Genetic diversity in Italian *Lactobacillus sanfranciscensis* strains assessed by multilocus sequence typing and pulsed-field gel electrophoresis analyses

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Lactobacillus sanfranciscensis is a lactic acid bacterium that characterizes the sourdough environment. The genetic differences of 24 strains isolated in different years from sourdoughs, mostly collected in Italy, were examined and compared by PFGE and multilocus sequence typing (MLST). The MLST scheme, based on the analysis of six housekeeping genes (*gdh*, *gyrA*, *mapA*, *nox*, *pgmA* and *pta*) was developed for this study. PFGE with the restriction enzyme *Apal* proved to have higher discriminatory power, since it revealed 22 different pulsotypes, while 19 sequence types were recognized through MLST analysis. Notably, restriction profiles generated from three isolates collected from the same firm but in three consecutive years clustered in a single pulsotype and showed the same sequence type, emphasizing the fact that the main factors affecting the dominance of a strain are correlated with processing conditions and the manufacturing environment rather than the geographical area. All results indicated a limited recombination among genes and the presence of a clonal population in *L. sanfranciscensis*. The MLST scheme proposed in this work can be considered a useful tool for characterization of isolates and for in-depth examination of the strain diversity and evolution of this species.

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INTRODUCTION

Sourdough technology is widespread in Europe, and is traditionally used in bread making for a variety of baked products, such as rye and wheat bread, crackers, brioches, Italian panettone and similar cakes, some pizzas and numerous local baked goods (De Vuyst & Neysens, 2005). The type of cereal, recipe formulation, starter method (back-slopping or use of selected cultures), dough yield, redox potential, fermentation time and temperature are the environmental factors that affect the dominant microbiota, which is mainly constituted of lactic acid bacteria and yeasts (Vogel *et al.*, 1996, 1999; De Vuyst *et al.*, 2009). In particular, the metabolic activities of heterofermentative lactobacilli involved in most sourdough production provide beneficial effects on flavour (Thiele *et al.*, 2002; Hansen & Schieberle, 2005), nutritional properties (Liljeberg *et al.*, 1995; Liljeberg & Björck, 1996; Di Cagno *et al.*, 2002, 2004), texture

(Korakli *et al.* 2001; Tieking & Gänzle, 2005; Arendt *et al.*, 2007) and shelf-life of the end products (Corsetti *et al.*, 1998a; Schnürer & Magnusson, 2005).

Lactobacillus sanfranciscensis is considered a key micro-organism (Gobbetti *et al.*, 1996), being the predominant bacterial species in type I sourdoughs for San Francisco bread and many traditional Italian and German baked products (Corsetti *et al.*, 2001; Foschino *et al.*, 2001; Meroth *et al.*, 2003). The increasing knowledge of sourdough ecology is leading to the improvement of the quality of the end product through the use of starter cultures with enhanced technological capabilities, such as exopolysaccharide production (Korakli *et al.*, 2003; Lacaze *et al.*, 2007), antifungal activity (Corsetti *et al.*, 1998b; Schnürer & Magnusson, 2005), antistaling properties (Corsetti *et al.*, 2000; Moore *et al.*, 2007) and decreased gluten intolerance (Di Cagno *et al.*, 2005; Gobbetti *et al.*, 2007). The identification of *L. sanfranciscensis* at strain level proves to be crucial as a consequence of the rising interest in the above-mentioned strain-related functional traits and for monitoring patented technologies in sourdough production. Moreover, the accurate detection of a bacterial strain could be a valuable criterion for traceability systems, in order to support the typicality of the traditional fermented foods by providing information for consumers and safeguarding the autochthonous microbiota. In fact, the possibility of extracting

Abbreviations: MLST, multilocus sequence typing; ST, sequence type; UPGMA, unweighted pair group method with arithmetic means.

The GenBank/EMBL/DDBJ accession numbers for the *gdh*, *gyrA*, *mapA*, *nox*, *pgmA* and *pta* sequences of the *Lactobacillus sanfranciscensis* strains analysed in this study are GU454588–GU454611, GU454612–GU454635, GU454636–GU454659, GU454660–GU454683, GU454684–GU454707 and GU454708–GU454731, respectively.

amplifiable microbial DNA directly from panettone cake after heat treatment, and consequently of identifying the species involved in the fermentative process, has recently been demonstrated (Picozzi *et al.*, 2006).

