KMITL Sci. Tech. J. Vol. 8 No. 1 Jan. - Jun. 2008

SCREENING AND IDENTIFICATION OF LACTIC ACID BACTERIA PRODUCING ANTIMICROBIAL COMPOUNDS FROM PIG GASTROINTESTINAL TRACTS

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ABSTRACT

A total of 87 lactic acid bacteria were isolated from 15 samples of pig gastrointestinal tract. Antimicrobial activities of all isolates grown under oxygen-restricted conditions to eliminate the effect of hydrogen peroxide were tested against 5 pathogenic bacterial strains using agar well diffusion technique. Seventeen isolates exhibited antimicrobial activity against at least one indicator strain tested. Among the 17 isolates, two strains of *Lactobacillus amylovorus/ L. sobrius* and one strain of *Lactobacillus reuteri* were found to produce antimicrobial compounds other than organic acids. The neutralized supernatants from two *L. amylovorus/ L. sobrius* strains inhibited the growth of *Escherichia coli* and *Listeria monocytogenes*, respectively. Therefore, these strains may have potential use as an alternative to antibiotics for pigs. The strains can also be used to produce antimicrobial compounds which can be a substitute for chemical preservatives in food industry. Diversity of lactic acid bacteria with antimicrobial activities was studied by identification to the species level of the 17 isolates. This was achieved by sequencing of approximately 500 bp of 16S rRNA gene which contained V1 and V2 variable regions and comparing the sequences with existing data in GenBank. Of the 17 isolates, 7 isolateswere identified as *Lactobacillus reuteri*, the other 10 isolates were identified as *Lactobacillus amylovorus/L. sobrius* (4 isolates), *Lactobacillus mucosae* (3 isolates), *Lactobacillus acidophilus johnsonii*/*L. johnsonii* (2 isolates) and *Streptococcus alactolyticus* (1 isolate).

KEYWORDS: antimicrobial compounds, lactic acid bacteria, pig gastrointestinal tract

1. INTRODUCTION

In pig farming, antibiotics are generally used in suckling and weaned pigs for prevention of intestinal infection caused by enteric pathogens and for growth promotion [1]. Most of antibiotics used are tetracycline, penicillin G, bacitracin, oxytetracycline and chloramphenicol. However, after prolonged treatment, accumulation of antibiotics in meat products occurs and becomes aserious problem. Some intestinal microflora have also developed resistance to these antibiotics [2].

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Moreover, antibiotics can be harmful to beneficial microorganisms in gastrointestinal tract of pigs. Since an EU Regulations ban antibiotics as feed additives, other natural biological control agents including probiotic bacteria have been used as an alternative to antibiotics. Probiotics are live-microorganisms which give health benefit to their host. A number of *Lactobacillus* sp., *Enterococcus* sp., *Bifidobacterium* sp. and *Bacillus* sp. have been reported to be used as probiotics in human and animal [3-4].

One important reason for screening probiotic lactic acid bacteria is the ability of some strains to produce antimicrobial compounds, including organic acids, hydrogen peroxide and bacteriocins, that can inhibit the growth of enteric pathogens. A number of studies have reported the antagonistic properties of probiotics against many common gastroenteric pathogens, e.g. *Salmonella* sp. [5], *Escherichia coli* O157:H7 [6], *Clostridium perfringen* [7], *Campylobacter jejuni* [8], *Listeria monocytogenes* and *Helicobacter pylori* [9].

Identification of lactic acid bacteria based on carbohydrate fermentation patterns is unreliable and not accurate enough to distinguish closely related strains due to their similar nutritional requirements [10]. Nowadays, several molecular techniques have been used for bacterial identification [11]. Amongst these techniques, sequence analysis of the partial 16s rRNA gene has been used for identification of lactobacilli to the species level [12-13].

The aim of this work is to isolate and screen for lactic acid bacteria with antimicrobial activity against enteric pathogenic bacteria. Because of the ability of probiotic organisms to adhere to mucous and epithelial surface, potential probiotic lactic acid bacteria were isolated from gastrointestinal tracts of pigs. Diversity of lactic acid bacteria producing antimicrobial compounds was also studied using partial 16s rRNA gene sequence analysis for strain identification.

