



Research paper

The population structure of *Ochrobactrum* isolated from entomopathogenic nematodes indicates interactions with the symbiotic system



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ABSTRACT

Entomopathogenic nematodes (EPNs) form specific mutualistic associations with bioluminescent enterobacteria. In *Heterorhabditis indica*, *Ochrobactrum* spp. was identified beside the symbiont *Photorhabdus luminescens* but its involvement in the symbiotic association in the EPNs remains unclear. This study describes the population structure and the diversity in *Ochrobactrum* natural populations isolated from EPNs in the Caribbean basin in order to question the existence of EPN-specialized clones and to gain a better insight into *Ochrobactrum*-EPNs relationships. EPN-associated *Ochrobactrum* and *Photorhabdus* strains were characterized by multi-locus sequence typing, Pulsed-Field Gel Electrophoresis fingerprinting and phenotypic traits.

Population study showed the absence of EPN-specialized clones in *O. intermedium* and *O. anthropi* but suggested the success of some particular lineages. A low level of genetic and genomic diversification of *Ochrobactrum* isolated from the natural population of Caribbean nematodes was observed comparatively to the diversity of human-associated *Ochrobactrum* strains. Correspondences between *Ochrobactrum* and *P. luminescens* PFGE clusters have been observed, particularly in the case of nematodes from Dominican Republic and Puerto Rico. *O. intermedium* and *O. anthropi* associated to EPNs formed less biofilm than human-associated strains.

These results evoke interactions between *Ochrobactrum* and the EPN symbiotic system rather than transient contamination. The main hypothesis to investigate is a toxic/antitoxic relationship because of the ability of *Ochrobactrum* to resist to antimicrobial and toxic compounds produced by *Photorhabdus*.

1. Introduction

Soil dwelling entomopathogenic nematodes (EPNs) of the family *Heterorhabditidae* form specific mutualistic associations with bioluminescent enterobacteria present in their gut: *Photorhabdus luminescens*, *Photorhabdus temperata*, *Photorhabdus asymbiotica* (Fischer-Le Saux et al., 1999) and *Photorhabdus heterorhabditis* (Ferreira et al., 2014). Infective juveniles invade the insect prey, regurgitating a small number of *Photorhabdus* cells that evade the immune system and kill the insect. While the symbiotic association between *Photorhabdus* and *Heterorhabditis* was previously described as highly specific and monoxenic, non-symbiont bacteria have been reported to inhabit beside *Photorhabdus*. They include *Providencia rettgeri* (Jackson et al., 1995), *Paenibacillus* spp. (Enright et Griffin, 2004), *Ochrobactrum* spp. (Babic et al.,

2000), *Stenotrophomonas* spp. (Wollenberg et al., 2016) and *Pseudomonas aeruginosa* (Salgado-Morales et al., 2017).

Among them, the frequent occurrence of *Ochrobactrum* in *H. indica* together with its symbiont *P. luminescens* (Babic et al., 2000) (Romano et al., 2009) (Aujoulat et al., 2014) suggested a non-random association. The precise location of *Ochrobactrum* in the EPN is an open issue; it could be in the gut or between the two cuticles (Babic et al., 2000). The genus *Ochrobactrum* belongs to *Alphaproteobacteria* (*Rhizobiales*, *Brucellaceae*) and contains ubiquitous and versatile bacteria that occupy diverse ecological niches. *Ochrobactrum anthropi* and *Ochrobactrum intermedium*, the most studied species in the genus, are found in polluted soils, rhizosphere, rhizoplane and plants but they are also increasingly recognized as opportunistic human pathogens, particularly in health-care-associated infections.

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Except for *H. indica*, *Ochrobactrum* sp. is described as a non-symbiotic bacterium of the EPNs *Steinernema siamkayai* (Razia et al., 2011), *Steinernema scapterisci* (Aguillera et al., 1993) and *Heterorhabditis* sp. (Abouelhag and El-Sadawy, 2012). *Ochrobactrum* sp. is also associated to the free-living terrestrial nematode *Caenorhabditis elegans* (Dirksen et al., 2016) and to *Acroboloides maximus* (Baquiran et al., 2013). Testing experimental microbiota in *C. elegans* showed that *Ochrobactrum* MYb71 is enriched in worm gut, confirming its ability to persist in the nematode intestine (Dirksen et al., 2016). In *A. maximus* gut, *Ochrobactrum* is highly prevalent and authors hypothesized that the genus play mutualistic roles in host protection against pathogens and in facilitation of enzymatic digestion of other ingested bacteria (Baquiran et al., 2013). The genus *Ochrobactrum* also dominates the gut microbiota of the Asian corn borer (*Ostrinia furnacalis*) (Li et al., 2013).

The intraspecific diversity and population genetics of *O. anthropi* and *O. intermedium* are described on the basis of both gene sequences (Lebuhn et al., 2006), (Romano et al., 2009), (Aujoulat et al., 2014) and genome macrorestriction by Pulsed-Field Gel Electrophoresis (PFGE) (Romano et al., 2009), (Aujoulat et al., 2014). *O. anthropi* displays a sub-population specifically found in human samples suggesting the emergence of a human-adapted clone named CC4 (Romano et al., 2009) meeting with the ecotype paradigm (Koeppel et al., 2008). By contrast, the population structure of *O. intermedium* seems unrelated to the origin of strains but a main cosmopolitan *O. intermedium* clonal complex (CC68) has been identified. Its emergence could be driven by anthropic selection pressures, particularly in polluted and technological contexts (Aujoulat et al., 2014). *Ochrobactrum* strains identified in *Heterorhabditis indica* beside the symbiont *P. luminescens* are avirulent by injection in insect models *Galleria mellonella* and *Spodoptera littoralis* (Babic et al., 2000) and their involvement in the symbiotic association between *P. luminescens* and EPNs remains unclear. Studies focused on non-*Photorhabdus* and non-*Xenorhabdus* EPN-associated bacteria are still necessary for a better understanding of their role in EPN-bacterial symbiosis. Population genetics studies of EPN-associated bacteria can give valuable data about population structure, that accounts for bacterial life-style (Romano et al., 2009) (Aujoulat et al., 2014). The aim of this study was to describe the population structure and the diversity in *Ochrobactrum* natural populations isolated from EPNs in the Caribbean basin in order to question the existence of EPN-specialized clones and to gain a better insight into *Ochrobactrum*-EPNs relationships.

