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Inhibition of food-related bacteria by antibacterial substances produced by *Pseudomonas* sp. strains isolated from pasteurized milk

Inibição de bactérias associadas a alimentos por substâncias antimicrobianas produzidas por estirpes de Pseudomonas sp. isoladas de leite pasteurizado

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Summary

In this work, the production of antimicrobial substances by strains of *Pseudomonas* sp. isolated from pasteurized milk and their potential action against food-related bacteria were investigated. Samples of pasteurized milk were purchased from arbitrarily chosen commercial establishments in the city of Rio de Janeiro, Brazil. Of the four samples analyzed, three presented several typical colonies of *Pseudomonas*. About 100 colonies were chosen and subjected to biochemical tests for confirmation of their identity. Eighteen strains of the *Pseudomonas* genus were identified and submitted to tests for the production of antimicrobial substances. Twelve strains (66.7%) were identified as *Pseudomonas fluorescens*, four (22.2%) as *P. aeruginosa*, one (5.5%) as *P. mendocina* and one (5.5%) as *P. pseudoalcaligenes.* Only two *P. fluorescens* strains were unable to produce any antimicrobial substance against any of the indicator strains tested. Most of the strains presented a broad spectrum of action, inhibiting reference and food-related strains such as *Proteus vulgaris, Proteus mirabilis, Hafnia alvei*, *Yersinia enterocolitica*, *Escherichia coli* and *Salmonella typhi*. Five antimicrobial substance-producing strains, which presented the broadest spectrum of action, were also tested against *Staphylococcus aureus* reference strains and 26 *Staphylococcus* sp. strains isolated from foods, some of which were resistant to antibiotics. The producer strains 8.1 and 8.3, both *P. aeruginosa*, were able to inhibit all the staphylococcal strains tested. The antimicrobial substances produced by strains 8.1 and 8.3 did not seem to be typical bacteriocins, since they were resistant to the three proteolytic enzymes tested. Experiments involving the characterization of these substances are being carried out in order to evaluate their biotechnological application.

Key words: *Pseudomonas sp.; Antimicrobial substances; Milk; Foodborne pathogens; Staphylococcus spp.*

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Resumo

Neste trabalho, a produção de substâncias antimicrobianas por estirpes de *Pseudomonas* sp. isoladas de leite pasteurizado e seu potencial de ação contra bactérias associadas a alimentos foram investigados. As amostras de leite pasteurizado foram adquiridas em estabelecimentos comerciais, arbitrariamente escolhidos, da cidade do Rio de Janeiro-RJ, Brasil. Dentre as quatro amostras analisadas, três apresentaram várias colônias típicas de *Pseudomonas*. Cerca de cem colônias foram escolhidas e submetidas a testes bioquímicos para confirmação da identificação. Dezoito estirpes do gênero *Pseudomonas* foram identificadas e submetidas aos testes de produção de substâncias antimicrobianas. Doze estirpes (66,7%) foram identificadas como *Pseudomonas fluorescens*; quatro (22,2%) como *P. aeruginosa*; uma (5,5%) como *P. mendocina*, e uma (5,5%) como *P. pseudoalcaligenes*. Apenas duas estirpes de *P. fluorescens* não foram capazes de produzir qualquer substância antimicrobiana contra os microorganismos indicadores testados. A maioria das estirpes apresentou um amplo espectro de ação, inibindo estirpes de referência e associadas a alimentos, como *Proteus vulgaris*, *Proteus mirabilis*, *Hafnia alvei*, *Yersinia enterocolitica*, *Escherichia coli* e *Salmonella* Typhi. Cinco estirpes produtoras de substâncias antimicrobianas, que apresentaram o mais amplo espectro de ação, também foram testadas contra estirpes de referência de *Staphylococcus aureus* e contra 26 estirpes de *Staphylococcus* sp. isoladas a partir de alimentos, sendo, algumas delas, resistentes a antibióticos. As estirpes produtoras 8.1 e 8.3, ambas *P. aeruginosa*, foram capazes de inibir todas as estirpes de *Staphylococcus* testadas. As substâncias antimicrobianas produzidas pelas estirpes 8.1 e 8.3 não parecem ser bacteriocinas típicas, uma vez que foram resistentes às três enzimas proteolíticas testadas. Experimentos envolvendo a caracterização destas substâncias estão sendo realizados, a fim de avaliar a sua aplicação biotecnológica.

