
4	L41	76.33±1.25	26	LP621	25.88±1.42	48	GC	40.67±2.10
5	LB6	172.81±1.68	27	K1	81.54±1.72	49	S72	130.23±1.36
6	L5	75.25±2.3	28	K2	149.94±1.58	50	GD	22.88±1.35
7	L48	70.97±1.34	29	K5	81.54±0.82	51	S75	22±1.24
8	L54	71.68±1.96	30	K11	11.80±1.47	52	S79	15.52±0.84
9	La5	78.83±1.54	31	K15	33.26±0.67	53	S91	31.11±1.37
10	L60	74.18±1.63	32	L20	18.50±0.74	54	P71	13.47±1.32
11	L13	163.88±1.53	33	B56	118.76±1.39	55	P36	57.58±1.25
12	L64	133.50±1.48	34	B43	90.23±0.65	56	K4	98.61±0.75
13	LC	81.69±1.42	35	B62	237.14±0.59	57	K3	117.73±0.86
14	L57	65.25±0.75	36	B90	119.5±0.77	58	K12	20.23±1.11
15	L21	76.33±1.72	37	B25	114.94±0.92	59	K16	28.61±1.15
16	8014	132.43±1.32	38	B55	187.88±1.65	60	K13	19.5±1.24
17	7830	131.72±1.76	39	B82	94.35±0.59	61	K6	21.41±0.58
18	L4	78.47±0.98	40	B30	175.97±1.36	62	K7	105.67±0.99
19	L61	72.39±1.36	41	B26	107.58±1.45	63	K8	117.88±0.62
20	L24	53.81±0.85	42	S46	97.14±1.28	64	K9	108.61±0.59
21	L35	15.82±1.48	43	S47	117.58±1.53	65	K10	42±1.37
22	BB01	86.90±0.86	44	S73	90.38±1.49	66	K14	43.76±1.42

Figure 1. EPS production and antioxidant activity of the selected 11 LAB

Prasanna et al. (2012) screened 4 EPS-producing strains from 22 *Bifidobacterium* strains. The correlation analysis of the apparent viscosity and pH of fermented milk showed that the EPS produced by

these four strains helps increase the viscosity of dairy products during milk acidification.

A large number of studies have shown that some LAB-EPS have antioxidant effects. Xu et al. (2012) studied the antioxidant activity of EPS produced by

L. casei KW3. It showed that when the EPS concentration was $200 \mu\text{g}\cdot\text{mL}^{-1}$, the DPPH radical scavenging rate was 37.53% In vitro experiments. Meng et al. (2012) found that the antioxidant activity of EPS from *Lactobacillus paracasei* H9 increased with increasing EPS concentrations. The DPPH radicals rates was $(70.8\pm0.6)\%$ when the EPS concentration reached $200 \text{ mg}\cdot\text{L}^{-1}$. The above-mentioned studies on the yield of EPS and antioxidation performance obtained were much than this experiment, which mainly because this experiment did not optimize the culture medium. At the end of the experiment, the culture medium will be optimized to obtain more EPS production and antioxidant activity.

Phylogenetic analysis by 16S rDNA analysis

The results of the agarose gel electrophoresis (Figure 2) showed that after sequencing, the length of all amplicons exceeded expectations, reaching 1000 bp, 1394 bp (B55), 1396 bp (B30), 1410 bp (7830), 1431 bp (K2) and 1358 bp (B62). After amplification and purification of the target fragment, the five strains was preliminary identified as *L. fermentum* (B55, B62), *L. plantarum* (7830), *P. acidilactici* (B30) and *L. helveticus* (K2) via molecular characterization based on 16S rDNA homology of partial sequences (B55, B62, B30, 7830 and K2) with the sequences available in the NCBI database. In order to track the evolutionary patterns of isolates based on sequence similarity, a phylogenetic tree was constructed using the UPGMA adjacent ligation method between the 16S rDNA sequence of the strain in the NCBI database and the corresponding sequence of different probiotic lactic acid bacteria (Sun *et al.*, 2016). The resulting trees of Figure 3 showed that strain B55 had 100% homology with *L. fermentum* NCDO 1750, 63% homology with *L. fermentum* CIP 102980 and *L. fermentum* NBRC 15885. B62 had the 100% homology relationship with *L. fermentum*

strain CIP 102980 and *L. fermentum* strain NBRC 15885. Similarly, 80% homology with *P. acidilactici* strain DSM 20284 was shown in the tree of B30 and both had 99% homology with *P. acidilactici* strain NGRI 0510Q, 99% homology was found for strain 7830 with *L. plantarum* strain JCM 1149, *L. plantarum* strain NBRC 15891, *L. plantarum* strain CIP 103151 and *L. plantarum* strain NRRL B-14768, strain K2 had 100% homology with *L. helveticus* strain NBRC 15019 and *L. helveticus* strain DSM 20075. Overall, due to the high production of EPS potential, the five strains was selected for further study as *L. fermentum* (B55, B62), *P. acidilactici* (B30) *L. helveticus* (K2) and *L. plantarum* (7830) via molecular phylogenetic analysis.