2. Material and methods

2.1. Collection of *Ochrobactrum* sp. and *P. luminescens* strains isolated from *H. indica* and DNA extraction

The studied population corresponds to collections of *Ochrobactrum* sp. and *P. luminescens* from *H. indica* collected in 1996 (Babic et al., 2000) (Table 1). Additional strains of *Ochrobactrum* sp. and *P. luminescens* were isolated from *H. indica* collected in this study (2005) (Table 1). *H. indica* nematodes were harvested in the inland parts of Puerto Rico and the Dominican Republic in 1996 in various sites and ecosystems: croplands, orchards, grasslands, and forests (Fischer-Le Saux et al., 1998) (data not shown). In Guadeloupe and neighbour islands in 1996 and 2005, harvesting sites were beaches in coastal areas and were geographically separated from each other: Basse Terre and Grande Terre, and the neighbour islands, Les Saintes (Fig. 1) (Fischer-Le Saux et al., 1998). One specimen came from Saint-Barthelemy, an island at equal distance between Guadeloupe and Dominican Republic. Nematodes were collected using *Galleria mellonella* (Lepidoptera) baiting (“*Galleria* trap” technique) (Bedding and Akhurst, 1975; Fischer-Le Saux et al., 1998). The nematodes were grinded in Phosphate-Buffered Saline (PBS) buffer after decontamination of their tegument surface with bleach solution at 9.5 chlorometric degrees. Samples were then inoculated on Drigalski agar (Difco) supplemented with 4 mg l⁻¹ cef-tazidime, a selective medium for beta-lactam multiresistant, non-

exigent, Gram-negative bacteria such as *Ochrobactrum* spp. Inoculation onto the non-selective nutrient bromothymol blue agar (NBTA) (nutrient agar supplemented with 25 mg l⁻¹ bromothymol blue and 40 mg l⁻¹ triphenyltetrazolium chloride) allowed the detection of *Photorhabdus* spp. based on the particular aspect and dye of their colonies (Boemare et al., 1997). Subculture of colonies evocating *Ochrobactrum* spp. and *P. luminescens* and their identification were performed as previously described (Teyssier et al., 2005) (Boemare and Akhurst, 1988) (Boemare et al., 1997) (Fischer-Le Saux et al., 1999).

In this study, “pair” designated an *Ochrobactrum* isolate and a *Photorhabdus* isolate recovered from the same nematode specimen. The nomenclature of nematodes and bacterial strains was as follows: i) PR, DO and FRG corresponded to the islands Puerto Rico, Dominican Republic and Guadeloupe, respectively. ii) a number corresponding to the harvesting site (Fig. 1, for Guadeloupe), iii) *Ochrobactrum* strains are labelled with ‘/sat’. If nematodes have been collected from the same Guadeloupean site in 1996 and 2005, the strains isolated in 2005 were labelled with ‘b’.

Human associated *O. anthropi* and *O. intermedium* strains (Romano et al., 2009) included in the study of phenotype were presented in Supplementary Table 1 & 2.

2.2. 16S rRNA gene-based analyses

Genomic DNA of *Ochrobactrum* and *Photorhabdus* strains was obtained using the MasterPure™ DNA purification kit (EpiCentre) and used at 50 ng µl⁻¹. The 16S rRNA genes were amplified using primers (27f and 1492r) and PCR conditions previously described (Teyssier et al., 2003). PCR products and molecular weight marker (phage phiX DNA digested with *Hae*III, New England Biolabs) were separated in 1.5% (w/v) agarose gel in 0.5× Tris Borate EDTA (TBE) buffer. Sequencing was done using an ABI3730xl automatic sequencer (Cogenics, UK). Partial 16S rRNA gene sequences of at least 730 bp (accession numbers MG921332 to MG921366) were compared with sequences deposited in GenBank/EMBL/DDBJ using the standard nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequence identity of > 99.5% with a deposited type strain allowed the affiliation of the isolate to the corresponding species. Three species of *Ochrobactrum* (*O. anthropi*, *O. lupini* and *O. cytisi*) displayed the same 16S rRNA gene sequence and for *O. intermedium*, only one large insertion of 26 pb (Teyssier et al., 2003) differentiated this species from *O. ciceri*. Sequencing additional genetic markers included in the Multi Locus Sequence Typing (MLST) scheme (see below) allowed definite species identification. For *P. luminescens* identification, RFLP experiments on DNA were performed as follows: PCR products were digested with the endonuclease *Alu*I, *Hae*III or *Cfo*I (New England Biolabs, Ipswich, USA) as described previously (Fischer-Le Saux et al., 1998) and analysed on a 3% (w/v) agarose gel in 0.5× TBE buffer.

2.3. Multi locus sequence typing (MLST)

The genes *dnaK*, *recA*, *rpoB*, *trpE*, *aroC*, *omp25* and *gap* were amplified and sequenced as previously described (Romano et al., 2009) (Aujoulat et al., 2014). The combination of allele numbers for each isolate defined the sequence type (ST). Global optimal eBURST implemented by PHYLOViZ (Francisco et al., 2012) was used to cluster STs with double-locus variant (DLV) limitation, generating a goeBURST diagram to visualize relationships between STs. Clonal complexes (CC) included STs that differed by 1 or 2 alleles while singletons were defined as STs that differed by at least 3 loci from all other STs. STs obtained for strains isolated from nematode were compared to those previously published for collections of *O. anthropi* (Romano et al., 2009) and *O. intermedium* (Aujoulat et al., 2014) strains. The sequence references were given in Supplementary Table 1 & 2. The sequences determined in this study (28 EPN-associated strains) have been deposited

Table 1

Origin, species, sub-species and genotypes of the *Ochrobactrum* spp. and *P. luminescens* strains associated with nematodes from the Caribbean basin. ST: sequence type, CC: clonal complex. *from Les Saintes.

