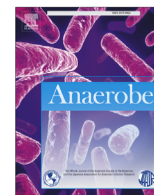
Anaerobe 48 (2017) 257–261

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe



Antimicrobial susceptibility of anaerobic bacteria

Identification of CfiA coding genes in *Bacteroides fragilis* isolates recovered in Argentina. Inconsistencies in CfiA organization and nomenclature

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A R T I C L E I N F O

Article history:
Received 27 June 2017
Received in revised form
4 October 2017
Accepted 6 October 2017
Available online 7 October 2017

Handling Editor: Jozsef Soki

Keywords:
CfiA metallo-β-lactamase
Bacteroides fragilis
Carbapenem resistance

A B S T R A C T

CfiA (CcrA) metallo-β-lactamase is the main carbapenem resistance mechanism in *B. fragilis*. From *cfiA* positive isolates detected in a previous surveillance study, 3 displayed resistance to imipenem while the remaining were susceptible. The aim of this study was to identify the *cfiA* alleles and to analyze the presence of IS elements in their upstream regions. CfiA-1, CfiA-4, CfiA-13, CfiA-19 and CfiA-22 were detected. IS elements belonging to IS*21* family and IS*942* group were identified upstream to *cfiA* in the 3 imipenem resistant isolates.

We present an exhaustive analysis of *cfiA*/CfiA registers in databases, illustrating the inconsistencies in both organization and nomenclature. According to this analysis CfiA family comprises nowadays 15 different CfiA variants coded by 24 *cfiA* sequences. Curation of CfiA database is mandatory, if not new *cfiA* admission at GenBank will contribute to make this classification more complex.

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1. Introduction

Human infections caused by anaerobic bacteria are mainly endogenous and usually polimicrobial [1]; among those anaerobes, *Bacteroides* spp. are one of the most frequently gram-negative rods recovered from clinical specimens [2]. *Bacteroides* spp. may be responsible of abscesses in the abdomen, brain, liver, pelvis and lungs, and also bacteremia [3–6].

Susceptibility studies performed worldwide have gradually reported increasing antibiotic resistance levels among anaerobic bacteria [3,7–16]. The genus *Bacteroides* stands out for its antimicrobial resistance, especially to β-lactam antibiotics [3,13,14,17–20], remaining the carbapenems as good therapeutic options. Three different β-lactamases have been described in *B. fragilis*: - CepA: an endogenous cephalosporinase that confers resistance to the majority of the β-lactam antibiotics with the exception of cephamycins and carbapenems, and susceptible to β-lactamase inhibitors [21]. - CfxA β-lactamase: also a cephalosporinase that confers resistance to cephaloridine, cefoxitin and other β-lactams with the exception of imipenem [22]. The third one, - CfiA (CcrA) metallo-β-lactamase, is active against penicillins, cephalosporins, including cephamycins, and even carbapenems. As expected, CfiA is neither inhibited by clavulanic acid nor sulbactam, while EDTA is a good inhibitor [23,24]. This metallo-β-lactamase was initially detected in 1986 in two *B. fragilis* isolates (TAL2480 and TAL3636) [25]. In 1990, the sequence of its coding gene was reported individually from *B. fragilis* TAL2480 and then from *B. fragilis* TAL3636, and named as *cfiA* and *ccrA*, respectively [26,27].

The *cfiA* gene may be silent or expressed at different levels, depending on the presence of IS upstream *cfiA* [28–30]. Among others, IS*942*, IS*1186*, IS*1187*, IS*1188*, IS*612*, IS*613*, IS*614*, IS*615*, IS*616*, IS*4351*, have been related to *cfiA* in *B. fragilis,* with varying promotion efficiency [31–33]. All these *Bacteroides*-specific ISs display a *Bacteroides*-specific promoter structure (-7 and -33 regions) which was firstly described by Bayley et al. [31,34].

In a national surveillance study carried out in our country during 2006–2009, CfiA coding genes were detected in 8/363 clinical

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https://doi.org/10.1016/j.anaerobe.2017.10.003
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B. fragilis Group isolates [18]. As this resistance marker was observed in resistant but also in imipenem susceptible *B. fragilis* isolates, the aim of this study was to identify the *cfiA* alleles and the associated IS elements in both groups.

