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► **To cite this version:**

Marion Dalmasso, Pierre Nicolas, H el ene Falentin, Florence Valence, Jarna Tanskanen, et al.. Multilocus sequence typing of *Propionibacterium freudenreichii*. *International Journal of Food Microbiology*, 2011, 145 (1), pp.113-120. 10.1016/j.ijfoodmicro.2010.11.037 . hal-01454161

HAL Id: hal-01454161

<https://hal.science/hal-01454161>

Submitted on 28 May 2020

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Multilocus sequence typing of *Propionibacterium freudenreichii*

Marion Dalmasso ^{a,b,1}, Pierre Nicolas ^{c,1}, H el ene Falentin ^{a,b}, Florence Valence ^{b,d}, Jarna Tanskanen ^e, Hanna Jatila ^e, Tuomas Salusj arvi ^e, Anne Thierry ^{a,b,*}

^a INRA, UMR1253 Science et Technologie du Lait et de l' euf, F-35042 Rennes, France

^b AGROCAMPUS OUEST, UMR1253 Science et Technologie du Lait et de l' euf, F-35042 Rennes, France

^c INRA, UR1077 Math ematique Informatique et G enome, F-78350 Jouy-en-Josas, France

^d CIRM-BIA, INRA, UMR1253 Science et Technologie du Lait et de l' euf, F-35042 Rennes, France

^e Valio Ltd, PO Box 30, FI-00039 VALIO, Helsinki, Finland

ARTICLE INFO

Article history:

Received 31 August 2010

Received in revised form 24 November 2010

Accepted 25 November 2010

Available online 1 December 2010

Keywords:

Multilocus sequence typing

Propionibacterium freudenreichii

Recombination

Mutation

Subspecies

ABSTRACT

Propionibacterium freudenreichii is used as a ripening culture in Swiss cheese manufacture. This study investigates the molecular diversity and the population structure of this bacterium via multilocus sequence typing (MLST). Internal fragments of seven genes sequenced for 113 strains of different subspecies and origins allowed the resolution of 46 sequence types (STs) with occurrence frequencies ranging from 1 to 11. The core genome of the species harbours a low level of nucleotide polymorphism. In our data, single nucleotide polymorphisms account for only 2.28% of the concatenated sequences, and the average polymorphism rate in pairwise comparisons is 0.46%. The analyses reveal quantitatively comparable contributions of recombination and mutation in nucleotide changes at core genome loci along cell lineages. Remarkably, the STs exhibit little if any dairy biotope specialization. Phenotypic characterisation of the strains, based on their aptitude to use lactose and nitrate, shows that the two previously identified subspecies (*freudenreichii* and *shermanii*) do not reflect the ancestral relationships in the *P. freudenreichii* population. The considerable phenotypic heterogeneity, found even at the ST level, suggests instead a history of recurrent switches between phenotypes.

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1. Introduction

Propionibacterium freudenreichii belongs to the dairy group of propionic acid bacteria included in the *Actinobacteria* class of high G+C content Gram-positive bacteria. The species has mostly been isolated from dairy products and especially from Swiss cheese in which its population density generally reaches 10⁹ colony-forming units (cfu)/g. By converting lactic acid into propionic and acetic acids and CO₂, it is the main bacterium involved in the holes and flavor formations in Swiss-type cheeses, like Emmental and Leerdammer  cheeses (Langsrud and Reinbold, 1973). It also contributes to the formation of other aroma compounds in cheese thanks to the conversion of branched-chain amino acids into short branched-chain fatty acids, and to the hydrolysis of milk fat to free fatty acids (Dherb ecourt et al., 2010; Thierry et al., 2002; Thierry et al., 2005). A growing attention is paid to the probiotic potential of *P. freudenreichii* such as modulation of the microbiota, enzymatic activities, immune system, inflammation and carcinogenesis, at the gut level (Cousin et al., 2010; Jan et al., 2007). *P. freudenreichii* is divided into

two subspecies, *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii*, on the basis of lactose fermentation and nitrate reductase activity (Cummins and Johnson, 1986). Many *P. freudenreichii* activities are strain-dependant, like its survival and metabolic activity in the digestive tract (Lan et al., 2007), its autolytic activity (Lem ee et al., 1995) and its production of aroma compounds (Dherb ecourt et al., 2008; Thierry et al., 2005). Despite this diversity of reported phenotypes, there is a lack of information concerning the molecular diversity in the species and the relationships between strains that could define its population structure. The few data concerning the molecular diversity of propionibacteria strains are based on fingerprinting characterizations such as Randomly Amplified Polymorphic DNA (RAPD)-PCR and Pulsed-Field Gel Electrophoresis (PFGE) (Gautier et al., 1996; Rehberger, 1993; Rossi et al., 1998). These methods have proven to be useful for the identification of *Propionibacterium* strains in some cases. Nevertheless, RAPD-PCR and PFGE remain clustering techniques that hardly provide information about *P. freudenreichii* nucleotide diversity and mode of evolution.

Multilocus sequence typing (MLST), a method based on partial nucleotide sequences of multiple genes, has emerged as an alternative typing tool to study bacterial populations. It is recognized for its high degree of resolution and its capacity to rapidly characterize large numbers of isolates (Maiden et al., 1998). As MLST is based on DNA sequence analysis, it allows a phylogenetic approach of bacterial population that other typing tools do not permit. Therefore, it has

* Corresponding author. Mailing address: INRA, AGROCAMPUS OUEST, UMR1253 Science et Technologie du Lait et de l' euf, 65 rue de Saint-Brieuc, F-35042 Rennes cedex, France. Tel.: +33 2 23 48 53 37; fax: +33 2 23 48 53 50.

E-mail address: anne.thierry@rennes.inra.fr (A. Thierry).

¹ Marion Dalmasso and Pierre Nicolas contributed equally to the manuscript.

been largely used for epidemiologic and phylogenetic studies of pathogenic bacteria such as *Neisseria* (Hanage et al., 2005) or *Staphylococcus aureus* (Sakwinska et al., 2009). MLST has more recently been applied to bacteria used in the food industry like *Oenococcus oeni* (de Las Rivas et al., 2004), *Lactobacillus casei* (Cai et al., 2007), *Lactobacillus plantarum* (de las Rivas et al., 2006), and *Streptococcus thermophilus* (Delorme et al., 2010).