Origin of nematode (reference)	Year	<i>Ochrobactrum</i> strains	ST	CC	Species identification	Pulsotype (<i>SpeI</i>)	PFGE cluster (<i>SpeI</i>) 80%	<i>P. luminescens</i> strains	Sub-species identification	Pulsotype (<i>NotI</i>)	PFGE cluster (<i>NotI</i>) 80%		
Dominican Republic 1996 (Babic et al., 2000)		DO02/sat	84	68	<i>O. intermedium</i>	1	F	DO02	<i>P. luminescens</i> subsp. <i>akhurstii</i> genotype 12	I	c3		
		DO07/sat	84	68		1	F	DO07		I	c3		
		DO09/sat	84	68		1	F	DO09		II	c3		
		DO13/sat	84	68		1	F	DO13		I	c3		
		DO19/sat	84	68		1	F	DO19		III	c3		
		DO20/sat	84	68		1	F	DO20		I	c3		
		DO23/sat	84	68		1	F	DO23		I	c3		
		DO24/sat	84	68		1	F	DO24		I	c3		
		DO09H/sat	84	68		1	F						
		DO21/sat	84	68		1	F						
Puerto Rico 1996 (Babic et al., 2000)		PR17/sat	84	68	<i>O. intermedium</i>	2	F	PR17		IV	c2		
		PR21/sat	84	68		2	F	PR21		V	c2		
		PR22/sat	84	68		2	F	PR22		V	c2		
		PR54/sat	84	68		2	F	PR54		VI	c1		
		PR63/sat	84	68		2	F	PR63		VI	c1		
		PR38/sat	1	1		<i>O. anthropi</i>	5	–	PR38		VII	c2	
Guadeloupe 1996 (Babic et al., 2000)		FRG05/sat	13	1	<i>O. anthropi</i>		6	B	FRG05		VIII	–	
		FRG11/sat	13	1			7	D	FRG11		IX	–	
		FRG19/sat	13	1			7	D	FRG19		X	–	
		FRG33/sat*	139	31			9	–					
		FRG05/satb	139	31			10	A	FRG05b		XI	–	
Guadeloupe 2005 (this study)		FRG09/sat	29	1	<i>O. anthropi</i>	11	C	FRG09		XII	a		
		FRG16/sat	139	31		10	A	FRG16		XIII	–		
		FRG17/sat	29	1		11	C	FRG17		XIV	a		
		FRG19/satb	30	S		12	–	FRG19b		XV	–		
		FRG44/sat	141	39		13	E	FRG44		XVI	b		
		FRG46/sat	141	39		13	E	FRG46		XVII	b		
		FRG72/sat	29	1		11	C	FRG72		XVIII	a		
		FRG10/sat	74	74		3	–						
		FRG14/sat	81	68		4	–						
		FRG04/sat	13	1		<i>O. anthropi</i>	6	B					
		FRG15/sat	29	1			11	C					
		FRG36/sat	139	31			14	A					
		FRG70/sat	13	1		15	B						
Saint-Barthélemy 1996 (Babic et al., 2000)		FRG23/sat	140	1	<i>O. anthropi</i>	8	–						

in the GenBank database (accession numbers MG921367 to MG921562). The sequences of the 7 other EPN-associated strains were obtained from Romano et al. (2009) and Aujoulat et al. (2014) (Supplementary Table 1 & 2).

2.4. Pulsed-field gel electrophoresis (PFGE)

High-molecular-weight genomic DNA was prepared in agarose plugs as previously described (Teyssier et al., 2003), except that *Photobacterium* strains were suspended in PBS instead of Tris-EDTA for plug preparation. Each plug contained about 2 µg of DNA. *Photobacterium* genomic DNA was restricted by *NotI* and separated in PFGE as described (Gaudriault et al., 2008). *Ochrobactrum* genomic DNA was restricted by *SpeI* and separated in PFGE as described (Romano et al., 2009). Molecular marker was a concatemer of phage λ (New England Biolabs). PFGE profiles were standardized and analysed with Photocapt software (Vilbert-Lourmat, Marne-la-Vallée, France). The profiles were scored for the presence or absence of band at every migration distance. Clustering was based on Dice coefficient and was predicted by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) using the dendroUPGMA on-line tool (<http://genomes.urv.cat/UPGMA/>). PFGE clusters were defined at 80% similarity.

2.5. Phenotypic assays

The determination of doubling times and the study of biofilm formation of *Ochrobactrum* spp. were conducted in flat bottom 96-well polystyrene microplates (Nunc, Roskilde, Denmark) in Tryptic Soy

Broth (TSB). The wells were inoculated by dilution (approximately 10E6 CFU/ml) of overnight culture. Growth curves of *Ochrobactrum* spp. were obtained from optical density measurements (OD600 nm) at 30 °C, 34 °C and 37 °C in an Infinite M200 microplate reader (TECAN). For each determination, the OD600 nm of 3 replicate wells containing 200 µl of diluted culture was measured every 30 min. Doubling times were determined from the exponential phase of growth. Ability of *Ochrobactrum* spp. strains to form biofilm was examined as described by Abdouchakour et al. (2015). Biofilm assay was performed three times. The averages and standard deviations were calculated for all repetitions of the experiment. Phenotypic analysis also included *Ochrobactrum* spp. strains ($n = 15$) non-associated to EPN (Supplementary Table 1 & 2). Results were compared by the Mann-Whitney test (GraphPad Prism 5 software) with p values < .05 revealing significant differences.

