Requirement of Borate Cross-Linking of Cell Wall Rhamnogalacturonan II for *Arabidopsis* Growth

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Turgor-driven plant cell growth depends on wall structure. Two allelic L-fucose–deficient *Arabidopsis thaliana* mutants (*mur1-1* and *1-2*) are dwarfed and their rosette leaves do not grow normally. *mur1* leaf cell walls contain normal amounts of the cell wall pectic polysaccharide rhamnogalacturonan II (RG-II), but only half exists as a borate cross-linked dimer. The altered structure of *mur1* RG-II reduces the rate of formation and stability of this cross-link. Exogenous aqueous borate rescues the defect. The reduced cross-linking of RG-II in dwarf *mur1* plants indicates that plant growth depends on wall pectic polysaccharide organization.

Boron (B) is an essential element of all higher plants (*1*, *2*). Recent evidence suggests that the predominant place B functions in plants is in primary cell walls (*3*, *4*), where it cross-links the pectic polysaccharide rhamnogalacturonan II (RG-II) (*5*, *6*). RG-II (Fig. 1A) has a conserved glycosyl sequence (*7*) and exists in all higher plant primary walls predominantly as a dimer that is covalently cross-linked by a borate di-ester (*8*). Dimer formation in muro results in a cross-linked pectic network because RG-II is covalently inserted within homogalacturonan chains (see Fig. 1B). This network contributes to the physical and biochemical properties of the wall because the initial phenotype of boron deficiency is structurally abnormal walls (*9*).

The aerial portions of *mur1* plants contain virtually no L-fucose. The altered gene in these plants encodes an isoform of guanosine diphosphate (GDP)–D-mannose-4,6-dehydratase that is required for the formation of GDP–L-Fuc (*10*). To date, *mur1* is the only mutation of *Arabidopsis* shown to affect the glycosyl composition of RG-II (*11*). The L-fucose (L-Fuc) and 2-*O*-methyl L-fucose (2-*O*-Me L-Fuc) residues of *mur1* RG-II are replaced by L-galactose (L-Gal) and 2-*O*-methyl L-galactose (2-*O*-Me L-Gal) residues, respectively (see Fig. 1A). The presence of L-Gal and 2-*O*-Me L-Gal on *mur1* RG-II can be accounted for if *Arabidopsis* fucosyltransferases can use GDP–L-Gal and GDP–2-*O*-Me L-Gal as substrates. This possibility is made more likely by the recent demonstration that GDP–L-Gal is an efficient substrate for mammalian fucosyltransferase V (*12*). Wild-type and *mur1* RG-II contain comparable amounts of galacturonic, glucuronic, aceric, 3-deoxyheptulosaric, and 3-deoxyoctulosonic acid (*11*). However, the 2-*O*-methyl xylose (2-*O*-Me Xyl) content of *mur1* RG-II is reduced by ~50% (Table 1). Nevertheless, wild-type and *mur1* RG-IIs have comparable molecular masses (*13*) even though their glycosyl residue compositions differ.

Mur1-1 and *mur1-2* plants (*14*) have smaller rosette leaves [Fig. 2, A (top) and B] than wild-type plants. These physiological symptoms are characteristic of a marginal B deficiency (*15*) even though the *mur1* plants were fertilized with normal amounts of borate (*16*). The RG-II dimer accounts for ~95% of the RG-II in wild-type plants but for only ~50% of the RG-II in the mutants (Table 1). This suggested that borate cross-linking of RG-II is reduced by replacing L-Fuc and 2-*O*-Me L-Fuc with L-Gal and 2-*O*-Me L-Gal, respectively, and that *mur1* plants would have a higher B requirement than their wild-type counterparts. Indeed, exogenous aqueous borate rescues the growth of *mur1* plants [Fig. 2, A (middle) and C] (*17*), and most of the RG-II exists as the dimer even though its glycosyl residue composition has not altered (Table 1). The mechanisms by which reduced borate cross-linking of RG-II affects the growth of *mur1* plants remain to be determined, although the cumulative results of several studies suggest that boron's primary role is in wall expansion rather than in cell division (*9*, *15*).