The present study was undertaken to investigate the population structure of *P. freudenreichii* by means of the first MLST scheme developed for a member of the *Propionibacterium* genus. The results of our analysis indicate that the core genome of *P. freudenreichii* displays a low level of nucleotide diversity and undergoes frequent recombination events, both characteristics contributing to the species cohesiveness. Our results also show that the lineages of recent common ancestry as defined by the sequence types exhibit broad dairy biotope ranges and phenotypic diversity.

2. Materials and methods

2.1. Bacterial strains, growth conditions and DNA extractions

A total of 113 *P. freudenreichii* strains from CIRM-BIA (Centre International de Ressources Microbiennes – Bactéries d'Intérêt Alimentaire, INRA, Rennes, France), Valio Ltd (Helsinki, Finland) and Actilait (Institut technique du lait et des produits laitiers, Rennes, France) collections were used (Table 1). Their origin is representative of the main biotopes where *P. freudenreichii* strains have been isolated so far: Swiss cheeses (58 strains), other cheeses (25 strains), milk (17 strains), other biotopes (5 strains) and unknown origin (8 strains). Strains originated from ten countries, the best represented being Finland (57 strains) and France (36 strains).

All the strains were routinely cultured in YEL broth (Malik et al., 1968) at 30 °C under microaerophilic conditions for 48 h. Cells were harvested from 2 ml of each culture (8000 g, 10 min, ambient temperature), suspended in 180 µl lysis buffer (lysozyme 20 mg/ml, mutanolysin 50 U/ml in 20 mM Tris–HCl [pH 8], 2 mM EDTA and 1% (v/v) Triton X 100), and incubated for 1 h at 37 °C. DNA was then extracted by means of DNeasy tissues kit (QIAGEN, Courtaboeuf, France).

2.2. Subspecies characterization

All the strains were tested for the presence of a nitrate reductase activity and for lactose fermentation. Nitrate reductase activity was detected by means of the Griess reagent (Biomérieux, Marcy l'Etoile, France) after strain incubation on broth containing nitrate (potassium nitrate 1.5 g/l (VWR International, Fontenay-sous-Bois, France), tryptone 10 g/l, yeast extract 5 g/l, glucose 1 g/l) for 48 h at 30 °C under microaerophilic conditions. Lactose fermentation was tested on modified API 50CH media (tryptone 10 g/l, yeast extract 5 g/l, K₂HPO₄ 0.25 g/l, MnSO₄ 0.05 g/l, and bromocresol purple 0.17 g/l). One milliliter of a lactose solution (200 g/l, Panreac, Lyon, France) was added to 9 ml of modified API 50CH medium. Strains were incubated at 30 °C for 48 h under microaerophilic conditions. All the tests were carried out in triplicate.

2.3. Loci, primers, PCR conditions and sequencing

The genome sequence of the type strain *P. freudenreichii* CIRM-BIA1^T (CIP103027^T) (Falentin et al., 2010) was used for choosing the genes encoding the following proteins: RNA polymerase β-subunit (*rpoB*), adenylate kinase (*adk*), carboxylic ester hydrolases (*pf169* and *pf1637*), DNA recombinase A (*recA*), fumarate hydratase (*fumC*), cell-wall polysaccharide synthase (*gtf*). Primers were designed with Primer3Plus software (Untergasser et al., 2007) and their sequences are specified in Table 2. PCR reactions were performed in 50 µl containing Taq DNA

polymerase buffer (100 mM Tris–HCl [pH 8.8], 500 mM KCl, 0.8% Nonidet P40), 2 mM MgCl₂, 200 µM of dNTPs, 0.5 µM of each primer, 0.5 U Taq DNA polymerase (Fermentas, Saint-Rémy-Les-Chevreuses, France) and 2 µl of template DNA (equivalent to 20–50 ng). The PCR amplifications were carried out with an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at the appropriate primer set temperature (Table 2) and extension at 72 °C for 30 s; and ending with 72 °C for 10 min. For each PCR product, both forward and reverse DNA strands were sequenced using the same primers as for PCR by the sequencing service of AGOWA genomics (Berlin, Germany).

2.4. Description of the sequence polymorphism

Each distinct gene sequence was arbitrarily assigned an allele number or allele type (AT) and each unique combination of the seven gene ATs was assigned a sequence type (ST) (Maiden, 2006). The mean G + C content at the seven loci was calculated. The detection of intra- and inter-loci recombination events and the minimal number of homoplasies were respectively assessed by means of the statistics R_{min} and h using LDhat and DNAPars (PHYLIP) (Nicolas et al., 2008). R_{min} is the Hudson and Kaplan's lower bound on the minimal number of recombination events needed to explain the sequence data in absence of homoplasy (Hudson and Kaplan, 1985). The number of apparent homoplasies, h , corresponds to the minimal number of homoplasies that would be needed to explain the data in absence of recombination. The associated statistical test evaluates whether or not h is compatible with the level of polymorphism if no recombination is involved. It is suitable for detecting recombination between fairly related strains with similar sequences, i.e. differing by less than 5% (Maynard Smith and Smith, 1998).