3. Results

3.1. Origin and identification of bacterial strains isolated from EPNs

Twenty-five *Ochrobactrum* spp. / *P. luminescens* pairs were included in the study. Ten *Ochrobactrum* spp. were isolated without their *P. luminescens* counterpart. The isolates of the genus *Ochrobactrum* belong to the species *O. intermedium* ($n = 17$) and *O. anthropi* ($n = 18$). *Ochrobactrum* species identification was then confirmed by multi-locus sequencing (see below). The 16S rRNA gene PCR-RFLP assay (Babic et al., 2000) affiliated the *P. luminescens* strains to *P. luminescens* subsp. *akhurstii* genotype 12. Origin, nomenclature and identification of *Ochrobactrum* and *Photobacterium* isolates in EPNs from Caribbean islands

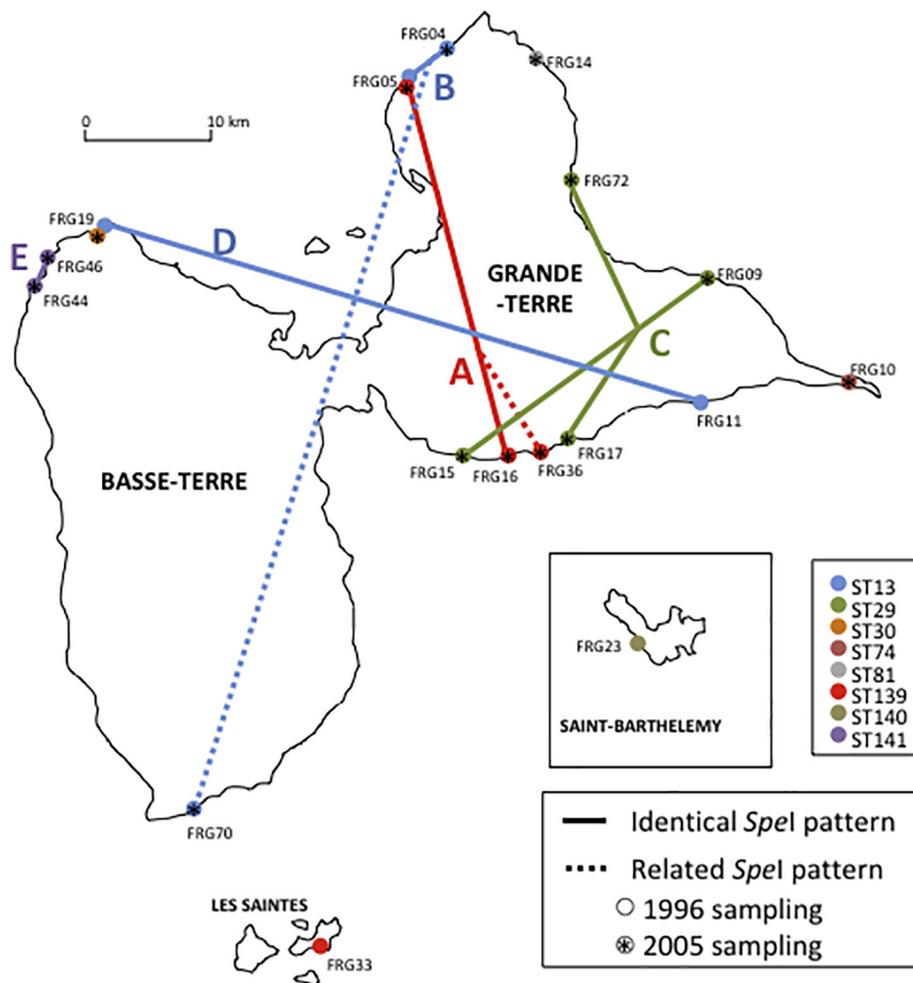


Fig. 1. Geographic map of Guadeloupe, Les Saintes and Saint Barthelemy, indicating the sites of nematode harvesting (named FRG and the number affected at every site) during in 1996 and 2005. Coloured circles represent sequence type (ST) of isolated strains. Coloured lines connect strains with identical (solid lines) or related (hatched lines) *Spel*-PFGE pattern. Letters correspond to *Spel*-PFGE patterns as noted in Table 1. Sites sampled in 2005 are marked with asterisk.

are presented in Table 1.

3.2. Population genetics of EPN-associated *O. anthropi* strains

Sixteen of 18 strains harvested in Guadeloupe and Les Saintes in 1996 and 2005 belonged to the species *O. anthropi* (Table 1). *O. anthropi* was also identified from EPNs in Saint-Barthelemy (strain FRG23/sat) and Puerto-Rico (strain PR38/sat). MLST showed that *O. anthropi* strains belonged to 7 STs, each grouping from one to 5 isolates (Table 1), 6 STs belonging to 3 CCs and the 7th being a singleton. The 3 most represented STs (ST13, ST29 and ST139) grouped strains isolated from distant sites in Guadeloupe (Fig. 1) and recovered during both sampling periods. For instance, strains FRG16/sat and FRG36/sat isolated in South Grande Terre in 2005 belonged to ST139 as strains FRG05/satb and FRG33/sat isolated in 1996 in Les Saintes and in 2005 in North Grande Terre (Guadeloupe), respectively (Fig. 1). Each of the 2 strains isolated in Caribbean islands away from Guadeloupe displayed unique ST.

Genotyping by PFGE of the 18 *O. anthropi* strains revealed 11 pulsotypes and 5 PFGE clusters (Fig. 2 and Table 1). Strains within a single ST could present different pulsotypes but strains belonging to the same PFGE cluster always shared the same ST (Table 1). PFGE clusters A, B, C, D and E grouped strains recovered from different sites and periods in Guadeloupe as observed in Fig. 1. By contrast, nematodes harvested in the same site in 1996 and in 2005 (FRG05/sat - FRG05/satb and FRG19/sat - FRG19/satb) carried *O. anthropi* isolates

displaying different MLST genotypes and PFGE patterns.

The comparison of multilocus sequences of Caribbean EPN-associated *O. anthropi* with those of a collection of clinical and environmental strains with origins encompassing the currently known habitats of *O. anthropi* (Romano et al., 2009) showed that strains formed two major and four minor CCs (Fig. 3). EPN-associated strains from the Caribbean basin were not clustered in specific CCs but were widely distributed among the *O. anthropi* population, except in CC4, which was confirmed herein to be human-specialized. However, the CC31 clusters together 6 nematode-associated strains without common geographical origin (4 from Guadeloupe and 2 from Europe, continental France and Italy) among 7 strains. The seventh strain (LMG2133) has been isolated from “thin-layer Sephadex-plates” in 1981 and may be a laboratory contaminant of unknown natural origin. Eleven among the 18 EPN-associated strains belonged to the major CC1, which appeared polyvalent because it grouped strains with a great diversity of habitats and life-styles (human clinical, soil, water, and nematode). Phenotypic comparison between EPN and non EPN-associated populations showed similar growth rate at 25, 30 and 37 °C (Supplementary Figure) while EPN-associated strains formed significantly less biofilm at 25 and 30 °C than other strains (Fig. 4).

