

# Comparison of Media for the Isolation of *Enterobacter sakazakii*<sup>∇</sup>

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*Enterobacter sakazakii* is associated with neonatal infections and is occasionally present at low levels (<1 CFU/g) in powdered infant formula milk (IFM). It has been previously reported that some *E. sakazakii* strains do not grow in standard media for *Enterobacteriaceae* and coliform bacteria; therefore, a reliable method is needed for recovery of the organism. Three *E. sakazakii* enrichment broths—*Enterobacteriaceae* enrichment broth (EE), *E. sakazakii* selective broth (ESSB), and modified lauryl sulfate broth (mLST)—were compared with a novel broth designed for maximum recovery of *E. sakazakii*, *E. sakazakii* enrichment broth (ESE). One hundred seventy-seven strains (100%) grew in ESE, whereas between 2 and 6% of strains did not grow in EE, mLST, or ESSB. *E. sakazakii* possesses  $\alpha$ -glucosidase activity, and a number of selective, chromogenic agars for *E. sakazakii* isolation based on this enzyme have been developed. *E. sakazakii* isolation agar produced fewer false-positive colonies than did Druggan-Forsythe-Iversen agar. However, the latter supported the growth of more *E. sakazakii* strains. It was also determined that 2% of *E. sakazakii* strains did not produce yellow pigmentation on tryptone soya agar at 25°C, a characteristic frequently cited in the identification of *E. sakazakii*. The recovery of desiccated *E. sakazakii* (0.2 to 2000 CFU/25 g) from powdered IFM in the presence of a competing flora was determined with various enrichment broths and differential selective media. Current media designed for the isolation and presumptive identification of *E. sakazakii* do not support the growth of all currently known *E. sakazakii* phenotypes; therefore, improvements in the proposed methods are desirable.

*Enterobacter sakazakii* is an occasional contaminant of powdered infant formula milk (IFM) and is a rare cause of neonatal infections (7, 9, 20, 22). Although not all cases have been attributed to the ingestion of IFM, the microbiological safety and preparation of IFM are of concern (4, 5). The Codex Alimentarius Commission is currently reviewing the code of hygienic practices for foods for infants and children, and the European Union has introduced microbiological criteria (2).

Several methods have been proposed for the enrichment and isolation of *E. sakazakii* (6, 11, 21). An integral part of all methods is the  $\alpha$ -glucosidase test (16). However, a number of other *Enterobacteriaceae* are  $\alpha$ -glucosidase positive (14, 15), and coisolation of these organisms lowers the efficiency of chromogenic media for the isolation of *E. sakazakii*. Although *E. sakazakii* can be recovered from 3 to 14% of IFM samples, reported levels have never exceeded 1 CFU g<sup>-1</sup> (10, 17, 18). Therefore, specific and sensitive enrichment is required for isolation of the organism.

Farmer et al. (3) reported reduced plating efficiency of *E. sakazakii* strains on media commonly used in enteric bacteriology. It has also been noted that some *E. sakazakii* strains are unable to grow in lauryl sulfate broth (LST) or brilliant green bile broth (12). As the latter strains also failed to grow in *Enterobacteriaceae* enrichment broth (EE), it was deemed necessary to design a modified enrichment medium to aid comparison of the selective media. This study reports a comparison between currently proposed enrichment and isolation media for the detection of *E. sakazakii*.

(A preliminary report of this work was presented at the 105th General Meeting of the American Society for Microbiology [13]).

## MATERIALS AND METHODS

**Microbiological strains.** Proposed media for the isolation of *E. sakazakii* were assessed with over 250 *Enterobacteriaceae* isolates from the culture collection of Nottingham Trent University, Nottingham, United Kingdom. The *E. sakazakii* isolates ( $n = 177$ ) were from clinical, food, and environmental sources and have been described previously (10, 11). The competing  $\alpha$ -glucosidase-positive *Enterobacteriaceae* were *Buttiauxella noakiae* and strains from two as-yet-unnamed species identified as distinct 16S cluster groups (14, 15). The remaining strains were *Enterobacter pyrinus*, *Enterobacter cloacae*, *Citrobacter koseri*, *Citrobacter freundii*, *Citrobacter braakii*, *Enterobacter asburiae*, *Enterobacter aerogenes*, *Enterobacter amnigenus*, *Escherichia hermanni*, *Escherichia coli*, *Hafnia alvei*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Raoultella terrigena*, *Kluyvera* sp., *Leclercia adecarboxylata*, *Pantoea* sp., *Proteus vulgaris*, *Providencia rettgeri*, *Salmonella enterica* serovar Enteritidis, *Serratia marcescens*, and *Serratia ficaria*.

