Regulation of cell wall plasticity by nucleotide metabolism in *Lactococcus lactis*

Ana Solopova\(^1\), Cécile Formosa-Dague\(^2\), Pascal Courtin\(^3\), Sylviane Furlan\(^3\), Patrick Veiga\(^3\,\,a\), Christine Péchoux\(^4\), Julija Armalyte\(^1\,\,b\), Mikas Sadauskas\(^3\,\,c\), Jan Kok\(^1\), Pascal Hols\(^2\), Yves F. Dufrêne\(^2\), Oscar P. Kuipers\(^1\), Marie-Pierre Chapot-Chartier\(^3\) and Saulius Kulakauskas\(^3\)\(^*\)

\(^1\)Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747AG, Groningen, the Netherlands

\(^2\)Institute of Life Sciences, Université catholique de Louvain, Croix du Sud, 4-5, bte L7.07.06., B-1348 Louvain-la-Neuve, Belgium

\(^3\)Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

\(^4\)GABI, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

\(^a\) Present address: Danone Nutricia Research, RD 128 - avenue de la Vauve, F-91767 Palaiseau Cedex - France

\(^b\) Present address: Department of Biochemistry and Molecular Biology, Faculty of Natural Sciences Vilnius University, M. K. Čiurlionio 21, LT-03101 Vilnius Lithuania

\(^c\) Present address: Department of Microbiology and Biotechnology, Faculty of Natural sciences, Vilnius University, Čiurlionio 21/27, 03101 Vilnius, Lithuania

Running title (50 characters max): Link between nucleotide synthesis and peptidoglycan plasticity

\(^*\) Correspondence: E-mail: Saulius.Kulakauskas@jouy.inra.fr; Phone: (+33)-1-34-65-2073; Fax: (+33)-1-34-65-20-65.

Keywords (6): *Lactococcus lactis*, peptidoglycan, cell wall plasticity, guaA, nucleotide synthesis, cell wall.
ABSTRACT

To ensure optimal cell growth and separation, and to adapt to environmental parameters, bacteria have to maintain a balance between cell wall (CW) rigidity and flexibility. This can be achieved by a concerted action of peptidoglycan (PG) hydrolases and PG synthesizing/modifying enzymes. In a search for new regulatory mechanisms responsible for the maintenance of this equilibrium in *Lactococcus lactis*, we isolated mutants that are resistant to the PG hydrolase lysozyme. We found that 14% of the causative mutations were mapped in the *guaA* gene, the product of which is involved in purine metabolism. Genetic and transcriptional analyses combined with PG structure determination of the *guaA* mutant enabled us to reveal the pivotal role of the *pyrB* gene in the regulation of CW rigidity. Our results indicate that conversion of L-aspartate (L-Asp) to N-carbamoyl-L-aspartate by PyrB may reduce the amount of L-Asp available for PG synthesis and thus cause the appearance of Asp/Asn-less stem peptides in PG. Such stem peptides do not form PG cross-bridges, resulting in a decrease in PG crosslinking and, consequently, reduced PG thickness and rigidity. We hypothesize that the concurrent utilization of L-Asp for pyrimidine and PG synthesis may be part of the regulatory scheme, ensuring CW flexibility during exponential growth and rigidity in stationary phase. The fact that L-Asp availability is dependent on nucleotide metabolism, which is tightly regulated in accordance with the growth rate, provides *L. lactis* cells the means to ensure optimal CW plasticity without the need to control the expression of PG synthesis genes.

INTRODUCTION

Peptidoglycan (PG) is the major component of the Gram-positive bacterial cell wall (CW), which envelops the cell as a multilayer sacculus. PG consists of a basic unit made up of N-acetylglucosamine-N-acetyl-muramic acid (GlcNAc-MurNAc) disaccharides bound to stem pentapeptides. Disaccharide pentapeptide units are synthesized intracellularly and transported through the cytoplasmic membrane as lipid-disaccharide pentapeptides, called lipid II. These blocks are covalently linked to the pre-existing PG polymers by high-molecular-weight penicillin binding proteins, or PBPs (1). Class A PBPs contain both transglycosylation and transpeptidation domains, whereas class B PBPs are involved only in transpeptidation. Transglycosylation links the disaccharide pentapeptide to the pre-existing PG chain, while transpeptidation connects the stem pentapeptides to neighboring chains, which ensures PG cross-linking through the formation of an interpeptide bridge. Cross-linking in *Lactococcus lactis* involves the synthesis of an interpeptide bridge made of one D-amino acid (D-Asp or D-Asn), and, in this species, the PG cross-linking index was estimated to be 35.5% (2). Studies of *Staphylococcus aureus* have revealed that this PG cross-linking correlates with CW rigidity (3).

The basic PG structure is often modified, as PG glycan chains can undergo N-deacetylation or O-acetylation, and free carboxyl-groups of amino acids of peptide chains may be amidated (4). In *L. lactis*, MurNAc O-acetylase is encoded by the *oatA* gene and is associated with resistance to peptidoglycan hydrolases (PGH) (5). N-Deacetylation of the GlcNAc present in PG is achieved by the PG-deacetylase PgdA and has also been shown to protect PG from PGH activity (6,7). The free carboxyl groups of PG-forming amino acids are amidated intracellularly, before the precursors are translocated through the cytoplasmic membrane (8). In *L. lactis*, these amino acids include D-Glu, found in stem peptides, and D-Asp, on side chains or cross-bridges (2). Amidation of D-Asp takes place after it has been added to the PG precursor by AslA and is catalyzed by an asparagine synthase (AsnH) (9), since D-Asn is not a substrate for the aspartate ligase AslA (10). In *L. lactis*, the D-Asp cross-bridge is only partially (75%) amidated during the exponential phase, in contrast to other bacteria in which amidation is almost complete (2). D-Asp amidation of *L. lactis* PG decreases sensitivity to cationic antimicrobials such as lysozyme or nisin, which may be related to a reduction of the net negative charge inside the cell wall (10). A strong PG sacculus is needed to
counteract both high turgor pressure and cell wall stress related to environmental factors. At the same time, cleavage of PG strands is needed to allow the insertion of newly synthesized PG blocks during bacterial cell growth and for daughter cell separation after division (2,11,12). PG flexibility is especially advantageous during exponential growth. Two opposing features of PG, i.e. rigidity and flexibility, require the coordinated and balanced action of PG synthesizing and degradation enzymes. The loss of this balance may cause growth arrest and/or cell lysis. In bacteria, such a balance is achieved mostly by regulating the activities of potentially lethal autolytic enzymes such as PGHs (13). The factors that affect CW sensitivity to autolysins include: i) their proteolytic degradation (14), ii) their specific localization within the cell, often at the septal region, iii) shielding of PG from PGH by secondary cell wall polymers such as teichoic acids or wall polysaccharides, iv) alanylation of (lipo-)teichoic acids, v) O-acetylation or N-de-acetylation of PG, vi) amidation of D-Glu and D-Asp in PG stem peptides, and vii) glycosylation of autolysins (2). In addition to the introduction of PG breaks by PGHs, PG flexibility in L. lactis could also result from defective PG synthesis and cross-linking due to a deficiency in Pna, one of the class-A PBPs (15). PG plasticity could also be enhanced by the incorporation of exogenously added non-canonical D-amino acids into PG (16). For example, the weakening of CWs by the achiral amino acid glycine is exploited for the preparation of competent cells of Gram-positive bacteria (17,18).

