Physical and genetic map of the *Weissella paramesenteroides* DSMZ 20288^T chromosome and characterization of different *rrn* operons by ITS analysis

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The *Weissella paramesenteroides* DSMZ 20288^T chromosome was analysed by pulsed-field gel electrophoresis, enabling the construction of a physical and genetic map. A total of 21 recognition sites of the restriction enzymes *Ascl*, *I-CeuI*, *NotI* and *SfiI* were mapped on the chromosome, which was found to be circular with an estimated size of 2026 kb. This is believed to constitute the first study into the genomic organization of a strain of this genus, addressing the localization of important chromosomal regions such as *oriC* and *terC*. A total of 23 genetic markers were mapped, including eight *rrn* operons that were precisely assigned in 37 % of the *W. paramesenteroides* chromosome. The transcription direction of *rrn* loci was determined and three different *rrn* clusters were recognized regarding the presence/absence of tRNA genes in ITS regions.

INTRODUCTION

Weissella paramesenteroides (formerly *Leuconostoc paramesenteroides*), described by Garvie (1986), is one of the predominant lactic acid bacteria (LAB) species in fresh vegetables and can even be found in processed meat substrates such as fermented sausages and dry salami (Björkroth *et al.*, 2002). Although it was initially placed in the genus *Leuconostoc*, DNA hybridization studies (Garvie, 1976) and phylogenetic analysis (Martinez-Murcia & Collins, 1990) soon revealed a closer relationship with some heterofermentative lactobacilli rather than with the other *Leuconostoc* species. The genus *Weissella* (Collins *et al.*, 1993) was created to include *L. paramesenteroides* and five species of atypical heterofermentative lactobacilli, namely *Lactobacillus viridescens* (the type species), *Lactobacillus confusus*, *Lactobacillus minor*, *Lactobacillus kandleri* and *Lactobacillus halotolerans*.

Until recently, information regarding the structure and organization of LAB genomes was scarce, mainly due to the lack of appropriate genetic tools (Le Bourgeois *et al.*, 1993, 2000). The introduction of 'top-down' approaches by the use of macrorestriction and pulsed-field gel electrophoresis techniques provided the means to estimate genome sizes

and to construct physical and genetic maps. The structure of the chromosome of several LAB has already been obtained in this way. Physical and genetic maps of the facultative heterofermentative lactobacilli *Lactobacillus sakei* (Dudez *et al.*, 2002), *Lactobacillus plantarum* (Chevallier *et al.*, 1994) and *Oenococcus oeni* strains PSU-1 (Zé-Zé *et al.*, 1998) and GM (Zé-Zé *et al.*, 2000) were reported and may constitute the closest genome organization to that of *Leuconostoc* or *Weissella*. The knowledge of LAB genomes has been greatly enhanced by sequencing projects. In fact, by 2002 five genomes of LAB had already been sequenced and 29 more projects were under way all around the globe in private and public institutions (Klaenhammer *et al.*, 2002). Although the number of completely sequenced LAB genomes has more than doubled (data from NCBI, JGI and other databases), no sequencing project for *Weissella* species has come to our attention.

In this study we analysed the chromosome structure and the localization of some relevant genes of *Weissella paramesenteroides* DSMZ 20288^T. The first physical and genetic map of a *Weissella* strain was constructed using restriction enzymes *Ascl*, *I-CeuI*, *NotI* and *SfiI* and electrophoretic separation by PFGE. The localization of several genetic markers was accomplished by Southern hybridization.

METHODS

Bacterial strains and growth conditions. Type strains of *Weissella paramesenteroides* (DSMZ 20288^T), *Weissella confusa*

Abbreviations: CHEF, counter-clamped homogeneous electric field; ITS, internal transcribed spacer; LAB, lactic acid bacteria.

The GenBank accession numbers for the sequences reported in this paper are given in Tables 1 and 2 and in the legend of Fig. 3.

Table 1. PCR conditions used to amplify DNA sequences used directly as probes or in plasmid construction

