APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2005, p. 1223–1230 Vol. 71, No. 3
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.3.1223–1230.2005

Functional Analysis of Three Plasmids from *Lactobacillus plantarum*

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Received 26 March 2004/Accepted 29 September 2004

***Lactobacillus plantarum* WCFS1 harbors three plasmids, pWCFS101, pWCFS102, and pWCFS103, with sizes of 1,917, 2,365, and 36,069 bp, respectively. The two smaller plasmids are of unknown function and contain replication genes that are likely to function via the rolling-circle replication mechanism. The host range of the pWCFS101 replicon includes *Lactobacillus* species and *Lactococcus lactis*, while that of the pWCFS102 replicon also includes *Carnobacterium maltaromaticum* and *Bacillus subtilis*. The larger plasmid is predicted to replicate via the theta-type mechanism. The host range of its replicon seems restricted to *L. plantarum*. Cloning vectors were constructed based on the replicons of all three plasmids. Plasmid pWCFS103 was demonstrated to be a conjugative plasmid, as it could be transferred to *L. plantarum* NC8. It confers arsenate and arsenite resistance, which can be used as selective markers.**

Lactic acid bacteria are used for the preservation of food and feed raw materials like milk, meat, and vegetables or other plant materials. Certain strains of lactic acid bacteria, in particular, strains from the genus *Lactobacillus*, have been attributed probiotic activities in humans and animals (30). Several lactic acid bacteria, including lactococci, streptococci, lactobacilli, and pediococci, are known to harbor plasmids. These may encode important traits like resistance to phages or antibiotics, lactose catabolism, and production of proteolytic enzymes or bacteriocins. *Lactobacillus plantarum* species often harbor several plasmids (49). Several of these have been sequenced (6, 11, 12, 20, 31, 40, 52, 58). Although most of them are of unknown function, one plasmid encoding phage resistance (pMD5057, 10,877 bp) and another plasmid (pLKS, 2,025 bp) probably introduced from another source and coding for tetracycline resistance have been described previously (12, 20). All are smaller than 11 kb and are predicted to replicate via the rolling-circle replication mechanism, except for the largest plasmid, pMD5057, which is predicted to replicate via the theta mechanism (12).

The capacity for conjugal transfer is an important characteristic for plasmids. Self-transmissible conjugative plasmids have the ability to form effective cell-to-cell contact, while mobilizable plasmids are only able to prepare their DNA for transfer (38). Mobilization involves the action of a specific DNA-protein structure called the relaxosome to produce single-stranded cleavage at the nicking site (*nic*) within the origin of transfer (*oriT*) of the plasmid (38). To date, there is very little information on conjugation in lactobacilli. Sasaki et al. demonstrated conjugational transfer of the promiscuous theta-replicating plasmid pAMβ1 from *Streptococcus faecalis* to *L. plantarum* (51). Ahn et al. described an 8.5-kb chloramphenicol resistance plasmid which had been comobilized with pAMβ1 from *L. plantarum* to *Carnobacterium maltaromaticum* (3), previously known as *Carnobacterium piscicola* (43). To our knowledge, no conjugative *L. plantarum* plasmids have been reported.

Recently, we determined the complete nucleotide sequence of the genome of *L. plantarum* WCFS1 (35). The sequences of the three endogenous plasmids of WCFS1 were obtained from this genome sequencing project. Here, we present the nucleotide sequence analyses and characterization of the plasmids from this strain.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Bacillus subtilis* were grown in Luria-broth-based medium at 37°C (50). *L. plantarum* and *Leuconostoc lactis* were grown in MRS broth (Difco Laboratories) at 37 and 30°C, respectively. *Lactococcus lactis* was grown in M17 broth (Difco Laboratories) supplemented with 0.5% glucose at 30°C. *Streptococcus thermophilus* was grown in M17 broth supplemented with 0.5% lactose at 42°C. *C. maltaromaticum* was grown in brain heart infusion medium at 30°C. If appropriate, the media contained chloramphenicol (10 μg/ml), rifampin (50 μg/ml), or erythromycin (5 μg/ml).

Heavy-metal resistance. The sensitivity of *L. plantarum* strains toward heavy metals was tested by inoculating MRS broth with a 0- to 50-mg/ml concentration series of arsenite ($NaAsO_2$), arsenate ($Na_2HAsO_4 \cdot 7H_2O$), cadmium ($CdSO_4 \cdot 8/3H_2O$), cobalt ($CoCl_2$), copper ($CuCl_2 \cdot 2H_2O$), iron ($FeSO_4 \cdot 7H_2O$), lead [$Pb(NO_3)_2$], mercury ($HgCl_2$), nickel ($NiCl_2 \cdot 6H_2O$), or zinc ($ZnCl_2$) with 2% of an overnight culture and determining the turbidity of the culture at 600 nm after 16 h of incubation.