It is important to note that too-rigid PG also has deleterious consequences for bacterial growth. Overexpression of the L. lactis oatA gene leads to PG that is excessively resistant to PGH, and consequently results in growth arrest (7). The lethal effect of overly strong PG is consistent with findings that several PGHs are collectively essential for the growth of Escherichia coli, Bacillus subtilis, and Staphylococcus aureus. This lethality could be related to the need for PGH-mediated PG remodeling during key steps in the cell cycle (19-22).

To identify factors that may be involved in maintenance of the balance between CW flexibility and CW rigidity during cell growth, we isolated mutants of L. lactis that were resistant to hen egg white lysozyme. Analysis of one class of resistant mutants, which had a deletion of a large chromosome fragment encompassing the guaA gene, led us to the potential mechanism that allows the adjustment of CW rigidity to bacterial growth rate requirements without the transcriptional regulation of gene expression. This mechanism is based on the utilization of L-Asp for both PG and pyrimidine synthesis. To the best of our knowledge the presented data are the first indication of a link between nucleotide metabolism and cell wall plasticity in bacteria.

RESULTS

Screening for spontaneous lysozyme-resistant mutants reveals a deficiency in guanine biosynthesis – To select mutants with affected CW structure we chose to use lysozyme for its double antibacterial activity. First, it is a muramidase and hydrolyzes the β-1,4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of PG, ultimately resulting in cell lysis. Second, lysozyme acts as a cationic antimicrobial that creates pores in the cytoplasmic membrane, leading to leakage of intracellular ions from the cell (23). We reasoned that using such “bicideal” antimicrobial would allow isolation of a larger spectrum of different mutants. We selected 59 independent spontaneous lysozyme-resistant mutants of L. lactis strain MG1363. Of these, 8 (14%) were unable to efficiently grow in rich M17G medium. This deficiency was corrected by the addition of guanine (20 µg/ml). The guanine deficiency was also confirmed using chemically defined medium [SA, (24), results not shown]. Since it has been reported that guanine auxotrophy could be due to inactivation of the guaA gene that encodes GMP synthase (25,26), we verified the presence of mutations in this gene in the guanine auxotrophs. The guaA gene has been shown to be involved in acid resistance in L. lactis, a phenotype that could be linked to CW modification (26). Unexpectedly, we failed to PCR-amplify the DNA fragment carrying the guaA gene using primers gua1 and gua2 from the chromosomal DNA of the obtained mutants (Fig. 1). In the L. lactis chromosome, the large 33 893-bp DNA fragment that carries the guaA gene is flanked by two IS905 elements oriented in the same direction (27). This organization allows the deletion of this fragment by homologous intra-chromosomal
rearrangements due to intra-chromosomal recombination. Indeed, large chromosomal rearrangements due to intra-chromosomal recombination between IS905 elements were previously shown to occur in *L. lactis* (28). We tested this possibility by PCR with primers gua3 and gua4, located outside of the IS905 elements, and obtained a 3590-bp fragment from all eight spontaneous guanine auxotrophs. DNA nucleotide sequence determination of the amplified DNA fragment from one of these mutants, VES2824, showed that it contained one copy of IS905 DNA, thus confirming that an intra-chromosomal recombination event was the basis of the deletion. Since the deleted fragment included other genes involved in purine metabolism (Fig. 1), we constructed a deletion mutant of only the *guaA* gene (strain VES4883, Table 1). As was the case for VES2824, the mutant VES4883 required guanine for growth in M17G and SA media and exhibited identical lysozyme resistance. The lysozyme resistance of strains VES2824 and VES4883 decreased when guanine was added to the medium (Fig. 2).

**Transcriptome analysis of the ΔguaA mutant shows down-regulation of pyrimidine biosynthetic genes** - To determine which genes may be involved in the lysozyme resistance of strain VES4883 (*ΔguaA*), we compared its transcriptional profiles with that of the control strain MG1363. In order to identify which genes are induced by cell wall stress, we included in this assay MG1363 cells treated with lysozyme (Table 2). In the *ΔguaA* mutant, we observed that, first, the *dlt*-operon genes *dltA*, *dltB*, and *dltD*, which are responsible for D-alanylation of teichoic acids (29), were up-regulated more than three-fold. Second, expression of *ponA*, which encodes the PG-synthesis enzyme PBP1A (30), was up-regulated four-fold in the *ΔguaA* mutant. Since this gene is involved in PG assembly, its increased expression could result in more cross-linked PG and thus contribute to lysozyme resistance. Third, expression of *pyrR*, which encodes the transcriptional regulator of the pyrimidine biosynthetic genes, was markedly decreased. This may explain the observation of strong down-regulation of other genes involved in pyrimidine biosynthesis: *pyrB*, *pyrP*, *carA*, *pyrK*, *pyrDb*, *pyrF*, *pyrE*, and *pyrC* (25). The only PG-synthesis genes that were affected in the *ΔguaA* mutant were *murB* and *murC*, which encode enzymes involved in the synthesis of UDP-MurNAc pentapeptide precursors (2). Notably, the cell-envelope-stress genes cesSR and *spxB* were not up-regulated in the *ΔguaA* mutant, indicating that the lysozyme resistance of this strain is not monitored by the cesSR regulon.

The cesSR and *spxB* genes were induced in lysozyme-treated MG1363 cells as expected. This corroborated the hypothesis that lactococci react to cell wall stress by inducing genes of the cesSR operon, which leads to resistance to PG hydrolysis (7,31). The expression of PG-synthesis genes was not affected in lysozyme-treated cells, indicating that PG synthesis is not regulated in response to lysozyme-provoked CW stress. Despite its apparent role in lysozyme resistance, *dlt* operon expression was also not affected under these stress conditions.