Palavras-chave: *Pseudomonas sp.; Substâncias antimicrobianas; Leite; Patógenos associados a alimentos; Staphylococcus spp.*

1 Introduction

Although designed to supply complete nutrition for growing calves, bovine milk also provides an appropriate growth medium for a variety of microorganisms. The abundance of carbohydrates, proteins and fats in association with the neutral pH allows for the development of a microbial community which may be highly variable (ALI and ABDELGADIR, 2011).

Amongst the main reasons causing milk contamination are the poor conditions of hygiene during milking, insufficient cleaning procedures of the utensils and equipments and also problems related to the storage and transport (SILVA et al., 2011).

A variety of microorganisms with human pathogenic potential, including *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* and *Mycobacterium tuberculosis*, can be found in raw milk (ARCURI et al., 2006). However the presence of psychrotolerant bacteria belonging to various genera are extremely important in relation to the shelf life of milk and its derivatives, since they may develop even during long periods of cooling. During the storage of raw milk, these microorganisms can produce many proteolytic and lipolytic enzymes responsible for the spoilage of milk and dairy products, reducing both the quality and shelf life of the processed milk and resulting in important economic losses (FRANZETTI and SCARPELLINI, 2007; DE JONGHE et al., 2011).

Although the pseudomonas are also psychrotolerant microorganisms and are generally isolated from milk, they can also produce antimicrobial substances (AMS) able to inhibit other bacteria. Some *Pseudomonas aeruginosa* strains, for example, produce AMS called pyocins (MICHEL-BRIAND and BAYSSE, 2002; ELFARASH et al., 2012).

Since therapeutic antibiotics are prohibited for use in foods, the interest in these natural antimicrobial substances is increasing exponentially. Additives with antagonistic properties have become a trademark in the search for food safety and preservation. In food and drinks, the addition of antimicrobial compounds to processed products has become a powerful weapon in the arsenal of food preservation. These compounds, especially the bacteriocins, can be interesting strategies against the growth of undesirable microorganism (RILEY and WERTZ, 2002; ELFARASH et al., 2012, NISHIE et al., 2012).

Given the relevance of the research on antagonistic additives, this work aimed to characterize strains of *Pseudomonas* sp. isolated from pasteurized milk, and investigate the production of antimicrobial substances and their potential for action against the Gram-negative bacteria and staphylococci strains associated with food.

2 Material and Methods

2.1 Milk samples and strains

Four samples of pasteurized milk were purchased in arbitrarily chosen commercial establishments in the city of Rio de Janeiro, Brazil, and immediately taken for analysis. The staphylococcal strains presented in Table 1 were isolated in a previous study carried out by our research group. The other strains belong to the collection of the Laboratory of Microbiology of the Federal Institute of Rio de Janeiro, Brazil. All the strains used as indicators in this work were cultivated on Casoy agar (Himedia, Brazil) at 36 °C for 18 hours and stock cultures maintained in Casoy broth at –20 °C with the addition of 40% glycerol.

2.2 Isolation and identification of the bacteria

The bacteria were isolated and identified according to Dworkin et al. (2006) and Franzetti and Scarpellini (2007). When necessary, bacterial identification was also carried out using the Bactray® commercial kit for bacterial identification (Laborclin, São Paulo, Brazil).