Probe	Function	Primer pair (5'-3')	PCR cycles*	Origin; Accession no.	Reference for primers
<i>rrs</i>	16S rRNA	AGAGTTTGATCCTGGCTCAG and AAGGAGGTGATCCAGCCGCA	ID+33 cycles 94 °C 1 min, 55 °C 1 min, 72 °C 1 min 30 s+FE	<i>W. paramesenteroides</i>	Massol-Deya <i>et al.</i> (1995)
<i>rrl</i>	23S rRNA	CAGTAAACGGCGGCCGTAAC and GAGCTTAACTTCTGTGTTCCGG	ID+33 cycles 94 °C 1 min, 55 °C 1 min, 72 °C 1 min 30 s+FE	<i>W. paramesenteroides</i>	This study
<i>gyrB</i>	DNA gyrase subunit B	GAAGTCATCATGACCGTTCTGCA-YGCNNGNGGNAARTTYGA and AGCAGGGTACGGATGTGCGAGC-CRTCACRTCNCGTCAT	ID+30 cycles 94 °C 1 min, 48 °C 2 min, 72 °C 2 min+FE	<i>W. paramesenteroides</i>	Yamamoto & Harayama (1995)
<i>rpoC</i>	RNA polymerase β' subunit	TGYTCWTGYGGWAARTAYAA and CATWACYTCWACRTGYTTRTC	ID+30 cycles 94 °C 1 min, 48 °C 1 min, 72 °C 1 min+FE	<i>W. paramesenteroides</i>	Morse <i>et al.</i> (1996)
<i>harosynth</i>	Putative phosphoshikimate carboxyvinyltransferase and chorismate synthase	GCATGCAATTGGGAATCA and ACTGTTGTATGGTCACGA	ID+33 cycles 94 °C 1 min, 55 °C 1 min, 72 °C 1 min+FE	<i>W. paramesenteroides</i> ; AY661804	This study†
<i>dnaA</i>	Replication initiator protein	ACATYTAATGCARGCTATCGG and GWATGATCKCGYCCRCCAA	ID+30 cycles 94 °C 1 min, 56 °C 1 min, 72 °C 1 min+FE	<i>W. paramesenteroides</i>	This study
<i>recA</i>	Recombinase A	CAAACCDGATACHGGHGAACAAG and CCAAYTTTTTCACGAATYTG	ID+35 cycles 94 °C 1 min, 48 °C 1 min, 72 °C 35 s+FE	<i>W. paramesenteroides</i>	This study
<i>dnaK</i>	HSP70 (chaperone protein)	ATGWCWAAAATTATTGGTATTGATTTAGG and CTCTTYAAARTCR-SCATCAAC	ID+33 cycles 94 °C 1 min, 50 °C 1 min, 72 °C 1 min 30 s+FE	<i>W. confusa</i>	This study
intC8	Oxoacylreductase and RNA methyltransferase	CGGAATTCTYCCTGCGAGGATAGG-ACG and CGGAATTCATGTATTAG-GCAGCCG	ID+33 cycles 94 °C 1 min, 57 °C 1 min, 72 °C 2 min 30 s+FE	<i>W. paramesenteroides</i> ; AY661800	This study
<i>hrpA</i>	RNA polymerase α subunit	ACATYTAATGCARGCTATCGG and GWATGATCKCGYCCRCCAA	ID+33 cycles 94 °C 1 min, 57 °C 1 min, 72 °C 45 s+FE	<i>Leuconostoc pseudomesenteroides</i> ; AY661806	This study
GACA3	Unknown; no coding sequence revealed	GACAGACAGACAGACA	ID+33 cycles 94 °C 1 min, 40 °C 1 min, 72 °C 1 min 30 s+FE	<i>W. paramesenteroides</i> A3 fragment; AY661799	Meyer <i>et al.</i> (1993)

*ID, Initial denaturation: 94 °C 5 min; FE, final extension 72 °C 5 min.

†Primers were provided by P. Duwat (deceased; Unité de Recherches Laitières et de Génétique Appliquée, INRA, Jouy-en-Josas, France), directed for *dnaA* amplification in *Lactococcus lactis*.

(DSMZ 20196^T) and *Leuconostoc pseudomesenteroides* (CECT4027^T) were grown in MRS medium at 30 °C. *Escherichia coli* JM109 and XL-1 Blue MRF' (Stratagene) were grown at 37 °C in LB broth, supplemented with 100 µg ampicillin ml⁻¹ when required.

Isolation and cleavage of chromosomal DNA, and DNA fragment nomenclature. Intact genomic DNA was prepared in agarose plugs and single or double digested with the restriction enzymes *AscI*, *I-CeuI*, *NotI* and *SfiI* as previously described (Tenreiro *et al.*, 1994; Zé-Zé *et al.*, 1998, 2000). Restriction fragments produced by digestion with a single enzyme are indicated by the initial letter of the endonuclease. Those obtained by double digestion are designated using the names of the respective single-digestion overlapping fragments joined by a hyphen. All fragments are numbered in size order, from the largest to the smallest. Co-migrating fragments are numbered with sequential numbers; when presenting hybridization results, following the enzyme letter, the numbers of the fragments are separated by a slash (indicating the possibility of hybridization of both or just one of the fragments). DNA extraction and purification for general purposes was carried out as described by Pitcher *et al.* (1989). Some DNA fragments were recovered after gel electrophoresis using the Jet Quick-Gel extraction spin Kit (Genomed).

PFGE. PFGE was carried out in the Gene Navigator system (Pharmacia) with contour-clamped homogeneous electric field (CHEF) as previously described (Zé-Zé *et al.*, 1998). The mean size of each fragment was estimated from at least two (fragments larger than 1000 kb) or 15 (fragments smaller than 1000 kb) runs by linear interpolation with two or more flanking size standards using KODAK 1D 2.0 software (Kodak). Lambda ladder and Low-Range PFG Markers (New England Biolabs) were used as molecular mass standards as well as intact chromosomes of *Saccharomyces cerevisiae* (Bio-Rad).