Mur1 plants grown in the presence of L-Fuc contain normal amounts of L-Fuc and are not dwarfed (*18*). Our data [Fig. 2, A (bottom) and D] confirm that L-Fuc rescues the growth of *mur1* plants (*19*) and show that most of the RG-II exists as the dimer and that this RG-II

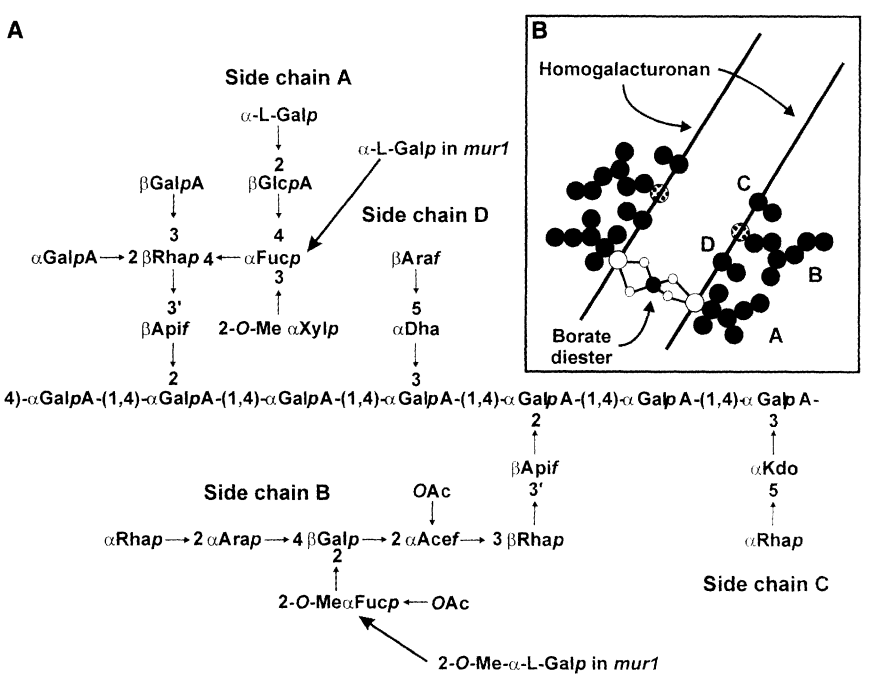

Fig. 1. The structure of RG-II. (**A**) The RG-II backbone is composed of 1,4-linked α-D-galactopyranosyluronic acid (GalpA) residues. Four oligoglycosyl side chains (A to D) are attached to the backbone. The aerial portions of *mur1* plants synthesize RG-II that has no L-Fuc but contains a 3,4-linked α-L-Gal residue in side chain A and a terminal nonreducing 2-*O*-Me α-L-Gal residue in side chain B. The 2-O-Me Xyl content of *mur1* RG-II is reduced by ~50% (see Table 1). No other discernible changes were found in the glycosyl residue composition of *mur1* RG-II (*11*). (**B**) Two RG-II molecules are covalently linked by a borate ester that is formed between the apiosyl residues (open circle) in the A side chain of each RG-II monomer.

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contains L-Fuc and 2-*O*-Me L-Fuc (Table 1). The 2-*O*-Me Xyl content of *mur1* RG-II is also restored to normal levels (Table 1) by L-Fuc treatment, which suggests that L-Fuc is more effective than L-Gal as an acceptor for the glycosyltransferase that attaches 2-*O*-Me Xyl to these residues.