2.3 Antibiotic resistance profile

The antibiotic resistance profile of the strains isolated in this work was determined by disc diffusion in Mueller-Hinton agar according to the CLSI procedures (CLSI, 2012). The following antibiotics (Sensifar, São Paulo, Brazil) were used: aztreonam (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg) and tetracycline (30 µg).

2.4 Production of antimicrobial substances and determination of the action spectrum

This experiment was carried out as described by Giambiagi-Demarval et al. (1990). The pseudomonas bacteria isolated from milk were grown in 5 mL of Casoy broth (Himedia, Brazil) at 36 °C for 18 h. Five microlitres of each culture were spotted onto Casoy plates. After 18 h at 37 °C, the bacteria were killed by chloroform fumes and the plates sprayed with the indicator strain culture (0.3 mL of a previously grown culture in 3 mL of Casoy soft agar). The plates were incubated for a further 18 h at 37 °C and the diameters of the inhibition zones measured (in mm). Different Gram-negative and Gram-positive bacteria were used as the indicator strains for the production of antimicrobial substances.

2.5 Susceptibility of the inhibitory substances to proteolytic enzymes

The effect of the proteolytic enzymes trypsin (Sigma-Aldrich, São Paulo, Brazil), pronase XXIII (Sigma-Aldrich, São Paulo, Brazil) and proteinase K

Table 1. Inhibition of staphylococcal strains isolated from food by the *Pseudomonas* producer strains 8.1 and 8.3.

Indicator strains	Identification	Antibiotic resistance profile	Inhibition by the producer strains	
			8.1	8.3
S4	*S. equorum*	-	+++	+++
S5	*S. equorum*	-	++	++
S10	*S. epidermidis*	-	++++	+++
S11	*S. epidermidis*	Amp, Pen	+	+
S12	*S. epidermidis*	Amp, Pen	+	+
S14	*S. epidermidis*	Tet	+	+
S15	*S. epidermidis*	Amp, Pen	+	+
S16	*S. epidermidis*	Pen	+	+
S17	*S. lentus*	Eri	+	+
S18	*S. epidermidis*	-	+++	+++
S19	*S. epidermidis*	-	+	+
S23	*Staphylococcus* sp.	Amp, Eri, Pen	+	+
S24	*S. epidermidis*	-	+	+
S25	*S. carnosus*	-	+	+
S26	*S. equorum*	Amp, Eri, Pen	+	+
S29	*S.epidermidis*	-	+	+
S31	*Staphylococcus* sp.	-	+	+
S32	*Staphylococcus* sp.	-	+	+
S33	*Staphylococcus* sp.	-	+	+
S34	*Staphylococcus* sp.	-	+	+
S35	*S. hominis*	-	+	+
S36	*Staphylococcus* sp.	-	++	++
S37	*S. epidermidis*	-	+	+
S38	*Staphylococcus* sp.	Ami, Amp, Ctx, Eri, Gen, Pen	+++	+++
S39	*Staphylococcus* sp.	Ctx	+++	++
S40	*S. hominis*	-	+++	+++

+, diameter of inhibition zones ≤ 20 mm; ++, diameter of inhibition zones between 21 and 30 mm; +++, diameter of inhibition zones between 31 and 40 mm; ++++, diameter of inhibition zones > 40 mm; -, no inhibition; Ami, amicacin; Amp, ampicillin; Ctx, cefotaxime; Eri, erythromycin; Pen, penicillin; Tet, tetracycline.

(Sigma-Aldrich, São Paulo, Brazil) on the antimicrobial substance activity was determined according to Giambiagi-Demarval et al. (1990). Forty microlitres of the enzymes (1 mg/mL, prepared in 0.05 M Tris (pH 8.0) with 0.01 M $CaCl_2$) were applied around the producer strain after chloroform treatment. The plates were incubated at 37 °C for 4 h and then sprayed with the indicator strain. After treatment with the enzymes, the absence of inhibition zones indicates that the antimicrobial substance presents an active proteinaceous compound.