PCR conditions. PCR was performed in a Thermocycler (Stratagene). The reaction mixtures contained 100–500 ng template DNA, 50 pmol each primer, 200 µM each dNTP, 1.5 mM MgCl₂ and 2.5 U *Taq* polymerase in the buffer supplied (all from Invitrogen). A list of conditions and primers used for DNA amplification can be found in Table 1.

Plasmid construction. Plasmids containing fragments used as

probes are listed in Table 2. Standard cloning procedures were used as described by Sambrook *et al.* (1989). Plasmids containing PCR-generated inserts (pICP) were based on pGEMT-easy (Promega) or pKSII (Stratagene) cloning vectors. T-vector construction used for pICP3, pICP4 and pICP5 was described previously (Marchuk *et al.*, 1991). In the construction of pICR plasmids, all the cloned fragments were obtained through genomic restriction. A total of 5–10 µg genomic DNA was digested with *EcoRI*, *EcoRI/NotI* and *XhoI/NotI* and cloned into plasmid vectors digested with the same set of enzymes (see Table 2).

DNA probes. All probes used in this work (see Table 5) were labelled with digoxigenin using PCR Dig labelling mix (Roche). Most PCR probes were amplified using specific primers for conserved regions in gene sequences. The specific probe for *rriI* was made using PCR-specific primers for the amplification of the downstream sequence from the *I-CeuI* restriction site in the 23S gene up to the 5S gene. *harosynth* and *hrpO*A were amplified with different primers designed for *dnaA* amplification in *Lactococcus lactis* and LAB, respectively. The GACA3 PCR product was obtained from fragment A3 with a single primer generally used for RAPDs (random amplified polymorphic DNA) and cloned into modified plasmid pKSII. The hypothetically corresponding genes or gene products can be found in Table 5. For the labelling of cloned probes, plasmid primers such as T3, T7 and SP6 were used in the PCR reaction.

Southern blotting. In Southern blotting, gel pre-treatment and capillary transfer was done using standard procedures (Sambrook *et al.*, 1989). The supplier's procedures (Roche) were observed during all the stages of hybridization, stringency washes, immunochemical detection and chemiluminescent visualization. Results were observed by autoradiographic exposure, and treated with Kodak imaging software. Hybridizations were performed at 40 °C using formamide at 50 %, for high stringency (homologous probes) in pre-hybridization and hybridization solutions, and at 25 % for low stringency (heterologous probes). Probes from *W. confusa* and *L. pseudomesenteroides* were used as homologous probes.

DNA sequencing. All probes used in this work, with the exception of the *rrs* probe, were sequenced in an automated DNA capillary sequencer CEQ 2000-XL (Beckman Coulter) by a dye-labelled dideoxy termination method (DTCS, Dye Terminator Cycle sequencer start

Table 2. Plasmids

Plasmid	Vector used in construction	Relevant markers in insert	Origin of cloned insert; Accession no.	Reference
pICP1	pGEMT-easy	<i>aroA</i>	<i>W. paramesenteroides</i> DSMZ 20288 ^T	This study
pICP2	pGEMT-easy	23S rDNA 3' fragment	<i>W. paramesenteroides</i> DSMZ 20288 ^T	This study
pICP3	pKSII with T overhang	<i>dnaK</i>	<i>W. confusa</i> DSMZ 20196 ^T	This study
pICP4	pKSII with T overhang	<i>rpoA</i>	<i>Leuconostoc pseudomesenteroides</i> CECT 4027 ^T	This study
pICP5	pKSII with T overhang	GACA3	<i>W. paramesenteroides</i> A3 fragment	This study
pICR1	pKSII digested with <i>EcoRI</i>	CTP synthase	<i>EcoRI</i> fragment of <i>W. paramesenteroides</i> DSMZ 20288 ^T ; AY661801	This study
pICR2	pKSII digested with <i>XhoI/NotI</i>	Aldo-keto reductase and putative transposase	<i>XhoI/NotI</i> fragment of <i>W. paramesenteroides</i> DSMZ 20288 ^T ; AY661803	This study
pICR3	pKSII digested with <i>EcoRI/NotI</i>	Putative 5'-nucleotidase	<i>EcoRI/NotI</i> fragment of <i>W. paramesenteroides</i> DSMZ 20288 ^T ; AY661805	This study
pICR4	pKSII digested with <i>EcoRI/NotI</i>	DNA polymerase III	<i>EcoRI/NotI</i> fragment of <i>W. paramesenteroides</i> DSMZ 20288 ^T ; AY661802	This study
pZN12	pKSII digested with <i>NotI</i>	<i>tgt</i> (queuine tRNA-ribosyltransferase)/ <i>clpB</i> (CLPB ATP-binding protein)	N12 restriction fragment of <i>O. oeni</i> PSU-I	Zé-Zé <i>et al.</i> (2000)

Table 3. Sizes (kb) of restriction fragments obtained by cleavage of the *W. paramesenteroides* DSMZ 20288^T chromosome

The observed (Obs.) sizes are mean values, calculated from 15–17 determinations, except for fragments A1 and S1 (2–5 determinations). The maximal deviation in size was estimated to be less than 2.6 %. The map sizes are theoretical sizes of fragments calculated by placing restriction sizes on the map.