**Growth media.** The following media were prepared according to the manufacturers' instructions: buffered peptone water (BPW) (CM0509; Oxoid, Basingstoke, United Kingdom), EE (CM0317; Oxoid), modified LST (mLST) (CM0451; Oxoid) with 0.5 M NaCl and 10 mg l<sup>-1</sup> vancomycin (6), violet red bile glucose agar (VRBGA) (CM0485; Oxoid), violet red bile lactose agar (VRBL) (CM0107; Oxoid), *E. sakazakii* chromogenic agar (Druggan-Forsythe-Iversen [DFI] formulation) (CM1055; Oxoid) (11), and tryptone soya agar (TSA) (CM0131; Oxoid). *E. sakazakii* isolation agar (ESIA) (AEB520010; AES Laboratoire) (8) and *E. sakazakii* selective broth (ESSB) (AEB611448; AES Laboratoire) were purchased as prepared media. Milk agar was prepared as follows: bacteriological agar (3.0 g) (LP0011; Oxoid) and ammonium sulfate were dissolved in 40 ml of distilled water. After autoclaving, 200 ml of warm (55°C) liquid IFM was added and the mixture was dispensed into petri dishes.

*E. sakazakii* enrichment broth (ESE) was composed of disodium hydrogen phosphate (6.5 g), potassium dihydrogen phosphate (2.0 g), yeast extract (1.5 g), neutralized peptone (4.0 g), base tryptone (12.0 g), sodium chloride (4.0 g), sucrose (100.0 g), and sodium deoxycholate (0.5 g) dissolved in distilled water (1,000 ml). The complete medium (pH 7.0  $\pm$  0.1) was autoclaved at 121°C for 15 min.

**Growth measurement.** Enrichment broths were inoculated (1  $\times$  10<sup>4</sup> CFU ml<sup>-1</sup>) with overnight cultures diluted in sterile saline. Initially, growth was determined by measuring the change in optical density at 590 nm (OD<sub>590</sub>) at

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TABLE 1. Methods for the recovery of desiccated *E. sakazakii* from powdered IFM

Method	Preenrichment	Enrichment	Primary isolation	Presumptive identification
FDA (21)	BPW (37°C)	EE broth (37°C)	VRBGA (37°C)	Yellow pigment on TSA at 25°C for 48-72 h
DFI (11)	BPW (37°C)	ESE broth (37°C)	DFI (37°C)	Blue-green colonies
mLST (6)	BPW (37°C)	mLST + vancomycin (45°C)	TSA (37°C with enhanced light exposure)	Yellow, α-glucosidase-positive colonies
AES	ESSB broth (37°C)		ESIA (44°C)	Blue-green colonies

37°C and 44°C with a TECAN SPECTRA Fluor instrument (TECAN United Kingdom Ltd., Reading, United Kingdom). Due to the inability to detect growth of some *E. sakazakii* strains in selective media with the OD measurements, 10 ml of EE, ESSB, and mLST were inoculated ( $1 \times 10^7$  CFU ml<sup>-1</sup>) from overnight cultures into BPW (18 *E. sakazakii* strains and 21 strains of other *Enterobacteriaceae*). After a 24-h incubation, the viable counts were determined by decimal dilutions on TSA incubated at 37°C.