2.5. Phylogenetic analysis

Phylogenetic analysis was performed using ClonalFrame software version 1.1 (Didelot and Falush, 2007). ClonalFrame grounds the reconstruction of the genealogical relationship between sequences on a model that accounts for both vertical transmission of the genetic material in cell lineage (the clonal frame) and the occurrence of recombination tracts of variable length. Divergence is generated by mutations as well as and by recombination events that consists of the localized replacement of the sequence by a divergent sequence. In addition to the genealogy of the clonal frame, the parameters of this model are the population mutation rate (θ), the population recombination rate (R), the average length of the recombination tracts (δ) and the fraction of nucleotide divergence in sequences incorporated by recombination (v). Population mutation and recombination rates correspond to the rates of accumulation of these events in two diverging lineages when time is measured in coalescent units; one coalescent unit of time being the average time to the last common ancestor for a pair of isolates sampled randomly within the population. Inference is carried out in a Bayesian framework using a MCMC (Markov Chain Monte Carlo) algorithm. The number of burn-in cycles of the MCMC algorithm and further iterations were both fixed at a number of 250,000 (five times the default settings). The thinning interval was set to 50, resulting in a posterior sample of size 1000. In this analysis, each ST was represented by a unique sequence and the algorithm was run simultaneously on the 7 loci without concatenating the sequences. Posterior samples were simulated using the default settings where all parameters are estimated or with given values for some parameters other than R . Robustness of the inferred topology for the genealogy with respect to the starting point of the algorithm was verified by comparing the results obtained with three available options for the initial tree: a fully unbalanced tree, a uniformly chosen coalescent tree and the scaled UPGMA tree. The estimated matrix of pairwise-distances was used to draw a phylogenetic tree using

Table 1
Strains, allele types (ATs), sequence types (STs), biotopes, countries of isolation and subspecies.

Strain number ^a	Strain name	AT							ST	Biotopes	Country of isolation	Subsp.
		<i>rpoB</i>	<i>adk</i>	<i>pf1637</i>	<i>recA</i>	<i>pf169</i>	<i>fumC</i>	<i>gtf</i>				
1102	CIRM-BIA472	1	1	3	4	1	3	1	43	Swiss cheese	Finland	S
150	CIRM-BIA122	1	1	3	4	1	3	2	28	NA ^b	NA	S
177	CIRM-BIA556	1	1	3	4	1	3	2	28	Swiss cheese	Finland	S
111	CIRM-BIA512	1	1	3	2	1	2	2	8	Morbier cheese	France	S
132	CIRM-BIA692	1	1	3	2	1	2	2	8	Morbier cheese	France	S
137	TL1001	1	1	3	2	1	2	2	8	Cow milk	France	S
198	CIRM-BIA630	1	1	3	2	1	2	2	8	Swiss cheese	Finland	S
168	CIRM-BIA547	1	1	3	2	1	6	2	32	Swiss cheese	Finland	S
141	TL1263	1	1	3	2	1	3	2	25	Milking machine	France	S
191	CIRM-BIA623	7	5	3	1	1	2	2	39	Swiss cheese	Finland	P+
1108	CIRM-BIA121	1	2	6	1	1	1	2	44	Swiss cheese	USA	F
127	CIRM-BIA693	1	2	3	1	1	4	2	21	Ras cheese	Egypt	S
164	CIRM-BIA543	7	2	3	1	1	1	2	30	Swiss cheese	Finland	F
196	CIRM-BIA628	7	2	3	1	1	1	1	41	Swiss cheese	Finland	S
1110	ITG P9	1	1	3	1	5	1	2	45	NA	NA	S
13	CIRM-BIA685	1	1	3	1	1	1	2	3	Cow milk cheese	Brazil	S
145	TL566	1	1	3	1	1	1	2	3	Sbrinz cheese	Switzerland	P+
167	CIRM-BIA546	1	1	3	1	1	1	2	3	Swiss cheese	Finland	S
174	CIRM-BIA553	1	1	3	1	1	1	2	3	Cow milk	Finland	P-
1105	CIRM-BIA478	1	1	3	1	1	1	2	3	Swiss cheese	Finland	P+
1107	CIRM-BIA488	1	1	3	1	1	1	2	3	Swiss cheese	Finland	P-
129	CIRM-BIA527	1	1	3	1	1	4	2	22	Fribourg cheese	Switzerland	F
162	CIRM-BIA541	1	1	3	1	1	4	2	22	Swiss cheese	Finland	S
114	CIRM-BIA516	3	1	3	1	1	1	2	11	Yak milk cheese	Nepal	S
121	CIRM-BIA684	3	1	3	1	3	1	2	15	Cow milk cheese	Brazil	S
112	CIRM-BIA513	1	1	3	2	1	1	1	9	Ras cheese	Egypt	S
142	TL600	1	1	3	2	1	1	1	9	Swiss cheese	Australia	P+
1101	CIRM-BIA701	1	1	3	2	1	1	1	9	NA	Greece	S
136	CIRM-BIA481	1	1	3	2	1	1	1	9	Swiss cheese	Finland	S
154	CIRM-BIA533	1	1	3	2	1	1	1	9	Swiss cheese	Finland	S
175	CIRM-BIA554	1	1	3	2	1	1	1	9	Swiss cheese	Finland	S
194	CIRM-BIA626	1	1	3	2	1	1	1	9	Swiss cheese	Finland	S
122	CIRM-BIA688	1	1	3	2	4	1	1	16	Cow milk	France	S
119	CIRM-BIA682	1	1	3	1	1	1	1	14	Cow milk	France	P+
131	CIRM-BIA686	1	1	3	1	1	1	1	14	Goat milk cheese	France	S
140	TL1079	1	1	3	1	1	1	1	14	Cow milk	France	S
128	CIRM-BIA511	1	1	3	1	1	1	1	14	Swiss cheese	Switzerland	P+
155	CIRM-BIA534	1	1	3	1	1	1	1	14	Swiss cheese	Finland	S
179	CIRM-BIA611	1	1	3	1	1	1	1	14	Swiss cheese	Finland	P+
192	CIRM-BIA624	1	1	3	1	1	1	1	14	Swiss cheese	Finland	P+
123	CIRM-BIA689	1	1	3	1	1	5	1	17	Tomme Agen Cheese	France	S
146	TL1089	1	1	3	1	1	5	1	17	Cow milk	France	P+
187	CIRM-BIA619	1	1	3	1	1	4	1	37	Swiss cheese	Finland	S
144	TL929	1	1	3	2	1	5	2	27	Cow milk	France	P+
158	CIRM-BIA537	1	1	3	2	1	5	2	27	Swiss cheese	Finland	P-
163	CIRM-BIA542	1	1	3	2	1	5	2	27	Swiss cheese	Finland	P+
165	CIRM-BIA544	1	1	3	2	1	5	2	27	Swiss cheese	Finland	P+
169	CIRM-BIA548	1	1	3	2	1	5	2	27	Swiss cheese	Finland	S
180	CIRM-BIA612	1	1	3	2	1	5	2	27	Swiss cheese	Finland	P+
184	CIRM-BIA616	1	1	3	2	1	5	2	27	Swiss cheese	Finland	S
185	CIRM-BIA617	1	1	3	2	1	5	2	27	Swiss cheese	Finland	F
1106	CIRM-BIA482	1	1	3	2	1	5	2	27	Swiss cheese	Finland	S
166	CIRM-BIA545	1	1	3	2	1	1	2	31	Swiss cheese	Finland	P+
143	CIRM-BIA456	6	1	3	2	1	1	4	26	Raclette cheese	France	S
188	CIRM-BIA620	2	2	3	1	1	1	1	38	Cow milk	Finland	S
199	CIRM-BIA528	2	1	3	1	1	1	2	42	Swiss cheese	Finland	S
178	CIRM-BIA610	1	1	4	5	1	1	2	34	Swiss cheese	Finland	S
124	CIRM-BIA704	2	1	2	3	1	1	2	18	Beaufort cheese	France	S
18	CIRM-BIA687	2	2	1	1	1	3	3	6	Morbier cheese	France	P-
116	CIRM-BIA530	1	1	2	1	1	4	2	12	Swiss cheese	Finland	P-
130	CIRM-BIA531	1	1	2	1	1	4	2	12	Swiss cheese	Finland	S
153	CIRM-BIA532	1	1	2	1	1	4	2	12	Swiss cheese	Finland	S
156	CIRM-BIA535	1	1	2	1	1	4	2	12	Swiss cheese	Finland	P-
160	CIRM-BIA539	1	1	2	1	1	4	2	12	Swiss cheese	Finland	S
161	CIRM-BIA540	1	1	2	1	1	4	2	12	Swiss cheese	Finland	F
170	CIRM-BIA549	1	1	2	1	1	4	2	12	Swiss cheese	Finland	F
172	CIRM-BIA551	1	1	2	1	1	4	2	12	Swiss cheese	Finland	P-
176	CIRM-BIA555	1	1	2	1	1	4	2	12	Swiss cheese	Finland	P-
1104	CIRM-BIA476	1	1	2	1	1	4	2	12	Swiss cheese	Finland	P-
171	CIRM-BIA550	1	4	2	1	1	4	2	33	Swiss cheese	Finland	S
12	CIRM-BIA123	1	1	2	1	1	2	1	2	Morbier cheese	France	S
118	CIRM-BIA8	1	1	2	1	1	2	1	2	Cow milk	France	S
189	CIRM-BIA621	1	1	2	1	1	2	1	2	Swiss cheese	Finland	P+