**Inactivation of pyrB confers lysozyme resistance and links pyrimidine biosynthesis to peptidoglycan assembly** - The genes *pyrP*, *pyrR*, *pyrB*, and *carA*, which are strongly repressed in the *ΔguaA* mutant (Table 2), constitute an operon in *L. lactis* MG1363 (32). The *pyrP* gene encodes a uracil permease, required for utilization of exogenous uracil. The other two genes in the operon, *pyrB* and *carA*, encode pyrimidine biosynthetic enzymes. In *L. lactis*, *PyrB* is a unique aspartate transcarbamoylase, which converts L-Asp to L-carbamoyl-L-aspartate (25). L-Asp is also used for CW biosynthesis: it is converted to D-Asp by RacD racemase, and is subsequently attached to the stem peptide of PG by AslA ligase (10) and then converted to D-Asn by AsnH (9). Simultaneous utilization of L-Asp for PG and for pyrimidine biosynthesis could make the D-Asp/Asn content in the CW dependent on pyrimidine biosynthesis. In this case, *pyrB* could play a pivotal role in the tradeoff between L-Asp utilization for PG or for pyrimidine biosynthesis. Therefore we focused further studies on this gene by constructing a *pyrB* deletion mutant of MG1363 and testing this strain for lysozyme resistance.

Since the *L. lactis* *pyrB* mutant is a uracil auxotroph (25), the lysozyme resistance test was performed in M17G medium supplemented with uracil (100 µg/ml). We also included *L. lactis* *dltD* and *ponA* mutants, since the expression of these genes was affected in the transcriptomics experiment described above (Table 2). We observed that a *ponA* mutation only slightly affected lysozyme
resistance while the inactivation of \textit{dltD} in the \textit{ΔguaA} mutant markedly abolished lysozyme resistance (Fig. 3). This indicates that increased TA alanylation may indeed play an important role in this phenotype of the \textit{ΔguaA} mutant. Inactivation of \textit{pyrB} resulted in lysozyme resistance, albeit slightly weaker than that of the \textit{ΔguaA} mutant and of strain VES2824, which carried the large chromosomal deletion. Inactivation of \textit{pyrB} in the \textit{ΔguaA} mutant did not affect the lysozyme resistance phenotype, which was consistent with the transcriptomics data showing that \textit{pyrB} is already strongly repressed in the \textit{ΔguaA} mutant.

\textit{Inactivation of pyrB results in a thicker and more rigid cell wall} - The cell envelope of the \textit{ΔpyrB} mutant, growing exponentially in M17G medium was considerably thicker (41±1.3 nm, Fig. 4B) than that of parental strain MG1363 (36±1.6 nm), as observed in transmission electron micrographs (Fig. 4A). Cell wall thickening was also observed in the \textit{ΔpyrB} mutant when cells were grown in M17G supplemented with uracil (44±2.6 versus 35±1.7 nm for MG1363, Fig. 4B). As the TEM results pointed towards possible changes in cell wall rigidity in the \textit{ΔpyrB} mutant, strains VES6497 (\textit{ΔpyrB}) and MG1363 (WT) were grown in M17G medium with and without uracil (100 µg/ml) and examined by atomic force microscopy (AFM) in order to measure cell wall rigidity (33,34).

Using an innovative method for sample immobilization in micro-structured polydimethylsiloxane (PDMS) stamps (35), combined with multi-parametric imaging, we were able to image \textit{L. lactis} cells collected in the exponential growth phase while simultaneously probing their nanomechanical properties (rigidity of the cell wall). Height images (Fig. 5A, B, C, and D) showed that the cell morphology was not modified by either \textit{pyrB} inactivation or the addition of uracil to the culture medium. On the corresponding rigidity images (Fig. 5E, F, G, and H), each pixel corresponds to a Young’s modulus (YM) value which reflects the rigidity of the cell wall. These images thus revealed that global stiffness varied between the two strains, with darker MG1363 cells being softer than the lighter \textit{ΔpyrB} mutants. However, two dividing cells did not always present the same nanomechanical properties (see Fig. 5G), which indicates that exponentially growing cells are heterogeneous in their cell wall rigidity. To precisely quantify the variations in cell stiffness, local force measurements were performed in small areas (500 × 500 nm) of the cells (36). Fig. 5I shows representative force vs. indentation obtained from the two strains in the two growth conditions, and fitted with a Hertz model to extract the YM values. As was already observed in the multiparametric imaging data, indentation curves (Fig. 5I) clearly show that MG1363 cells are softer than \textit{ΔpyrB} mutant cells. Indeed, MG1363 cells display an average YM value that is eight-fold lower than that of \textit{ΔpyrB} mutant cells, both in the absence (226.9 ± 160.5 kPa vs. 1707.4 ± 920.4 kPa, respectively) and the presence of uracil (95.8 ± 61.7 kPa vs. 780.1 ± 560.7 kPa, respectively) (Fig. 5I). Despite the large standard variations, which are due to the heterogeneity found in exponentially growing cells, the differences in YM values are highly significant \((p < 0.0001)\). This quantitative analysis also showed that the presence of uracil resulted in an approximately two-fold decrease in the rigidity of both wild type and mutant cells (Fig. 5J), which indicates that growth conditions have a slight impact on the nanomechanical properties of cell walls. Overall these nanomechanical data connected the increase in CW thickness in the \textit{ΔpyrB} mutant that was observed in TEM with the rigidity of the cell wall, i.e. thicker cell walls are more rigid.

Since it has been reported (7) that CW thickening is related to severe growth arrest of \textit{L. lactis} cells, we further examined the growth characteristics of the \textit{ΔpyrB} mutant.

\textit{Growth defect of the ΔpyrB mutant is linked to cell wall rigidity} - As reported previously (25), the \textit{ΔpyrB} mutant was not able to grow in minimal SA medium without the addition of uracil (results not shown). Therefore we evaluated the growth characteristics of the \textit{ΔpyrB} mutant in rich M17G medium. In this medium, the mutant exhibited a 5-hour lag phase compared to its parent strain (Fig. 6A). This phenotype was complemented by introducing the positive allele of \textit{pyrB}, cloned in a plasmid under control of the nisin-inducible promoter. Note that in the medium without nisin, partial complementation was observed, in keeping with reports about the modest leakiness of this promoter (37). Interestingly, the growth delay was also restored by the addition of uracil to
the medium, indicating that even in rich medium the ΔpyrB mutant is starved for nucleotides (Fig. 6). We further examined whether the growth impediment of the ΔpyrB mutant was due only to starvation for uracil, or also to changes in the CW, possibly influencing its thickening and/or rigidity and consequently cell division and separation. To this end, we introduced into the ΔpyrB mutant a plasmid that carried a nisin-inducible gene encoding PGH Lc-P40 of *Lactobacillus casei* (K. Regulski and M.-P. Chapot-Chartier, personal communication). Lc-P40 (Lcabl_00230) is a PG-specific peptidase that hydrolyzes PG at the interpeptide bridges, which are formed by D-Asp in both *Lb. casei* and *L. lactis* (2,38). We expected that Lc-P40 would “relax” the overly thick and rigid CW of the ΔpyrB mutant by creating breaks in PG. Indeed, the expression of Lc-P40 in the ΔpyrB mutant markedly increased growth, especially when the nisin inducer was added. It is important to note that the improvement in growth was observed in M17G medium without uracil, indicating that the growth delay of the ΔpyrB mutant was not only due to starvation for uracil.