Genetic characterization at the strain level has already been evaluated using different techniques (Zapparoli *et al.*, 1998; Randazzo *et al.*, 2005; Catzeddu *et al.*, 2006; Corsetti *et al.*, 2007; De Angelis *et al.*, 2007; Valcheva *et al.*, 2007). Although random amplified polymorphic DNA (RAPD)-PCR assays have been presented as a suitable tool to detect genetic polymorphism within a species, it is often difficult to compare the results from different studies, since they are heavily dependent on the experimental operating conditions. At the same time, repetitive element PCR (rep-PCR) has proved to be a simple fingerprinting application with high discriminatory power, but with the same difficulties as RAPD-PCR.

PFGE analysis has been universally recognized as suitable to determine intra-specific diversity, having been shown to be the most appropriate technique for the typing of bacterial isolates (Tenover *et al.*, 1995); nevertheless, it also has some disadvantages, such as high consumable costs, taking a long time to perform, and complexity. Moreover, some strains have been shown to be difficult to handle due to problems with cell wall disruption and in the generation of 'readable' restriction patterns.

Recently, multilocus sequence typing (MLST) has been used as a novel approach for typing non-pathogenic food bacteria, such as *Oenococcus oeni* (De las Rivas *et al.*, 2004; Bihère *et al.*, 2009), *Lactobacillus casei* (Cai *et al.*, 2007; Diancourt *et al.*, 2007) and *Lactobacillus plantarum* (De las Rivas *et al.*, 2006; Tanganurat *et al.*, 2009), in order to obtain a precise and unambiguous characterization of strains collected from autochthonous microbiota in natural fermentations or used in commercial starter cultures.

The aims of this work were to explore the polymorphisms in nucleotide sequences of six housekeeping genes in *L. sanfranciscensis*, to develop an MLST protocol and to compare its discriminatory power with a PFGE technique for this species.

METHODS

Bacterial strains and growth conditions. In this study, a total of 24 *L. sanfranciscensis* strains were examined (Table 1), including strains previously isolated as dominant populations from sourdoughs collected in Italy at different times and places and reference strains obtained from an international collection. One strain was isolated from a Brazilian sourdough sample. Bacteria were grown at 30 °C for 24–48 h in modified SanFrancisco medium (SFM) broth or agar (Picozzi *et al.*, 2005). Concentrated cultures were stored at –80 °C in the same medium with 20 % (w/v) glycerol.

Table 1. Information on *L. sanfranciscensis* strains isolated from sourdoughs and their allele profiles at each locus

Strain	End product	Country, town	Firm	Date	PFGE genotype	Allele						ST
						<i>gyrA</i>	<i>mapA</i>	<i>gdh</i>	<i>pta</i>	<i>nox</i>	<i>pgmA</i>	
DSM20451 ^T	San Francisco bread	USA, San Francisco		1971	XI	1	1	1	1	1	1	1
2B4	Wheat bread	Italy, Catania	N	2007	XVI	1	6	3	3	6	1	2
B2001	Pandoro cake	Italy, Verona	B	2001	XIX	1	6	1	1	6	1	3
MBA1	Panettone cake	Brazil, Ipiranga	O	2006	VI	1	6	1	6	7	1	4
PB213	Wheat bread	Italy, Ancona	P	2003	IX	1	5	1	1	2	1	5
3MLN	Panettone cake	Italy, Milan	A	2007	I	2	3	2	1	5	1	6
B92	Panettone cake	Italy, Milan	A	2001	XI	2	2	1	1	5	1	7
MDC	Panettone cake	Italy, Verona	M	2007	III	2	2	2	3	5	1	8
8M2A3	Panettone cake	Italy, Milan	A	12/11/2004	XII	3	4	1	1	6	2	9
83ML2	Panettone cake	Italy, Milan	A	23/06/2005	XII	3	4	1	1	6	2	9
MT0T	Panettone cake	Italy, Milan	A	21/11/2006	XII	3	4	1	1	6	2	9
83MV2	Panettone cake	Italy, Verona	A	23/06/2005	IV	3	4	1	1	6	2	9
BAT0408	Panettone cake	Italy, Parma	Q	2008	XXI	3	4	1	1	6	2	9
B48	Pugliese bread	Italy, Verona	F	1998	X	4	5	1	4	3	1	10
CB1	Wheat bread	Italy, Terni	C	1991	VII	5	6	3	1	8	1	11
D15	Panettone cake	Italy, Pavia	D	1994	XVII	6	3	1	1	6	1	12
DSM20663	Rye bread	Germany		1978	XX	7	6	3	1	4	1	13
H2A	Wheat bread	Italy, Campobasso	H	1998	V	7	6	1	2	2	1	14
E20	Wheat bread	Italy, Perugia	E	1999	XIV	8	6	1	1	6	1	15
I2	Panettone cake	Italy, Milan	I	2001	XV	8	6	1	1	6	1	15
S133	Brioche	Italy, Verona	L	1998	XIII	8	4	1	1	2	1	16
MG1	Panettone cake	Italy, Sondrio	G	2005	VIII	8	6	1	1	7	1	17
8P4A1B1	Brioche	Italy, Perugia	B	2003	II	8	6	1	1	2	1	18
8V4A2B2	Brioche	Italy, Verona	B	2003	XVIII	8	6	4	1	2	1	19