(continued on next page)

Table 1 (continued)

Strain number ^a	Strain name	AT							ST	Biotoxes	Country of isolation	Subsp.
		<i>rpoB</i>	<i>adk</i>	<i>pf1637</i>	<i>recA</i>	<i>pf169</i>	<i>fumC</i>	<i>gtf</i>				
I9	CIRM-BIA41	1	1	2	1	1	2	2	7	Comté cheese	France	S
I39	TL1041	1	1	2	1	1	2	2	7	Cow milk	France	S
I47	TL1122	1	1	2	1	1	2	2	7	Ewe milk	France	S
I49	TL1045	1	1	2	1	1	2	2	7	Cow milk	France	S
I82	CIRM-BIA614	1	1	2	2	1	2	2	36	Swiss cheese	Finland	S
I25	CIRM-BIA690	1	1	2	2	1	5	2	19	Leerdammer cheese	France	F
I26	CIRM-BIA691	5	3	2	2	1	1	2	20	Abondance cheese	France	S
I59	CIRM-BIA538	5	1	2	2	1	1	2	29	Swiss cheese	Finland	S
I112	ITG P20	1	1	2	2	6	1	2	46	NA	NA	S
I5	CIRM-BIA134	1	1	2	2	1	1	2	5	Raclette cheese	France	P–
I6	CIRM-BIA135	1	1	2	2	1	1	2	5	Ewe milk	France	S
I10	CIRM-BIA510	1	1	2	2	1	1	2	5	Beaufort cheese	France	S
I48	TL1004	1	1	2	2	1	1	2	5	Cow milk	France	P–
I83	CIRM-BIA615	1	1	2	2	1	1	2	5	Swiss cheese	Finland	S
I93	CIRM-BIA625	1	1	2	2	1	1	2	5	Swiss cheese	Finland	P–
I81	CIRM-BIA613	1	1	5	2	1	1	2	35	Cow milk	Finland	S
I17	CIRM-BIA7	4	1	2	2	1	1	2	13	Cow milk	France	P+
I52	CIRM-BIA119	4	1	2	2	1	1	2	13	Gruyere cheese	France	P+
I73	CIRM-BIA552	4	1	2	2	1	1	2	13	Swiss cheese	Finland	P+
I38	TL1014	4	1	2	2	1	1	1	24	Cow milk	France	S
I51	TL214	4	1	2	2	1	1	1	24	Fribourg cheese	France	P+
I113	ITG P23	4	1	2	2	1	1	1	24	NA	NA	S
I1	CIRM-BIA1 ^T	1	1	1	1	1	1	1	1	NA	The Netherlands	S
I34	CIRM-BIA9	1	1	1	1	1	1	1	1	Cheese	The Netherlands	S
I100	CIRM-BIA702	1	1	1	1	1	1	1	1	NA	Greece	S
I7	CIRM-BIA141	1	1	1	1	1	1	1	1	Wheat	France	S
I20	CIRM-BIA683	1	1	1	1	1	1	1	1	Wheat	France	S
I33	CIRM-BIA515	1	1	1	1	1	1	1	1	Straw	France	S
I15	CIRM-BIA529	1	1	1	1	1	1	1	1	Swiss cheese	Finland	S
I86	CIRM-BIA618	1	1	1	1	1	1	1	1	Swiss cheese	Finland	S
I90	CIRM-BIA622	1	1	1	1	1	1	1	1	Swiss cheese	Finland	S
I97	CIRM-BIA629	1	1	1	1	1	1	1	1	Swiss cheese	Finland	S
I103	CIRM-BIA474	1	1	1	1	1	1	1	1	Swiss cheese	Finland	S
I4	CIRM-BIA132	1	1	1	1	2	1	1	4	Cow milk cheese	USA	S
I13	CIRM-BIA514	1	1	1	1	1	1	2	10	Hay	France	F
I111	ITG P18	1	1	1	1	1	1	2	10	NA	NA	F
I95	CIRM-BIA627	1	1	1	1	1	5	2	40	Swiss cheese	Finland	F
I35	CIRM-BIA118	1	1	1	2	1	1	2	23	Gruyere cheese	France	S
I109	CIRM-BIA508	1	1	1	2	1	1	2	23	Gruyere cheese	France	S
I57	CIRM-BIA536	1	1	1	2	1	1	2	23	Swiss cheese	Finland	S