Following the same reasoning, we mutated the ponA gene to create another “relaxation” factor in the ΔpyrB mutant. The ponA gene encodes PBP1A, an enzyme needed for the formation of PG cross-links, and mutations in it have been reported to favor the appearance of breaks in PG (15). As expected, the ponA mutation reduced the growth lag of the ΔpyrB mutant in M17G medium without uracil, indicating that the growth delay of the ΔpyrB mutant was not only due to starvation for uracil.

We also examined acmA, the main lactococcal autolysin (14), as another factor that affects the number of breaks in PG (15). Deletion of acmA in the ΔpyrB mutant had a similar effect on growth delay, albeit less pronounced, as introduction of the plasmid carrying pgdA did. Only a slight effect of the ΔacmA mutation was observable in the WT strain (Fig. 8 B). Note that growth retardation caused by pgdA overexpression or by acmA deletion in the ΔpyrB mutant was seen in a medium with uracil, indicating that starvation for uracil is not responsible for the observed effects in the ΔpyrB mutant. In conclusion, the observed results are consistent with the increased CW rigidity of the ΔpyrB mutant compared to WT.

*Inactivation of pyrB increases D-Asp/Asn-containing muropeptides in PG and cross-linking* - We determined the PG structure of exponentially growing *L. lactis* strains VES4883 (*guaA*) and VES6497 (*pyrB*) by separation of the constituent muropeptides with RP-HPLC, and compared their muropeptide profiles with that of their parent, MG1363, which was grown to the exponential and early stationary phases. In keeping with the role of pyrB in the regulation of L-Asp availability for PG synthesis, more D-Asp/D-Asn was present in PG of both mutants (Table 3).

In particular, the RP-HPLC profiles of the ΔguaA and ΔpyrB mutants had fewer muropeptides that lacked D-Asp/Asn, linked to their stem peptide (2.1% and 1.9%, respectively) relative to the control strain MG1363 in the same growth phase (4.0%). Furthermore, these two strains displayed elevated PG cross-linking (34.1 and 34.7%, respectively, versus 31.7% in WT). The MG1363 cells in early stationary phase (OD$_{600}$ 1.2) behaved similarly to ΔpyrB or ΔguaA mutants in the exponential growth phase (both at OD 0.2). Among the muropeptides that contained D-Asp/Asn, a relative increase in D-Asn was seen in the CW of ΔpyrB or ΔguaA mutants and MG1363 in the early stationary phase: the Asn/Asp ratio in these strains was 3.1, 4.1, and 3.8, respectively, versus 2.2 for exponentially grown MG1363 (Table 3). Overall, the PG muropeptide analysis confirmed that there was
more D-Asp/Asn in the PG of ΔpyrB and ΔguaA mutants, supporting the hypothesis that pyrB plays a pivotal role in the switch between L-Asp utilization for PG and its use in pyrimidine biosynthesis.

DISCUSSION

The bacterial PG sacculus needs to be strong in order for cells to be able to withstand high turgor pressure. Rigid and thick PG is advantageous in the stationary phase, when bacterial cells are exposed to different stress conditions, e.g., exhaustion and acidification of the medium (39). However, during exponential growth, PG must be flexible to allow fast cell division and enlargement. A balance between CW rigidity and flexibility may be maintained through the concerted activities of various enzymes involved in CW synthesis (PBPs) and hydrolysis (PGHs) (22,40). Despite recent achievements in this field, the elucidation of the mechanisms that govern the maintenance of this balance remains a challenge. Notwithstanding extensive studies on bacterial PG modifications in response to PG-hydrolysis-induced CW stress (41), little is known about the regulation of these processes under non-stress conditions.

Through the isolation of lysozyme-resistant mutants of L. lactis and the identification of the genes responsible for this phenotype, we were able to shed light on the mechanisms that govern the equilibrium between hydrolysis and synthesis of PG in this organism. Of the independent lysozyme-resistant isolates we obtained, 14% were guanine auxotrophs, which indicated that guaA was involved in the resistance phenotype. Whole-genome expression studies of the ΔguaA mutant showed decreased expression of the gene that encodes the regulator PyrR. This may be the reason why these mutants exhibited repression of the whole pyr operon (pyrK, pyrF, pyrB and carA) and other genes involved in pyrimidine metabolism (32,42). More studies are required to understand the precise regulatory mechanism that links the down-regulation of genes involved in pyrimidine metabolism with the inactivation of guaA. Since guaA is involved in the synthesis of guanosine monophosphate, and consequently of the global regulatory alarmones (pppGpp (43), it might be that the latter participates in the regulatory scheme.

Most importantly, studies of the ΔguaA mutant led us to discover the contributions of pyrB to CW structure and properties. The aspartate carbamoyltransferase PyrB is responsible for the utilization of L-Asp for pyrimidine synthesis. L-Asp is also a precursor for CW synthesis (see Fig. 9); it is converted to D-Asp by racemase RacD, then attached to the stem peptide of the PG precursor by AslA ligase (10) and converted to D-Asn by AsnH (9). Simultaneous utilization of L-Asp for PG and for pyrimidine biosynthesis could provide the means of coordinating CW structure in a manner dependent on pyrimidine biosynthesis, in which PyrB could play a pivotal role. We hypothesize that down-regulation of pyrB expression, as in the ΔguaA mutant, or its absence, as in the case of pyrB deletion, results in more L-Asp, which is transformed into D-Asp and used for the formation of PG cross-bridges. This would lead to the observed increase in PG rigidity and possibly to lysozyme resistance. To our knowledge, the data presented here indicate for the first time that a link exists between pyrimidine metabolism and CW plasticity in bacteria.

This hypothesis is supported by the exceptional CW-related features of the ΔpyrB mutant: i) the observed growth defects, which were restored by the introduction of additional mutations designed to weaken the CW (Fig. 6, Fig. 7, Fig. 8), ii) increased PG cross-linking and D-Asp/D-Asn content (Table 3), iii) elevated lysozyme resistance (Fig. 3), and iv) increased CW thickness (Fig. 4) and rigidity (Fig. 5).