The evaluation of the generation time of an *L. sanfranciscensis* dominant strain in a 'mother' dough for panettone was carried out at one firm (firm A) according to the existing operating conditions. Approximately 10 g sample was diluted in 90 ml sterile peptone water and homogenized in a Stomacher 400 Circulator (Seward) for 5 min at 260 r.p.m. Aliquots of the appropriate dilutions were plated on modified SFM agar and incubated at 30 °C for 48 h. Microbial counts were replicated in three independent experiments.

Species identification. Bacterial DNA was extracted using an Archive Pure DNA Purification System (5 PRIME) according to the manufacturer's instructions. A preliminary identification was performed through the recognition of the electrophoretic profile generated from the amplification of internal ribosomal spacers according to Valcheva *et al.* (2007). Furthermore, all the strains were subjected to the sequencing of a portion of the 16S rRNA-coding gene in order to confirm that they belonged to the species. The amplification reaction was performed as described by Borgo *et al.* (2007) using a Biometra thermocycler. The amplification products were purified on QIAquick spin columns (Qiagen) for direct sequencing with the PCR forward primer using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech), in accordance with the manufacturer's protocol, and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

PFGE analysis. DNA samples were prepared according to Zapparoli *et al.* (1998). Briefly, fresh cell suspensions of OD₆₀₀ 1.0 in SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.7) were mixed with 2% (w/v) Certified Molecular Biology Agarose (Bio-Rad) and pipetted into a plug mould. The blocks were treated with lysozyme (3 mg ml⁻¹) at 37 °C for 12 h, then with 1% (w/v) *N*-lauryl sarcosine and proteinase K (1 mg ml⁻¹) at 37 °C for 16 h. Plugs were washed several times in 1 × TE buffer (Tris/HCl 10 mmol l⁻¹, pH 8.0, EDTA 100 mmol l⁻¹) and subjected to overnight DNA digestion at 37 °C with 10 U *Apal* (MBI Fermentas). Plugs were subjected to PFGE on a CHEF-DR II apparatus (Bio-Rad). DNA fragments were separated in 1% (w/v) Pulsed Field Certified Agarose (Bio-Rad) in 0.5 × TBE buffer (45 mM Tris/HCl, 45 mM boric acid, 1 mM EDTA, pH 8.0). A switch time ranging from 0.5 to 8 s for 17 h at a constant voltage of 6.0 V cm⁻¹ at 14 °C was adopted.

The DSM20663 strain was used as an internal reference strain. After migration, gels were stained with ethidium bromide, photographed under UV light, and analysed with a GelDoc XR system (Bio-Rad).

Sequence analysis. The following six loci were chosen for the sequence analysis scheme: *gdh*, encoding glucose-6-phosphate dehydrogenase

(accession no. AJ810804), *gyrA*, encoding the A subunit of DNA gyrase (AY912125), *mapA*, encoding maltose phosphorylase (AJ224340), *nox*, encoding an NADH oxidase (AB035801), *pgmA*, encoding a phosphoglucomutase (AJ224340), and *pta*, encoding phosphotransacetylase (AB035800). These housekeeping genes were selected on the basis of their putative function and on the availability of sequence data for *L. sanfranciscensis*.

Primers were designed with the online software Primo Pro 3.4 (<http://changbioscience.com/primo/primo.html>), using sequences of highly conserved DNA regions of these *L. sanfranciscensis* genes obtained from GenBank (Table 2).

A 25 µl reaction mixture for PCR amplification containing 20 ng template DNA, 10 mM Tris/HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate, 1 µM of each primer and 1 U *Taq* polymerase (Expand High Fidelity PCR System, Roche) was prepared. The reaction was performed using a Biometra thermocycler. The temperature profile used the following cycling parameters: 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 55, 60 or 63 °C (see Table 2), 1 min at 72 °C, and 10 min at 72 °C. Amplified products were resolved on a 1.5% (w/v) agarose gel in 1 × TAE (40 mM Tris/HCl, pH 8.2, 1 mM EDTA), stained with ethidium bromide and photographed under UV illumination.