^a Strains are listed according to their order in the phylogenetic tree (Fig. 1). Abbreviations for subspecies: F, *P. freudenreichii* subsp. *freudenreichii*; S, *P. freudenreichii* subsp. *shermanii*; P+, *P. freudenreichii* “pheno+” able to use lactose and nitrate; P–, *P. freudenreichii* “pheno–” unable to use lactose and nitrate.

^b Not available.

neighbor-joining. From the posterior sample, the support for each internal branch was assessed as the proportion of trees possessing the branch. The respective contribution of recombination and mutation to the nucleotide changes was computed from the output of the MCMC algorithm as the “r/m” ratio (Guttman and Dykhuizen, 1994), with r expressed as the product of δ , ν and the recombination rate R, and m as the mutation rate θ .

2.6. Nucleotide sequence accession numbers

Nucleotide sequences have been deposited in EMBL under accession number FN650812 to FN650924 (*rpoB*), FN650925 to FN651037 (*adk*), FN651038 to FN651150 (*pf1637*), FN651151 to FN651263 (*recA*), FN651264 to FN651376 (*pf169*), FN651277 to FN651489 (*fumC*) and FN651490 to FN651602 (*gtf*).

Table 2
Genes, primer sequences, annealing temperatures and amplicon sizes.

Gene	Primer		Annealing temperature (°C)	Amplicon size (bp)	References		
	Forward	Reverse					
<i>rpoB</i>	rpoB2-F	GAGTTCGGCGAGTACGACTC	rpoB2-R	GTGCCGAAGAAGCTCCTTGAG	58	530	Deutsch et al. (2008)
<i>adk</i>	adk-F3	GCGATCTCAACCGGAGAC	adk-R3	GACTTGTAGGCGTCGAGCAG	58	482	
<i>pf1637</i>	pf1637-F	TTCCTGGATGCCTATTCA	pf1637-R	CGACTCCATGTCGTAGAGCA	58	580	
<i>recA</i>	recA-F	CAATGAGATCGTGGGAAACC	recA-R	ACCGTAGCCTTACCGGACTT	58	414	
<i>pf169</i>	pf169-F	TGATCGAATATCCGGTGCAG	pf169-R	GATAGCAGGCAAGCCATAG	59	411	
<i>fumC</i>	fumC2-F	GAACGCCAATGAGGTGATCT	fumC2-R	GGTCTCGTCTGATCTTCT	58	448	
<i>gtf</i>	Gtf-F2	CAGTCGGTGAAGAACCGCTACC	Gtf-R1	CGGCAGGCGATAGGTGAACAAC	60	342	

3. Results

3.1. MLST scheme and allelic diversity

A MLST database was created to host the data generated from the 113 *P. freudenreichii* strains (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/>) (Jolley et al., 2004). The size of the seven gene fragments considered in this MLST scheme ranged from 234 bp (*gtf*) to 477 bp (*pf1637*) (Table 3). The mean G + C content of genes varied from 62.30% (*recA*) to 67.32% (*adk*). The number of allele types (ATs) was as follows: 4 ATs for *gtf*, 5 ATs for *adk* and *recA*, 6 ATs for *pf1637*, *pf169* and *fumC*, and 7 ATs for *rpoB*. The combination of the ATs at the seven loci permitted the detection of 46 sequence types (STs) among the 113 strains. Seventeen STs were identified more than once (Table 1). ST-1 was the most frequent ST and gathered 11 strains, directly followed by ST-12 (10 strains) and ST-27 (9 strains).

The values of the summary statistics depicting polymorphisms at the seven loci are reported in Table 3. Considering the 2409 nucleotides of the seven gene fragments together, there were 55 single nucleotide polymorphisms (SNPs), which represented a proportion of 2.28% of the concatenated sequences. The number of SNPs varied from 3 (1.28% of the allele sequence) at *gtf* locus to 15 (3.14% of the allele sequence) at *pf1637* locus. The level of pairwise nucleotide diversity (π), i.e. the percentage of positions that differed on average in two strains, was 0.46% for the concatenated sequences but fluctuated from 0.03% (*pf169*) to 1.33% (*pf1637*). Only 15 SNPs among the 55 were non-synonymous ($S_{\text{non-syn}}$).

3.2. Evidence for recombination events

The contribution of recombination events to the sequence polymorphisms was detected by means of two statistics, the Hudson and Kaplan's lower bound on the number of recombination events (R_{min}) and the minimal number of apparent homoplasies (h) (see materials and methods). Values were computed for each locus and for the whole dataset as reported in Table 3. R_{min} was 1 for *pf1637* and *rpoB* and 0 for all the other loci. The low values of R_{min} at the different loci can be interpreted as an absence of evidence for significant intra-locus recombination but R_{min} reached 7 for the concatenated sequences, indicating recombination between loci. The statistic h is the minimal number of homoplasy (reversions or parallel mutations in independent lineages) that would be needed in absence of recombination. It is computed as the difference between the number of polymorphisms and the minimal number of mutations along a tree for explaining the sequences. As for R_{min} , h was equal to 0 or 1 when

the loci were considered separately. However, h obtained for the concatenated sequences ($h = 54$) was greatly higher than the sum of the seven independent h values at the different loci ($h = 2$). This value was significantly higher than the expected value in absence of recombination (p -value < 0.005) and confirmed the non ambiguous contribution of recombination between loci to the generation of the STs.