2.6 Susceptibility of the inhibitory substances to NaOH

The antimicrobial substances were also treated with 0.2 M NaOH to rule out the possibility that the inhibition exhibited could have been due to the production of organic acids by the producer strain during its metabolism. This assay was carried out according to Giambiagi-Demarval et al. (1990).

3 Results and Discussion

Of the four pasteurized milk samples analyzed, three presented several typical colonies suggestive of *Pseudomonas* sp.. About 100 colonies were arbitrarily chosen and subjected to biochemical tests for confirmation of their identity.

Of the eighteen strains analyzed belonging to the *Pseudomonas* genus, twelve (66.7%) were identified as *P. fluorescens*, four (22.2%) as *P. aeruginosa*, one (5.5%) as *P. mendocina* and one (5.5%) as *P. pseudoalcaligenes* (Table 2). The significant presence of *P. fluorescens* was expected, since other studies have also reported this species as the dominant spoilage species in refrigerated raw and pasteurized milk (DOGAN and BOOR, 2003; MUNSCH-ALATOSSAVA and ALATOSSAVA, 2006; 2007).

All the 18 strains selected were tested for resistance to different classes of antimicrobials. Five strains were only resistant to aztreonam, another only to cefotaxime and four to aztreonam and cefotaxime (Table 2). According to the

literature, resistance to antibiotics has become common amongst strains isolated from milk and dairy products (ARSLAN et al., 2011; BEENA et al., 2011). Straley et al. (2006) verified that *Pseudomonas* spp. isolated from bulk tank milk samples were the dominant non-coliform Gram-negative bacteria and showed the highest levels of resistance to antibiotics. In a recent study, Arslan et al. (2011) verified that all 32 *Pseudomonas* strains isolated from cheese were susceptible to ciprofloxacin, gentamicin and imipenem. Antibiotic-resistant *Pseudomonas* spp. have significant importance in the dairy industries, since they are able to grow at low temperatures and have the potential to form biofilms (MARCHAND et al., 2012).

Table 2. Description of the *Pseudomonas* spp. strains isolated from milk.

Producer strains	Mik sample	Identification	Characteristics
8.1	1	*P. aeruginosa*	AMS^+
8.2	1	*P. aeruginosa*	AMS^+
8.3	1	*P. aeruginosa*	AMS^+
E2A	2	*P. fluorescens*	AMS^+
E2B	2	*P. fluorescens*	AMS^+
E2C	2	*P. fluorescens*	AMS^+
E2D	2	*P. fluorescens*	CFT^R
E2E	2	*P. pseudoalcaligenes*	AMS^+, AZT^R, CFT^R
CC2G	2	*P. fluorescens*	AMS^+, AZT^R, CFT^R
CC2H	2	*P. fluorescens*	AMS^+, AZT^R
CC2I	2	*P. fluorescens*	AMS^+, AZT^R
CC2J	2	*P. fluorescens*	AMS^+, AZT^R, CFT^R
CC2K	2	*P. fluorescens*	AMS^+, AZT^R
CC2L	2	*P. aeruginosa*	AMS^+, AZT^R
CC2M	2	*P. fluorescens*	AMS^+, AZT^R
CC2N	2	*P. mendocina*	AMS^+
CC2O	2	*P. fluorescens*	AMS^+, AZT^R, CFT^R
CC3P	3	*P. fluorescens*	AMS^+

AMS^+, antimicrobial substance producer; AZT^R, resistant to aztreonam; CFT^R, resistant to cefotaxime.

The eighteen *Pseudomonas* sp. strains were also submitted to assays for the production of antimicrobial substances. All were able to produce antimicrobial substances against at least one of the indicator strains tested (Table 3). Differently from some of the antimicrobial substance-producer *Pseudomonas*, the antimicrobial spectrum of action of the strains isolated in this work was not restricted to pseudomonas (LAVERMICOCCA et al., 1999; RILEY and WERTZ, 2002).