Fragment	<i>AscI</i>		<i>I-CeuI</i>		<i>NotI</i>		<i>SfiI</i>	
	Obs. size	Map size	Obs. size	Map size	Obs. size	Map size	Obs. size	Map size
1	1236	1230	637	642	879	870	1059	1055
2	632	629	637	638	483	494	500	505
3	166	167	292	295	405	412	314	315
4			207	205	160	157	151	151
5			109	109	49	49		
6			91	91	44	44		
7			38	38				
8			8	8				
Total	2034	2026	2019	2026	2020	2026	2024	2026

kit, Beckman Coulter). DNA sequences were compared with National Center for Biotechnology Information GenBank entries using the BLAST algorithm (Altschul *et al.*, 1990).

RESULTS

Macrorestriction fragments and genome size of *W. paramesenteroides* DSMZ 20288^T

The restriction of *W. paramesenteroides* chromosome with *AscI*, *NotI*, *SfiI* and the homing endonuclease *I-CeuI* yielded three, six, four and eight fragments, respectively. The estimated mean sizes of the fragments produced by each enzyme are listed in Table 3 and restriction profiles are shown in Fig. 1. Restriction with *SfiI* sometimes also gave rise to two fragments of 820 kb and 650 kb (mean observed sizes). These fragments were considered to be the result of partial digestion because of their occasional appearance and lower intensity when compared to others. In *I-CeuI* restriction, the presence of a double fragment of about 640 kb was revealed by the following: the apparent ‘loss’ of genome size relative to restriction with other enzymes; the production of two fragments in double digestion analysis with *AscI*, one of 640 kb (supposedly C1) and another of 563 kb (resulting from a *AscI* restriction site within C2 fragment; see Table 4); and the results from hybridizing probes which indicate that the target fragment C1/2 can be associated with different locations in the chromosome.

The genome size of *W. paramesenteroides* DSMZ 20288^T was determined to be 2026 kb, the mean of the sizes obtained with the four enzymes.

Double digestions and co-localization of restriction sites

The analysis of double digestions provides the relative localization of almost all restriction sites in the *W.*

Fig. 1. Macrorestriction fragments of *W. paramesenteroides* DSMZ 20288^T. Lane 1, Lambda Ladder PFG Marker (sizes in kb). Lanes 2–5, *W. paramesenteroides* DNA digested with *AscI*, *I-CeuI*, *NotI* and *SfiI*, respectively.

Table 4. *Ascl*, *NotI*, *SfiI* and *I-CeuI* double restriction fragments of the *W. paramesenteroides* chromosome