3.3. Population structure

In order to attempt the reconstruction of a phylogenetic tree despite the presence of recombination, the sequences of the seven loci were analyzed all together with ClonalFrame. This software explicitly accounts for recombination in the history of the sample. The consensus phylogenetic tree derived from the posterior sample obtained with the default settings, where all parameters are estimated, is presented in Fig. 1. This tree contains 14 internal branches with posterior support greater than 80%, suggesting that the level of recombination was not high enough to obliterate the signal on the relationships between the cell lineages identified by the STs. The topology of the tree was robust with respect to the starting point of the algorithm and to the set of parameters left free in the estimation. ClonalFrame generates parameter estimates that allow evaluating the relative contributions of recombination and mutation to the nucleotide changes in the evolution of a cell lineage, summarized in the r/m ratio (Guttman and Dykhuizen, 1994). Fig. 2 shows the posterior distribution of the r/m ratio. The algorithm was also run with fixed values for the population mutation rate (θ) and for the fraction of nucleotide divergence in recombination tracts (ν) (Vos and Didelot, 2009). The two parameters were set to 0.005 nucleotide changes per site. This particular value was selected to agree with two estimators of θ : the pairwise nucleotide diversity estimator (π), and the Watterson's estimator (θ_w) that relies on the comparison between the number of SNPs and the sample size (Watterson, 1978). The value of π is 0.0046 bp^{-1} (Table 3) and the value of θ_w varies between 0.0043 bp^{-1} , when multiple occurrences of the same ST are taken into account, and 0.0052 bp^{-1} , when multiple occurrences are omitted. As shown in Fig. 2, the posterior distribution of r/m has its mode ranging from 1 to 2, depending on whether the default settings or fixed values for θ and ν are used.

3.4. Phenotypic and dairy origin variability

The differentiation of the two *P. freudenreichii* subspecies was performed to investigate the possible relationships between STs and

Table 3
Sequence variations and polymorphisms at seven loci.

Gene	Locus			Polymorphism				Recombination	
	Position (kbp) ^a	Length (bp)	Mean G + C content (mol%)	S ^b	S _{non-syn} ^c	No. of AT	π (bp^{-1}) ^d	R _{min} ^e	h ^f
<i>rpoB</i>	656	429	65.22	11	2	7	0.0020	1	1
<i>adk</i>	695	303	67.32	7	1	5	0.0013	0	0
<i>pf1637</i>	1438	477	66.07	15	3	6	0.0133	1	1
<i>recA</i>	1471	312	62.30	4	3	5	0.0018	0	0
<i>pf169</i>	1577	309	66.34	5	3	6	0.0003	0	0
<i>fumC</i>	1784	345	65.30	10	2	6	0.0071	0	0
<i>gtf</i>	2089	234	65.81	3	1	4	0.0021	0	0
Total									
Sum		2409	–	55	15	–	0.0046	2	2
Concatenation		2409	–	55	15	46 ^g	0.0046	7	54 ^{**}

^a Chromosomal position on the complete genome (2,616,384 bp) of strain CIRM-BIA1^T.

^b Number of single nucleotide polymorphisms (SNPs).

^c Number of non-synonymous SNPs.

^d Average level of pairwise nucleotide diversity per site.

^e Hudson and Kaplan's lower bound for the number of recombination.

^f Minimal number of apparent homoplasies (**, $P = 0.005$).

^g Number of STs.