Conjugation. Conjugation was performed using filter matings. Cells from overnight cultures of the donor (2 ml) and recipient (200 μl) were mixed, collected on a sterile 0.45-μm-pore-size filter in a plastic filter holder by using a syringe, and washed with 25 ml of sterile H_2O. The filters were placed on MRS agar plates and incubated anaerobically at 37°C for 20 h. The cells were resuspended in 5 ml of 0.25× Ringers solution. Dilutions were plated in triplicate on MRS agar plates containing 10 mg of sodium arsenate/ml for determining the donor count and on MRS agar plates containing rifampin and 10 mg of sodium arsenate/ml for determining the transconjugant count. The plates were incubated anaerobically at 37°C for 2 days. Cells from the overnight culture of the donor were treated similarly and plated on MRS agar plates containing rifampin to determine the number of spontaneous rifampin-resistant mutants. The frequency of appearance of spontaneous rifampin-resistant mutants was 20- to 100-fold lower in all conjugation experiments (ranging from 2.0×10^{-8} to 1.8×10^{-9}) than the conjugation frequencies. The transconjugant numbers reported here have all been corrected with the spontaneous rifampin-resistant donor numbers.

DNA isolation and manipulation. Isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as described by Sambrook et

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristic(s)[a]	Reference
Strains		
Escherichia coli DH5-α	Plasmid-free strain	25
Lactobacillus plantarum WCFS1	Single-colony isolate of NCIMB8826, sequenced strain	35
Lactobacillus plantarum WCFS2	Strain WCFS1 with plasmid pWCFS110 instead of pWCFS103	This study
Lactobacillus plantarum WCFS3	Strain WCFS1 with plasmid pWCFS111 instead of pWCFS103	This study
Lactobacillus plantarum WCFS4	Strain WCFS1 with plasmid pWCFS112 instead of pWCFS103	This study
Lactobacillus plantarum NC8	Plasmid-free strain	5
Lactobacillus plantarum NC8R	Rif derivative of NC8	This study
Lactobacillus plantarum NZ7109	Ery^r derivative of WCFS1	9
Lactobacillus casei LMG6904	*Lactobacillus casei* type strain (ATCC 393)	26
Lactobacillus helveticus CNRZ 32	Plasmid-free strain	33
Lactococcus lactis MG1363	Plasmid-free strain	22
Leuconostoc lactis NZ6091	Plasmid-free strain	13
Streptococcus thermophilus ST11	Plasmid-free strain	42
Carnobacterium maltaromaticum LV17B	Plasmid-free strain	1
Bacillus subtilis 168	Plasmid-free strain	53
Plasmids		
pUC18Ery	Amp^r Ery^r, 3.6-kb pUC18 integration vector carrying the erythromycin resistance gene from pAMβ1	57
pNZ124	Cm^r, 2.8-kb pSH71 replicon	46
pWCFS101	1,917-bp cryptic plasmid	This study
pWCFS102	2,365-bp cryptic plasmid	This study
pWCFS103	36,069-bp conjugative arsenate or arsenite resistance plasmid	This study
pWCFS104	Cm^r, 3.0-kb, pWCFS101-based cloning vector	This study
pWCFS105	Cm^r, 3.4-kb, pWCFS102-based cloning vector	This study
pWCFS106	Cm^r, 4.2-kb, pWCFS103-based cloning vector	This study
pWCFS107	Ery^r, *arsB* integration vector	This study
pWCFS108	Ery^r, *cadD* integration vector	This study
pWCFS109	Ery^r, *traA* integration vector	This study
pWCFS110	Ery^r, *arsB*, pWCFS107 integrated into the *arsB* gene of pWCFS103	This study
pWCFS111	Ery^r, *cadD*, pWCFS108 integrated into the *cadD* gene of pWCFS103	This study
pWCFS112	Ery^r, *traA*, pWCFS109 integrated into the *traA* gene of pWCFS103	This study

[a] Amp^r, ampicillin resistant; Ery^r, erythromycin resistant; Tet^r, tetracycline resistant, Cm^r, chloramphenicol resistant; Rif^r, rifampin.

al. (50). Small-scale isolation of plasmid DNA from the gram-positive bacteria was performed using log-phase cultures as described previously (16). Large-scale plasmid DNA isolation for *L. plantarum* was performed by the method of Anderson and McKay (4). Electrotransformation was performed using a Gene Pulser apparatus (Bio-Rad). Electrocompetent cells of *E. coli* (18), *L. plantarum* (5), *Lactococcus lactis* (16), *Leuconostoc lactis* (46), *S. thermophilus* (42), *Lactobacillus helveticus* (7), *C. maltaromaticum* (2), and *B. subtilis* (36) were prepared as previously described.

Nucleotide sequence analysis. *L. plantarum* WCFS1 plasmid sequence data were obtained by a random shotgun approach as was applied for the determination of the chromosomal sequence of this strain (35). Sequence data were assembled and analyzed using the PHRAP assembler (21), and open reading frames were predicted by using Genemark (28) and Glimmer version 2.0 (15) trained on known *L. plantarum* genes. The SWALL (www.ebi.ac.uk) and EMBL prokaryote libraries were screened for homologies by using the Fasta3 service at the European Bioinformatics Institute website (www.ebi.ac.uk) (45). The multiple-sequence alignment was performed using the ClustalW service at the European Bioinformatics Institute website (55).