First enzyme	Fragment*	Products after digestion with second enzyme†
<i>Ascl</i>	A1	<i>NotI</i> : N1, A1-N3 (198), A1-N4 (69), N5, N6; <i>SfiI</i> : A1-S1 (857), A1-S2 (222), S4; <i>I-CeuI</i> : C1, C3, C5, C6, A1-C2 (75), A1-C4 (10), C8
	A2	<i>NotI</i> : A2-N2 (415), A2-N3 (214); <i>SfiI</i> : S3, A2-S1 (198), A2-S2 (116); <i>I-CeuI</i> : A2-C2 (563), C7, A2-C4 (28)
	A3	<i>NotI</i> : A3-N2 (79), A3-N4 (88); <i>SfiI</i> : A3; <i>I-CeuI</i> : A3
<i>I-CeuI</i>	C1	<i>Ascl</i> : C1; <i>NotI</i> : C1-N1 (534), C1-N4 (59), N5; <i>SfiI</i> : C1-S1 (279), C1-S2 (212), S4
	C2	<i>Ascl</i> : C2-A2 (563), C2-A1 (75); <i>NotI</i> : C2-N2 (349), C2-N3 (289); <i>SfiI</i> : S3, C2-S1 (273), C2-S2 (50)
	C3	<i>Ascl</i> : C3; <i>NotI</i> : C3-N1 (245), N6, C3-N3‡ (6); <i>SfiI</i> : C3
	C4	<i>Ascl</i> : A3, C4-A2 (28), C4-A1 (10); <i>NotI</i> : C4-N4 (98), C4-N2 (107); <i>SfiI</i> : C4
	C5	<i>Ascl</i> : C5; <i>NotI</i> : C5; <i>SfiI</i> : C5
	C6	<i>Ascl</i> : C6; <i>NotI</i> : C6; <i>SfiI</i> : C6
	C7	<i>Ascl</i> : C7; <i>NotI</i> : C7; <i>SfiI</i> : C7
	C8	<i>Ascl</i> : C8; <i>NotI</i> : C8; <i>SfiI</i> : C8
<i>NotI</i>	N1	<i>Ascl</i> : N1; <i>SfiI</i> : N1-S1 (615), S4, N1-S2 (104); <i>I-CeuI</i> : N1-C1 (534), N1-C3 (245), C6
	N2	<i>Ascl</i> : N2-A2 (415), N2-A3 (88); <i>SfiI</i> : N2-S3 (299), N2-S2 (195); <i>I-CeuI</i> : N2-C2 (349), N2-C4 (107), C7
	N3	<i>Ascl</i> : N3-A2 (214), N3-A1 (198); <i>SfiI</i> : N3-S1 (396), N3-S3 (16); <i>I-CeuI</i> : N3-C2 (280), C5, C8, N3-C3‡ (6)
	N4	<i>Ascl</i> : N4-A1 (69), N4-A3 (88); <i>SfiI</i> : N4; <i>I-CeuI</i> : N4-C4 (98), N4-C1 (59)
	N5	<i>Ascl</i> : N5; <i>SfiI</i> : N5; <i>I-CeuI</i> : N5
	N6	<i>Ascl</i> : N6; <i>SfiI</i> : N6; <i>I-CeuI</i> : N6
<i>SfiI</i>	S1	<i>Ascl</i> : S1-A1 (857), S1-A2 (198); <i>NotI</i> : S1-N1 (615), S1-N3 (396), N6; <i>I-CeuI</i> : C3, S1-C1 (279), S1-C2 (273), C5, C6, C8
	S2	<i>Ascl</i> : S2-A1 (222), A3, S2-A2 (116); <i>NotI</i> : S2-N2 (195), N4, S2-N1 (104), N5; <i>I-CeuI</i> : S2-C1 (212), C4, S2-C2 (50), C7
	S3	<i>Ascl</i> : S3; <i>NotI</i> : S3-N2 (299); S3-N3 (16); <i>I-CeuI</i> : S3
	S4	<i>Ascl</i> : S4; <i>NotI</i> : S4; <i>I-CeuI</i> : S4

*Fragment sizes are presented in Table 3.

†Numbers in parentheses refer to theoretical sizes in kb obtained after localization of restriction sites on the physical map.

‡Fragment not observed but deduced from physical map construction.

paramesenteroides DSMZ 20288^T chromosome. The order of *Ascl* fragments is arbitrary, since there are only three fragments, and was considered to be A1-A2-A3 (clockwise orientation in circular map; Fig. 2). The presence of two partial digests of 820 kb and 650 kb in *SfiI* restriction resolves for itself the order of *SfiI* fragments. The 650 kb fragment was attributed to the sum of the fragments S2 (505 kb) and S4 (151 kb), and the fragment of 820 kb to the sum of S2 (505 kb) and S3 (315 kb). In this way the linkage S3-S2-S4 was deduced, leading to the linkage of S1 to S3 and S4. The relative position of *Ascl* and *SfiI* fragments was deduced from double-restriction analysis (Table 4) and Southern hybridization with *rpoC* that placed S4 in fragment A1. The lack of *Ascl* restriction of S3 indicated that A1 overlapped with S1 (A1-S1, 857 kb) and S2 (A1-S2, 222 kb). Once *SfiI* mapping of A1 was done, S3 was assigned to A2 (confirmed by hybridization with the *hred/htransp* probe). Since the order of *SfiI* fragments had already been obtained, the relative location of *Ascl* and *SfiI* fragments and restriction sites was achieved. The clockwise linkage of *SfiI* fragments is thus S1-S3-S2-S4, the location of A3 in S2 being established.

Regarding the physical mapping of *NotI* fragments, only N5 and N6 did not have any *Ascl* or *SfiI* restriction site. Allocation of N5 to A1 and S2 was possible by the hybridization of the h5'*nucl* probe, and the assignment of N6 to A1 and S1 was due to *hpolC* hybridization. Fragment N1 was not cut by *Ascl* and due to its size (870 kb) it had to be located within A1. In *SfiI* digestion N1 generated a fragment of 615 kb that represented an overlap of N1 and S1. The hybridization of *rpoC* in N1 and S4 (which had no *NotI* site) allowed the precise location of N1 in *SfiI* and *Ascl* maps. The remainder of S1 (apart from N6) was found to be a fragment of 396 kb shared also by N3, which was confirmed by hybridization with *dnaK* and *harosynth*. These hybridizations revealed also that N3 and A2 partially overlapped (fragment A2-N3 of 214 kb). With this, the clockwise linkage of N3-N2, that provided the *NotI* site in S3, was also established, taking into consideration the assignment of *hred/htransp* to both A2 and N2. N2 extended over to S2 (195 kb) and had in common with A3 a fragment of 88 kb. N4, which was not restricted by *SfiI*, was partially allocated to A3 by double digestion analysis and GACA3 hybridization. With this last identification of a *NotI*