Finally, both multilocus genetics and genomic pattern comparisons showed an overall diverse and non-specialized population of EPN-associated *O. anthropi* but the identity or the relatedness of some strains with diverse origins and/or periods of sampling in Guadeloupe suggested the successful spread and persistence of some clones among a

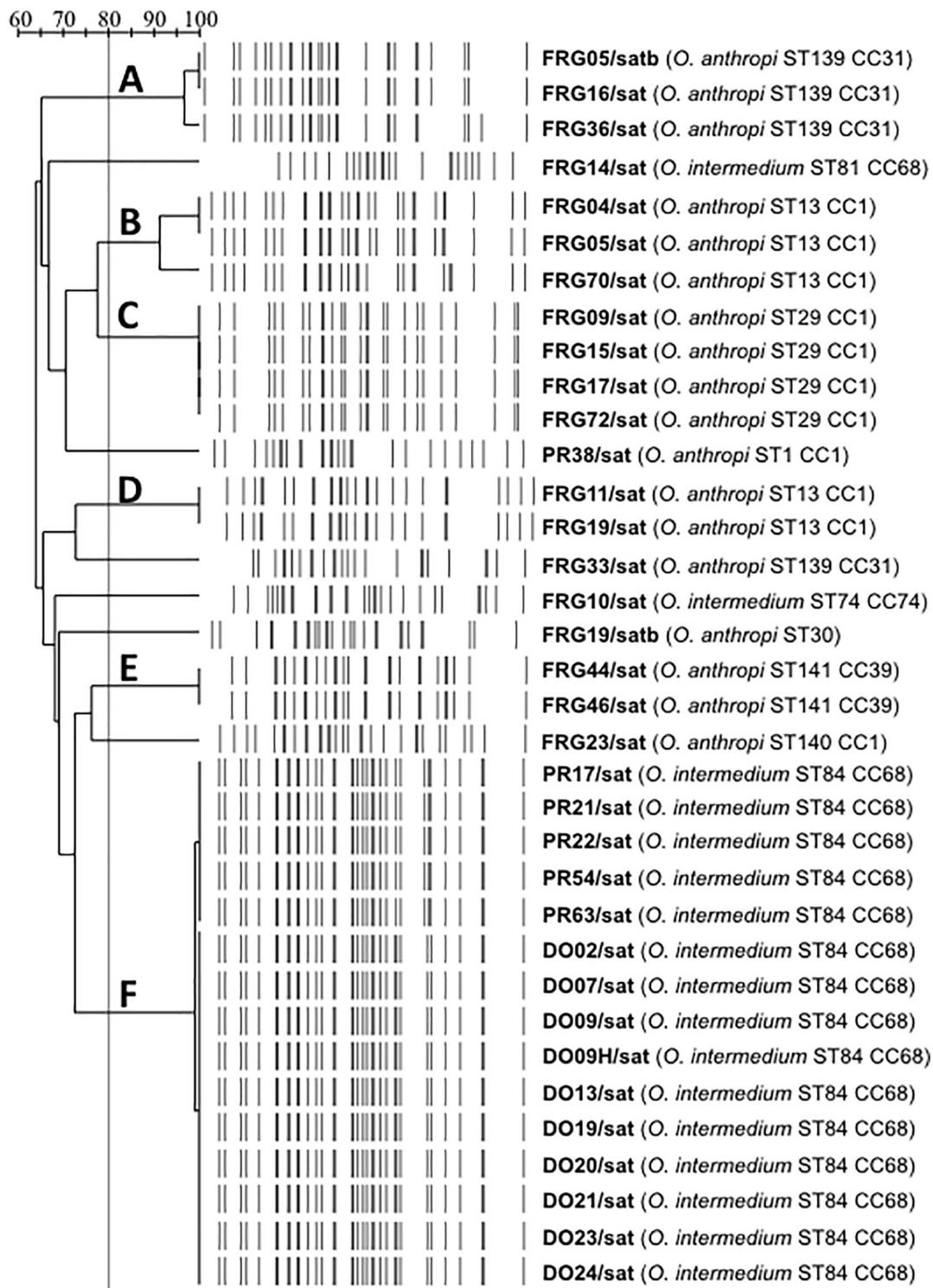


Fig. 2. Dendrogram constructed by using the UPGMA method from the standardized fingerprints after PFGE migration of *Spe*I-digested genomic DNA from *Ochrobactrum* isolates. Letters (from A to F) correspond to PFGE clusters defined at 80% similarity.

diversified population.

3.3. Population genetics of EPN-associated *O. intermedium* strains

Seventeen strains of *O. intermedium* were studied, 15 were isolated from EPNs harvested in Puerto Rico and Dominican Republic in 1996 and two from EPNs from Guadeloupe (2005) (FRG10/sat and FRG14/sat). The EPN associated *O. intermedium* population was less diverse than that of *O. anthropi* since the 17 strains were grouped in only 3 STs

(Table 1). The 15 *O. intermedium* strains isolated from Puerto Rican and Dominican EPNs belonged to the same multilocus genotype, ST84. They displayed very related PFGE patterns named 1 and 2, which formed the homogeneous cluster F (Table 1) (Fig. 2). The two other isolates originating from Guadeloupe (FRG10/sat and FRG14/sat) belonged to ST74 and ST81, respectively and presented individual pulsotypes (Table 1) (Fig. 2). The pattern 1 was observed for Dominican Republic strains and differed from pattern 2 observed for Puerto Rico strains by two fragments of 140 and 160 kb probably resulting from a single event