Construction of plasmids. To construct the cloning vectors based on the replicons of the endogenous *L. plantarum* plasmids, pWCFS101 and pWCFS102 were digested with XbaI and blunted. The replicon from pWCFS103 was obtained by PCR using the high-fidelity *Pwo* polymerase (Boehringer Mannheim). Cloning sites were introduced in the primers (underlined) with the sequences 5′-GCCGCGGTCGACAAGCCCCTATTCTTCTGTTT-3′ and 5′-GCCGCGCTCGAGTAAGCAAAGCCTGTATGTAA-3′, and the PCR product was digested with XhoI-SalI. A fragment containing the multiple-cloning site and chloramphenicol resistance gene was isolated from pNZ124 as an XhoI-SalI fragment, blunted for pWCFS101 and pWCFS102, and ligated with the three replicons. For each ligation, one orientation of the resulting plasmids was used for further research and designated pWCFS104 (from pWCFS101), pWCFS105 (from pWCFS102), and pWCFS106 (from pWCFS103).

Construction of single-crossover integrants. To construct single-crossover disruption mutants of *arsB*, *cadD*, or *traA*, internal gene fragments of approximately 0.4 to 0.5 kb were generated by PCR. KpnI and BamHI cloning sites (underlined) were introduced in the primers 5′-CCGCGCGGTACCGTTACTGGGATCGTTTG-3′ and 5′-CCGCGCGGATCCACATTCTTAGGGCATAC-3′ (*arsB*), 5′-CCGCGCGGTACCATTCTTTATACATCAACTGCAATCGA-3′ and 5′-CCGCGCGGATCCAACAAACAGCGTTACGATTAGCTG-3′ (*cadD*), and 5′-CCGCGCGGTACCAAAATGGGCGAGTGATCGGGAGA-3′ and 5′-CCGCGCGGATCCCTTGATCGACAAATGATTTTTCGCT-3′ (*traA*). The PCR fragments were cloned into pUC18Ery (28), and the resulting plasmids were designated pWCFS107 (*arsB*), pWCFS108 (*cadD*), and pWCFS109 (*traA*), respectively. These plasmids were electroporated to *L. plantarum* WCFS1. Erythromycin-resistant colonies harbored pWCFS103 single-crossover integrant derivatives, the integrity of which was checked by Southern blot hybridization. For each knockout, a single-copy integrant was selected for further research.

Plasmid stability tests. Plasmid stability of pWCFS104, pWCFS105, and pWCFS106 was tested in the erythromycin-resistant WCFS1 derivative NZ7109. Strains were cultured by serial transfer (1/1,000) in MRS broth supplemented with erythromycin. After approximately 110 generations (11 transfers), dilution series were plated on MRS plates supplemented with erythromycin. From each strain, 100 colonies were transferred to MRS plates supplemented with erythromycin and erythromycin plus chloramphenicol.

Relative-copy-number determination. Plasmid copy number was assessed using real-time PCR detection. For that purpose, plasmids pWCFS104, pWCFS105, and pWCFS106 were transformed to *L. plantarum* strain NZ7109, which harbors a single copy of a chromosomally inserted erythromycin resistance gene and was obtained via a standard two-step homologous recombination strategy (9). This chromosomal marker was used as a chromosomal copy number reference to which all plasmid copy numbers were compared. Real-time PCR amplification was performed using exponentially grown *L. plantarum* containing pWCFS104, pWCFS105, or pWCFS106. Cells from 1 ml of culture were harvested by cen-