2. MATERIALS AND METHODS

2.1 Isolation of lactic acid bacteria and culture conditions

Pig small intestines and caeca were collected immediately after slaughter from commercial slaughterhouses in Nakornprathom province, Thailand. Samples were kept on ice and immediately transferred to the laboratory for bacterial isolation. After dissection, surface mucus was removed from the tissue to obtain adhering bacterial isolates. Ten grams of epithelial tissue were scraped with a sterile blade, resuspended in 90 ml of phosphate buffered saline (PBS) and vigorously shaken using a Stomacher (Smasher, AES Chemunex, France) for 1 min. Homogenized samples were serially diluted and plated on MRS medium (Oxoid) supplemented with 0.5% $CaCO_3$ and 0.05% (w/v) L-cysteine-hydrochloride (MRSC). After incubation for 2 days at 37 °C under anaerobic conditions in Anaerobic jars with Anaerocult A gas packs (Merck, Darmstadt, Germany), only acid producing bacterial colonies were selected. This can be observed from clear zones around the colonies which indicated the dissolving of $CaCO_3$ by an acid. Colonies with different morphology were counted, picked up and purified by restreaking on the same medium. Cell morphology, Gram staining and catalase test were performed as a preliminary screening for lactic acid bacteria. Gram-positive, non-spore forming and catalase-negative strains were selected for further studies. Seven DSMZ type strains, *Lactobacillus johnsonii* DSM 10533, *Lactobacillus reuteri* DSM 20016, *Lactobacillus amylovorus* DSM 20531, *Lactobacillus sobrius* DSM 16698, *Lactobacillus acidophilus* DSM 20079, *Lactobacillus mucosae* DSM 13345 and *Streptococcus alactolyticus* DSM 20728 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany and were grown in MRS medium. The selected lactic acid bacteria were maintained as stock cultures at -80 °C in 10% skim milk (Oxiod). Indicator pathogens, *Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Bacillus*

cereus and *Staphylococcus aureus* were grown in Tryptic Soy Broth (TSB, Oxoid) and Tryptic Soy Agar (TSA, Oxiod) and incubated at 30°C.

2.2 Screening of lactic acid bacteria for antimicrobial activity

An overnight culture of each isolate grown in MRSC broth at 37 °C was standardized to an optical density of 0.5 at a wavelength of 600 nm. (Hitachi U-2010 model spectrophotometer). One percent of standardized culture was used to inoculate MRSC broth. After incubation at 37 °C for 24 hrs, cells were removed by centrifugation at 10,000 x g for 15 min. The pH of one portion of supernatant was adjusted to 7.0 and filtered through 0.22 μm membranes. The filtrates of both pH and non-pH adjusted were used to evaluate antimicrobial activity using agar well diffusion method. Positive results were recorded when the zone of inhibition of at least 1 mm around the wells was observed [14].

2.3 Detection of antimicrobial activity by agar well diffusion method

An agar well diffusion method as described by Barefoot and Klaenhammer [15] was used with some modifications. An overnight culture of pathogens including *E. coli, L. monocytogenes, S. typhimurium, B. cereus* and *S. aureus* grown in TSB medium at 30 °C was diluted to a turbidity equivalent to that of a 3.0 McFarland standard (bioMerieux, France) with a sterilized 0.85% NaCl solution. A lawn of an indicator strain was made by spreading the cell suspension over the surface of TSA plates with a cotton swab. The plates were allowed to dry and a sterile cork borer of diameter 7.0 mm was used to cut uniform wells in the agar plates. Each well was filled with 70 μl of filter–sterilized supernatant obtained from culture grown in MRSC medium. All the assays were carried out in triplicate. After incubation at 37°C for 24 hrs, the diameter (mm) of the inhibition zone around the well was measured.

2.4 Identification of lactic acid bacteria using API 50CHL system

Carbohydrate fermentation patterns were determined using API50 CHL test kit (bioMerieux, France). The strains were identified using the APILAB Plus software version 3.3.3 from bioMerieux.