Mur1 plants require a higher concentration of B for normal growth than their wild-type counterparts (Fig. 2A). This suggested that essential structural and conformational features of RG-II are altered in *mur1* plants and that such changes affect the formation or stability of the borate cross-link. Thus, we compared the in vitro rate of formation and pH stability of wild-type and *mur1* RG-II dimers (*20*). The *mur1* RG-II dimer forms less rapidly than the wild-type dimer irrespective of whether the reaction is performed in the presence or absence of divalent cations or with increased concentrations of boric acid (Fig. 3, A and B). The RG-II dimer from *mur1* plants, whether grown in the presence or absence of boric acid, is less stable at low pH than the dimer obtained from either wild-type plants or L-Fuc–treated *mur1* plants (Fig. 3, C and D). Thus, the replacement of L-Fuc and 2-*O*-Me L-Fuc with L-Gal and 2-*O*-Me L-Gal, respectively, together with a reduction in the amount of 2-*O*-Me Xyl, reduces the ability of *mur1* RG-II to form a dimer, and this dimer once formed is less stable than its wild-type counterpart. Hydrophobic interactions may have a role in dimer formation and stability because L-Fuc only differs from L-Gal by having a methyl group rather than a hydroxymethyl group at C-6. The hydrophobicity of *mur1* RG-II is also likely to be altered by reducing its 2-*O*-Me Xyl content. Nevertheless, these structural changes do not completely prevent dimer formation, which suggests that a mechanism has evolved in *Arabidopsis* that allows survival in the virtual absence of L-Fuc.