The amplification products were purified on QIAquick spin columns (Qiagen) for sequencing with the respective forward and reverse primers (Table 2), by Primm.

The DNA sequences of the *L. sanfranciscensis* strains analysed have been deposited in GenBank (Table 2).

Data analysis. For the PFGE data, the densitometric values from the electrophoretic bands were transferred to BioNumerics 5.10 software (Applied Maths). The fingerprints obtained were compared by means of the Dice coefficient, and cluster analysis was performed by the unweighted pair group method with arithmetic means (UPGMA).

For the MLST analysis, the sequences obtained for each locus were compared for all isolates, and an allele number was given to each distinct sequence variant. Each isolate was defined by an allele profile or sequence type (ST) derived from the combination of alleles at the six loci analysed (Table 1). The STs were identified by arbitrary numbers assigned in order of description.

Sequence alignments and comparison were done with CLUSTAL_X (<http://www.clustal.org/>) and converted into MEGA and NEXUS files

Table 2. Primers used for MLST, and accession numbers of sequences of investigated loci

Protein	Gene	Primers	Sequence (5'→3')	PCR product length (bp)	Annealing temperature (°C)	Accession nos
Glucose-6-phosphate dehydrogenase	<i>gdh</i>	GDH1F	AAGTTATATCCTGCAATCTTT	489	55	GU454588–GU454611
		GDH1R	TTACCTAAGTAATGATCAATTC			
Subunit A of DNA gyrase	<i>gyrA</i>	GYRA1F	CGAATATTCGCCTCACA	484	55	GU454612–GU454635
		GYRA1R	GAGCACCTTCAATAATAGCA			
Maltose phosphorylase	<i>mapA</i>	MAP2F	ACCCAGACTACTTCGGTAA	735	63	GU454636–GU454659
		MAP2R	GAAGCGAATGCCTTGTTG			
NADH oxidase	<i>nox</i>	NOX1F	GTAGGTTGTACTCACGCT	734	55	GU454660–GU454683
		NOX1R	CAGTGTTAGGGCGGAAAC			
Phosphotransacetylase	<i>pta</i>	PTA1F	GTTTCAGGTGTGCTCAC	583	55	GU454708–GU454731
		PTA1R	TGATACTTTATAAACGTCCTC			
Phosphoglucomutase	<i>pgmA</i>	PGM2F	GACTTGGATGGCGTCATC	409	60	GU454684–GU454707
		PGM2R	ATGTAGCGTTGCTGGGTC			

with START2 (Sequence type Analysis and Recombinatorial Tests; <http://www.medawar.ox.ac.uk/maiden/software.shtml>). Phylogenetic trees were assembled with MEGA version 4.1 software (<http://www.megasoftware.net>). Dendrograms were constructed by application of UPGMA. The percentage of bootstrap confidence levels for internal branches, as defined by the MEGA program (Kumar *et al.*, 1994), was calculated from 1000 random resamplings.

To assess the degree of tree-like structure in the alleles found for each locus in the complete set of 24 isolates, the method of split decomposition, performed with SPLITSTREE 4.0, was used (Huson & Bryant, 2006). The START2 program was used to calculate the ratio of non-synonymous to synonymous substitutions (the d_N/d_S ratio; Nei & Gojobori, 1986) and the Sawyer's run test for intragenic recombination. The measure of linkage equilibrium between alleles at the six housekeeping genes was assessed by calculating the index of association (I_A) (Smith *et al.*, 1993). To avoid dependence on the number of loci, the standardized I_A (I_A^S) was also calculated (Haubold & Hudson, 2000).

RESULTS

Characterization of *L. sanfranciscensis* strains by PFGE

A pattern repeatability of 91 % was assessed by determining the similarity percentage joining independent replicates of

L. sanfranciscensis DSM20663 (data not shown), and this value was considered as a threshold to define identical pulsotypes (Tenover *et al.*, 1995; Ravelo *et al.*, 2003).