Fig. 2. Physical and genetic map of *W. paramesenteroides* DSMZ 20288^T using the enzymes *AscI*, *I-CeuI*, *NotI* and *SfiI*. The chromosome map was oriented based on the location of *dnaA*. Direction of transcription of *rrn* operons is indicated by an arrow. The scale is in kb.

restriction site in A3, the order of *NotI* fragments was completely assessed as being N1-N6-N3-N2-N4-N5, starting from N1 and in a clockwise direction. Similar analysis was applied to *I-CeuI* mapping, providing the location of all but one (C8) *I-CeuI* fragments. C1, C3, C5, C6 and C7 have no *AscI* restriction site and, with the exception of C7, were allocated to A1. By double restriction with *I-CeuI*, A2 was found to be constituted by a fragment of 563 kb common to C2 (confirmed by hybridizations of *rrl*, *dnaK* and *hred/htransp* probes), an A2-C4 fragment of 28 kb and C7. This could also be confirmed by the fact that C7 had to be placed in N2 since it was not cleaved by *NotI*, pZN12 showed hybridization to both fragments and A3 had no *I-CeuI* restriction site. Assignment of A3 to C4 and therefore the clockwise linkage C7-C4 was confirmed by the hybridization of GACA3 (a probe generated from A3). *I-CeuI* restriction of fragment N4 into two fragments common to C4 (98 kb) and C1 (59 kb) provided the evidence of C4-C1 linkage. A clockwise linkage order of C1-C6-C3 and C3-C5 was deduced both from double digests of *I-CeuI/NotI* (in which N5 and N6 are not restricted) and from the allocation of the *hctpsynth* probe in C3 and N1. The clockwise order of *I-CeuI* fragments was thus determined to be C1-C6-C3-C5-C2-C7-C4.

At this point the only unknown location was that of the C8 fragment, since due to its very small size (8 kb) it could be adjacent to any *I-CeuI* restriction site. This was resolved later using a specific PCR amplification, as described below under 'rrn organization'.

Mapping of genetic markers

The probes used have been assigned to the restriction fragments to which they hybridized in Southern experiments

(Table 5, Fig. 2). In most cases their exact positioning and transcription direction in the fragment remained unknown. Their mapping position corresponds to the median position in the smaller fragment where they were located, be it from single or double digestion. Since *h5'nucl* and *hred/htransp* were obtained through genomic restriction with *NotI*, they could be precisely located in the chromosome. Although the *hpolC* probe was obtained the same way as *h5'nucl*, its precise location remains unknown as it can be at either extremity of N6 (N1-N6 or N6-N3, clockwise), since N6 is not restricted by any of the other enzymes. The need to obtain a probe that hybridized to A3 fragment led to the use of a random amplification approach, which resulted in the amplification of a 580 bp product (*GACA3* probe).

ITS assignment

Amplification of the ITS was done using primers PS1490 and PL132 (Massol-Deya *et al.*, 1995), revealing three different products of about 600 bp, 500 bp and 400 bp. This difference in ITS sizes was determined, by sequencing, to be due to the presence/absence of different tRNAs. The larger product has both tRNA^{Ile} and tRNA^{Ala} genes, the 500 bp ITS has only tRNA^{Ala} and the smallest one has no tRNA gene. PCR amplification with specific *I-CeuI* fragments allowed us to assign to each ribosomal operon its specific ITS. This was possible because, apart from C1, which had two 16S rRNA genes, and C2, which contained no 16S rRNA gene (explained in the following section), all the other *I-CeuI* fragments contained only one 16S rRNA gene and only one ITS region. Additionally, the ITS amplification of C1 gave rise to only the smallest ITS, and so the two ITS present in C1 were considered to be of this type. As can be seen in Fig. 2, tRNA^{Ile} and tRNA^{Ala} genes are present in *rrn*

Table 5. DNA sequences used as probes in this study and their location on the *W. paramesenteroides* DSMZ 20288^T chromosome