2.5 16S rDNA sequencing and sequence analysis

Almost complete 16S rDNA sequence was amplified using primers 27f (5'-AGAGTTTGATC-CTGGCTCAG-3') and 1525r (5'-AAGGAGGTG(A/T)TCCA(A/G)CC-3') [16] purchased from Bioservice Unit (BSU), National Science and Technology Development Agency, Thailand. After amplification, the PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Hilden,Germany) according to the manufacture's instructions. The purified products were checked by electrophoresis (Sub-Cell GT Agarose Gel Electrophoresis systems. Bio-Rad, USA) on a 1 % agarose gel, stained with ethidium bromide, visualized and photographed on a Gel Doc (UVIdoc System, model GAS9000/9010).

The sequences of approximately 500 bp from 5' terminal part of the amplified 16S rDNA of 17 selected isolates were determined from both strands using primers 27f and 530r (5'-GGCAGAATGGTAACACCAGAGT-3') [16]. Corresponding partial sequences of the type strains obtained from DSMZ was also determined. DNA was sequenced using an ABI Big Dye Terminator Cycle Sequencing Ready Reaction Mix kit on the Perkin Elmer Model 9400 thermal cycler. The sequencing reaction products were analyzed in an automated 310 DNA sequencer (Applied Biosystem/Perkin-Elmer). Sequence homology and analysis were performed using the Blast program available online at the National Center for Biotechnology Information, NCBI [17].

3. RESULTS AND DISCUSSION

3.1 Isolation of lactic acid bacteria from pig gastrointestinal tracts

Lactic acid bacteria were isolated from 15 samples of pig small intestines and caeca using modified MRS supplemented with 0.05% L-cysteine-hydrochloride (MRSC). L-cysteine-hydrochloride was used as a reducing agent to provide more strict anaerobic conditions to MRS medium which will promote the growth of intestinal lactic acid bacteria. Lactic acid bacteria could be isolated from all samples and four typical colony morphologies were observed. The number of each colony type varies between samples ranging from 10^4 to 10^7 CFU/g. Four to five distinct colony morphologies were selected from each sample and screened for Gram-positive and catalase negative bacteria. A total of 87 lactic acid bacteria were finally selected for further analysis. Microscopic examination of the 4 common colony types showed different cell morphologies, cocci, long-rods in chain, rods and short rods. In all intestinal samples, short rod-shaped bacteria were found to be the predominant type and the strain was later identified as *Lactobacillus reuteri*.

3.2 Screening of lactic acid bacteria for antimicrobial activity

An agar well diffusion method was used to assess the production of antimicrobial compounds by the selected lactic acid bacteria isolated from pig gastrointestinal tracts against 5 pathogens including Gram-positive *L. monocytogenes, B. cereus, S. aureus* and Gram-negative *E. coli* and *S. typhimurium*. Out of 87 isolates tested, 17 isolates were found to exhibit antimicrobial activity against indicator strains. As shown in Table 1, the spectra of inhibition were different among the isolates tested. Isolate S5-2 showed the largest antimicrobial spectrum, exhibiting inhibitory activity against 4 pathogens, *L. monocytogenes*, *B. cereus, E. coli* and *S. aureus* and followed by isolate S9-2 that inhibited 3 pathogens. Isolates S5-2 and S9-2 could inhibit *L. monocytogenes*, a significant food-borne pathogen in food industry especially in meat, poultry, and seafood products [18].

To rule out the possibility that the inhibition was due to the effect of organic acids, the supernatants were neutralized to pH 7.0 before the assay. Only 3 out of 17 isolates including isolates S7-4, S5-2 and S9-2 still retained antimicrobial activity against some pathogens tested. The possibility of inhibition due to hydrogen peroxide production was also considered by growing the isolates under oxygen-restricted conditions in MRSC medium. Moreover, anaerobic conditions also favored bacteriocin production over aerobic and oxidative stress conditions [19]. Therefore, antimicrobial activities of these 3 isolates might be due to the production of bacteriocin-like compounds. These bacteriocin-like compounds might be useful as biological control agents, an alternative to chemical preservatives in food industry.