2. Material and methods

2.1. Antimicrobial susceptibility and clonally testing of B. fragilis isolates

In the present study, 7 out of the 8 *cfiA* positive *B. fragilis* were included, as one isolate could not be recovered [18,35]. Carbapenem MICs were determined according to the CLSI reference agar dilution method, with brucella agar supplemented with 5 g/ml hemin, 1 g/ml vitamin K, and 5% laked sheep blood [36]. Different carbapenems such as imipenem, meropenem, ertapenem and doripenem, were included. Clonal relationship among these isolates was investigated by PCR (REP-PCR) based methods (Table 1).

2.2. Identification of cfiA/CfiA alleles

The presence of *cfiA* was confirmed using the primers GBI-1 and GBI-2 (Table 1), according to Kato et al. [35]. Full amplification of *cfiA* was carried out using primers CfiA-START-F and CfiA-END-R (Table 1) and heated extracted total DNA as template. Purified *cfiA* amplicons (750 bp) were sequenced in both strands using the primers mentioned above. To assess the sequence of the 5′ and 3'ends, there were included primers E-R and G-F, and CfiA-DS-F and GBI-6, respectively (Table 1). Complete nucleotidic sequences were compared with all *cfiA* alleles deposited in Gene Bank using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/).

The following *cfiA* alleles were downloaded and aligned using the ClustalW Tool of VECTOR NTI 11.5 software program: *ccrA* (NG050389), *cfiA* (M34831), *cfiA-1* (AB087225), *cfiA-2* (AB087226), *cfiA-2b* (KU559621), *cfiA-3* (AB087228), *cfiA-4* (AB087229), *cfiA-4b* (KT989373), *cfiA-5* (AB087230), *cfiA-6* (AB087231), *cfiA-7* (AB087232), *cfiA-8* (AB087233), *cfiA-9* (AB087234), *cfiA-10* (AB087227), *cfiA-11* (FM200784), *cfiA-12* (FM200786), *cfiA-13* (FM200787), *cfiA-14* (FM200789), *cfiA-14b* (KT318729), *cfiA-14c* (KT318731), *cfiA-15* (FM200790), *cfiA-16* (FM200792), *cfiA-17* (NG_054674.1), *cfiA-18* (NG054673), *cfiA-18a* (KT318727.1), *cfiA-18b* (KT318728), *cfiA-19* (NG054664), *cfiA-20* (KT989375), *cfiA-21* (KU206762), *cfiA-22* (KU559622), *cfiA-23* (KU559623) and *cfiA-24* (KU559624) (S1).

All nucleotide sequences (including those detected in this study) were translated and aligned using the ClustalW Tool of the VECTOR NTI 11.5 program, including the signal peptide. The SignalP 4.1 Server on line Tool (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the presence and location of its cleavage site.

2.3. Analysis of cfiA upstream regions

Screening for IS elements in the region immediately upstream *cfiA* was performed by PCR, using the primers G-F and E-R (Table 1), according to Kato et al. [35]. As suggested, those strains where *cfiA* is linked to IS elements generate amplicons of about 1.6–1.7 kb. In these case it was further investigated the presence of IS*942*, IS*1186*, IS*4351*, IS*21*, IS*612* and IS*614* by PCR amplification using specific primers (Table 1). Moreover in those isolates in which these IS could not be detected upstream *cfiA*, inverse PCR was carried out using the *Eco*RV enzyme and primers CfiA-DS-F and CfiA3-R (Table 1) [37]. Purified amplicons were sequenced on both strands and analyzed using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/) and VECTOR NTI 11.5 software program. In those strains which yielded fragments of about 350 bp, indicating the absence of IS elements upstream *cfiA*, the amplicons were also analyzed. Furthermore, promoter regions (-33 and -7) involved in *cfiA* expression were investigated *in silico* supported by the online tool BPROM available in http://molbiol-tools.ca/Promoters.htm.

3. Results

3.1. Antimicrobial susceptibility and clonally testing of B. fragilis isolates

MICs for different carbapenems are shown in Table 2. Three isolates (3091, 3189 and 3409) were categorized as resistant to imipenem and the remaining 4 isolates (3116, 3527, 3178 and 3010) as susceptible.