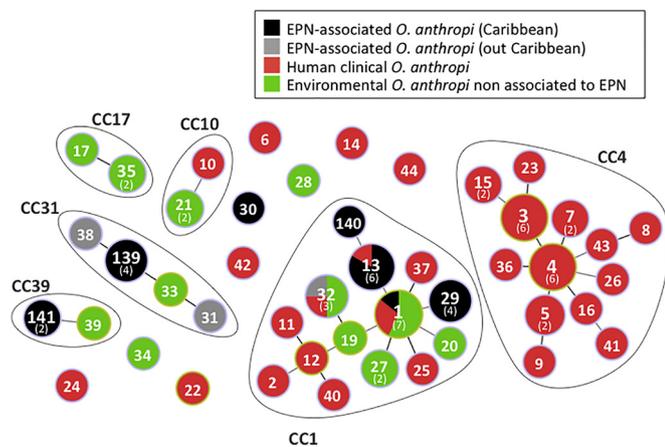


Fig. 3. goeBURST diagram based on MLST data for the 18 *O. anthropi* EPN strains and 70 previously published strains of different origins (Romano et al., 2009) (Supplementary Table 1). Each circle corresponds to a sequence type (ST). The number given in the circle corresponds to the ST designation. The size of each circle is proportional to the number of isolates in each ST and, if greater than one, the number of isolates is indicated in parenthesis under the ST number. Links between STs indicate single (SLV) or double locus variants (DLV). SLV and DLV were linked to form clonal complexes (surrounded by a grey line). Colour in circles indicates origin of the strains as indicated in the cartouche.

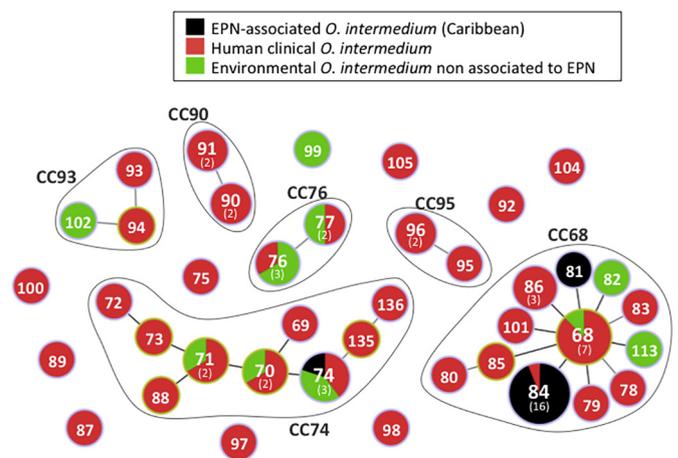


Fig. 5. goeBURST diagram based on MLST data for 17 *O. intermedium* EPN isolates and 61 previously described isolates of different origins (Aujoulat et al., 2014) (Supplementary Table 2). Each circle corresponds to a sequence type (ST). The number given in the circle corresponds to the ST designation. The size of each circle is proportional to the number of isolates of each ST and, if greater than one, the number of isolates is indicated in parenthesis under the ST number. Links between STs indicate single (SLV) or double locus variants (DLV). SLV and DLV were linked to form clonal complexes (surrounded by a grey line). Colour in circles indicates origin of the strains as indicated in the cartouche.

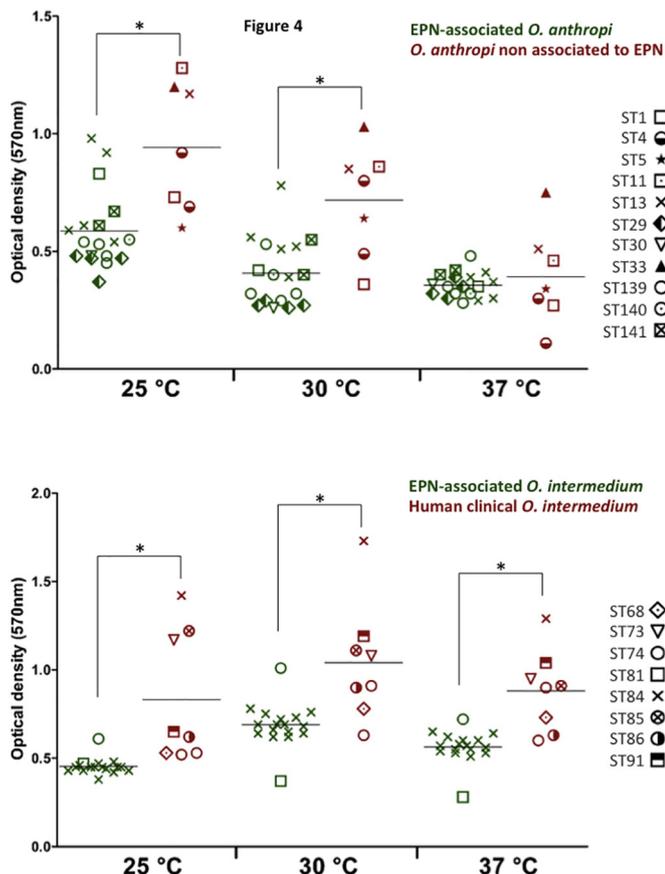


Fig. 4. Biofilm formation according to the temperature (25, 30 and 37 °C) of EPN-associated *O. anthropi* (A) and *O. intermedium* (B) strains (green) in comparison with strains of other origins (red). Horizontal line corresponds to the mean biofilm formation. STs are symbolized by a graphical code. Significant differences of mean biofilm formation are indicated by * ($p < .005$).

of genomic rearrangement (Fig. 2). Finally, PFGE after *SpeI* macro-restriction, which is the more discriminative typing method for *O. intermedium*, revealed a remarkable lack of genomic variations for the EPN-associated strains originating from Puerto Rico and Dominican Republic.

The goeBURST diagram in Fig. 5 showed the genetic links between EPN-associated *O. intermedium* strains and strains from diverse habitats (collection presented in Aujoulat et al., 2014). ST84 and ST81 belonged to the clonal complex CC68 previously described as cosmopolitan because worldwide distributed while ST74 belonged to the CC74, which included strains mostly isolated from continental France. These two large CCs included strains from diverse origins (nematode, human and environmental habitats). These results confirmed the polyvalence of *O. intermedium* and suggested that EPN-associated *O. intermedium* strains formed less biofilm than clinical strains despite similar growth rate (Fig. 4 and Supplementary Figure). This result was also observed within the ST84 for EPN-associated and clinical strains.