From 24 *L. sanfranciscensis* strains considered in this work, 22 pulsotypes (PTI to PTXXII) were obtained (Fig. 1); this outcome was expected, since *L. sanfranciscensis* strains were recovered from different locations, types of sourdough and years. All the strains were clearly subdivided into two clusters, named A and B, composed of 11 and 13 PFGE patterns that shared more than 54 and 55 % similarity, respectively. In general, no relationships among geographical origin, sourdough technology or year of isolation were observed. However, the isolates 8M2A3, 83ML2 and MT0T (cluster B), which were collected from the ‘mother’ dough from the same firm (A) but in three consecutive years (2004, 2005 and 2006), proved to be closely related (PTXXII). On the other hand, B92 and 3MLN strains, still collected from samples from firm A in different years (2001 and 2007, respectively), belonged to different pulsotypes (PTXI and PTI) and were both located in cluster A. Moreover, the restriction profiles generated from 8P4A1B1 (cluster A) and 8V4A2B2 (cluster B) isolates were shown to be completely different (PTII and PTXVIII), even though

Fig. 1. PFGE analysis of *L. sanfranciscensis* strains after digestion with *Apal*. The dendrogram was constructed by UPGMA cluster analysis of the restriction profiles. The scale bar shows percentage similarity.

these micro-organisms were isolated from sourdough samples originated from the same 'mother', which was back-slopped in two different sites, Perugia and Verona, of the same firm (B).

Diversity in housekeeping genes

Since the genome sequence of *L. sanfranciscensis* had not been completed when this study was performed, a number of candidate loci were identified by searching the *L. sanfranciscensis* gene sequences deposited (<http://ncbi.nlm.nih.gov>). These genes can be differentiated into two functionally distinct classes: informational (*gyrA*), which

are involved in information processing; and operational (*gdh*, *mapA*, *pgmA*, *pta* and *nox*), which function in day-to-day processes of cell maintenance (Rivera *et al.*, 1998).

The six genes were successfully amplified for all 24 *L. sanfranciscensis* strains, and sequenced in forward and reverse direction to confirm the order of the nucleotides. Fig. 2 shows the position of the polymorphic sites for each locus, indicating the changed nucleotides with respect to the consensus sequence reported in GenBank for the DSM20451^T strain. For all loci the 24 sequences aligned without gaps or insertions. All loci were polymorphic, and

<i>gdh</i>				<i>gyrA</i>														
	241	283	337				34	84	106	176	182	213	214	289				
Consensus	A	G	C			Consensus	C	A	T	G	A	C	A	T				
Allele 1 (18)	T	.	.			Allele 1 (5)	.	.	.	.	.	.	.	.				
Allele 2 (2)	.	A	.			Allele 2 (3)	T	G	A	A	.	A	.	C				
Allele 3 (3)	.	.	T			Allele 3 (5)	T	G	.	.	.	.	.	C				
Allele 4 (1)						Allele 4 (1)	T	G	.	.	.	A	.	.				
						Allele 5 (1)	.	G	.	.	.	.	G	.				
						Allele 6 (1)	.	.	.	.	G	.	.	.				
						Allele 7 (2)	.	G	.	.	.	.	.	C				
						Allele 8 (6)	.	G	.	.	.	.	.	.				
<i>mapA</i>				20	135	148	445	470	607	631	<i>pta</i>				9	34	66	427
Consensus	G	T	G	G	T	A	G				Consensus	C	T	C	G			
Allele 1 (1)	A	.	.	A	C	.	.				Allele 1 (19)	.	.	.	.			
Allele 2 (2)	.	C	A	A	C	.	.				Allele 2 (1)	T	.	.	.			
Allele 3 (1)	.	.	.	A	C	G	.				Allele 3 (2)	.	G	.	.			
Allele 4 (6)	.	.	.	A	C	.	A				Allele 4 (1)	.	.	T	.			
Allele 5 (2)	.	.	.	A	C	.	.				Allele 5 (1)	.	.	.	A			
Allele 6 (12)																		
<i>nox</i>				14	105	113	116	183	481	484	489	<i>pgmA</i>				52		
Consensus	C	C	C	G	A	G	C	A				Consensus	G					
Allele 1 (1)	.	.	.	.	.	.	.	.				Allele 1 (19)	.					
Allele 2 (5)	.	.	.	A	.	.	.	.				Allele 2 (5)	A					
Allele 3 (1)	T	.	.	A	.	.	.	.										
Allele 4 (1)	.	T	T	A	.	.	.	.										
Allele 5 (3)	.	.	.	A	G	.	A	.										
Allele 6 (10)	.	.	.	A	.	T	.	.										
Allele 7 (2)	.	.	.	A	.	.	A	.										
Allele 8 (1)	.	.	.	A	.	.	.	G										

Fig. 2. Polymorphic nucleotide sites in *L. sanfranciscensis* MLST genes. Only the variable sites are shown. The number of strains possessing an allele is indicated in parentheses.