These 7 isolates displayed different banding patterns in the REP-PCR approach, indicating that they are not clonally related (data not shown).

Table 1
Primers used in this study.

Gene	Primer name	Primer sequence (5′→3′)	Ref no	Fragment (bp)
cfiA	GBI-1	CCCAACTCTCGGACAAAA GTG	[35]	390
	GBI-2	AGT GAA TCG GTG AAT CCA TG		
	GBI-6	AAAGCATCCGGCAATCGTTA	[35]	
	CfiA-START-F	ATGAAAACAGTATTTATCCTTATCT	This study	750
	CfiA-END-R	CTATGGTTTTGAGGTGCTTTCTA		
	CfiA-DS-F	CATACCAAGCAGATCGTGAACC	This study	–
Region upstream *cfiA*	G-F	CGC CAA GCT TTG CCT GCC ATT AT	[35]	~350/~1500
	E-R	CTTCGAATTCGGCGAGGGATACATAA		
IS*942*	IS942-F	TCCTCAATACATGAGCCGC	[41]	940
	CfiA3-R	GGTTGTTGATAACAATCATCCC	This study	
IS*1186*	IS1186-F	GAGAATCAAGCT TCTCGCC	[42]	1319
	E-R	CTTCGAATTCGGCGAGGGATACATAA	[35]	
IS*4351*	IS4351-F	AACCGAGGATCCAAGGTATGCAATTTCT	[42]	1042
	E-R	CTTCGAATTCGGCGAGGGATACATAA	[35]	
IS*21*	IS21-F	GCTGGTTGA ATATGCACGGC	This Study	1014
	E-R	CTTCGAATTCGGCGAGGGATACATAA	[35]	
IS*614-12*	IS614-12-F	CCTTACCCCACA ATGCGACTTGAG	This Study	1670
	CfiA3-R	GGTTGTTGATAACAATCATCCC	[41]	
REP-PCR	REP-1		[43]	-
	REP-2	IIIGCGCCGICATCAGGC ACGTCTTATCAGGCCTAC		

3.2. Identification of the *cfiA*/CfiA alleles

The presence of *cfiA* was confirmed in the 7 *B. fragilis* isolates included. As a first impression, *cfiA* alleles could not be identified using BLASTn tool as they displayed 100% identity with more than one allele deposited in GeneBank. Consequently, as mentioned above, all deposits were downloaded and aligned. The resulting dendrogram is shown in Fig. 1A. Sequences of *ccrA and cfiA* were identical as it was the case for *cfiA-4*, *cfiA-5*, *cfiA-7* and *cfiA-11; cfiA-12*, *cfiA-13* and *cfiA-15; cfiA-6* and *cfiA-16;* and *cfiA-18* and *cfiA-18a* (Fig. 1A). So, considering the first registered complete allele, only *cfiA, cfiA-1, cfiA-2, cfiA-2b, cfiA-3, cfiA-4, cfiA-4b, cfiA-6, cfiA-8, cfiA-9, cfiA-10, cfiA-13, cfiA-14, cfiA-14b, cfiA-14c, cfiA-17, cfiA-18a, cfiA-18b, cfiA-19, cfiA-20, cfiA-21, cfiA-22, cfiA-23* and *cfiA-24* correspond to different *cfiA* sequences (Fig. 1A). A dendrogram including all CfiA enzymes is shown in Fig. 1B. Cleavage site for signal peptide was predicted between position 18 and 19 (VMA-KQ). Inclusion of signal peptide in protein analysis is controversial, although no changes were observed in this region. *cfiA*-1, *cfiA*-2, *cfiA*-2b, *cfiA*-3 and *cfiA*-6 translate into the same the CfiA metallo-β-lactamase, which will be referred as CfiA-1. *cfiA* codes for CfiA while *cfiA-13* codes for CfiA-13, as these enzymes are identical between them, they will be referred as CfiA-13 (as a CfiA-1 is already assigned and in current use to another variant). *cfiA-14, cfiA-14b* and *cfiA-14c* which code for CfiA-14, CfiA-14b and CfiA-14c, respectively, will be referred as CfiA-14. Finally *cfiA-18a* and *cfiA-18b,* coding for CfiA-18a and CfiA-18b, respectively, will be mentioned as CfiA-18. The other alleles code for different CfiA variants. In summary, at the moment all CfiA registered in database correspond to 15 different proteins (S2).