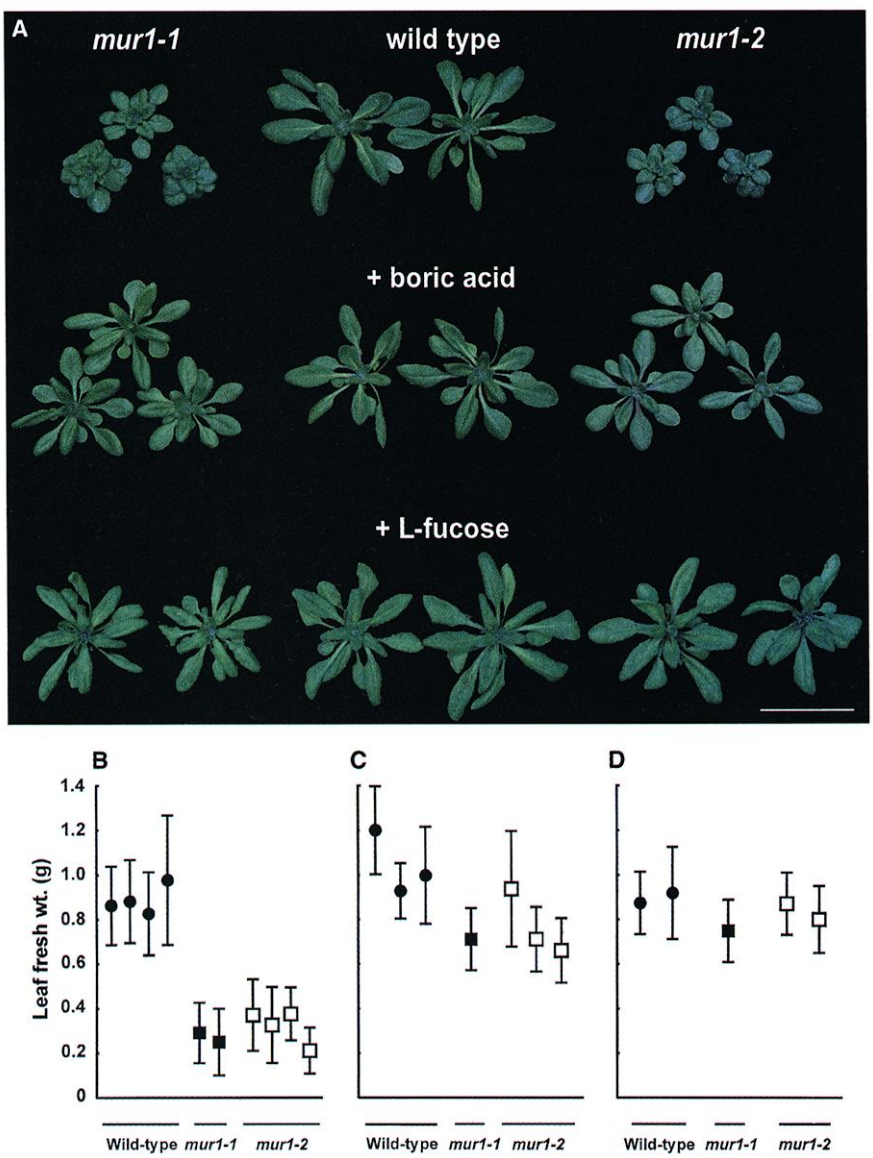

Fig. 2. The effect of boric acid and L-Fuc on the growth of *A. thaliana* wild-type, *mur1-1*, and *mur1-2* plants. (**A**) Plants were sprayed every 7 days with fertilizer solution only (top row), fertilizer containing added boric acid (middle row), and fertilizer containing added L-Fuc (bottom row). The photographs were taken 4 weeks after planting. Scale bar, 4 cm. Photographs were digitized and manipulated with Photoshop (Adobe, Mountain View, CA) to prepare the figure. (**B**) The rosette fresh weights of 4-week-old control *A. thaliana* wild-type and *mur1* plants. (**C**) The rosette fresh weights of 4-week-old *A. thaliana* wild-type and *mur1* plants grown in the presence of added boric acid. (**D**) The rosette fresh weights of 4-week-old *A. thaliana* wild-type and *mur1* plants grown in the presence of added L-Fuc. The data are the mean fresh weights ± SD. The data from at least 15 plants were averaged for the data points in each growth experiment.

Mur1-1 and *mur1-2* are members of a family of eight phenotypically distinguishable allelic mutants that differ in the amounts of L-Fuc in their walls (*21*). For example, the aerial portions of *mur1-3* plants contain ~30% of the amount of L-Fuc in wild-type plants, yet the *mur1-3* plants have no visible phenotype. The fact that *mur1-3* grows normally is consistent with our observation that (i) most of the RG-II in *mur1-3* walls is cross-linked, (ii) the RG-II contains L-Fuc and 2-*O*-Me L-Fuc as well as 2-*O*-Me L-Gal (Table 1), and (iii) the RG-II dimer from *mur1-3* plants and from L-Fuc–treated *mur1* plants have similar stabilities at pH 2 (Fig. 3D).

Xyloglucan is another primary wall polysaccharide that contains L-Fuc residues that are replaced by L-Gal residues in *mur1* plants (*22*). The L-Fuc–treated *mur1* plants synthesize xyloglucan containing ~50% of the normal amount of L-Fuc. However, fucosylation of xyloglucan itself is not likely to be responsible for the increased growth of L-Fuc–treated *mur1* plants because boric acid treatment promotes the growth of *mur1* plants (see Fig. 2A) but does not result in an increase in the L-Fuc content of xyloglucan (*23*). Moreover, *Arabidopsis mur2* plants, which are deficient in a xyloglucan-specific fucosyl transferase (*24*), synthesize xyloglucan that contains ~2% of the normal amounts of L-Fuc but grow normally (*25*). *Mur2* and wild-type plants grown in the absence of added boric acid are visibly indistinguishable and do not exhibit symptoms of B deficiency (*13*). The *mur2* mutation does not cause a reduction in the extent of RG-II cross-linking and has no effect on the L-Fuc and 2-*O*-Me L-Fuc contents of RG-II (Table 1). Thus, *mur2* and wild-type *Arabidopsis* are unlikely to differ substantially in their B requirements. The absence of L-Fuc residues on the N-linked complex glycan side chains of glycoproteins synthesized by *mur1* plants (*26*) is also unlikely to result in the dwarf phenotype because an *Arabidopsis* mutant (*cgl1*) that synthesizes glycoproteins that lack L-Fuc grows normally (*27*).