**Recovery of desiccated *E. sakazakii* from powdered IFM containing competing organisms.** Three strains of *E. sakazakii* (NCTC 11467<sup>T</sup>, ATCC 12868, and SK90) were grown overnight on milk agar at 37°C. These strains are the species type strain, the ATCC Perceptrol quality control strain, and a clinical strain kindly supplied by Franco Pagotto (19), respectively. The cells were harvested from the plates and resuspended in sterile infant formula to give cell densities of ca.  $10^{11}$  CFU/ml prior to freeze-drying. The freeze-dried samples were stored for

4 weeks prior to use for the bacterial concentrations to stabilize. The bacterial viable cell counts in the desiccated samples were estimated by a most-probable-number technique ( $n = 8$ ) in BPW prior to inoculation of powdered IFM. Appropriate quantities of desiccated cells were used to inoculate triplicate 25-g quantities of commercial milk-based powdered infant formula (Cow & Gate Premium Stage 1) at 0.2 to 2,000 CFU 25g<sup>-1</sup>. The IFM contained endogenous *Bacillus* spp., and one aliquot contained endogenous *Raoultella terrigena*. All aliquots were additionally inoculated with yellow-pigmented *Enterobacteriaceae* isolates comprising an α-glucosidase-positive strain and an α-glucosidase-negative *Pantoea* strain at 0.4 CFU g<sup>-1</sup>.

Four recovery methods were compared: FDA (<http://www.cfsan.fda.gov/~comm/mmesakaz.html>), DFI (11; also this study), mLST (6), and AES (<http://www.aeslaboratoire.com/>). For convenience, the preenrichment, enrichment, primary isolation, and presumptive identification steps for each of these methods

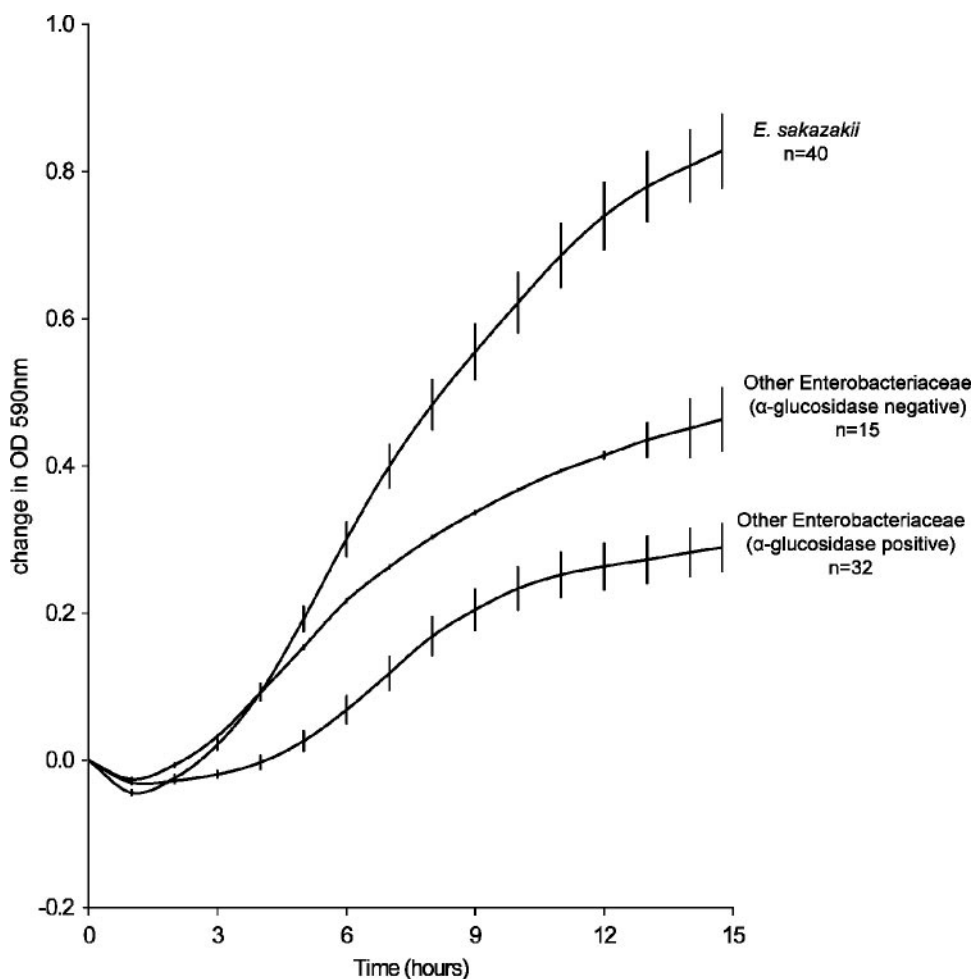


FIG. 1. Comparative growth of *E. sakazakii* and other organisms in ESE. Error bars represent the mean ± the standard error of the sample mean, calculated from the standard deviation of the sample mean divided by  $\sqrt{n}$ . A difference in OD<sub>590</sub> of 0.5 is equivalent to ca.  $0.5 \log_{10}$  CFU ml<sup>-1</sup>.