Considering what is mentioned above, CfiA-13 was identified in both *B. fragilis* 3189 and 3409, while *cfiA* from *B. fragilis* 3189 corresponded 100% to *cfiA-13,* in *B. fragilis* 3409 it was observed a single substitution respect to *cfiA*-13 (A744G) (AN: LT714129 and AN: LT714130). In *B. fragilis* 3091 and 3178, *cfiA* presented 2 silent substitutions respect *cfiA*-4b (T738C and G744A) coding for CfiA-4 (AN: LT714126 and AN: LT714128). In *B. fragilis* 3527 the sequence of *cfiA* displayed a single substitution (C81T) respect to *cfiA-2,* corresponding to CfiA-1 (AN: LT714124). In *B. fragilis* 3010 it was identified a silent substitution in *cfiA-19* (G744A), coding for CfiA-19 (AN: LT714125). In *B. fragilis* 3116, *cfiA* differed from *cfiA-22* in 2 silent substitutions (AN: LT714127), corresponding to CfiA-22 (Fig. 1B, Table 2 and Table S2).

3.3. Analysis of *cfiA* upstream region

In those *B. fragilis* susceptible to imipenem (3178, 3527, 3116 and 3010) amplification with primers G-F and E-R yielded 350 bp fragments. In *B. fragilis* 3527 and *B. fragilis* 3178 the sequence of these amplicons showed 99% identity with the target sequence for IS*1186* (AN: X72300). In *B. fragilis* 3010 and *B. fragilis* 3116 the amplified fragments displayed 100% identity with the upstream *cfiA* region of a susceptible prototype strain (AN: AY373495) [30,38]. In all of them, *cfiA* -33 and -7 promoter sequences were identified however neither of them corresponded to those reported as strong promoters for *cfiA* expression [31,34].

In *B. fragilis* 3409 the amplification with primers G-F and E-R yielded a 1500 bp amplicon, indicative of the presence of an IS element. A 1670 bp fragment was obtained with specific primers IS614-12-F and CfiA3-R. Its sequence displayed 99% identity with IS*612B* (AN: AY682395.1); promoter sequences could be detected using BPROM online tool. Using the IS Finder Blast (https://www-is.biotoul.fr/) it was observed that this IS corresponded to IS*1380* family and IS*942* group (Table 2).

No amplicons could be obtained with primers G-F and E-R in *B. fragilis* 3091 and 3189. In *B. fragilis* 3091 a 1000 bp fragment was amplified using primers IS21-F and E-R. It sequence showed 99% identity with IS*21* (AN: AF303352) and the presence of -33 and -7 promoter sequences was detected. The IS Finder Blast revealed identity with IS*Bf1* element of the IS*21* family. In *B. fragilis* 3189 the *cfiA* upstream region was accessed by inverse PCR, rendering a 1800 bp fragment. Blastn tool and IS Finder Blast detected, with 86% identity, an IS*613*-like element (AN: AB646744.1), which belongs to IS*1380* family and IS*942* group. Despite the low identity observed, -33 and -7 promoter sequences were detected.

4. Discussion

Here we present an exhaustive analysis of *cfiA*/CfiA registers in databases, illustrating the inconsistencies in both organization and nomenclature. Even if as early as in 2003, Kato et al. reported that some *cfiA* alleles such as *cfiA-4*, *cfiA-5* and *cfiA-7* had the same nucleotide sequence, this information has not been taken into account and these still persists in databases [35]. As mentioned above, to recognize the different *cfiA* alleles in *B. fragilis* isolates included in this study, it was necessary to depurate the alleles available on the GeneBank. According to this analysis CfiA family comprises nowadays 15 different CfiA variants coded by 24 different *cfiA* sequences (Table S2).