Probe	Gene (function/description)*	Approx. fragment size (kb)	G + C content (mol%)	Hybridizing fragments			
				<i>Ascl</i>	<i>I-CeuI</i>	<i>NotI</i>	<i>SfiI</i>
<i>rrs</i>	Small-subunit rRNA	1.5	51.4	A1, A2	C1, C3, C4, C5, C6, C7, C8	N1, N2, N3, N4	S1, S2
<i>rrl</i>	Large-subunit rRNA	1.1	50.3	A1, A2	C1, C3, C4, C5, C6, C7, C8	N1, N2, N3, N4	S1, S2
<i>dnaA</i>	Replication initiator protein	0.7	37.7	A1	C1	N1	S1
<i>dnaK</i>	HSP70 (chaperone protein)	1.8	45.6	A2	C1/2	N3	S1
<i>gyrB</i>	DNA gyrase subunit B	1.4	42.3	A1	C1/2	N1	S1
GACA3	Undetermined, no coding	0.6	51.4	ND	C4	N4	ND
<i>recA</i>	Recombinase A	0.25	49.6	A1	C1	N1	S2
<i>rpoC</i>	RNA polymerase β' -subunit	3.5	43.8	A1	C1/2	N1	S4
intC8	3-Oxoacyl-reductase [<i>Oceanobacillus iheyensis</i> HTE 831] $I=44\%$, $e=7e-57$; possible RNA methyltransferase [<i>Mycobacterium leprae</i>] $I=30\%$, $e=3e-18$	2.8	34.1	A1	ND	N3	S1
<i>tgt, clpB</i>	Queuine tRNA-ribosyltransferase/CLPB ATP-binding protein	6	42.2	ND	C7	N2	ND
<i>harosynth</i>	<i>aroA</i> putative 3-phosphoshikimate 1-carboxy-vinyltransferase [<i>Streptococcus pyogenes</i>] $I=50\%$, $e=2e-47$. Chorismate synthase [<i>Leuconostoc mesenteroides</i> ATCC 8293] $I=44\%$, $e=2e-06$	0.8	40.9	A2	ND	N3	S1
<i>hctpsynth</i>	CTP synthase [<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>] $I=61\%$, $e=1e-53$	0.6	41.7	A1	C3	N1	S1
<i>hred, htransp</i>	Aldo-keto reductase [<i>Helicobacter hepaticus</i>] $I=53\%$, $e=6e-63$; putative transposase [<i>Xanthomonas campestris</i> pv. <i>campestris</i>] $I=53\%$, $e=1e-16$	1.4	32.8	A2	C2	N2	S3
<i>hpolC</i>	Polymerase III, PolC-type [<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>] $I=77\%$, $e=2e-48$	0.4	43.0	A1	C3	N6	S1
<i>hrpoA</i>	DNA-directed RNA polymerase, α subunit [<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>] $I=98\%$, $e=3e-49$	0.4	42.0	A1	C1	N1	S2
<i>h5'nucl</i>	Putative 5'-nucleotidase [<i>Lactobacillus plantarum</i>] $I=41\%$, $e=3e-42$	0.7	39.8	A1	C1	N4	S2

ND, Not detected.

*When the only reference to the possible function of the DNA sequences was obtained by BLAST searches (BLASTX or BLASTP) identity values (I) and expected value (e) are shown.

operons B and D, and *rrn* operons E and G contain a tRNA^{Ala} gene, whereas *rrn* operons A, C, F and H contain no tRNA gene.

***rrn* organization**

Since the *W. paramesenteroides* chromosome has eight *I-CeuI* restriction sites, this was considered to be the number of *rrn* operons present in the genome. This result was confirmed by hybridizations with *rrs* and *rrl* probes. Hybridization with *rrn* probes provided not only the confirmation of

I-CeuI restriction sites in the other restriction fragments but also the organization and direction of transcription of ribosomal operons. Since the *rrl* probe is in fact specific for the *I-CeuI* downstream sequence of the 23S gene (see Methods), any *I-CeuI* fragment that hybridizes to both *rrs* and *rrl* probes indicates the presence of two transcriptionally co-orientated *rrn* operons (assuming the classical 16S-23S-5S arrangement). This was the case for all *I-CeuI* fragments obtained, with the exception of C1 and C2. In fact, the observable fragment C1/2 hybridized to both probes but, from the hybridization of *I-CeuI* double digests with *Ascl*

Fig. 3. Fragment C8 and PCR products that allowed its positioning relative to C3 and C5 and identification of its specific ITS. ITS accession numbers are AY454527, AY454528, AY454529 for the ITS of 592 bp (with tRNA^{Ile} and tRNA^{Ala} genes), 495 bp (with tRNA^{Ala} gene) and 399 bp (with no tRNA), respectively.

and *Sfi*I, we can deduce that C1 hybridizes only with *rrs* and C2 only with *rrl* (see Table 5). Taking into account the position of *I-Ceu*I restriction sites in other fragments, the overall *rrn* positioning and orientation presented in Fig. 2 was determined.

The localization of C8 was obtained from the result of the hybridization of a C8-specific probe and the analysis of the ITS of the *rrn* operon located upstream. Since the observed size for C8 fragment was 8 kb, and C8 hybridized with both *rrl* and *rrs* probes, the presence of two transcriptionally co-orientated operons no more than 8 kb apart was indicated. Two primers were designed (5Sf and 16Sr; see Table 1 and Fig. 3) which allowed the amplification of the entire region between the 5S rRNA gene and the downstream 16S rRNA gene. This amplification gave rise to a 2.8 kb fragment (intC8) that was used as a probe and also confirmed the estimated size of C8 as 8 kb (considering the 16S, 23S and 5S sizes, as well as the ITS mean size of 500 bp). Hybridization of intC8 in *Not*I fragments revealed the localization of C8 in N3. Fragment N3 also contains C5, and so C8 could be either upstream or downstream from C5 (orientation regarding the direction of *rrn* transcription). Using primers 16Sr and PS1490 (see Fig. 3), an amplification product of about 5.7 kb was obtained, corresponding to the region between the beginning of the 16S gene present in C8 and the 3' end of the 16S gene from the *I-Ceu*I fragment located upstream (intC8us in Fig. 3). A nested PCR using this last fragment of DNA as template and primers PS1490 and PL132 resulted in the amplification of the 400 bp specific ITS allocated to C3 instead of C5 (which was determined to contain an ITS of 500 bp). This result allowed the exact location of fragment C8. The determination of the ITS in C8 as being of 600 bp was obtained following an identical PCR strategy but using primers 5Sf and PL132 (intC8ds in Fig. 3) in the first PCR reaction.