In particular, we observed a longer lag-phase and lower final optical density in ΔpyrB mutant cultures than in the control strain MG1363 (Fig. 6). This growth defect was suppressed by introducing CW “relaxing” determinants in the ΔpyrB mutant, such as PGH of L. casei Lc-P40, which introduces breaks in PG interpeptide bridges, or a mutation in the ponA gene (Fig. 7). In addition, we exploited the fact that the growth defect was corrected by uracil to show that the opposite was also true, namely that the introduction of genetic factors that potentially lead to a decrease in PG breaks resulted in growth retardation. Thus, overexpression of the PG deacetylase PgdA, which leads to resistance to autolysis and lysozyme through increased N-deacetylation of PG (6,7) resulted in growth retardation in the ΔpyrB mutant.
Removal of the main lactococcal autolysin AcmA (14) produced similar albeit less pronounced effect.

RP-HPLC analysis revealed that PG of the pyrB-negative strain as well as of the ΔguaA mutant (in which expression of pyrB was drastically decreased), contained fewer muropeptides that lacked D-Asp or D-Asn (1.9% and 2.1%, respectively) than the control strain (4.0%) in the exponential growth phase (Table 3). This was true regardless of whether the medium was supplemented or not with uracil (data not shown). The increased amount of D-Asp and D-Asn content corresponded to an increased cross-linking index value (31.7% for WT versus 34.7% and 34.1% for ΔpyrB and ΔguaA mutants, respectively). Remarkably, the D-Asn/D-Asp ratios in PG of the ΔpyrB and the ΔguaA mutants were higher (3.1 and 4.1, respectively) than in the WT strain (2.2). Preferential accumulation of D-Asn in PG could be explained by the simultaneous increase in D-Asp supply in ΔguaA and ΔpyrB mutants, by the activity of AsnH, and/or increased affinity of flippase for disaccharide pentapeptides carrying D-Asn. It has been reported that amidation of D-Asp renders L. lactis more resistant to lysozyme due to a decrease in the net negative charge of the CW (9). Therefore a higher amount of D-Asn in PG could result in a less negative CW charge, leading to elevated resistance to this cationic antimicrobial.

Interestingly, a relatively small increase in PG cross-linking and D-Asn/D-Asp content and ratio corresponded to considerable differences in other phenotypes, such as CW rigidity (Fig. 5), thickness (Fig. 4), growth defects (Fig. 7), and lysozyme resistance (Fig. 3). This might be explained by assuming that more intensively cross-linked PG is specifically localized, which, if true, would not have been observable in our experiments because of the sample-averaging nature of HPLC analyses (44). Similarly, it has been reported that a relatively moderate shift in PG O-acetylation (from 2.6% to 4.2%) caused complete growth arrest in L. lactis (7).

The pyrB mutant phenotype could in part explain the high lysozyme resistance of the guaA mutant. The results presented here imply that the AguaA mutant possesses several regulatory mechanisms that enable it to prevent lysozyme activity, all potentially affecting cell envelope charge and/or PG cross-linking. The first is induction of the dlt operon, which is involved in alanylation of anionic TA (29,45). The consequent decrease in negative charge of the CW increases resistance to the positively charged lysozyme (7). Second, an increase in the D-Asp/Asn supply due to pyrB down-regulation could result in elevated PG cross-linking and thus counteract the muramidase activity of lysozyme. Third, the presence of a higher amount of D-Asn in PG also results in a decrease in the net negative charge of the CW, which would increase lysozyme resistance. Moreover, the ponA gene, which encodes PBP 1A (30), was four-fold over-expressed in the lysozyme-resistant ΔguaA mutant. It has been reported that up-regulation of PBP expression is a common strategy of Gram-positive bacteria responding to cell wall stress (41). Despite this, the ponA mutation did not increase the sensitivity of MG1363 to lysozyme activity (Fig. 3), suggesting that ponA over-expression only marginally affects this phenotype. Strikingly, the deletion of only one gene, namely guaA, protected against both types of lysozyme activities, the muramidase enzyme and the cationic antibacterial peptide.

The link described here between pyrB expression and CW rigidity could have an important role in maintaining the balance between CW flexibility and rigidity during the growth of a bacterial community. This function is possible because the expression of pyrimidine metabolism genes is tightly linked to pyrimidine availability in the medium, which in turn depends on the growth phase of the culture (25,32). Differential pyrB expression during culture growth was documented in chrono-transcriptomics studies of L. lactis MG1363 grown in milk (46) or in M17G (47,48). Notably, it was observed that the pyr operon is considerably more expressed during the exponential growth than it is in the stationary phase. If the D-Asp/D-Asn content in PG is dependent on pyrB expression, this would enable the availability of flexible PG during exponential growth and more rigid and thick PG in the stationary phase. In keeping with this reasoning, we observed a higher amount of Asp/Asn-less muropeptides in PG of MG1363 in the exponential phase than we did in the stationary phase. Correspondingly, the cross-linking index of MG1363 PG was lower in the exponential than in the stationary phase, where it was comparable to that of the ΔpyrB mutant (Table 3).
Such “metabolic programming” would enable the maintenance of the needed CW plasticity without the need to transcriptionally control CW-synthesis genes. It could be considered advantageous because the majority of CW-synthesis genes are essential, and it has been suggested that essential genes in bacteria generally are not regulated at the transcriptional level (49). To avoid the transcriptional regulation of genes, encoding autolytic PGHs could also be advantageous, since these are secreted extracellular enzymes and thus their regulation via intracellular transcription could be challenging. In keeping with this interpretation, the lactococcal response to CW hydrolysis proceeds by modulating the target (O-acetylation of PG) rather than by decreasing transcription of the gene that codes for the secreted autolysin AcmA (7). Indeed, no decrease in expression of lactococcal-PGH-encoding genes was observed in response to lysozyme treatment in our transcriptomics study (Table 2).

PG hydrolases are necessary for CW synthesis in growing cells (21,50). The decrease of L-Asp availability in exponential phase, regulated by PyrB, leads to greater PG sensitivity to hydrolysis, which would be beneficial in terms of making PG more apt for incorporation of newly synthesized.

The described hijacking of the regulatory circuit of pyrimidine metabolism, which is tightly regulated by growth requirements, in order to maintain an optimal balance between CW rigidity and flexibility, may also take place in various other Gram-positive bacteria with D-Asp/D-Asn in their PG cross-bridges, such as Enterococcus faecium, L. casei, Lactobacillus delbrueckii, Lactobacillus brevis, and others (51). Interestingly, this mechanism is not used by L. lactis to respond to CW stress, since pyrB is not down-regulated in cells treated with lysozyme (Table 2). It is probable that the tight regulation of genes involved in nucleotide metabolism precludes the possibility of changing pyrB expression in response to CW stress.