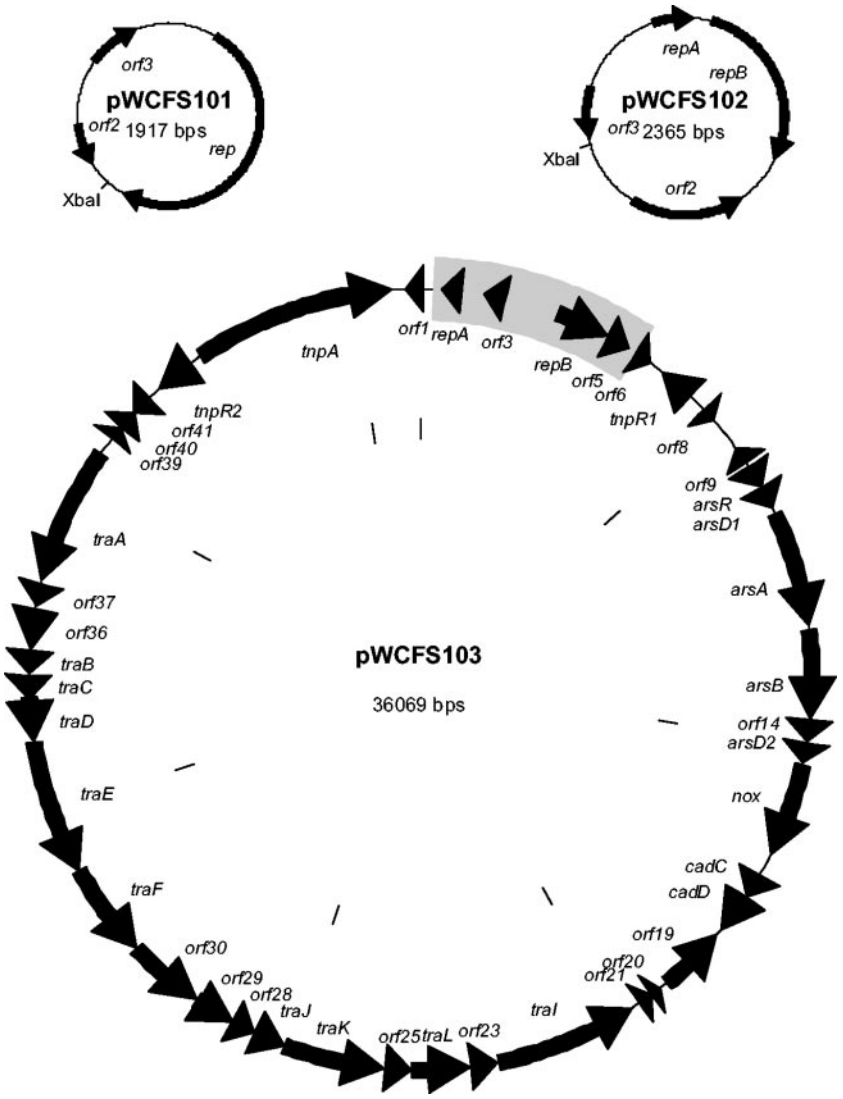

FIG. 1. Physical and genetic maps of plasmids pWCFS101, pWCFS102, and pWCFS103. The unique XbaI sites used to construct pWCFS104 and pWCFS105 are indicated. The replicon region of pWCFS103 used to construct pWCFS106 is boxed grey.

trifugation, washed with water, and pelleted by centrifugation. Cells were lysed by microwave treatment (3 min, 800 W) and resuspended in 400 μl of water. Appropriate solutions of these suspensions were used as templates for PCRs. These reactions contained the primer pairs designed on the chromosomal marker *ery* (TM-ery-F99, 5′-TTCACCGAACACTAGGGTTGC-3′; TM-ery-R100, 5′-ATTCCGCTGGCAGCTTAAG-3′) combined with the FAM (6-carboyfluorescein) reporter and TAMRA (6-carboxytetramethylrhodamine) quencher dye containing and *ery* probe (TM-ery-FAM, 5′-FAM-TGCACACTCAAGTCTCG ATTCAGCA-TAMRA-3′) and the primers designed for detection of the plasmid-derived chloramphenicol resistance gene *cat* (TM-cat-F96, 5′-TCAAATAC AGCTTTTAGAACTGG-3′; TM-cat-R97, 5′-ACCATCAAAAATTGTATA AAGTGGC-3′) combined with the VIC reporter and TAMRA quencher dye containing *cat* probe (TM-cat-VIC98, 5′-VIC-GCGACGGAGAGTTAGGTTATT GGG-TAMRA-3′). Reactions were performed with the TaqMan core reagent kit (Applied Biosystems, Nieuwekerk a/d IJsel, The Netherlands). The threshold cycle number (C_t) was determined (27) using ABI Prism 7700 sequence detection system software and was used to calculate the relative gene copy number (N_{relative}) for each plasmid in relation to the chromosomal copy number with the formula $N_{\text{relative}} = 2^{(C_{t,cat} - C_{t,ery})}$, where $C_{t,cat}$ is the C_t for the reaction with *cat* and $C_{t,ery}$ is the C_t for the reaction with *ery*. These experiments were performed in triplicate.

Nucleotide sequence accession numbers. The complete nucleotide sequences of plasmids pWCFS101, pWCFS102, and pWCFS103 have been submitted to the EMBL database and are available under accession numbers CR377164, CR377165, and CR377166, respectively.

RESULTS AND DISCUSSION

Sequence analysis of pWCFS101, pWCFS102, and pWCFS103. The organization of the three plasmids found in *L. plantarum* WCFS1 is shown Fig. 1. The average G+C contents were 39.5, 34.3, and 40.8% for pWCFS101, pWCFS102, and pWCFS103, respectively, compared to 44.5% for the chromosome of strain WCFS1 (35). All open reading frames larger than 40 amino acids were compared to those in the SWALL database (www.ebi.ac.uk). The results are depicted in Table 2. Plasmids pWCFS101 and pWCFS102 are cryptic, while pWCFS103 contains several genes for heavy-metal resistance, NADH-oxidase activity, and conjugation and a complete transposon.

Replicons. Plasmids pWCFS101 and pWCFS102 are homologous to rolling-circle replicating plasmids. pWCFS101 has a pC194-type replicon (34) with a single replication gene, *repA*,

TABLE 2. Putative genes and their products, deduced from the plasmid nucleotide sequences