the number of polymorphic nucleotide sites varied between one in *pgmA* and eight in *gyrA* and *nox*, suggesting a different evolution rate. Fragments with a length between 353 (*pgmA*) and 648 bp (*nox*) allowed the identification of the number of alleles per locus as between two (*pgmA*) and eight (*gyrA* and *nox*) (Table 3). The observed frequencies showed that one allele was dominant for most of the loci (*mapA*, *gdh*, *pta* and *pgmA*) (Table 1). The mean G+C content of the different gene fragments varied from 34.79 (*nox*) to 47.64 and 48.38 mol% (*mapA* and *pgmA*, respectively). By combining the sequence results of the six loci, 19 STs were distinguished. The UPGMA dendrogram in Fig. 3 shows the genetic relatedness among the *L. sanfranciscensis* strains investigated. In most cases, the identified ST profiles allowed the strains to be distinguished by a unique combination of alleles, though with limited variation. In fact, ST3 (B2001) diverged from ST15 (E20 and I2) only at the *gyrA* locus, and ST16 (S133) and ST18 (8P4A1B1) differed only at the *mapA* locus. Furthermore, the ST18 profile diverged from ST19 (8V4A2B2) only at the *gdh* locus, and ST15, ST17 (MG1) and ST18 can be distinguished only by a polymorphism in *nox*. With respect to the sequence reported in GenBank for the DSM20451^T *nox* gene, a thymine instead of a cytosine is present at position 32.

No significant clusters that correlated with geographical origin, type of sourdough or year of isolation were observed. However, ST9 grouped five isolates, four of which were collected from sourdoughs from firm A in different years, and one of which was from firm Q.

The values of d_N (number of non-synonymous changes per non-synonymous site) and of d_S (number of synonymous changes per synonymous site) were calculated for each gene. The d_N/d_S ratios were evaluated and, for all loci but two (*gyrA* and *mapA*), were <1 (Table 3), indicating that a high selective pressure against amino acid changes was present.

In-frame concatenated gene sequence fragments from all the loci were analysed and drawn in a graphic using SPLITSTREE software (Fig. 4a); the tree-like structure that was obtained suggested a clonal descent. The split decomposition analysis, afterwards calculated for the alleles of each locus, matched the clonal nature of the evolution in

the tested genes, since tree-like structures were observed. Only the graph for *gyrA* presented a parallelogram-like structure, suggestive of intragenic recombination (Fig. 4b). The split graph for *pgmA* showed a line, since only two alleles were examined. When the sum of squares of condensed fragments was considered, Sawyer's test revealed no evident occurrence of intragenic recombination in the samples (Table 4). This result was supported by the values obtained with the maximum condensed fragment method ($P=1.000$). The linkage disequilibrium among alleles was estimated from the I_A value. Since this index showed a value of 0.4344, significantly higher than 0, a clonal population could be identified. Furthermore, the standardized I_A value (I_A^S), which is independent of the number of loci, was 0.0869, indicative of a limited recombination among genes.

Comparative results for PFGE versus MLST

The discriminatory power of the two typing analyses was compared: PFGE differentiated 22 pulsotypes, whereas the MLST protocol recognized 19 STs. Some strains that proved to be indistinguishable by PFGE analysis, such as isolates 8M2A3, MT0T and 83ML2 (PTXII), revealed identical sequences for all the gene fragments that were analysed (ST9). On the other hand, isolates 83MV2 and BAT0408, belonging to the same ST (ST9), were located in different PFGE clusters (A and B) and were not related to PTXII.

For each of the other strains (except E20 and I2, see below), a single pulsotype and ST were determined. In fact, strains showing very similar restriction profiles, such as MBA1 and CB1 or D15 and 8V4A2B2, presented distant allelic STs (ST4 and ST11 or ST12 and ST19, respectively).

An interesting outcome, validated through results from both PFGE and MLST analyses, was the inconsistency of the geographical factor in the evolution of the dominant bacterial populations naturally selected in sourdough fermentations. In fact, strains isolated in the same town (Verona) but from six different firms (A, B, F, L, M and P) showed dissimilar pulsotypes and distinct STs. On the other hand, strains E20 and I2, which exhibited a unique ST profile (ST15) and closely related PFGE patterns, were isolated in two different Italian regions.

Table 3. Descriptive analysis of MLST data

Gene	Fragment analysed (bp)	Mean G+C content (mol%)	Polymorphic sites*	Alleles	d_N/d_S
<i>gyrA</i>	357	37.87	8 (2)	8	1.1522
<i>mapA</i>	632	47.64	7 (1)	6	1.5710
<i>gdh</i>	396	36.07	3 (0)	4	—
<i>pta</i>	459	40.08	4 (2)	5	0.2997
<i>nox</i>	648	34.79	8 (3)	8	0.5504
<i>pgmA</i>	353	48.38	1 (1)	2	0.0000

*Number of silent polymorphic sites in parentheses.