TABLE 2. Strains showing increases in OD after 24 h of incubation in enrichment media

Organism (no. of strains)	% of strains <sup>a</sup>							
	37°C				44°C			
	ESE	EE	mLST	ESSB	ESE	EE	mLST	ESSB
<i>E. sakazakii</i> (177)	100	97	96	96	99	95	94	87
$\alpha$ -Glucosidase-positive <i>Enterobacteriaceae</i> (40)	100	100	95	93	98	95	78	65
$\alpha$ -Glucosidase-negative <i>Enterobacteriaceae</i> (34)	100	100	100	97	100	100	91	88

<sup>a</sup> Values are percentages of strains showing increases in OD after 24 h of incubation at the indicated temperatures. See Materials and Methods for enrichment medium manufacturers' details.

are summarized in Table 1. Up to five presumptive isolates were selected and identified by biochemical profiles with an API20E instrument (bioMérieux United Kingdom Ltd.) according to the manufacturer's instructions.

## RESULTS

**Enrichment broth evaluation.** Preliminary experiments had shown that a number of *E. sakazakii* strains were sensitive to brilliant green, lauryl sulfate, crystal violet, and/or novobiocin and that all *E. sakazakii* strains studied were able to ferment sucrose. In contrast, the majority of non-*E. sakazakii*  $\alpha$ -glucosidase-positive *Enterobacteriaceae* did not utilize sucrose. Therefore, with sucrose in place of dextrose and/or lactose, *E. sakazakii* was able to outgrow other *Enterobacteriaceae* in ESE (Fig. 1). Sodium deoxycholate was included as a selective agent to suppress gram-positive organisms.

All 177 *E. sakazakii* strains grew well in ESE at 37°C (Table 2). In contrast, growth was not detected for 3 to 13% ( $n = 177$ ) of *E. sakazakii* strains in EE, mLST, or ESSB at 37°C and 44°C. The viable cell counts after a 24-h incubation in selective

broths are presented in Fig. 2. All *Enterobacteriaceae* strains grew in ESE. In the three selective enrichment broths—EE, mLST, and ESSB—the viability of four to six *E. sakazakii* strains decreased and some were unrecoverable (>6 log decline). mLST was the most selective broth, with only two non-*E. sakazakii* strains able to grow. *Bacillus cereus* ( $n = 1$ ), *Bacillus subtilis* ( $n = 2$ ), *Staphylococcus aureus* ( $n = 2$ ), and *Lactobacillus* spp. ( $n = 2$ ) were not recoverable from any broths (data not shown).

**Selective agar assessment.** Table 3 shows that 2% ( $n = 177$ ) of *E. sakazakii* strains did not produce yellow pigmentation on TSA after 3 days of incubation at 25°C, a criterion which has been recommended for the presumptive identification of *E. sakazakii* (8, 21). All of the *E. sakazakii* strains grew at 37°C and produced characteristic colonies on the *Enterobacteriaceae* (VRBGA) and coliform (VRBL) agars; however, one strain (NTU 531) grew very poorly on these media. This isolate was also the only *E. sakazakii* strain in this study that did not grow and produced characteristic (blue-green) colonies on DFI after