Plasmid and gene	Codon		No. of amino acids	Best homolog (organism, GenBank accession no.)	% Identity (no. of amino acids overlapping)	Proposed function of gene product
	Start	Stop[a]				
pWCFS101						
rep	163	1122	319	Rep protein pLAB1000 (*Lactobacillus hilgardii*, E973099)	71 (314)	Replication protein
orf2	1406	1260 C	48	No hits		Hypothetical protein
orf3	1627	1809	60	No hits		Hypothetical protein
pWCFS102						
repB	102	761	219	RepB-like protein pLH2 (*Lactobacillus helveticus*, Q48562)	88 (219)	Replication protein
orf2	1393	953 C	146	No hits		Hypothetical protein
orf3	1889	1686 C	67	No hits		Hypothetical protein
repA	2242	35	52	CopA pPSC22 (*Lactobacillus plantarum*, Q48820)	60 (50)	Copy number control protein
pWCFS103						
orf1	51	35839 C	93	DinJ (*Escherichia coli*, Q47150)	30 (80)	DNA-damage-inducible protein
repA	610	302 C	102	RepA-like protein pSAK1 (*Lactobacillus sakei*, Q48860)	53 (76)[b]	Replication protein
orf3	925	1257 C	110	No hits		Hypothetical protein
repB	2061	2861	266	RepB pAD1 (*Enterococcus faecalis*, Q52229)	39 (276)	Copy number control protein
orf5	2863	3213	116	Hypothetical protein (*Lactobacillus sakei*, Q9AED4)	33 (113)	Hypothetical protein
orf6	3344	3586	80	No hits		Hypothetical protein
tnpR1	4370	3816 C	184	Resolvase-like protein pMD136 (*Pediococcus pentosus*, Q9WW47)	83 (184)	Putative resolvase
orf8	4649	4855	68	No hits		Hypothetical protein
orf9	5641	5378 C	87	No hits		Hypothetical protein
arsR	5713	6072	119	ArsR-like protein pL1100 (*Listeria innocua*, Q926M3)	48 (119)	Regulator of arsenical resistance operon
arsD1	6059	6421	120	ArsD-like protein pL1100 (*Listeria innocua*, Q926M4)	42 (123)	Repressor of arsenical resistance operon
arsA	6505	8235	576	ArsA-like protein pL1100 (*Listeria innocua*, Q926M2)	65 (568)	Arsenical pump-driving ATPase
arsB	8294	9589	431	ArsB-like protein (*Listeria monocytogenes*, P96678)	73 (427)	Arsenical pump membrane protein
orf14	9606	9935	109	No hits		Hypothetical protein
arsD2	9958	10257	99	ArsD-like protein pL1100 (*Listeria innocua*, Q926M4)	43 (95)	Repressor of arsenical resistance operon
nox	10281	11681	466	Hypothetical protein pL1100 (*Listeria innocua*, Q926L9)	36 (450)	Putative NADH oxidase
cadC	12031	12399	122	Transcriptional regulator ArsR family (*Chlorobium tepidum*, Q8KE78)	37 (97)	Putative positive regulator of cadmium resistance
cadD	12401	13015	204	CadD pUB101 (*Staphylococcus aureus*, Q8GNY9)	50 (205)	Putative cadmium resistance protein
orf19	14131	13100 C	344	Transposase (*Lactobacillus delbrueckii*, Q93L10)	41 (338)	Putative transposase
orf20	14404	14240 C	54	LtrC pMRC01 (*Lactococcus lactis*, O87224)	61 (49)[c]	Hypothetical protein
orf21	14623	14408 C	71	Hypothetical 8.0-kDa protein pMRC01 (*Lactococcus lactis*, O87223)	57 (70)	Hypothetical protein
tra1	16881	14746 C	711	Trs1 pMRC01 (*Lactococcus lactis*, O87222)	60 (722)	DNA topoisomerase
orf23	17298	16888 C	136	No hits		Hypothetical protein
traL	18170	17313 C	285	TrsL pMRC01 (*Lactococcus lactis* O87221)	44 (277)	Conjugation protein
orf25	18565	18176 C	129	Hypothetical 14.7-kDa protein pMRC01 (*Lactococcus lactis*, O87220)	32 (126)	Hypothetical protein
traK	20076	18565 C	503	TraK pMRC01 (*Lactococcus lactis*, O87219)	76 (503)	Conjugation protein
traJ	20548	20078 C	156	TrsJ pMRC01 (*Lactococcus lactis*, O87218)	48 (150)	Conjugation protein
orf28	20917	20549 C	122	Hypothetical protein pMRC01 (*Lactococcus lactis*, O87217)	42 (118)	Hypothetical protein
orf29	21521	20904 C	205	Hypothetical 23.3-kDa protein pMRC01 (*Lactococcus lactis*, O87216)	48 (204)	Hypothetical protein
orf30	22690	21536 C	384	Hypothetical protein pMRC01 (*Lactococcus lactis*, O87215)	54 (395)	Hypothetical protein
traF	24109	22691 C	472	TrsF pMRC01 (*Lactococcus lactis*, O87214)	50 (471)	Conjugation protein
traE	26120	24102 C	672	TrsE pMRC01 (*Lactococcus lactis*, O87213)	82 (671)	Conjugation protein
traE	26791	26132 C	219	TrsD pMRC01 (*Lactococcus lactis*, O87212)	80 (219)	Conjugation protein
traC	27122	27760 C	120	TrsC pMRC01 (*Lactococcus lactis*, O87211)	73 (115)	Conjugation protein
traB	27478	27143 C	111	TrsB pMRC01 (*Lactococcus lactis*, O87210)	72 (107)	Conjugation protein
orf36	28094	27480 C	204	Hypothetical 23.2-kDa protein pMRC01 (*Lactococcus lactis*, O97209)	51 (201)	Hypothetical protein
orf37	28471	28133 C	112	Hypothetical 13.1-kDa protein pMRC01 (*Lactococcus lactis*, O87208)	46 (90)	Hypothetical protein
traA	30584	28524 C	686	TraA pMRC01 (*Lactococcus lactis*, O87207)	49 (677)	Nickase
orf39	30856	31065	69	Hypothetical 10.9-kDa protein pMRC01 (*Lactococcus lactis*, O87206)	56 (66)	Hypothetical protein
orf40	31088	31366	92	Hypothetical 10.6-kDa protein pMRC01 (*Lactococcus lactis*, O87204)	50 (88)	Hypothetical protein
orf41	31356	31670 C	104	No hits		Hypothetical protein
tnpR2	32466	31880 C	195	TnpR transposon Tn*551* (*Staphylococcus aureus*, TNR7_ENTFA)	40 (181)	Putative resolvase
tnpA	32645	35638	997	Transposase pAM373 (*Enterococcus faecalis*, Q9F117)	64 (987)	Putative transposase

[a] C, complementary sequence.
[b] Homologous to C-terminal part of protein.
[c] Homologous to N-terminal part of protein.