3.4. Population structures of EPN-associated pairs of *Ochrobactrum* and *P. luminescens* strains

Ochrobactrum SpeI-PFGE clusters were compared to *P. luminescens NotI*-PFGE clusters (Fig. 6). Some correspondences were observed between the clusters of the two groups of bacteria. Clusters in bold in the *P. luminescens* UPGMA tree in Fig. 5 corresponded to *Ochrobactrum SpeI*-PFGE clusters.

The *NotI*-PFGE cluster c clustered all the Dominican (c3) and Porto Rican *P. luminescens* strains (c1 and c2). The profiles of strains from Dominican Republic were strictly identical except for strains DO09 and DO19 whose pulsotypes differed from others by only one faint band of 200–250 kb in size. This sort of faint band in a PFGE pattern could correspond to circular plasmids. *P. luminescens* symbiotic strains were therefore considered as genomotypically identical in *H. indica* harvested throughout the Dominican Republic and this was related to the observation of identical pulsotypes for their *O. intermedium* paired strains (*SpeI*-PFGE cluster A) (Fig. 6). *P. luminescens* from Puerto Rico showed a very limited polymorphism (PFGE clusters c1 and c2) and their *O.*

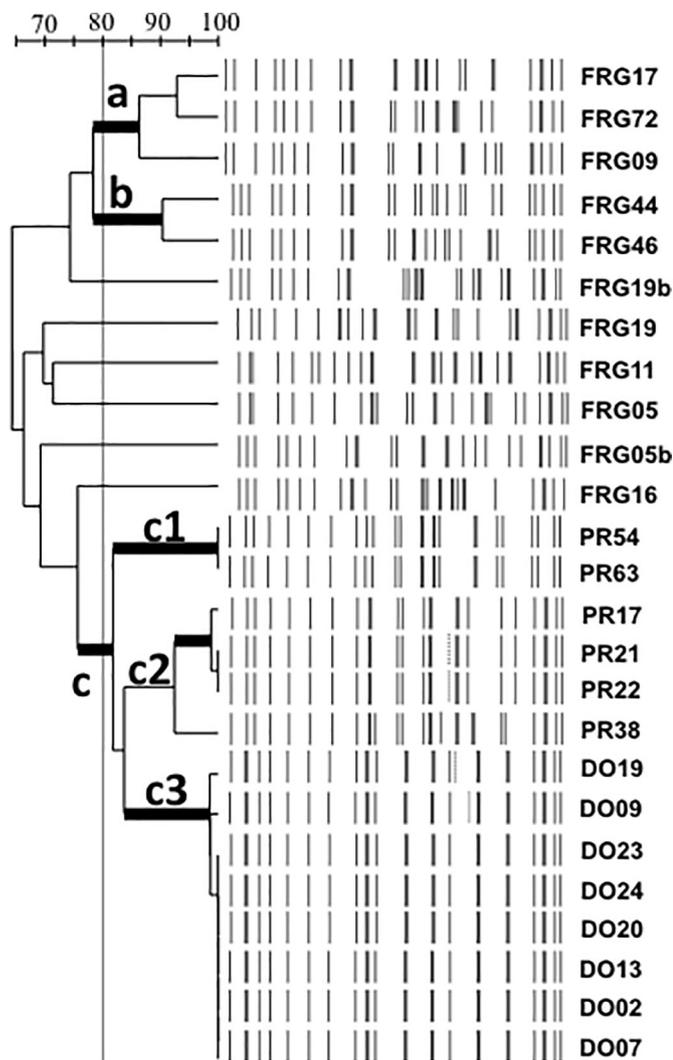


Fig. 6. Dendrogram constructed by using the UPGMA method from the standardized fingerprints after PFGE migration of *NotI*-digested genomic DNA from *P. luminescens* isolates. Letters (from A to F) correspond to PFGE clusters defined at 80% similarity. When *P. luminescens* clusters of strains corresponded to an *Ochrobactrum* clusters defined in Fig. 2, their roots are indicated in bold.

intermedium paired strains were identical (*SpeI*-PFGE pulsotype 2) (Fig. 6).

Small PFGE clusters a and b corresponding to trio and pair of *P. luminescens* from Guadeloupe were also observed. They corresponded to *O. anthropi* PFGE clusters C and E, respectively.

Altogether, we observed an overall correspondence in infra-species clustering between *P. luminescens* and their *Ochrobactrum* companion isolated from the EPN *H. indica* with a surprising high level of intra-species genotyping similarity for the bacterial couples (*O. intermedium*/*P. luminescens*) isolated in Dominican Republic, and to a lesser extent, in Puerto Rico.

4. Discussion

We proposed a precursory population study on a unique collection of natural EPN associated *Ochrobactrum*, particularly 25 pairs of *Ochrobactrum* and *P. luminescens* isolated from the same *Heterorhabditis indica*. Two typing methods were used for structuring *Ochrobactrum* and *Photorhabdus* populations associated to EPNs. First, housekeeping genes-based population typing (MLST) provides the backbone of the evolution divergence of sub-populations forming

bacterial species. This approach recognizes high-level similarities among geographically and temporally separated bacterial isolates. Second, genomic macro-restriction and PFGE explore both core and accessory genome structures giving an overview of the genomic flexibility related to fast adaptation processes within a species or a population (Rocha, 2004). As an illustration, MLST shows the worldwide spread of a human-adapted clonal sub-population in the species *O. anthropi* (Romano et al., 2009) while PFGE detected a genomic deletion during the *in vivo* fast evolution of an *O. intermedium* strain persisting for one year in the respiratory tract of a patient (Teyssier et al., 2003). As another example of fast evolution detected by PFGE, variations in genomic architecture were observed among phenotypic variants of the strain *P. luminescens* TT01 obtained from laboratory-maintained EPNs (Gaudriault et al., 2008). Because PFGE revealed fast evolution processes, it is among the most discriminative methods for typing bacteria and is still the gold standard in molecular epidemiology despite being a surviving method of the pre-genomic era (Miller, 2013).