This work shows that, in order to maintain optimal CW plasticity, bacteria regulate substrate availability (in this case L-Asp) for CW synthesis. The availability of L-Asp for CW synthesis might also be affected by expression of the aspartyl-tRNA synthetase gene aspS. This possibility is supported by the down-regulation of aspS in the stationary growth phase, as observed in chronotranscriptomics assays of L. lactis MG1363 grown in M17G (47,48); this activity could also favor the availability of L-Asp for CW synthesis.

An analogous mechanism to the one described here might be at play in bacteria that have L-amino acids in their PG cross-bridges, such as, e.g., Streptococcus pneumoniae (L-Ala-L-Ser or L-Ala-L-Ala in the cross-bridge). Like the competitive “switch” between PyrB and RacD for L-Asp and the resulting change in CW of L. lactis, in this case the competition between the protein synthesis machinery and tRNA-dependent aminoacyl ligases for aminoacylated-tRNA (52) could be a means of regulating PG plasticity. Ribosomal content is strictly regulated according to nutrient availability and is higher in the exponential phase than in the stationary phase (53). This would raise the probability of L-amino acids being incorporated in the PG cross-bridges of bacteria in stationary phase. The incorporation of amino acids in PG cross-bridges could therefore be viewed as an evolutionary adaptation enabling bacteria to tune PG plasticity to growth requirements.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions** - The bacterial strains and plasmids used in this study are listed in Table 1. All L. lactis strains used here are derivatives of strain MG1363 (54). L. lactis was grown at 30°C in M17 medium (BD Biosciences) that contained 0.5% glucose (M17G). Erythromycin (2.5 μg/ml) and chloramphenicol (5 μg/ml) (Sigma) were added when needed. E. coli was grown in LB medium (BD Biosciences) at 37°C, unless otherwise indicated, in the presence of ampicillin (100 μg/ml), erythromycin (100 μg/ml), or chloramphenicol (10 μg/ml), when needed. Nisin was prepared in Me2SO (Sigma) and added at a final concentration of 0.1 ng/ml. Growth was monitored by optical density measurement at 600 nm (OD600) with a spectrophotometer (Spectronic 20 Genesys).

**Lysozyme resistance tests** - A concentrated hen egg white lysozyme (Fluka, Buchs, Switzerland) solution was freshly prepared in GM17 medium and then diluted in molten GM17 agar (1.5%) at 45°C. Overnight
bacterial cultures were successively diluted 10-fold and 5 µl of each dilution was spotted on GM17 agar plates supplemented with different concentrations of lysozyme.

**Strain constructions -** A 1347-bp fragment containing the guaA gene was deleted from the chromosome using the pORI280 (lacZ)/pVE6007 two-plasmid system (55,56). First, the fragments upstream (578 bp) and downstream (607 bp) of the deletion site were PCR-amplified from *L. lactis* MG1363 genomic DNA using primer pairs guaA-bgl (5’- atgatgagatctcagttctacctgce, restriction site is underlined), guaA-xmab (5’- atgatgccccggatggttcagaaacacce); and guaA-xmax (5’- atgatgccccggatggttcagaaacacce), guaA-xba (5’- atgatgtagttctagtctcctgcc). Then, both fragments were digested with the restriction endonuclease SmaI (NEB) and PCR-amplified using primers guaA-bgl and guaA-xba. The amplified region was digested with *Bgl*II and *Xba*I and ligated to the pORI280 digested plasmid, and the ligation mixture was used to transform *E. coli* JIM4646. The pORI280 derivative that was needed for construction of a guaA-deletion mutant was obtained as an erythromycin-resistant transformant. The resulting plasmid, pVES4848, and a thermo-sensitive plasmid encoding chloramphenicol resistance, pVE6007, were introduced into MG1363; transformants were selected by erythromycin (2.5 µg/ml) and chloramphenicol (2.5 µg/ml) resistance. Plasmid pVES4848 was integrated into the resulting strain following overnight growth in *E. coli* liquid medium at 37°C, a temperature that prevents pVE6007 replication. The culture was then plated on GM17 agar containing lysozyme.

**DNA nucleotide sequence determination.** To inactivate the pyrB gene we followed the same procedure, using the primer pairs pyrBBeglII (5’- tgtgtgtagatctcagttctacctgce) and pyrBXmA R (5’- tgtgtgtagatctcagttctacctgce) for amplification of the upstream 588-bp fragment, and pyrBXmA R (5’- tgtgtgtagatctcagttctacctgce) and pyrBXbglI (5’- tgtgtgtagatctcagttctacctgce) for amplification of the downstream 576-bp fragment. The resulting strain, VES6497, carried a deletion of the 888-bp fragment that contained *pyrB*.

Similarly, the *guaA* deletion was introduced in strain VEL1378 (delD), creating strain VES5160; and the *pyrB* deletion in strain VES4883 (*guaA*), yielding VES6530. Also, the ΔacmA mutation was introduced in strain VES6497 by using the plasmid pINTAA and following the procedure described in (14), thus creating strain VES6831.

The ponA *pyrB* double mutant VES6949 was constructed by transforming strain VES6497 with plasmid pVE1837 [pRV300 carrying a 210-bp internal fragment of ponA (30)] and selecting for erythromycin resistance.

**Mapping of spontaneous guaA mutation -** With primers gua1 (5’- cggacttttgcaccttataa) and gua2 (5’- gcgttaatagaattatag cg) we PCR-amplified a 1786-bp fragment of *L. lactis* MG1363 genomic DNA, but were unable to obtain the corresponding DNA fragment using DNA of the mutant VES2824 (Fig. 1). However, in the latter strain we were able to amplify a 3590-bp DNA fragment using primers gua3 (5’- tttatacgggaatcgttgcg) and gua4 (5’- tggcatattaacctgc). DNA sequence determination of this fragment revealed that it contained the IS905 transposon DNA sequence, flanked by sequences that are situated upstream and downstream of IS905 transposons, indicating that VES2824 is missing the 33893-bp chromosomal fragment situated between the two IS905s.

**Cloning of pyrB under control of the nisin-inducible promoter -** The DNA fragment carrying the pyrB gene was PCR-amplified from *L. lactis* MG1363 genomic DNA using the primer pair 7.gibs.pyrB1 (5’- aaataattaattataggcaagttatacATGTACAGAAA AAATGGATTAGTTCC) and 8.gibs.pyrB2 (5’- agtgtagcgcagttatacagttatacAGTTACTTGC GTTTTTTCCAGCAAG). The fragment was ligated to the *Nco*I-digested plasmid pMSP3545 using iso thermal assembly (57) and introduced in VES6497. The clone that carried plasmid pMSP3545:pyrB+ (nisin-inducible *pyrB*), VES6953, was verified by PCR and DNA nucleotide sequence determination.