There is increasing awareness that the structures of cell-surface carbohydrates are impor-

Table 1. The proportion of RG-II dimer and the neutral glycosyl residue compositions of the RG-II from the rosette leaf cell walls of *A. thaliana* wild-type and mutant plants.

Plant	RG-II dimer (%)†	Glycosyl residue (mol %)*							
		MeFuc	Rha	Fuc	MeXyl	Ara	Api	MeGal	Gal‡
Wild type	95 ± 2	6 ± 0.8	22 ± 2.1	4 ± 0.7	6 ± 0.8	27 ± 3.3	9 ± 1.0	ND§	26 ± 3.0
+boric acid	94 ± 3	5 ± 1.0	22 ± 0.6	4 ± 0.6	6 ± 0.6	28 ± 2.6	9 ± 0.6	ND	26 ± 2.5
+L-fucose	96 ± 2	6 ± 0.6	21 ± 0.6	4 ± 0.6	6 ± 0.6	28 ± 1.2	10 ± 1.0	ND	25 ± 3.1
Mur1-1	56 ± 7	tr‖	24 ± 2.0	tr	2 ± 0.1	28 ± 2.1	10 ± 0.6	6 ± 0.1	30 ± 2.6
+boric acid	78 ± 4	tr	23 ± 1.5	tr	2 ± 0.2	29 ± 1.8	10 ± 0.5	5 ± 0.1	31 ± 2.0
+L-fucose	82 ± 5	3 ± 0.5	22 ± 2.0	3 ± 0.3	5 ± 0.2	30 ± 2.5	9 ± 0.6	2 ± 0.2	26 ± 2.0
Mur1-2	50 ± 5	tr	23 ± 1.4	tr	3 ± 0.8	30 ± 3.0	10 ± 2.0	5 ± 0.7	29 ± 2.7
+boric acid	74 ± 3	tr	23 ± 1.0	tr	2 ± 1.0	29 ± 4.6	9 ± 1.2	5 ± 0.6	32 ± 3.5
+L-fucose	81 ± 7	5 ± 0.6	23 ± 1.2	3 ± 0.6	5 ± 0.6	29 ± 1.2	10 ± 1.0	2 ± 0.1	23 ± 4.0
Mur1-3	75 ± 5	2 ± 0.1	23 ± 1.1	1 ± 0.5	2 ± 0.5	28 ± 1.5	10 ± 1.0	4 ± 0.5	30 ± 2.0
Mur2-1	97 ± 1	6 ± 0.6	22 ± 1.3	4 ± 0.1	6 ± 0.1	29 ± 1.7	9 ± 1.0	ND	24 ± 1.7

*Data are the mean value ± SD of at least three independent experiments. The RG-IIs isolated from wild-type and mutant plant rosette leaves contain comparable amounts of GalA, GlcA, AceA, Dha, and Kdo (*11*). †The percentage of RG-II dimer was determined by SEC. The data are the mean value ± SD of at least three independent experiments. ‡Values include D- and L-galactose. §ND, not detected. ‖tr, less than 1% of wild-type amount detected.