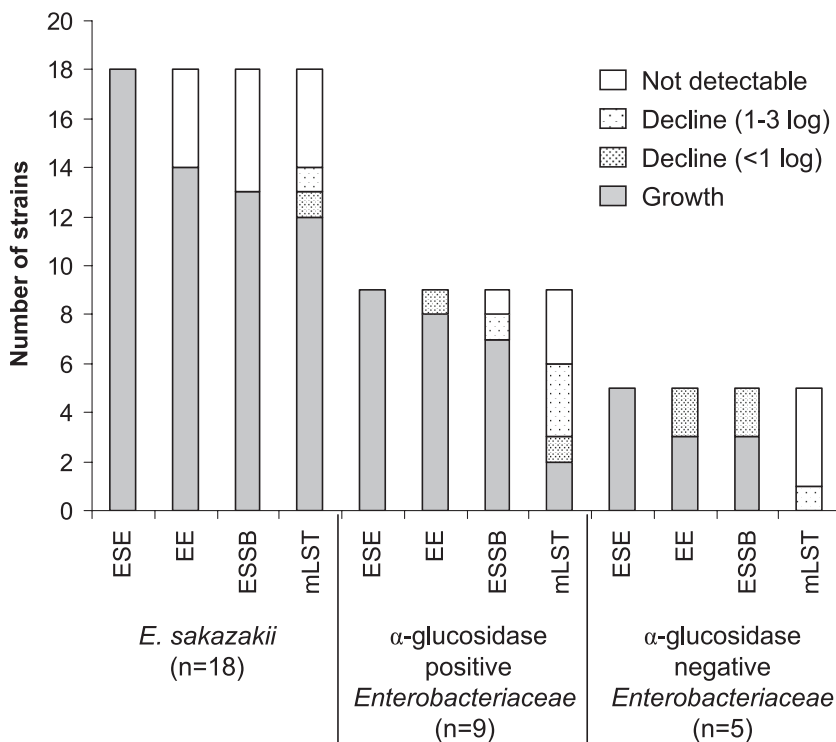


FIG. 2. Growth, persistence, and death of *E. sakazakii* and competitive organisms in enrichment broths.

TABLE 3. Growth of *E. sakazakii* and other *Enterobacteriaceae* on various selective and differential agars

Organism (no. of strains)	Yellow pigment production 25°C (TSA, 48–72 h)	% of strains <sup>a</sup>						
		Incubation temp for 24 h						ESIA
		37°C				44°C		
		TSA	DFI	VRBGA	VRBL	TSA	DFI	
<i>E. sakazakii</i> (177)	98	100	99	100	100	99	93	96
α-Glucosidase-positive <i>Enterobacteriaceae</i> (40)	93	100	100	100	92	95	45	63
α-Glucosidase-negative <i>Enterobacteriaceae</i> (34)	32	100	0	100	96	100	0	0

<sup>a</sup> Values are percentages of strains showing the following growth parameters: TSA, growth; DFI, blue-green colonies; VRBGA, red colonies; VRBL, red colonies. See Materials and Methods for agar manufacturers' details.

24 h of incubation at 37°C. NTU 531 appears to be more sensitive to sodium deoxycholate than the other isolates tested; decreasing the concentration of this compound in the media to 0.3g l<sup>-1</sup> improved the growth of this strain. At 44°C, 1% (*n* = 177) of *E. sakazakii* strains did not grow on TSA, whereas on ESIA and DFI, three and five *E. sakazakii* strains (respectively) were unable to grow. In addition, rather than blue-green colonies, eight strains produced white or partially colored colonies on DFI and four strains produced mauve, rather than blue-green, colonies on ESIA at this temperature.

#### Recovery of desiccated *E. sakazakii* from powdered IFM.

The numbers of samples positive for recovery of three desiccated *E. sakazakii* strains from powdered IFM are presented in Table 4. There were three replicates per strain at each inoculation level (2,000 to 0.2 CFU/25 g). The AES method recovered only the *E. sakazakii* type strain (NCTC 11467<sup>T</sup>). Endogenous *R. terrigena* was the only organism recovered by the FDA method (21) for one of the samples inoculated with 2,000 CFU 25 g<sup>-1</sup> *E. sakazakii*; therefore, *R. terrigena* had overgrown *E. sakazakii*. The mLST method recovered both *R. terrigena* and *E. sakazakii* in the corresponding sample, whereas the DFI method recovered only *E. sakazakii*. Fewer presumptive positive isolates were found to be false positive with the mLST method than with the other methods.

## DISCUSSION

ESE broth was developed to facilitate comparison of the performance of *E. sakazakii* selective enrichment broths. Preliminary experiments had shown that all *E. sakazakii* strains were able to ferment sucrose, whereas the majority of non-*E. sakazakii* α-glucosidase-positive *Enterobacteriaceae* did not. Therefore, ESE broth was formulated to support good growth

of *E. sakazakii* compared with competing organisms (Fig. 1). Although other *Enterobacteriaceae* also utilize sucrose, these are mainly α-glucosidase-negative organisms and so can be differentiated on current chromogenic media. As *E. sakazakii* has been shown to have greater desiccation tolerance than most other *Enterobacteriaceae* (1; J. Caubilla-Baron and S. J. Forsythe, submitted for publication), a high concentration of sucrose was incorporated into the broth to act as a humectant, lowering the available water. Sodium deoxycholate was incorporated to suppress the growth of gram-positive bacteria.