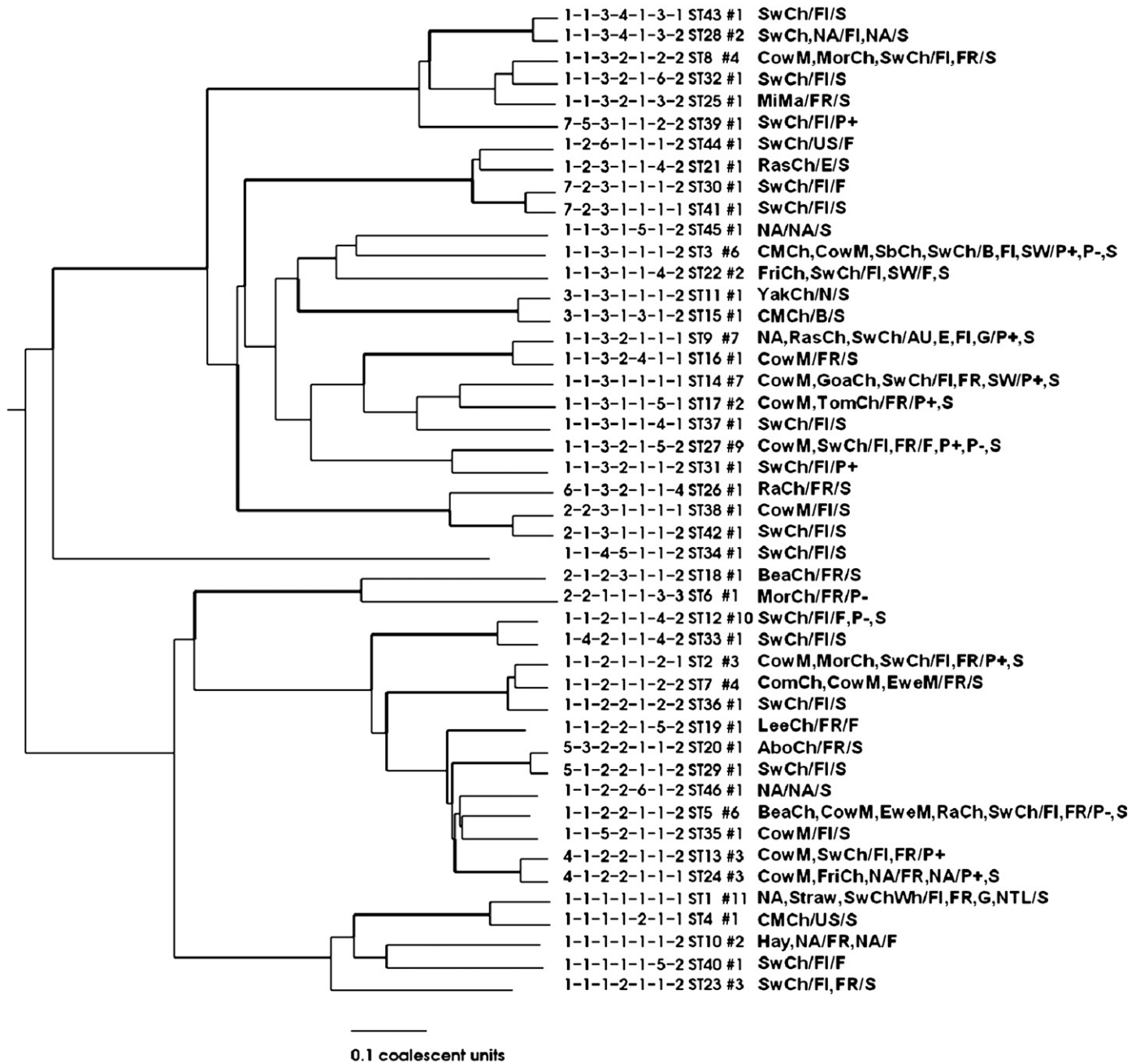


Fig. 1. Consensus phylogenetic tree showing the relationships of the 46 sequence types (STs) of *P. freudenreichii* obtained from the posterior sample of trees with the default settings of ClonalFrame software. Each ST was represented by a unique sequence and the algorithm was run on non-concatenated sequences of the 7 loci. Bold lines indicate internal branches with posterior support greater than 80%. Information at each tree leaf is as follows: allele types (*rpoB-adk-pf1637-recA-pf169-fumC-gtf*), ST number, # number of strains in the ST, biotope of strains/country of collection/subspecies. Abbreviations for biotopes of strains: AboCh, Abundance cheese; BeaCh, Beaufort cheese; CMCh, cow milk cheese; ComCh, Comté cheese; CowM, cow milk; EweM, ewe milk; FriCh, Fribourg cheese; GoaCh, goat milk cheese; GrCh, Gruyere cheese; Hay, hay; LeeCh, Leerdammer cheese; MiMa, milking machine; MorCh, Morbier cheese; NA, not available; RaCh, Raclette cheese; RasCh, Ras cheese; SbCh, Sbrinz cheese; Straw, straw; SwCh, Swiss cheese; TomCh, Tomme Agen cheese; Wh, wheat; and YakCh, yak milk cheese. Abbreviation for countries of isolation of strains: AU, Australia; B, Brazil; E, Egypt; FR, France; FI, Finland; G, Greece; N, Nepal; NA, Not available; NTL, The Netherlands; SW, Switzerland; and US, USA. Abbreviations for strain subspecies: F, *P. freudenreichii* subsp. *freudenreichii*; S, *P. freudenreichii* subsp. *shermanii*; P+, *P. freudenreichii* “pheno+” able to use lactose and nitrate; and P-, *P. freudenreichii* “pheno-” unable to use lactose and nitrate.

subspecies. It was conducted by testing the strains ability to reduce nitrate and to ferment lactose. Namely, *P. freudenreichii* subsp. *shermanii* is identified by its ability to use lactose but does not show a nitrate reductase activity, whereas *P. freudenreichii* subsp. *freudenreichii* cannot use lactose but shows a nitrate reductase activity. The results are reported in Table 1. As a control, the type strains CIRM-BIA1^T (CIP103027^T) and CIRM-BIA121 (CIP103026) were respectively recognized as *P. freudenreichii* subsp. *shermanii* and *P. freudenreichii* subsp. *freudenreichii*. A total of 72 *P. freudenreichii* subsp. *shermanii* and 10 *P. freudenreichii* subsp. *freudenreichii* strains could be

unambiguously recognized. For 31 strains, subspecies assignment was not possible because either they fermented lactose and reduced nitrate (19 strains labeled “pheno+”), or neither fermented lactose nor reduced nitrate (12 strains labeled “pheno-”). The strains exhibiting the different combinations of phenotypes were distributed randomly over the tree (Fig. 1). For example, ST-27 brought together strains displaying the four subspecies phenotypes. Moreover, no specific group of strains was noticeable and no link could be observed between STs and the biotopes of strains (Swiss cheese, other cheeses, or milk). For example, strains from Swiss cheese displayed various STs

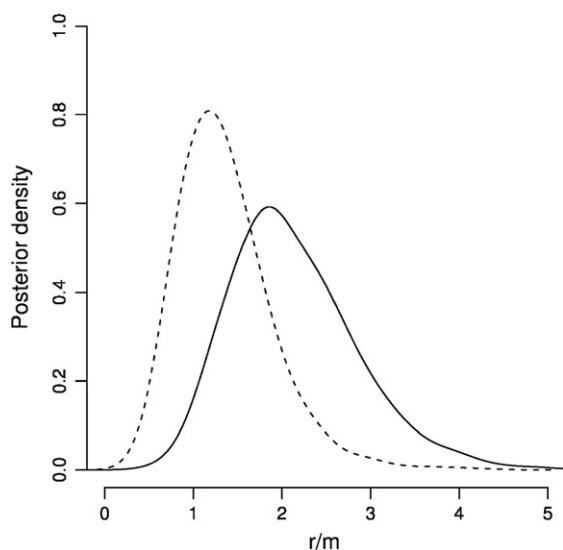


Fig. 2. Posterior distribution of the r/m ratio estimated with ClonalFrame software using (---) the default settings for parameter estimation or (—) fixed values for the population mutation parameter (θ) and the amount of nucleotide divergence in sequences incorporated by recombination (ν).

and were spread all over the phylogenetic tree. Four strains isolated from plants and one strain isolated from milking equipment were also included in the study for comparison with strains of dairy origin. They exhibited no particular trend with respect to their position in the tree.