Fig. 3. UPGMA tree generated from allelic profiles showing the genetic relatedness of the *L. sanfranciscensis* strains examined in this study. The scale bar indicates genetic relatedness.

Generation time of an *L. sanfranciscensis* strain under industrial conditions

Samples were aseptically collected every 2 h during the whole ripening time of the ‘mother’ (16 h) and immediately analysed at the laboratory of firm A. Through the elaboration of results obtained from plate counts the generation time was calculated to be 4 h. A few colonies at the highest dilutions were randomly picked at each monitoring time. Species identification was carried out by the analysis of the internal ribosomal spacers, as described by Valcheva *et al.* (2007).

DISCUSSION

L. sanfranciscensis is considered the dominant bacterial species in sourdough technology, and requires a daily propagation of the so-called ‘mother’ dough (type I process) (De Vuyst *et al.*, 2009; Foschino *et al.*, 2001; Gobetti *et al.*, 1996; Vogel *et al.*, 1999). Nevertheless, the use of this micro-organism in starter cultures for bakery has not yet been fully exploited, and its presence in the form of viable selected strains has never been successfully applied in starter programme management.

The use of PFGE analysis has proved to be helpful in discriminating *L. sanfranciscensis* isolates collected from

different fermentation processes (Zapparoli *et al.*, 1998). However, the need for dedicated tools and skilled staff, presence of strains that do not generate restriction profiles, and the possibility of recombination events within the same strain make the application of PFGE as a routine technique difficult. A suitable typing protocol that can allow a precise identification and that can be easily used in an inter-laboratory data comparison is necessary. MLST, a method that is based on partial nucleotide sequences of multiple housekeeping genes, has recently been shown to be a powerful technique for bacterial typing in food fermentations (De las Rivas *et al.*, 2006; Tanganurat *et al.*, 2009). Housekeeping genes are preferred, because an analysis of mutations in such genes is more likely to properly reflect the phylogeny of strains, since such genes evolve slowly and therefore can indicate the genetic relationships among bacterial isolates more accurately than genes under selective pressure. MLST uses variation that accumulates slowly, which is expected to be selectively neutral, and achieves very high resolution by analysing multiple loci.

Thus, we characterized a collection of 24 *L. sanfranciscensis* strains through MLST and compared the results with those of PFGE analysis. The high discriminatory power of PFGE was confirmed, since we identified 22 pulsotypes and detected two major clusters (A and B). Even though we sequenced six genes, we were not able to obtain the same discrimination level with MLST analysis. This lack of discrimination has been reported in other studies (Cai *et al.*, 2007; Johnson *et al.*, 2007), and is probably due to the fact that, since PFGE is based on restriction patterns, every single point mutation in the few enzyme recognition sites throughout the genome could significantly alter the profile. Moreover, this could explain why the cluster analysis obtained with the two techniques generates different and not similar results, considering that the evolution rate of housekeeping genes is different from the rearrangement rate of the whole genome. Notably, micro-organisms that cluster together in a single pulsotype (PTXII) were collected from the same firm (A) in three consecutive years. We assumed that these three isolates (8M2A3, 83ML2 and MT0T) belonged to the same strain and that, therefore, a single bacterial population might be predominant, at least for 32 months, in a ‘mother’ dough refreshed every day under standardized and controlled conditions of temperature, redox potential, moisture, recipe formulation and time of ripening (Picozzi *et al.*, 2009). Furthermore, this evidence was strengthened by the results of MLST, which revealed the same ST (ST9) for the three isolates considered above. Taking into account that the generation time for *L. sanfranciscensis* was calculated as approximately 4 h, under the operating conditions at the plant, we estimated that both the genome structure and the nucleotide sequences of the examined loci were unchanged for approximately 5900 cellular duplications. On the other hand, the evidence that when a ‘mother’ dough is transferred from one firm to another the dominant population changes is proof that environmental conditions and industrial practices strongly

Fig. 4. (a) Split decomposition analysis based on concatenated sequences of six housekeeping genes. The numbering refers to the ST profile. (b) Split decomposition analysis of alleles obtained for six loci. The parallelogram structures are suggestive of recombination. All branch lengths are drawn to scale. The numbering refers to allele numbers. The bars indicate the scale for the branch lengths.

affect the structure of the bacterial communities in a type I sourdough process. In fact, the original strain recovered from the 'mother' dough for brioche production sampled in Perugia was no longer found as the dominant strain in the 'mother' dough after 4 months of back-slopping in Verona.