The main result of this population study is the absence of EPN-specialized clones in the populations of *O. intermedium* and *O. anthropi*. Indeed, despite the relative low number of EPN-associated strains studied, they share genotypes with strains of other origin and, their genotypes and clonal complexes are widely distributed among the whole genetic structure of the species (Romano et al., 2009) (Aujoulat et al., 2014). However, as for other population studies, results should be interpreted considering potential sampling bias and some particular clusters deserve to be confirmed by studying larger population. For instance, a focus onto CC31 showed the clustering of 6 EPN-associated strains from diverse geographical origin among 7 strains. Moreover, the recently available genome of *O. anthropi* ML7 strain isolated from the nematode *Steinernema longicaudum* in Vietnam (Tobias et al., 2015) belongs to a new ST, which is a double locus variant of the ST139 and therefore also belongs to the CC31. Despite the absence of obvious EPN-specialized clones, our data suggest the success of some lineages, probably driven by complex interactions in EPN or in insect cadaver. This hypothesis prompts to go towards comparative genomics of a larger range of strains isolated from nematode and from other sources in order to define “EPN-associated core genome” in *Ochrobactrum* species or in the genus *Ochrobactrum*.

The multilocus genetics suggests that population structure of EPN-associated *Ochrobactrum* is not linked to biogeography, with strains from the Caribbean basin mixed with the whole population of *Ochrobactrum*. More locally, and according to PFGE data, all *O. intermedium* strain from Puerto-Rico and Dominican Republic were related with slightly different pulsotypes between islands. Some examples of biogeography-dependent distribution of eukaryote-associated bacteria have been described in neighbour islands. For example, bacterial communities associated to aphids, phytophagous insects specialized to feed on plant sap, differed among Hawaiian islands (Jones et al., 2011). Endophytic bacterial community of a Mediterranean marine angiosperm (*Posidonia oceanica*) also differed among Balearic Islands (Garcias-Bonet et al., 2012). For these cases, high-resolution biogeography suggests strong and stable relationships between eukaryote and bacterial members of the biological system. Our results suggested that it is also probably the case for *O. intermedium* in EPNs.

Generally in bacteria, the level of specialization in a particular niche or host is correlated to the potential of genetic/genomic diversification within a species or ecotype (Rocha, 2004), (Thomson et al., 2003), (Méthot and Alizon, 2014). In most cases, niche/host specialization leads to a decreased genetic diversity. For instance, the level of genetic diversity of *Ochrobactrum* was higher in bulk soil than in wheat rhizosphere (Bathe et al., 2006). In some extreme cases, host restriction leads to padlock and reduce bacterial genome non-reversibly (Moran and Plague, 2004) (Teyssier et al., 2003). We evaluated the genetic and genomic diversification within the natural populations of Caribbean nematode-associated *Ochrobactrum* and *Photorhabdus* strains. PFGE after *SpeI* macrorestriction revealed a remarkable absence of genomic

variations for the *O. intermedium* isolates originating from Puerto Rican and Dominican nematodes. For comparison, a collection of 32 clinical strains isolated in the same city, which is another local population of strains isolated from a single host, displayed 30 different *SpeI* patterns (data from Aujoulat et al., 2014). More contrasted situations were observed among the *O. anthropi* population. Some clusters strains persisted temporally and spread spatially while the PFGE pattern diversity was high among clusters in the same island. However, compared to another local population of strains isolated from a single host, EPN-associated *O. anthropi* strains in Guadeloupe displayed a limited diversity of genotypes: 16 strains from nematodes in Guadeloupe (1996 and 2005) displayed 10 *SpeI*-PFGE profiles while 22 human strains from the Montpellier hospital (1999–2007) displayed each a unique *SpeI*-PFGE profile (Romano et al., 2009).

Correspondences between *Ochrobactrum* and *P. luminescens* PFGE clusters have been observed. In the particular case of nematodes from Dominican Republic and Puerto Rico, the diversification of *O. intermedium* and *P. luminescens* is minimal. The success or the selection of certain genotypes of *Ochrobactrum* in the stable symbiotic system *Heterorhabditis/Photorhabdus* evokes strong interactions. The more obvious hypothesis to investigate is toxic/antitoxic relationships because *Ochrobactrum* are known for their huge ability to resist to antimicrobial (Furlan and Stehling, 2017) and various toxic compounds (Firdous et al., 2018) while *Photorhabdus* produce a range wide of antimicrobial products (Muangpat et al., 2017). A recent study, the first that used metatranscriptomics to analyse bacterial community in EPN-infected *G. mellonella* cadavers, showed enrichment in bacteria of the genus *Stenotrophomonas*. On the basis of its resistance to stilbene antibiotics and competition with *Photorhabdus*, *Stenotrophomonas* is considered as involved in complex interactions in the theoretically monoxenic environment formed by *Heterorhabditis* and *Photorhabdus* in the parasitized insect cadaver (Wollenberg et al., 2016). This could be also hypothesized for the role of *Ochrobactrum* in the EPNs. Babic et al. (2000) who described for the first time the presence of *Ochrobactrum* in *Heterorhabditis* qualified it as a natural dixenic association, particularly for *O. intermedium* strains that resist the broad-spectrum antibiotics produced by *P. luminescens* (Babic et al., 2000) (Muangpat et al., 2017). Another hypothesis is a role for the presence of *Ochrobactrum* or for the association *Ochrobactrum* / *Photorhabdus* in insect cadaver degradation and nutrient availability for the EPN thanks to the wide range of enzymatic functions encoded by the pangenome of the genus *Ochrobactrum*.

In an applicative perspective, more than a dozen EPNs have been commercialized for use in biological control (Shapiro-Ilan et al., 2016). Biosafety linked to EPN-associated bacteria should be evaluated not for their intrinsic pathogenicity but mostly for their multidrug-resistance and the potential infectious risk for immunocompromised patients. Among EPN-associated bacteria, *Ochrobactrum* deserve attention because it is the closest phylogenetic neighbour of *Brucella*, a strict pathogen for mammals. Recent comparative genomics of a new *Brucella* clade isolated from frogs suggested horizontal gene transfer between the genus *Brucella* and *Ochrobactrum* (Scholz et al., 2016). For these reasons, and because some EPN-associated *O. anthropi* and *O. intermedium* belong to genotypes that also include clinical isolates, a careful monitoring of associated bacteria during the mass production of commercialized EPN is needed to limit biohazard and diffusion of antimicrobial resistances.

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