DISCUSSION

Through a 'top-down' approach, using macrorestriction and PFGE analysis as well as hybridization of genetic

probes, a physical and genetic map of *W. paramesenteroides* DSMZ 20288^T was obtained (Fig. 2). From the overall characteristics of this chromosome some important features regarding genome structure and organization can be observed. The *W. paramesenteroides* chromosome is circular and has an estimated size of 2026 kb. Restriction with endonucleases *Asc*I, *Not*I and *Sfi*I, which recognize eight GC-pair sequences, resulted in a somewhat reduced number of fragments (13). When comparing with results from strains of phylogenetically close genera such as *Oenococcus oeni* PSU-1 and *O. oeni* GM with 32 and 36 fragments each (Zé-Zé *et al.*, 1998, 2000) and *Lactobacillus sakei* 23K with 38 (Dudez *et al.*, 2002), the difference becomes evident. However, this cannot be related to genome characteristics such as total GC content, which is about 40 % both in *W. paramesenteroides* and *O. oeni* (Schillinger *et al.*, 1989), or overall gene number, since the number of restriction sites differs considerably, depending on the enzyme. Compared to *O. oeni* strain GM, with 18 *Sfi*I and 3 *Asc*I fragments, more than a fourfold decrease in *Sfi*I restriction sites but the same number of *Asc*I fragments is observed. The physical map of *W. paramesenteroides* presented here has a mean fragment size of 96.5 kb, with few fragments of more than 200 kb. This fact imposes limitations on the precise localization of some genetic markers, namely those situated in the larger fragments. Nevertheless, in chromosomal regions with a concentration of restriction sites, such as the S2 fragment, the map resolution is greatly enhanced (mean fragment size of 56 kb). This variation in map resolution obtained with restriction enzymes that recognize eight GC-pair sequences suggests a non-random distribution of GC regions and may indicate the presence of chromosomal regions with different gene content.

Taking into consideration a general association of *gyrB*, *dnaA* and the chromosomal localization of the origin of replication *oriC* (Gasc *et al.*, 1998; Krause *et al.*, 1997; Zawilak *et al.*, 2001), positioning of both genes in C1 indicates fragment C1-S1 (279 kb) as the most probable site of the chromosomal origin of replication. The terminus

of replication *terC*, which is usually about 180° from *oriC*, is most likely in fragment C2. This functional arrangement description is reinforced by rRNA operon organization and transcription direction (Cole & Saint Girons, 1994). Ribosomal operons are usually more or less symmetrically disposed around *oriC*, with their transcription being towards *terC* (Le Bourgeois *et al.*, 2000). The presence of eight *rrn* operons, given by the number of I-CeuI fragments, may indicate an adaptation to an unstable environment with periodic resource fluctuations, where high growth rates are advantageous (Klappenbach *et al.*, 2000).

Apart from its practical use in determining I-CeuI fragment order, the analysis and discriminative amplification of ITS regions could be useful in determining homology with rRNA operons present in species of closely related genera such as *Lactobacillus*, *Leuconostoc* and *Oenococcus*. This is based in the fact that in *Leuconostoc* and *Oenococcus* strains only ITS with tRNA^{Ala} have been found (Nour, 1998a) whereas tRNA^{Ile+Ala} and no tRNA ITS seem to be prevalent in *Lactobacillus* species (Nour, 1998b; Tannock *et al.*, 1999). Nevertheless, the apparently random disposition of the different ITS regions could be the result of intra-chromosomal recombination events between different rRNA operons (Hashimoto *et al.*, 2003), which would undermine any homology assessment. Although the identification of conserved structures in the *W. paramesenteroides* chromosome cannot be done at this point, beyond the disposition of *oriC*, *terC* and *rrn*, the relative order of some genes such as *gyrB*, *rpoC*, *recA*, *tgt/clpB* and the probable *oriC* region seems to be similar to that seen in the PSU-1 and GM strains of *O. oeni* (Zé-Zé *et al.*, 2000).

The physical map of *W. paramesenteroides* DSMZ 20288^T reported here represents the first study into the genomic mapping of a *Weissella* strain. Further studies in evolutionarily close species may help in clarifying the common and distinctive features in genomic organization of lactic acid bacteria, in strain improvement, and in the establishment of evolutionary relationships.

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