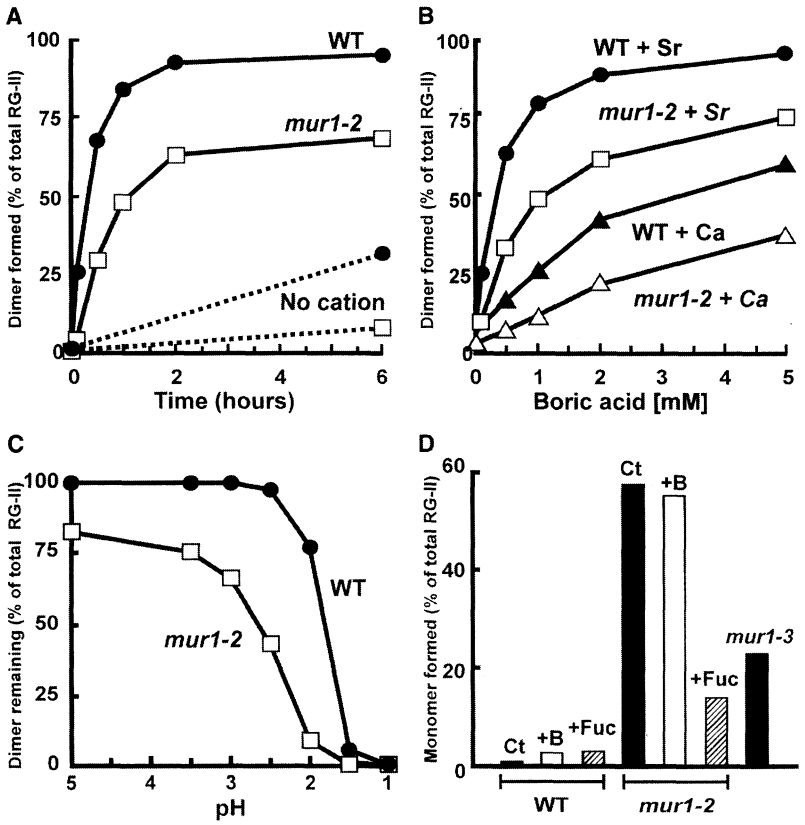

Fig. 3. The in vitro formation and stability of the RG-II dimers generated from wild-type (WT) and *mur1-2* plants. (**A**) The amount of RG-II dimer formed when wild-type and *mur1-2* RG-II monomers were reacted at pH 3.6 with boric acid (1 mM) in the presence and absence of $SrCl_2$ (1 mM). (**B**) The amount of RG-II dimer formed after 1 hour when wild-type and *mur1-2* RG-II monomers were reacted at pH 3.6 with boric acid in the presence of $SrCl_2$ (1 mM) or $CaCl_2$ (10 mM). (**C**) The amount of dimer remaining when the wild-type and *mur1-2* RG-II dimers were treated for 1 hour between pH 1 and 5. (**D**) The amount of RG-II monomer formed when the EPG-soluble, RG-II–containing fractions of the walls of wild-type and *mur1-2* plants grown in the presence of boric acid (+B) or L-Fuc (+Fuc) and from the walls of *mur1-3* plants (*mur1-3*) were treated for 1 hour at pH 2. Ct is control plants grown in the absence of added boric acid and L-Fuc.

tant for normal growth and development of eukaryotes. For example, altering the structures of glycosaminoglycans markedly affects animal cell morphogenesis and growth (*28*). Our report has shown that a modest change in the structure of a cell wall pectic polysaccharide affects plant growth and also illustrates how a mutation in a gene that encodes for an isoform of GDP-D-mannose-4,6-dehydratase may have unexpected biochemical and functional consequences.

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17. *A. thaliana mur1-1*, *mur1-2*, and wild-type plants were grown as described (*16*), except that after germination the seedlings were thinned to between four and six plants per pot. The plants were sprayed every 7 days with a solution (15 ml per pot) of commercial fertilizer (Jacks 20:20:20, 1 g/liter) containing added boric acid (152 mg/liter). Control plants were sprayed with a solution of the fertilizer that contained no added boric acid. Leaves were harvested from

4-week-old plants and RG-II was solubilized from their cell walls by treatment with EPG (*16*).
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19. *A. thaliana mur1-1*, *mur1-2*, and wild-type plants were grown as described (*17*). Plants were sprayed every 7 days with a solution (15 ml per pot) of commercial fertilizer (Jacks 20:20:20, 1 g/liter) containing added L-Fuc (1.64 g/liter). Control plants were sprayed with fertilizer containing no added L-Fuc. Cell walls were prepared from leaves and RG-II were solubilized as described (*16*).
20. Details of the methods used to determine the in vitro rates of formation and stabilities of wild-type and *mur1* RG-IIs are available as supplementary material (*13*).
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29. We thank W.-D. Reiter (University of Connecticut) and the *Arabidopsis* Biological Resource Center at Ohio State University for providing *Arabidopsis* seeds and J. Rose of Cornell University for critical reading of the manuscript. Supported by grants from the U.S. Department of Energy (DE-FG05-93ER20115 and DE-FG09-93ER20097).