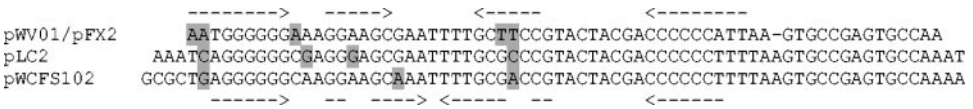

FIG. 2. Alignment of the double-stranded origins of plasmids pWV01/pFX2 (23) and pLC2 (37) and the putative double-stranded origin of pWCFS102. Dashed arrows indicate the inverted repeats. The arrowheads indicate the nick sites in pFX2 (23). Alignment mismatches are boxed in grey.

encoding a 37-kDa protein that was very similar to the Rep proteins from *Lactobacillus hilgardii* plasmid pLAB1000 (29) and *L. plantarum* plasmid pLP1 (8). Downstream of the *repA* gene at position 1798, there began a sequence (5′-TTCTT ATCTTGATA-3′) which was identical to the double-stranded origin of pC194 (24). Plasmid R1162 carries a set of three and a half 20-bp repeats that are essential for the expression of incompatibility and copy number control (41). Likewise, plasmid pLP1 contains a region with 13 contiguous direct repeats of 17 bp that are suggested to have a similar function (8). pWCFS101 has a region with five and a half direct repeats of 17 bp (5′-AGTGCGCATTATCATGT-3′) that are identical to those of pLP1 and may serve the same function.

pWCFS102 has a pMV158-type replicon (34) and carries two replication genes. The *repA* gene encodes a 6-kDa protein that is homologous to copy control proteins like RepC of pWV01 (39). The *repB* gene encodes a 25-kDa protein that is highly homologous to the Rep proteins from *L. helveticus* plasmid pLH2 and *Lactobacillus curvatus* plasmid pLC2 (37, 47). A putative double-stranded origin is formed by a sequence starting at position 2003, with high similarity to the double-stranded origins of pWV01, pFX2, and pLC2 (Fig. 2) (23, 37). These sequences are able to form a stem-loop structure and contain the nick site at which the Rep protein cleaves the DNA. The Rep binding region of pMV158-type replicons is composed of a set of two or three iterons which are separated from the nick site by an intervening region of 13 to 91 bp (34). Unlike the nick regions, the Rep binding regions are not conserved in their nucleotide sequence (34). We could not detect any clear direct repeats in the region close to the putative nick site nor elsewhere on pWCFS102.

Plasmid pWCFS103 has a replicon that is homologous to theta replicons. The *repA* gene encodes a 12-kDa protein that shares homology with the C-terminal part of the replication proteins from the *Lactobacillus sakei* plasmid pSAK1, the *Lactococcus lactis* plasmid pCI2000, and the *L. helveticus* plasmids pLJ1 and pLH1 (32, 54, 56). Compared to those replication proteins, RepA lacks approximately 260 amino acids at its N terminus, the region that has strongest conservation and includes the helix-turn-helix motif that is considered to be involved in DNA binding (56). The *repB* gene encodes a 30-kDa protein and is homologous to proposed plasmid copy control proteins from the *Enterococcus faecalis* plasmids pAD1 and pAM373 and the *Bacillus thuringiensis* plasmid pAW63 (14, 60, 61). Downstream of *repB* starts *orf4*, of which the start codon overlaps with the *repB* stop codon, suggesting transcriptional linkage of *orf4* to *repB* and a role for *orf4* in replication. For pAD1 and pAW63, a similar small gene that partly overlaps *repB* was found (60, 61). In between the *repA* and *repB* genes, an iteron region with 11 and 10 copies of imperfect repeats of the sequence 5′-TGTATCCT-3′ spaced by 37 nucleotides was found (Fig. 3). Plasmid pAD1 also has a series of 25 8-bp direct repeats located in between *repA* and *repB*, which is predicted to function as the origin of replication (60).

Host range. Based on the three replicons, three cloning vectors that contain the chloramphenicol resistance gene and multiple-cloning site from pNZ124 were constructed (see Materials and Methods). For pWCFS106, the 3.2-kb fragment with *repA*, *repB*, and *orf5* contained the complete replicon and could be stably maintained in *L. plantarum* NC8. To gain insight into the host range of the three cloning vectors, plasmid DNA isolated from *L. plantarum* NC8 was transformed to various gram-positive organisms and *E. coli* (Table 3). The theta-type pWCFS106 demonstrated a narrow host range and seemed restricted to *L. plantarum*. Rolling-circle-type pWCFS104 and pWCFS105 had a broader but limited host range, as both were able to replicate in *L. helveticus*, *Lactobacillus casei*, and *Leuconostoc lactis* and pWCFS105 was able to replicate also in *C. maltaromaticum* and *B. subtilis*.