Several lactic acid bacteria isolated from pig gastrointestinal tracts and pig faeces have been reported to have antagonistic activity against various pathogenic bacteria. *Lactobacillus salivarius, L. reuteri* and *L. amylovorus* isolated from pig gastrointestinal tracts could inhibit the growth of *Clostridium perfringens*, the cause of gas gangrene and necrotic enteritis in human and animals [7]. Twenty six lactic acid bacteria with anti-*Salmonella* activity were isolated from pig faeces and caeca. Those isolates were identified as *Lactobacillus murinus, L. salivarius* subsp. *salivarius, L. acidophilus* complex, *Pediococcus pentosaceus* and 5 members of the genus *Enterococcus* [5]. An antimicrobial compound produced by 28 strains of *L. reuteri* isolated from pig faeces was identified as reuterin. Reuterin could inhibit the growth of Gram-positive and Gram-negative bacteria as well as yeast and fungi [20]. Besides reuterin, some strains of *L. reuteri* also produced other bacteriocin-like compounds, e.g. reutericin and reutericyclin [21]. Three strains of *Enterococcus* sp, *E. faecalis, E. faecium* and *E. mundii* isolated from gastrointestinal tract of pigs, exhibited a high antagonistic activity against *E.coli* K88, a strain that causes diarrhea in pigs [22].

Table 1 Antimicrobial effects of the supernatants of lactic acid bacteria isolated from pig gastrointestinal tracts before and after neutralization

Tested LAB	**Inhibition of indicator strains**									
	B. cereus		***E. coli***		***L. monocytogenase***		***S. aureus***		***S. typhimurium***	
	Non-adj	**pH adj.**	**Non-adj**	**pH adj.**	**Non-adj**	**pH adj.**	**Non-adj**	**pH adj.**	**Non-adj**	**pH adj.**
L. reuteri										
S1-3	+	-	+	-	-	-	-	-	-	-
S4-1	+	-	+	-	-	-	-	-	-	-
S7-4	**+**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
S8-3	+	-	-	-	-	-	-	-	-	-
S9-1	+	-	-	-	+	-	-	-	-	-
S9-3	-	-	+	-	-	-	-	-	-	-
S10.1-3	-	-	+	-	-	-	+	-	-	-
S. alactolyticus										
S6-4	-	-	-	-	-	-	+	-	-	-
L. amylovorus / L. sobrius										
S1-2	+	-	+	-	-	-	+	-	+	-
S2-2	+	-	-	-	-	-	+	-	+	-
S5-2	**+**	**-**	**+**	**+**	**+**	**-**	**+**	**-**	**-**	**-**
S9-2	**+**	**-**	**+**	**-**	**+**	**+**	**-**	**-**	**-**	**-**
L. mucosae										
S2-1	-	-	-	-	-	-	-	-	+	-
S2-3	+	-	-	-	-	-	-	-	-	-
S4-3	-	-	+	-	-	-	+	-	-	-
L. acidophilus johnsonii/ L. johnsonii										
S2-4	+	-	-	-	-	-	-	-	-	-
S14-6	+	-	-	-	-	-	-	-	-	-

Positive results were recorded when the zone of inhibition of at least 1 mm around the wells was observed. Bold type indicates bacteriocin-like producing isolates

3.3 Identification of lactic acid bacteria using API 50CHL system

Carbohydrate fermentation patterns of 3 bacteriocin-like producing isolates (S5-2, S7-4, S9-2) and 3 DSMZ type strains were tested using API50 CHL kit (Table2). Based on carbohydrate fermentation pattern analysis, the two type strains, *L. amylovorus* DSM20531 and *L. sobrius* DSM 16698 were identified as *Lactobacillus crispatus* while *L. reuteri* DSM 20016 was identified as *Lactobacillus fermentum* 1, which is synonymous with *L.reuteri*. The results agreed with other studies that the kit is not accurate enough to identify to the species level in some cases [23].

Similar carbohydrate fermentation patterns were observed for isolates S5-2 and S9-2 and they were identified as *L. acidophilus* 3. Isolate S7-4 was identified as *L. fermentum* 1. *Lactobacillus acidophilus* group have been frequently found in pig gastrointestinal tract and can be divided into 6 subgroups based on DNA sequence homology and cell wall composition. The group comprises 6 closely related species including *L. acidophilus* (A_1), *L. crispatus* (A_2), *L. amylovorus* (A_3), *L. gallinarum* (A_4), *L. gasseri* (B_1) and *L. johnsonii* (A_2). Therefore strains S5-2 and S9-2 which were classified as *L. acidophilus* 3 could not be identified to subgroup level using API50 CHL system. Identification of the 3 bacteriocin-like producing isolates and 3 DSMZ type strains was then confirmed using partial 16s rDNA sequence analysis (Table 2). In addition, this technique can also be used to identify the remaining isolates which did not produce bacteriocins.