7 May 2001; accepted 27 August 2001

Comparative Genomics of *Listeria* Species

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Listeria monocytogenes is a food-borne pathogen with a high mortality rate that has also emerged as a paradigm for intracellular parasitism. We present and compare the genome sequences of *L. monocytogenes* (2,944,528 base pairs) and a nonpathogenic species, *L. innocua* (3,011,209 base pairs). We found a large number of predicted genes encoding surface and secreted proteins, transporters, and transcriptional regulators, consistent with the ability of both species to adapt to diverse environments. The presence of 270 *L. monocytogenes* and 149 *L. innocua* strain-specific genes (clustered in 100 and 63 islets, respectively) suggests that virulence in *Listeria* results from multiple gene acquisition and deletion events.

Listeria monocytogenes is the etiologic agent of listeriosis, a severe food-borne disease. It survives in the extreme conditions encountered in the food chain, such as high salt concentrations and extremes of pH and temperature. These characteristics are shared by *L. innocua*, a nonpathogenic species often associated with *L. monocytogenes* in food and the environment. The clinical features of listeriosis include meningitis, meningoencephalitis, septicemia, abortion, perinatal infections, and gastroenteritis (*1*). After ingestion of contaminated food, *Listeria* disseminates from the intestinal lumen to the central nervous system and the fetoplacental unit. The key role of the surface protein internalin (InlA) in the crossing of the intestinal barrier was recently reported (*2*). Other virulence factors include the invasion protein InlB; the proteins LLO and PlcA, which promote escape from the phagocytic vacuole; and the proteins ActA and PlcB, which are necessary for intracellular actin-based motility and cell-to-cell spread (*1, 3*). These genes are clustered on a 10-kb virulence locus that is absent from *L. innocua* (*3, 4*).

Two strains were selected for comparison: *L. monocytogenes* EGD-e (serovar 1/2a), a derivative of strain EGD used by Mackaness in his studies on cell-mediated immunity (*5*), and *L. innocua* strain CLIP 11262 (serovar 6a), used for heterologous expression of *L. monocytogenes* genes (*6*). The whole-genome random sequencing method was chosen (*7, 8*). *Listeria monocytogenes* contains one circular chromosome of 2,944,528 base pairs (bp) with an average G+C content of 39% (Table 1 and Fig. 1) (GenBank/EMBL accession number AL591824). The *L. innocua* chromosome has a similar size (3,011,209 bp) and a similar G+C content (37%) (Table 1 and Fig. 1) (GenBank/EMBL accession number AL592022). The *L. innocua* strain also contains a plasmid of 81,905 bp (GenBank/EMBL accession number AL592102). We identified 2853 protein-coding genes in the *L. monocytogenes* chromosome and 2973 in that of *L. innocua* (*8*). Encoded proteins revealed a striking similarity to those of the soil bacterium *Bacillus subtilis*. Genes were thus classified according to the functional categories defined for *B. subtilis* (*9*) [Web table 1 (*8*)]. No function could be predicted for 35.3% of *L. monocytogenes* genes and 37% of *L. innocua* genes, a proportion similar to that found in other sequenced bacterial genomes.

Both genomes encoded many putative surface proteins belonging to six families [Web fig. 1 (*8*)], and expansion of these families seems to be partly due to gene duplications. Internalin and InlB belong to a family of proteins characterized by an NH_2-terminal domain containing leucine-rich repeats (LRRs). Seven other members of this family have already been identified (*1, 3*). Except for InlB, which is loosely attached to the bacterial surface, and InlC, which is secreted, the five other LRR proteins have a Leu-Pro-X-Thr-Gly (LPXTG) motif that mediates their covalent linkage to peptidoglycan (*10*). The *L. monocytogenes* genome sequence revealed the presence of 41 proteins containing an LPXTG

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