Plasmid stability. The plasmid stability of the three cloning vectors pWCFS104, pWCFS105, and pWCFS106 in rifampin-resistant *L. plantarum* NZ7109 was determined after approximately 110 generations of growth without selection pressure. One hundred colonies were replica plated to plates supplemented with erythromycin and with erythromycin and chloramphenicol. All grew on the erythromycin plates, and 97 (pWCFS104), 100 (pWCFS105), and 98 (pWCFS106) colonies grew on the chloramphenicol-containing plates, illustrating that all three replicons are stably maintained in *L. plantarum*.

Plasmid copy numbers. Relative plasmid copy numbers were determined by real-time PCR analysis as described in the Materials and Methods section. These experiments revealed that the relative copy numbers of pWCFS104, pWCFS105, and pWCFS106 were 11.9 ± 1.2, 2.8 ± 0.3, and 4.7 ± 0.4, respectively. Therefore, these plasmids all generate a different gene dosage of genetic elements cloned on these vectors.

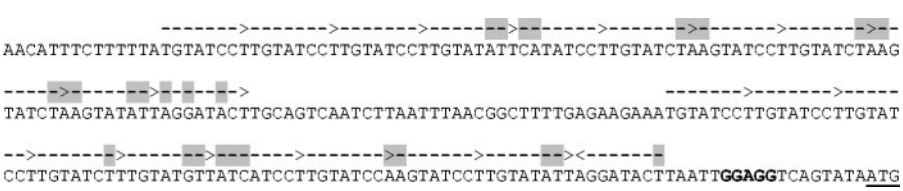

FIG. 3. Iteron region of pWCFS103. Dashed arrows indicate the direct and inverted repeats. Mismatches from the consensus sequence are boxed grey. The ribosome binding site (boldface) and start codon (underlined) of *repB* are indicated.

TABLE 3. Host range of *L. plantarum* WCFS1 cloning vectors[a]

Host	pWCFS104	pWCFS105	pWCFS106
Lactobacillus plantarum NC8	+	+	+
Lactobacillus helveticus CNRZ 32	+	+	−
Lactobacillus casei LMG6904	+	+	−
Leuconostoc lactis NZ6091	+	+	−
Lactococcus lactis MG1363	−	−	−
Streptococcus thermophilus ST11	−	−	−
Carnobacterium maltaromaticum LV17B	−	+	−
Bacillus subtilis 168	−	+	−
Escherichia coli DH5-α	−	−	−

[a] Transformants observed (+) or not observed (−).

Mobilization. Plasmid pWCFS103 carries a large gene cluster that has high homology with the mobilization gene cluster of the *Lactococcus lactis* plasmid pMRC01 (17). The *Lactococcus lactis* Tra region comprises an origin of transfer (*oriT*) sequence flanked by a gene cluster of 18 genes (*traA*, *orf6*, *orf7*, *traB*, *traC*, *traD*, *traE*, *traF*, *orf13*, *orf14*, *orf15*, *traJ*, *traK*, *orf18*, *traL*, *traI*, *orf21*, and *ltrC*) and a gene cluster of two genes in the opposite orientation (*orf4* and *orf3*) (17). The pWCFS103 Tra region runs from *orf40* (*orf3* in pMRC01) to *orf20* (*ltrC* in pMRC01) and is flanked by two transposase genes. Compared to pMRC01, it has an additional open reading frame, *orf23*, of unknown function inserted between *traL* and *traI*. Although there is no clear homology in the *oriT* region, the CGAAG sequence, conserved among several conjugative plasmids (17, 59) and preceded by a TTAAG sequence, is found upstream of *traA* and may function as the *oriT*.

To study the conjugal transfer of pWCFS103, *L. plantarum* WCFS1 and NC8R, a rifampin-resistant derivative of NC8, were used in filter matings. Conjugants were selected on plates containing rifampin to select for the recipient and containing sodium arsenate to select for plasmid pWCFS103 (see below). Typical frequencies of conjugation were between 4.4×10^{-6} and 3.1×10^{-7} per donor. Conjugation of the erythromycin-resistant derivatives of pWCFS103, pWCFS110, pWCFS111, and pWCFS112 resulted in similar frequencies of transfer. For pWCFS112, this was unexpected, as this derivative contains a single-crossover integration in *traA*, thought to be involved in conjugation by encoding the nicking enzyme. As TraA is a large protein, the possibility that an additional start site still gives rise to a functional nicking enzyme cannot be ruled out.