**Peptidoglycan structure analysis -** PG
was extracted from cultures in the exponential [OD\(_{600}\) 0.4 for MG1363, 0.2 for VES6497 (\(\Delta\)pyrB) and VES4883 (\(\Delta\)guaA)] and early stationary growth phase (OD\(_{600}\) 1.2 for MG1363) as described previously (58). PG was then hydrolyzed with mutanolysin, and the resulting soluble muropeptides were reduced and separated by RP-HPLC with an Agilent UHPLC 1290 system using an ammonium phosphate buffer and linear methanol gradient, as described previously (59). The eluted muropeptides were detected by UV absorbance at 202 nm. Muropeptides were identified according to their retention time by comparison with a reference chromatogram (58). The eluted muropeptides were quantified by integration of the peaks on the chromatogram. The relative amount of each muropeptide was expressed as the ratio of its peak area over the sum of all of the peak areas. The PG cross-linking index was calculated as described in (60).

**DNA microarray analysis** - For DNA microarray experiments, *L. lactis* MG1363 and *L. lactis* VES4883 (\(\Delta\)guaA) cells were grown in M17G medium and harvested at the mid-exponential growth phase. In order to assess the effect of the lysozyme treatment on the transcriptome, 5 mg/ml of lysozyme (Sigma-Aldrich, St. Louis, MO) was added to the culture. Cells were collected after 20 min of incubation with lysozyme. Total RNA of lysozyme-treated MG1363 and of VES4883 was compared to RNA isolated from MG1363 (WT) cells. Slides were scanned with a Genepix 4200 laser scanner at 10-µm resolution. ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD) was used to analyze slide images; processing and normalization were performed using MicroPrep software (61,62) as described in (62). Gene expression was considered to be significantly altered when the Cyber T Baysian P-value was \(\leq 0.001\).

**Transmission electron microscopy (TEM)** - Pellets of bacteria (OD\(_{600}\) 0.5 for MG1363, MG1363 + uracil, and \(\Delta\)pyrB mutant + uracil, and OD\(_{600}\) 0.2 for the \(\Delta\)pyrB mutant) were fixed with 2% glutaraldehyde in 0.1 M Na cacodylate buffer pH 7.2, for 3 hours at RT. Samples were contrasted with Oolong Tea Extract 0.5% in cacodylate buffer, fixed with 1% osmium tetroxide that contained 1.5% potassium cyanoferrate, gradually dehydrated in ethanol (30% to 100%), substituted gradually in a mixture of propylene oxide-epon, and embedded in Epon (Delta microscopic, Labège, France). Thin (70-nm) sections were collected onto 200 mesh copper grids, and counterstained with lead citrate. Grids were examined using a Hitachi HT7700 electron microscope operated at 80kV (Elexience, France). Images were acquired with a charge-coupled device camera (AMT, Japan). Cell wall thickness was measured on TEM micrographs of at least 3 cells at magnification 7000x, taking at least 5 measurements on each cell.

**Sample preparation and atomic force microscopy (AFM) experiments** - *L. lactis* strains MG1363 and its isogenic \(\Delta\)pyrB mutant were grown in M17G broth in the presence or absence of 100 µg/ml uracil at 30°C under static conditions, until the exponential phase was reached (OD\(_{600}\) 0.5 for MG1363, MG1363 + uracil, and \(\Delta\)pyrB mutant + uracil, and OD\(_{600}\) 0.2 for the \(\Delta\)pyrB mutant). Bacterial cells were concentrated by centrifugation, washed twice and resuspended in phosphate-buffered saline (PBS), and immobilized on PDMS stamps prepared as described previously (35). Briefly, microstructured PDMS stamps were covered by a total of 100 µl of the cell suspension. Cells were then deposited into the microstructures of the stamps by convective/capillary assembly.

Images were recorded in PBS in Quantitative Imaging\(^{(TM)}\) (QIT\(^{(TM)}\)) mode (33) using MSCT AU/WH (Bruker, Billerica, USA) cantilevers (nominal spring constants of 0.1 and 0.01 N/m), and with an applied force of 0.5 nN. Force spectroscopy mode was used to perform local nanoindentation measurements on areas of 200 × 200 µm\(^2\) on top of cells. The applied force was kept between 0.5 and 2 nN depending on the strains and growth conditions probed. The cantilevers’ spring constant was determined using the thermal noise method (63). For imaging and force spectroscopy we used a Nanowizard III (JPK Instruments, Berlin, Germany). For rigidity measurements, the force distance curves obtained in QIT\(^{(TM)}\) mode and during nanoindentation experiments were transformed into force-indentation curves by subtracting the cantilever deflection on a solid surface. The indentation curves were then fitted to the Hertz model, which links the force (F) as a function of the Young’s modulus value (E) with the square of the indentation (δ) for a conical indenter according to the following...
equation: \[ F = \left[2E \tan(\alpha/\pi(1-\nu^2))\right] \delta^2, \]
where \( \alpha \) is the tip opening angle (17.5°) and \( \nu \) the Poisson ratio, assumed to be 0.5. In each condition, the fitted indentation segment was kept constant at 50 nm. In each case, Young’s modulus values were measured on 12 cells (n = 12288 curves per conditions), and Young’s modulus medians were calculated from fits in a Gaussian model. All results were analyzed using the data processing software provided by JPK Instruments.

ACKNOWLEDGMENTS

We are indebted to A. Gruss, V. Fromion and J. Martinussen for valuable discussions. We thank Carmen Bulbarela-Sampier, Valeria Mikaelova, Aurelie Bobillot, and Pascal Quenée for help in different phases of the work. The work of P.C., S.F., P.V., J.A., M.S., M.-P.C.-C., and S.K. was supported by the Institut National de la Recherche Agronomique (INRA) and the Région Ile-de-France, and has benefited from the facilities and expertise of MIMA2 MET, UMR 1313 GABI, Equipe Plateformes, INRA, 78352 Jouy-en-Josas. The work of C.F.-D., Y.D., and P.H. was supported by the National Foundation for Scientific Research (FNRS), and the Research Department of the Communauté française de Belgique (Concerted Research Action). J.A. received a fellowship from the Région Ile-de-France (DIM Malinf). C.F.-D., P.H., and Y.D. are respectively Postdoctoral Researcher, Senior Research Associate, and Research Director at FNRS.