Since 2015, NCBI is responsible for the cure of β-lactamase databases, but this type of metallo-β-lactamase have not been processed yet (http://www.ncbi.nlm.nih.gov/projects/pathogens/submit_beta_lactamase). Recently, Hall et al. emphasized the need for a rational and accorded nomenclature system for resistance genes and proposed a threshold value of ≥2% difference in the DNA sequences, predicted proteins or both, to assign a new allele [39]. In response, Jacoby et al. argued that this cut off is unworkable with respect to β-lactamases nomenclature. These authors recommend that β-lactamase distinction should be based on protein but not nucleotidic sequence, independent whether it confers any relevant change in the substrate profile [40]. Meanwhile this topic is under discussion, we adopted the principles outlined by Jacoby et al. So, attempting to make *cfiA* database less

Table 2
Characterization of carbapenem resistance in *B. fragilis*.

Isolates	MIC (μg/ml)				CfiA	IS element
	Imipenem	Meropenem	Doripenem	Ertapenem		
B. fragilis 3091	32	>32	32	32	CfiA-4	IS*Bf1*/IS*21*family
B. fragilis 3189	32	>32	>64	>64	CfiA-13	IS*613*[a]/IS*942* group
B. fragilis 3409	>64	>32	>64	>64	CfiA-13	IS*612B*/IS*942*group
B. fragilis 3010	0.25	8	4	2	CfiA-19	–
B. fragilis 3116	1	8	4	4	CfiA-22	–
B. fragilis 3178	1	8	4	4	CfiA-4	–
B. fragilis 3527	0.25	4	8	4	CfiA-1	–

[a] The sequence analysis rendered 86% identity with IS*613*.

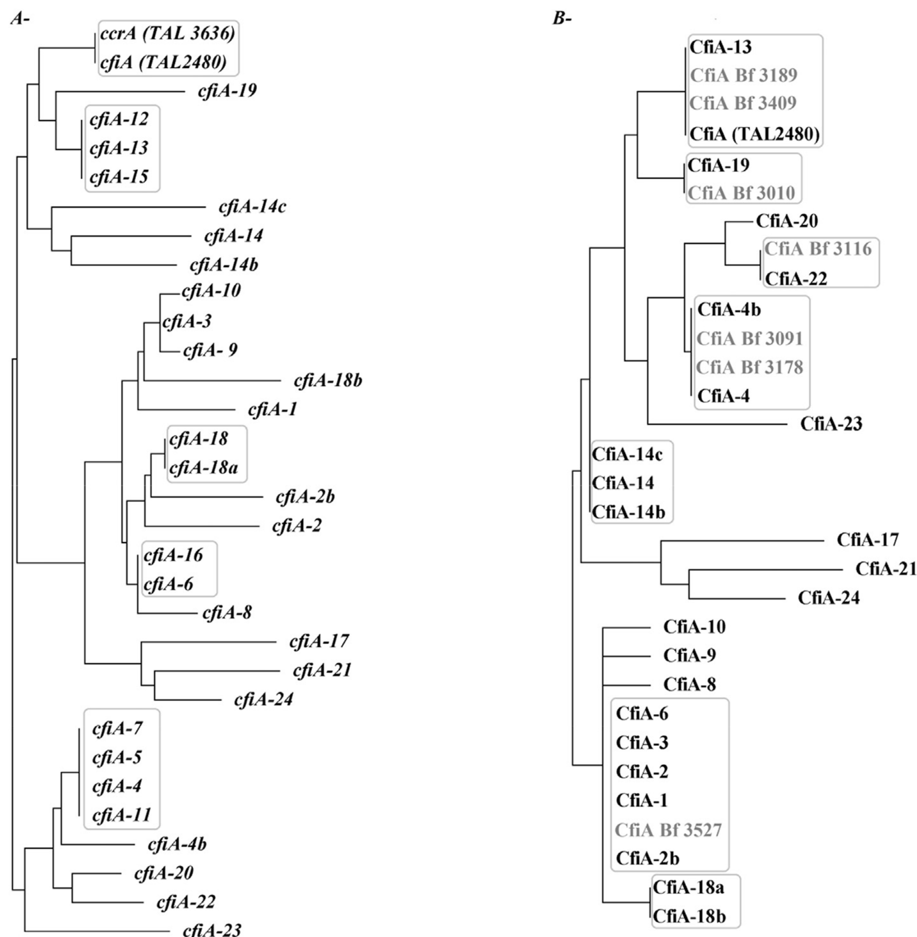

Fig. 1. A) Resulting dendrogram of *cfiA* nucleotide sequences. Grey squares show *cfiA* deposited sequences that are equal among them. B) Resulting dendrogram of CfiA protein sequences. CfiA sequences detected in the present study are shown in grey. Grey squares show CfiA that are equal among them.