Heavy-metal resistance. Plasmid pWCFS103 contains two gene clusters that are predicted to be involved in arsenate and/or arsenite resistance and cadmium resistance (Table 2). Bacterial arsenite transporters provide resistance to salts of the metalloids arsenic and antimonite (44). Arsenic mainly occurs as As(V) in arsenate and As(III) in arsenite. Arsenate is structurally related to phosphate and interferes with phosphate metabolism. It will be reduced to arsenite and exported (44). There are two types of arsenic resistance (*ars*) operons described to date. One consists of three genes, *arsR*, *arsB*, and *arsC*, coding for a regulator ArsR, the arsenate reductase ArsC, and a secondary transporter, ArsB, that couples extrusion of anions to the transmembrane electrochemical potential (19, 48). The other *ars* operon contains two additional genes, *arsD* and *arsA*, inserted between *arsR* and *arsB* (19, 48). ArsD is a second transcriptional regulator, and ArsA is an ATPase that associates with ArsB and converts it into a primary ATP-coupled arsenite transporter with improved resistance (19). The pWCFS103 *ars* operon is such a high-resistance gene cluster but lacks the arsenate reductase gene *arsC* and contains two copies of the *arsD* regulator gene. The *arsC* gene appeared to be present on the *L. plantarum* WCFS1 chromosome (35), thereby completing the set of *ars* genes in this strain. Moreover, the mobilization data suggest that the chromosomally encoded *arsC* function found in *L. plantarum* WCFS1 is conserved in other strains of this species. This makes the organization of the *L. plantarum ars* gene cluster unique compared to those of other bacteria where *arsC* is always present.

The pWCFS103 *cadD* gene is homologous to the *cadD* gene from *Staphylococcus aureus* encoded on plasmid pRW001 (10). *S. aureus cadD* confers low-level resistance to cadmium (below 10 μg per ml), which is increased approximately 10-fold when provided with a functional *cadC*-like gene, *cadX*, encoding a transcription regulator of the cadmium operon (10). The pWCFS103 *cadC* gene is homologous to transcription regulators, including *cadC* of *S. aureus*.

To test whether pWCFS103 confers heavy-metal resistance and if the *ars* and *cad* genes play a role in this resistance, *L. plantarum* WCFS1, *L. plantarum* WCFS2 (*arsB*), *L. plantarum* WCFS3 (*cadD*), and *L. plantarum* WCFS4 (*traA*) were incubated in media containing various concentrations of arsenite,

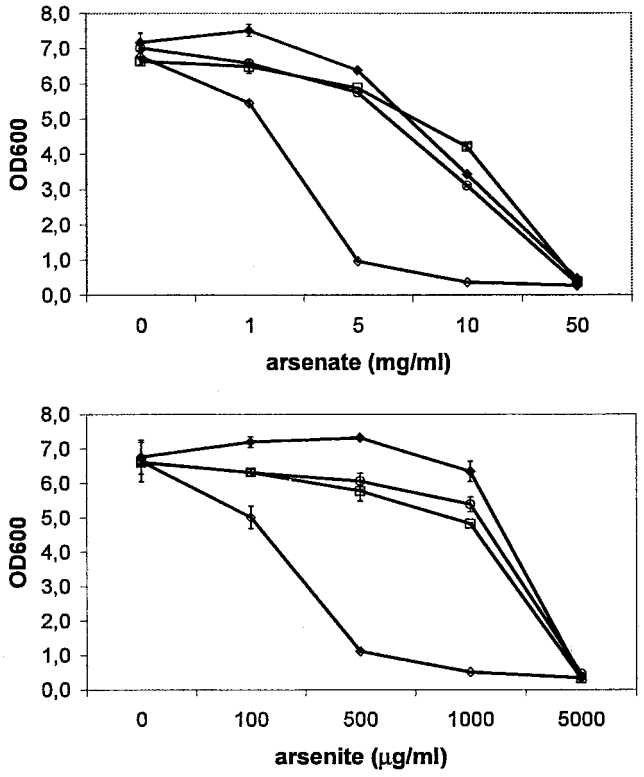

FIG. 4. Arsenate and arsenite resistance in *L. plantarum*. Filled diamonds, WCFS1; open diamonds, WCFS2 (*arsB*); open circles, WCFS3 (*cadD*); open squares, WCFS4 (*traA*). Each data point is the mean of duplicate cultures. Error bars indicate the deviation from the mean for single cultures. OD600, optical density at 600 nm.

arsenate, cadmium, cobalt, copper, iron, mercury, or nickel salts. Strain WCFS2 (with *arsB* disrupted) displayed retarded growth at lower arsenate and arsenite concentrations relative to the growth of strains WCFS1, WCFS3, and WCFS4 (Fig. 4). At 500 μg of arsenite per ml, WCFS2 barely showed growth after 16 h, while for the other strains, this result occurred at 5 mg of arsenite per ml. For the other heavy metals tested, no differences in final turbidity were observed for the four strains. However, a very limited effect on resistance toward iron and mercury was observed during growth. At close-to-lethal concentrations (10 mg per ml and 25 μg per ml for iron and mercury, respectively), the growth of WCFS4 was slightly better than that of WCFS3 (*cadD* disrupted) (data not shown). No effect of pWCFS103 on cobalt, copper, iron, or nickel resistance was observed (data not shown). From these experiments, we conclude that *arsB* is involved in arsenate or arsenite resistance. We have no evidence that the *cadD* gene is involved in cadmium resistance, but this may be obscured by the presence of another, more efficient cadmium resistance system encoded on the chromosome of WCFS1. In agreement with this suggestion is the prediction that several chromosomally encoded cation transporters have a predicted substrate range that includes cadmium (lp_1919, lp_3327, and lp_3435 [35]).

ACKNOWLEDGMENT

We are grateful to Bernadette Renckens for assistance with annotation of gene functions.

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