In relation to Gram-negative indicator strains, *P. aeruginosa* 8.1, 8.2 and 8.3 presented the broadest spectrum of action and also the largest inhibition zones, inhibiting *Proteus mirabilis, P. vulgaris, Hafnia alvei*, *Yersinia enterocolitica*, *Escherichia coli*, *Salmonella* spp. Figure 1 shows some examples of the antimicrobial activity exhibited by strains 8.1 ad 8.3.

The inhibition of *Salmonella* sp. by *Pseudomonas* strains was also verified by Hubert et al. (1998). A *Pseudomonas* strain isolated from well water sediment produced the bacteriocin named PsVP-10, which showed a wide range of antibacterial action, inhibiting bacterial species such as *S. typhi, S. typhimurium* and *S. sonnei,* besides other microorganisms.

Interestingly, all the Gram-positive reference strains used as indicators were inhibited by *P. aeruginosa* 8.1, 8.2 and also 8.3, including the three *Staphylococcus* reference strains (Table 3). So the inhibition ability of these three *Pseudomonas* producer strains against 26 strains of *Staphylococcus* spp. isolated from food in previous studies carried out by our research group was evaluated and the results are shown in Table 1. Some differences in the diameters of the inhibition zones were observed in this experiment, but all the staphylococcal strains were inhibited by these *Pseudomonas* strains. Strain 8.2 was not included in these tests, since its spectrum of action and the plasmidial DNA profile (data not shown) suggested that this strain was identical to strain 8.1.

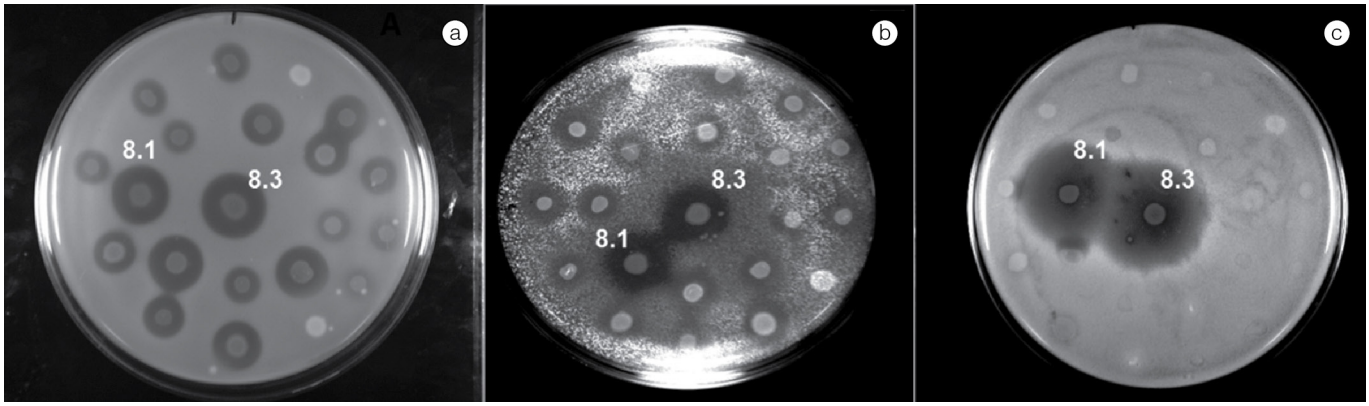

Figure 1. Agar-spot assay showing antimicrobial activity against (A) *Proteus mirabilis,* (B) *Salmonella* Typhi and (C) *Staphylococcus aureus* ATCC12600. The inhibitory activity is represented by a clear zone around microbial inoculums (spots). Strains 8.1 and 8.3 are highlighted.

Table 3. Inhibition of Gram-positive and Gram-negative bacteria by *Pseudomonas* spp. strains isolated from milk.