Table 2 Identification of Lactobacilli DSMZ type strains and bacteriocin-like producing strains by API 50 CHL kit and sequence analysis of V1-V2 region of 16s rRNA gene

Strains	**Identification with API 50 CHL**		**Identification based on V1-V2 sequences analysis**		
	Identification	**% identity**	**Identification**	**GenBank Accession No.**	**% similarity**
L. amylovorus DSM 20531	*L. crispatus*	53.1	*L. amylovorus* *L. sobrius*	EF120373 AY70006 3	99 99
L. sobrius DSM 16698	*L. crispatus*	72.1	*L. amylovorus* *L. sobrius*	EF120373 EF468103	99 99
L. reuteri DSM 20016	*L. fermentum 1* (*syn. L.reuteri*)	92.3	*L.reuteri*	X76328	99
S5-2	*L. acidophilus3*	97.9	*L. amylovorus* *L. sobrius*	AY94440 8 EF468103	99 99
S9-2	*L. acidophilus3*	89.6	*L. amylovorus* *L. sobrius*	EF120375 EF468103	99 99
S7-4	*L. fermentum1* (*syn. L.reuteri*)	99.7	*L.reuteri*	EF468094	99

3.4 Identification of lactic acid bacteria using V1-V2 16S rDNA sequencing

Since API50 CHL system failed to identify to species level of the *Lactobacillus,* especially within *L. acidophilus* complex, the diversity of lactic acid bacteria with antimicrobial activity isolated from gastrointestinal tract of pigs was then studied using partial 16S rRNA gene sequence analysis. Identification of the selected 17 isolates including 7 DSMZ type strains was performed by partial sequencing of approximately 500 bp containing V1 and V2 regions of 16S rRNA gene. It has been shown in many reports that the sequence of V1and V2 regions is sufficient for strain identification to the species level, even within the *L. acidophilus* complex [24-25]. Approximately 1.5 kb of 16S rDNA gene was amplified and the partial sequence of 500 bp was determined. Sequence analysis of 17 pig isolates using Blast program demonstrated the highest homology (99-100%) with 16S rDNA sequences of members of *Lactobacillus* and *Streptococcus* held in GenBank. Corresponding partial sequences of the 7 type strains obtained from DSMZ were also determined and the results showed that all the type strains had a good match (similarity of ≥ 99%) with sequences of their corresponding strains from GenBank. However, the sequence of this region could not be used to differentiate between *L. sobrius* and *L. amylovorus* as the sequences in this region of both strains shared almost 100% homology. Of the 17 identified isolates, isolates

S1-3, S4-1, S7-4, S8-3, S9-1, S9-3 and S10.1-3 were identified as *L. reuteri*. A cocci-shaped isolate S6-4 was identified as *S. alactolyticus*, while a group of 4 rod-shaped in chain, isolates S1-2, S2-2, S5-2 and S9-2 were identified as *L. amylovorus/L. sobrius*. Other 5 rod-shaped isolates, S2-1, S2-3, S4-3, S2-4 and S14-6 were identified as *L. mucosae, L. acidophilus johnsonii* and *L. johnsonii* (Table3).

As shown in Table 2, two bacteriocin-like producing isolates (S5-2 and S9-2), which were classified as *L. acidophilus* 3 by API 50CHL kit were re-identified as *L. amylovorus/ L. sobrius* based on partial 16s rDNA sequence analysis. Both methods gave the same identification results for isolate S7-4, which was identified as *L. reuteri*. Two type strains, *L. amylovorus* DSM 20531 and *L. sorbrius* DSM 16698 were incorrectly identified as *L. crispatus* by API50 CHL kit while partial 16s rDNA sequence analysis correctly identified the two type strains. However, this technique failed to differentiate between these two species.