4. Discussion

MLST has proven to be useful to characterize many bacterial species and has largely been described formerly (Maiden, 2006; Maiden et al., 1998). The aim of this study was to understand the structure of the dairy population of *P. freudenreichii*. This was achieved by developing the first MLST scheme for a *Propionibacterium* species. The seven selected loci are conserved at the species level, scattered on the chromosome, and exhibit a G + C content typical of *P. freudenreichii* genome (Falentin et al., 2010), suggesting that they have not been recently acquired in *P. freudenreichii*. In addition, the majority of SNPs reported here are synonymous, indicating that the selected genes are not under strong positive selection for amino acid changes. Thus, the seven genes are suitable for being used in population genetic analyses as representative of the core genome (Petersen et al., 2009).

This study revealed a low level of nucleotide polymorphism in the *P. freudenreichii* core genome, characterized by a small proportion of polymorphic sites (1.28% to 3.14%) and low pairwise nucleotide diversity ($\pi = 0.0046 \text{ bp}^{-1}$). The value of the Watterson's estimate of the population mutation rate ($\theta_w \sim 0.0043\text{--}0.0052 \text{ bp}^{-1}$) was lower than the estimates reported for the 16 bacterial species considered in the comparative analysis of Perez-Losada et al. (Perez-Losada et al., 2006) and can thus be considered as low. The level of nucleotide polymorphism in *P. freudenreichii* is nevertheless slightly higher than that reported for *Flavobacterium psychrophilum* (Nicolas et al., 2008) and *O. oeni* (Bilhere et al., 2009). The extent of the natural biotope of *P. freudenreichii* being unknown, it is tempting to interpret the small genetic diversity of the species as an indication of a limited effective population size that might be compatible, for example, with a natural biotope dependant on cattle. Recombination events are at least as important as mutation events in the accumulation of sequence changes with time along *P. freudenreichii* cell lineages. The value of the r/m ratio estimated to be comprised between 1 and 2 could be considered as intermediate when compared to 48 other species (Vos and Didelot, 2009). Characteristics that may be related to the exchange of genetic material by homologous recombination are

found in the *P. freudenreichii* genome. Particularly, the genome of the CIRM-BIA1^T strain carries 34 integrase loci and 73 transposase loci, and insertion sequences account for 3.5% of the total genome (Falentin et al., 2010). Some *P. freudenreichii* strains contain plasmids that can also play a role in genetic exchanges (Rehberger and Glatz, 1990).

The different lineages in the *P. freudenreichii* population as identified by the STs are not restricted to a particular dairy biotope (Swiss cheese, other cheeses, or milk). During the “cooking” of the curd in Swiss cheese manufacture, the heat treatment ($\sim 50\text{--}55^\circ\text{C}$ for about 30 min) is probably the main stress encountered by bacteria and the existence of thermotolerant and thermosensitive *P. freudenreichii* strains has clearly been demonstrated (Anastasiou et al., 2006). Strains isolated from Swiss cheese are thus likely to be the most thermotolerant strains among the studied strains. However, the strains from Swiss cheese were disseminated over the phylogenetic tree, as were the strains from other dairy biotopes. Different results have been reported regarding the connections between STs and strain origins in non-pathogenic bacteria. STs helped discriminating *L. casei* strains depending on their biotopes of isolation (cheese or silage) whereas they did not discriminate *L. plantarum* strains (Cai et al., 2007; de las Rivas et al., 2006). As for biotopes, geographical origins did not correlate with particular location in the tree. This could be a consequence of the probable impact of the worldwide trade of starters and cheeses on strain dissemination.

The relationships between the *P. freudenreichii* population structure and the subspecies definition were investigated. Two additional phenotypes (“pheno+” and “pheno–”) are detected along with the classical *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii*, based on the utilization of lactose and nitrate by strains. These two additional phenotypes have also occasionally been mentioned in former works (de Carvalho et al., 1994; Moore and Holdeman, 1974; Vorobjeva, 1999). According to nomenclature standards (Staley and Krieg, 1984), it would be conceivable to distinguish four subspecies rather than two. From an evolutionary perspective, an important finding is however that the lineages defined by the STs do not gather strains with the same phenotype. This gives a strong indication that the *P. freudenreichii* subspecies definition does not reflect the ancestral relationships between strains, and thereby provides an explanation for earlier reports on the difficulty to identify *P. freudenreichii* subspecies using molecular methods (Dasen et al., 1998; Fessler et al., 1999; Tilsala-Timisjarvi and Alatossava, 2001). This contrasts for instance with the case of *Lactococcus lactis* for which MLST results correlate with subspecies (*lactis* or *cremoris*) definition (Rademaker et al., 2007). Our results suggest that the phenotypic characteristics on which is based the subspecies definition are prone to recurrent changes in *P. freudenreichii*. The chromosomal context of the genes responsible for these phenotypes gives additional weight to this hypothesis. In the CIRM-BIA1^T strain, the genes coding for lactose fermentation are surrounded by integrases and transposases and the gene coding for nitrate degradation is disrupted by a frameshift (Falentin et al., 2010).

This study shows that the *P. freudenreichii* species constitutes a cohesive population with a low level of polymorphisms, and in which homologous recombination contributes to the generation of new allele combinations. Each *P. freudenreichii* lineage as defined by the STs is not restricted to a particular dairy biotope and can exhibit substantial phenotypic diversity.

Acknowledgments

We thank Pascal Le Bourgeois for his comments on the manuscript, Victoria Chuat for the preparation of strains, and Sylvain Brisse and Jean-Michel Thiberge (PF8 Genotyping of Pathogens and Public Health Platform, Institut Pasteur, Paris) for handling the MLST database. This study was partly supported by Valio Ltd (Helsinki, Finland) and Tekes (the Finnish Funding Agency for Technology and Innovation).

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