Wang et al. (2015) found that *Lactobacillus plantarum* YW11 can produce $90 \text{ mg}\cdot\text{L}^{-1}$ of EPS in semi-custom medium, which was isolated from Tibet Kefir. The results showed lower EPS yield than 7830 in this study, mainly because of differences in species and culture media.

The production, characterization and functional properties in vitro of EPS of *Lactobacillus helveticus* MB2-1 with whey solution as basal medium were investigated by Li et al. (2014) It is repoted that in the case of lactose and soy protein ept $80 \text{ g}\cdot\text{L}^{-1}$ and $20 \text{ g}\cdot\text{L}^{-1}$, respectively, the maximum yield of EPS was $753 \text{ mg}\cdot\text{L}^{-1}$. EPS also exhibits effective antioxidant effects such as the ability to scavenge hydroxyl groups, superoxide and DPPH free radicals, and the ability to chelate ferrous ions (related to dose). Since the fermentation conditions have not yet been optimized and the strains were not the same in this study, the amount of EPS produced by the *Lactobacillus helveticus* MB2-1 were higher than K2 (isolated from Kefir in this experiment). At the same time, Li et al. also confirmed that the polysaccharides have better antioxidant properties.

Figure 2. Agarose gel electrophoresis of PCR products from genomic DNA isolated from representative LAB strains.

Figure 3. Phylogenetic tree of concatenated LAB strain B62 (A), B55 (B), B30 (C) 7830 (D) and K2 (E) constructed by the Neighbour-joining method with Kimura 2-parameter as evolution model and based on 16S rDNA nucleotide sequences.

Fukuda et al. (2010) showed that a thermophilic LAB, *Lactobacillus fermentum* TDS030603, produced about $100 \text{ mg} \cdot \text{L}^{-1}$ of EPS in purified form when grew in MRS broth. Zhang et al. (2011) found a *Lactobacillus fermentum* F6 present in traditional dairy products in Inner Mongolia, China with an EPS yield of $20 \text{ mg} \cdot \text{L}^{-1}$. 1% (w/v) EPS aqueous solution exhibits higher viscosity, indicating that the EPS has potential as a food tackifier. The yields of the two strains (B62 and B55) of *Lactobacillus fermentum* which have the potential to produce polysaccharides in this study were all higher than the above studies.

Strain M76 was selected as an EPS-producing functional strain for the ability of DPPH free radicals to have antioxidant activity in makgeolli, a Korean fermented rice wine by Song et al. (Song et al., 2013). Strain M76 has a high identity (99.0%) with *P. acidilactici* according to the 16S rRNA gene sequencing analysis. And the soluble EPS produced by M76 can up to $200 \text{ mg} \cdot \text{L}^{-1}$, which EPS yield was higher than B30 in this study. Different strains have different EPS production, which is related to the strain itself. Identify the structure of the EPS and find out the relationship between yield and structure in the subsequent study.

CONCLUSIONS

The results of this study indicated that all 66 tested strains had some ability to produce EPS. Among the 66 strains, B62 had the highest EPS yield of 145.48 mg/L and had the strongest function of eliminating DPPH free radicals (66.43%). In addition, the EPS yields of B55, B30, 7830 and K2 were all greater than $100 \text{ mg} \cdot \text{L}^{-1}$, ie 10.66, 132.78, 122.11 and $111.72 \text{ mg} \cdot \text{L}^{-1}$, respectively. They also had the antioxidant function of eliminating DPPH free

radicals, respectively 56.29, 62.94, 68.71 and 61.87%. Basing in 16S rDNA homology of partial sequences, the molecular characterization were confirmed the preliminary identification as *L. fermentum* (B55, B62), *L. plantarum* (7830), *P. acidilactici* (B30) and *L. helveticus* (K2). These five LAB strains can be used as potential strains for the production of EPS which also can be applied to the products after optimized culture conditions and purification.

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