Our findings support the thesis of De Vuyst *et al.* (2009) and Scheirlinck *et al.* (2009) on the evolution of lactobacilli populations in a type I sourdough: they suggest that the processing conditions and the manufacturing environment, rather than the geographical area, are the factors mainly influencing the dominance of a strain.

As regards MLST analysis, the six investigated loci proved to be polymorphic. The sequencing of the *pgmA* fragment displayed only two alleles; the removal of this gene from the analysis led to the same number of STs. Furthermore, *pgmA* is adjacent to *mapA* on the chromosome of DSM20451, and may therefore be horizontally transferred by a single recombination event, introducing inaccuracies in evolution rate estimations. Nevertheless, we kept the *pgmA* gene in this work because of the differences shown

by allele 2, which remarkably are present in only five isolates (Table 1), confirming the high similarity among them. Notably, strains 83ML2 and 83MV2, which were isolated on the same day from samples from two different plants of the same firm (A), showed different PFGE profiles. This observation led to the hypothesis that a recombination event could have taken place at a previous time when the mother dough had been transferred from Milan to Verona. An optimization of the MLST scheme with another housekeeping gene with a higher number of alleles is suggested. Almost all the STs that were identified are represented by a single strain; the only two exceptions correspond to ST9 and ST15 (five and two isolates, respectively). The results obtained by split decomposition analysis of the six loci suggest a low rate of mutation and thus the occurrence of a rare recombination in these housekeeping genes. The same result could be obtained if we look at the lack of mosaic structure in the aligned sequences (Fig. 2). Our results differ from those obtained for similar species used in food fermentations, such as *O. oeni*, *L. casei* and *L. plantarum*, for which evidence of

panmitic populations has been demonstrated (De las Rivas *et al.*, 2006; Cai *et al.*, 2007; Bilhère *et al.*, 2009). The clonality of *L. sanfranciscensis* strains observed in this work could be explained by the emergence of a recent common ancestor, revealing the ability of this species to dominate the microbial consortia in sourdough production under harsh environmental conditions. The overall G+C content of 34.71 mol% estimated for the *L. sanfranciscensis* chromosome (Vogel *et al.*, 2009) is not a definitive value, since when we were writing this work, the whole genome

sequence of this species had not been published. Therefore, the differences between this value and those obtained from the housekeeping genes examined cannot be ascribed with confidence to a recent acquisition. Nevertheless, the *pgmA* and *mapA* allele sequences showed an unexpectedly high G+C content, confirming the stability of the amino acid sequences of these two enzymes and the significance of their catabolic abilities for survival in a sourdough environment. In fact, these two genes encode enzymes involved in the metabolism and transport of maltose into

Table 4. Sawyer's test analysis for evidence of intragenic recombination

Locus	SSCF* (P)	MCF† (P)
<i>gyr</i>	72 (1.000)	2 (1.000)
<i>mapA</i>	10 (1.000)	1 (1.000)
<i>gdh</i>	0 (1.000)	0 (1.000)
<i>pta</i>	18 (1.000)	2 (1.000)
<i>nox</i>	143 (1.000)	3 (1.000)
<i>pgmA</i>	0 (1.000)	0 (1.000)

*Sum of squares of condensed fragments.

†Maximum condensed fragment.

the cell; therefore, they are critical to the dominance of this species in sourdough, where maltose is the most available sugar. In fact, sourdough is the only habitat known to date where *L. sanfranciscensis* prevails, on account of its specialized metabolism and its high resistance to an acid environment. The high G + C content in *mapA* seems to be in disagreement with the results in Table 3, where it is shown that of seven polymorphic sites in this gene, only one silent site was found. This could be explained by considering that the sequenced fragment for this locus is positioned in a region located between the two catalytic sites of the enzyme. Thus, the non-silent changes do not affect the activity of the MapA protein. In the same way, *gyrA* shows an elevated number of non-silent polymorphic sites. This is an even more unexpected result, since this is an informational gene and for this reason is considered less malleable from an evolutionary point of view (Rivera *et al.*, 1998). Since there is no available information on the tertiary structure of the catalytic site for this species, we can only theorize that the mutations observed in almost 50 % of our strains do not lead to a conformational change of the protein and therefore to a loss of its activity.

In conclusion, the MLST scheme that we developed in this work could be useful for a precise discrimination at the strain level for this species. Considering industrial applications, this technique might appear quite expensive, but it could be a suitable tool for safeguarding brands or the Protected Designation of Origin of typical products.

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