All *E. sakazakii* isolates grew at 37°C in ESE, but 2 to 4% (*n* = 177) of the strains were undetected in EE, mLST, or ESSB. Assessment of the viability of these strains by standard plate counts showed that, for five strains, one or more of the selective media were bactericidal. There have been previous reports of *E. sakazakii* strains failing to grow in mLST (15), as well as in LST and brilliant green bile broth (12). Other α-glucosidase-positive organisms lost their viability in mLST to a greater extent than did *E. sakazakii*. Therefore, the selectivity of this medium is not necessarily a result of the increased growth of *E. sakazakii* but of the greater die-off of nontarget cells.

All strains produced characteristic red colonies on VRBGA and VRBLA. However, these media are selective only for *Enterobacteriaceae* and coliforms, respectively, and are not specific for *E. sakazakii*. They are therefore of use with respect to general hygiene monitoring but not for detection of specific pathogens, such as *Salmonella* and *E. sakazakii*. At the recommended incubation temperatures of 37 and 44°C, respectively, 99% of *E. sakazakii* strains grew on DFI agar but only 96% on ESIA. Incubation of DFI at 44°C (above the manufacturer's recommendation of 37°C) resulted in 7% of strains not showing the characteristic blue-green colony morphology. As 1% of *E. sakazakii* strains were unable to grow on nonselective medium (TSA) at 44°C, incubation at this temperature may not ensure the recovery of *E. sakazakii*.

Detection methods should be evaluated with desiccated *E. sakazakii* cells in the presence of competing flora to mimic environmental samples from manufacturing facilities. Comparison of four methods for the recovery of desiccated *E. sakazakii* from IFM indicated that the most sensitive method was pre-enrichment in BPW, followed by enrichment in ESE and plating on DFI agar (Table 4). However, at the lower inoculum levels, this method produced a large number of false-positive colonies on DFI. The presence of competing organisms reduced the sensitivity of the FDA method, and the AES method recovered only the *E. sakazakii* type strain. The mLST method was not as

TABLE 4. Recovery of *E. sakazakii* from powdered IFM in the presence of competing flora by four isolation methods

<i>E. sakazakii</i> concn (CFU 25 g <sup>-1</sup> )	No. of samples positive for <i>E. sakazakii</i> recovery ( <i>n</i> = 9) by the indicated method <sup>a</sup>			
	DFI (11)	FDA (21)	mLST (6)	AES <sup>b</sup>
2,000	9	7	9	3
200	8	6	8	1
20	6	3	3	1
2	3	1	0	0
0.2	0	0	0	0

<sup>a</sup> See Table 1 for details of the four methods.

<sup>b</sup> Only *E. sakazakii* NCTC 11467<sup>T</sup> was recovered by this method.

sensitive as the DFI method at low inoculum levels but produced fewer presumptive false positives. As one of the strains used in this experiment was sensitive to lauryl sulfate, the ability of the mLST method to recover it at the higher inoculum levels suggests that this method works better in the presence of the sample matrix (IFM) than when used for pure cultures. This may be due to divalent cations in the IFM counteracting the effects of the lauryl sulfate. Therefore, the performance of the mLST method may be reduced if used for other sample matrices.

This study has used a large number ( $n = 177$ ) of *E. sakazakii* strains to demonstrate that the levels of selective agents such as crystal violet, sodium lauryl sulfate, brilliant green, and sodium deoxycholate in media need to be reassessed to ensure the recovery of the organism, especially from mixed cultures. The use of sucrose (100 g/liter) in ESE promoted the growth of *E. sakazakii* relative to other  $\alpha$ -glucosidase-positive *Enterobacteriaceae*. However, this broth is not selective enough to be considered a viable alternative enrichment method, and further development of effective media for the isolation of *E. sakazakii* is needed.

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