Indicator strains	*Pseudomonas* **spp.**																	
	8.1	8.2	8.3	E2A	E2B	E2C	E2D	E2E	CC2G	CC2H	CC2I	CC2J	CC2K	CC2L	CC2M	CC2N	CC2O	CC2P
Gram-negative																		
Escherichia coli ATCC 25922	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hafnia alvei LMIFRJ	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae ATCC 4352	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC27853	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens ATCC13525	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Proteus mirabilis LMIFRJ	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	+	-	-
Proteus vulgaris LMIFRJ	+	+	+	-	-	-	-	-	-	+	-	-	+	+	+	+	+	-
Salmonella enterica Typhi ATCC 19214	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Salmonella spp. I-LMIFRJ	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+
Salmonella spp. VI-LMIFRJ	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+	-
Yersinia enterocolitica ATCC9610	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gram-positive																		
Bacillus cereus LMIFRJ	+	+	+	-	±	±	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus circulans LMIFRJ	+	+	+	-	±	±	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus megaterium LMIFRJ	+	+	+	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus sphaericus LMIFRJ	+	+	+	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus stearothermophilus NCTC10339	+	+	+	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus thuringiensis LMIFRJ	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus luteus LMIFRJ	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus epidermidis ATCC35984	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus ATCC12600	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus ATCC25923	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus xylosus LMIFRJ	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, inhibition (diameter of inhibition zones ≥ 20 mm); -, no inhibition; ±, small inhibition zones (diameter of inhibition zones ≤ 19 mm); ATCC, American Type Culture Collection; LMIFRJ, Laboratory of Microbiology of Instituto Federal do Rio de Janeiro; NCTC, National Type Culture Collection.

A few studies have described the inhibition of *Staphylococcus* sp. by *Pseudomonas*, generally related to clinical strains. Qin et al. (2009) found that the extracellular products of *P. aeruginosa* PAO1, mainly polysaccharides, disrupted established *S. epidermidis* biofilms. Iwalokun et al. (2006) and Saleem et al. (2009) also showed that some pyocins obtained from *Pseudomonas* sp. associated with patients had anti-staphylococcal activity. On the basis of the present results and those of the above mentioned studies, it appears that

antimicrobial substances produced by *Pseudomonas* sp. could have the potential to be used not only in medical situations but also in the food industry.

To evaluate the presence of an active proteinaceous compound, the sensitivity of the antimicrobial substances to the proteolytic enzymes trypsin, proteinase K and pronase XXIII was verified. There was no loss of the inhibitory activity, indicating that the substances were resistant to these enzymes.

This fact suggests that they are not typical bacteriocins, although some bacteriocins produced by *Pseudomonas* sp. are not susceptible to proteolytic enzymes, such as the pyocin SA188 described by Naz and Rasool (2013). This substance is produced by *P. aeruginosa* and is not digested by proteases, proteinase K, trypsin or papain. According to these authors, the findings are in agreement with the studies on pyocins, in which, of the three types of pyocin, S, R and F, only the S type is sensitive to proteolytic enzymes (NAZ and RASOOL, 2013).

The substances produced by strains 8.1 and 8.3 were also resistant to NaOH, ruling out the possibility that the inhibition of the indicator was the result of acids released by the producer strains.

4 Conclusions

The consumer demand for food without the addition of chemical preservatives and the spread of antibiotic-resistant bacteria in food has driven the research for natural inhibitory substances. The results presented in this work highlight the antimicrobial activity of *Pseudomonas* spp. isolated from pasteurized milk against Gram-negative and Gram-positive food-related pathogens, such as *Salmonella typhi* and *Staphylococccus* sp., which play important roles in foodborne diseases around the world, even in developed countries. Since the inhibitory compounds produced by strains 8.1 and 8.3 could have a potential application in reducing the levels of pathogens in foods, further studies are being carried out, including the determination of the best conditions for the production of antimicrobial substances that have proved promising.

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