Various species of lactic acid bacteria have been reported as being predominant in gastrointestinal tracts of pigs. However, different dominant species have been reported even though the isolated strains originated from the same source, gastrointestinal tracts of pigs. This difference is due to various factors, e.g. diet types, age of animal and other environmental factors [26]. The most frequently reported belongs to the genus *Lactobacillus* for example, *L. amylovorus* [7], *L. reuteri* [20], *L. crispatus*, *L. buchneri* [27], *L. ruminis* [28] and the new species *L. saerimneri* sp. nov. [29], *L. sobrius* [30] and *L. mucosae* [31].

4. CONCLUSIONS

In this report, lactic acid bacteria were isolated from pig gastrointestinal tracts and *L. reuteri* was found to be the predominant species. Carbohydrate fermentation pattern analysis using API50 CHL kit could not be used to identify some species of lactic acid bacteria isolated from pigs. Diversity of lactic acid bacteria with antimicrobial activity from pig gastrointestinal tracts was assessed by the analysis of V1 and V2 variable regions of 16S rRNA gene. Even though this technique proved to be an efficient, accurate and reliable method for strain identification, some strains such as *L. amylovorus* and *L. sobrius* could not be differentiated using this technique. Identification based on the analysis of partial 16S rDNA sequences of 17 lactic acid bacteria with antimicrobial activity, the strains found in pig gastrointestinal tracts were, *L. reuteri*, *S. alactolyticus*, *L. mucosae*, *L. amylovorus/ L. sobrius*, and *L. acidophilus johnsonii/ L. johnsonii*. Of the 17 antimicrobial producing isolates, 2 strains of *L. amylovorus*/ *L. sobrius* and 1 strain of *L. reuteri* were found to produce bacteriocin-like compounds. With the ability to produce bacteriocins and organic acids, these strains could be good candidates for potential application as probiotics in pig and also as natural food preservatives.

5. ACKNOWLEDGEMENTS

This project was financial supported by the research fund from the Faculty of Science, King Mongkut's Univrsity of Technology Thonburi.

Table 3 Identification of Lactobacilli DSMZ type strains and 17 selected strains with antimicrobial activity by sequence analysis of V1-V2 region of 16s rRNA gene

Strains	**Identification based on V1-V2 sequences analysis**		
	Identification	**GenBank Accession No.**	**% similarity**
L. amylovorus DSM 20531	*L. amylovorus* *L. sobrius*	EF120373 AY700063	99 99
L. sobrius DSM 16698 *	*L. amylovorus* *L. sobrius*	EF120373 EF468103	99 99
L. reuteri DSM 20016	*L.reuteri*	X76328	99
*L. mucosae*DSM13345	*L. mucosae*	AF126738	99
L. johnsonii DSM10533	*L. johnsonii*	AJ002515	99
L. acidophilus DSM20079	*L.acidophilus*	AB186321	99
S. alactolyticus DSM20728	*S. alactolyticus*	AF201899	99
S1-3	*L.reuteri*	DQ256277	100
S4-1	*L.reuteri*	DQ256277	99
S7-4	*L.reuteri*	EF468094	99
S8-3	*L.reuteri*	X76328	99
S9-1	*L.reuteri*	X76328	99
S9-3	*L.reuteri*	EF468094	99
S10.1-3	*L.reuteri*	AB289270	99
S6-4	*S. alactolyticus*	AF201899	99
S1-2	*L. amylovorus* *L. sobrius*	AY944408 AY700063	99 99
S2-2	*L. amylovorus* *L. sobrius*	AY944408 EF468103	99 99
S5-2	*L. amylovorus* *L. sobrius*	AY944408 EF468103	99 99
S9-2	*L. amylovorus* *L. sobrius*	EF120375 EF468103	99 99
S2-1	*L. mucosae*	AB186315	99
S2-3	*L. mucosae*	AF243148	99
S4-3	*L. mucosae*	EF462195	99
S2-4	*L. johnsonii*	AB295648	99
S14-6	*L.acidophilus* *johnsonii* *L. johnsonii*	M99704 AE017198	99 99

* = source of the strain : S. Konstantinov, Laboratory of Microbiology, Wageningen University and Research Centre Agrotechnology & Food Sciences, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherland as DSM 16698

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