confusing a good alternative should be as follows: **-** *cfiA-1, cfiA-2, cfiA-2b*, *cfiA-3* and *cfiA-6* that code for CfiA-1 could be re-named as *cfiA-1a, cfiA-1b, cfiA-1c, cfiA-1d* and *cfiA-1d*, respectively. **-** *cfiA-4* and *cfiA-4b* that code for CfiA-4 should be named as *cfiA-4a* and *cfiA-4b*, respectively. - *cfiA-13 and cfiA* which code for CfiA-13 should be named as *cfiA-13a* and *cfiA-13b*, respectively. - *cfiA-14, cfiA-14b* and *cfiA-14c* which code for CfiA-14 should be named as *cfiA-14a, cfiA-14b and cfiA-14c*, respectively (Table S2).

From the *cfiA* positive isolates recovered in a national survey [18], CfiA-13 was detected in 2 imipenem resistant isolates, whereas CfiA-4 was identified in one imipenem resistant and one imipenem susceptible isolate. The remaining susceptible isolates carried: CfiA-1, CfiA-19, and CfiA-22 (Table 2). In good agreement with previous descriptions, the imipenem resistant profile seems to be associated with the presence of IS elements upstream to *cfiA* (Table 2 and Table S1) [31]. ISs belonging to IS*942* group, were detected in 2/3 imipenem resistant isolates. IS*942* was previously described to be involved in *cfiA* expression in *B. fragilis*, harboring efficient promoter sequences that provide high level carbapenem resistance [32]. In the remaining resistant isolate an IS element of the IS*21* family could be detected upstream to *cfiA*. This mobile element was previously reported associated with the expression of *cepA* but not of *cfiA* in *B. fragilis* [29,31]. According with previously descriptions no IS elements were detected upstream *cfiA* in the imipenem susceptible isolates.

5. Conclusions

Different CfiA metallo-β-lactamases were identified in this study, independently of the imipenem resistance profile. In agreement with previous reports, it was observed that *cfiA* may be silent in susceptible isolates, as its expression depends on the presence of ISs elements.

There are too many inconsistencies in both organization and nomenclature for *cfiA*/CfiA. Here we present a working model for the organization of this metallo-β-lactamase family which could be considered by NCBI CfiA database curators and other researchers, whose suggestions would have impact on our model. What cannot be done is not taking care of the current inconsistencies, at a time when sequencing analysis is easily available, and profusion of new data will only make it more difficult to achieve a clean organization. Finally, we suggest that new admissions categorized as *cfiA* at GenBank should be hold until curation of this database, or at least statements alerting that corrected number or code assignments may appear in the future for them.

Acknowledgments

The present study was partially supported by UBACyT to M. Radice and G. Gutkind (20020150100174BA and 20020130100432BA); PICT to M. Radice (PICT-2013-0858); PIP to G. Gutkind and M. Radice (11220120100400CO); and MINCyT, ANPCyT, FONARSEC to G. Gutkind (0003/2011).

We thank to the members of Subcomisión de Bacterias Anaerobias, SADEBAC, Asociación Argentina de Microbiología (L. Fernández-Canigia, M.C. Legaria, L.Castello, S. C. Predari, A. Rossetti, R.Rollet, G.Carloni, and H. Bianchini) and to members of the Anaerobe Surveillance Team (M. Bottiglieri, M. Rocchi, I. A. Marqués, M. Machain, M. C. Mauro, M. R. Nuñez, and D. Ballester) for providing the isolates to conduct this study. We thank Dr. Karen Bush for her helpful discussion about this topic.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anaerobe.2017.10.003.

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