REFERENCES

1. Matsuhashi, M. (1994) Utilisation of lipid-linked precursors and the formation of peptidoglycan in the process of the cell growth and division: membrane enzymes involved in the final steps of peptidoglycan synthesis and the mechanism of their regulation. in Bacterial cell wall (Ghuysen, J. M., Hakenbeck, R. ed.), Elsevier, Amsterdam. pp 55-71
2. Chapot-Chartier, M. P., and Kulakauskas, S. (2014) Cell wall structure and function in lactic acid bacteria. Microbial cell factories 13 Suppl 1, S9
3. Loskill, P., Pereira, P. M., Jung, P., Bischoff, M., Herrmann, M., Pinho, M. G., and Jacobs, K. (2014) Reduction of the peptidoglycan crosslinking causes a decrease in stiffness of the Staphylococcus aureus cell envelope. Biophys J 107, 1082-1089
4. Vollmer, W. (2008) Structural variation in the glycan strands of bacterial peptidoglycan. FEMS microbiology reviews 32, 287-306
5. Moynihan, P. J., and Clarke, A. J. (2011) O-Acetylated peptidoglycan: controlling the activity of bacterial autolysins and lytic enzymes of innate immune systems. Int J Biochem Cell Biol 43, 1655-1659

FOOTNOTE

The abbreviations used are: PG, peptidoglycan; PGH, peptidoglycan hydrolase; CW, cell wall; PBP, penicillin binding protein; RP-HPLC, reverse phase high pressure liquid chromatography; WT, wild-type; PDMS, polydimethylsiloxane; GlcNAc, N-acetyl-glucosamine; MurNAc, N-acetyl-muramic acid; TEM, transmission electron microscopy; AFM, atomic force microscopy; YM, Young’s modulus.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

S.K. conceived and coordinated the study and wrote most of the paper. C.F.-D., P.H., and Y.F.D. designed, performed, and analyzed the AFM experiments and wrote the corresponding part of the paper. C.P. designed, performed, and analyzed TEM experiments. A.S., O.P.K., and J.K. designed, performed, and analyzed the transcriptomics experiments and participated in the writing of the manuscript. P.C. and M.-P.C.-C. designed, performed, and analyzed the experiments shown in Table 2. S.F., P.V., and J.A. constructed strains. M.S. performed and analyzed the experiments shown in Figures 6, 7, and 8. All authors reviewed the results and approved the final version of the manuscript.
6. Meyrand, M., Boughammoura, A., Courtin, P., Mezange, C., Guillot, A., and Chapot-Chartier, M. P. (2007) Peptidoglycan N-acetylg glucosamine deacetylation decreases autolysis in Lactococcus lactis. Microbiology 153, 3275-3285
7. Veiga, P., Bulbarela-Sampieri, C., Furlan, S., Maisons, A., Chapot-Chartier, M. P., Erkelenz, M., Mervelet, P., Noirot, P., Frees, D., Kuipers, O. P., Kok, J., Gruss, A., Buist, G., and Kulakauskas, S. (2007) SpxB regulates O-acetylation-dependent resistance of Lactococcus lactis peptidoglycan to hydrolysis. J Biol Chem 282, 19342-19354
8. van Heijenoort, J. (2007) Lipid intermediates in the biosynthesis of bacterial peptidoglycan. Microbiol Mol Biol Rev 71, 620-635
9. Veiga, P., Erkelenz, M., Bernard, E., Courtin, P., Kulakauskas, S., and Chapot-Chartier, M. P. (2009) Identification of the asparagine synthase responsible for D-Asp amidation in the Lactococcus lactis peptidoglycan interpeptide crossbridge. J Bacteriol 191, 3752-3757
10. Veiga, P., Piquet, S., Maisons, A., Furlan, S., Courtin, P., Chapot-Chartier, M. P., and Kulakauskas, S. (2006) Identification of an essential gene responsible for D-Asp incorporation in the Lactococcus lactis peptidoglycan crossbridge. Mol Microbiol 62, 1713-1724
11. Chapot-Chartier, M. P. (2010) Bacterial autolysins. in Procariotic Cell Wall Compounds (Koning, H., Claus, H., Varna, A. ed.), Springer Verlag Berlin, Heidelberg, Germany. pp 383-406
12. Vollmer, W., Joris, B., Charlier, P., and Foster, S. (2008) Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol Rev 32, 259-286
13. Lee, T. K., and Huang, K. C. (2013) The role of hydrolases in bacterial cell-wall growth. Curr Opin Microbiol 16, 760-766
14. Buist, G., Kok, J., Leenhouts, K. J., Dabrowska, M., Venema, G., and Haandrikman, A. J. (1995) Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of Lactococcus lactis, a muramidase needed for cell separation. J Bacteriol 177, 1554-1563
15. Mercier, C., Domakova, E., Tremblay, J., and Kulakauskas, S. (2000) Effects of a muramidase on a mixed bacterial community. FEMS Microbiol Lett 187, 47-52.
16. Cava, F., and de Pedro, M. A. (2014) Peptidoglycan plasticity in bacteria: emerging variability of the murein sacculus and their associated biological functions. Curr Opin Microbiol 18, 46-53
17. Buckley, N. D., Vadeboncoeur, C., LeBlanc, D. J., Lee, L. N., and Frenette, M. (1999) An effective strategy, applicable to Streptococcus salivarius and related bacteria, to enhance or confer electroporation competence. Appl Environ Microbiol 65, 3800-3804
18. Cruz-Rodz, A. L., and Gilmore, M. S. (1990) High efficiency introduction of plasmid DNA into glycine treated Enterococcus faecalis by electroporation. Mol Gen Genet 224, 152-154
19. Bisicchia, P., Noone, D., Jojou, E., Howell, A., Quigley, S., Jensen, T., Jarmer, H., and Devine, K. M. (2007) The essential YycFG two-component system controls cell wall metabolism in Bacillus subtilis. Mol Microbiol 65, 180-200
20. Singh, S. K., SaiSree, L., Amrutha, R. N., and Reddy, M. (2012) Three redundant murein endopeptidases catalyse an essential cleavage step in peptidoglycan synthesis of Escherichia coli K12. Mol Microbiol 86, 1036-1051
21. Vollmer, W. (2012) Bacterial growth does require peptidoglycan hydrolases. Mol Microbiol 86, 1031-1035
22. Wheeler, R., Turner, R. D., Bailey, R. G., Salamaga, B., Mesnage, S., Mohamad, S. A., Hayhurst, E. J., Horsburgh, M., Hobbs, J. K., and Foster, S. J. (2015) Bacterial Cell Enlargement Requires Control of Cell Wall Stiffness Mediated by Peptidoglycan Hydrolases. MBio 6, e00660
23. Ibrahim, H. R., Thomas, U., and Pellegrini, A. (2001) A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. J Biol Chem 276, 43767-43774
24. Jensen, P. R., and Hammer, K. (1993) Minimal Requirements for Exponential Growth of Lactococcus lactis. Appl Environ Microbiol 59, 4363-4366
25. Kilstrup, M., Hammer, K., Ruhdal Jensen, P., and Martinussen, J. (2005) Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiol Rev* **29**, 555-590

26. Rallu, F., Gruss, A., Ehrlich, S. D., and Maguin, E. (2000) Acid- and multistress-resistant mutants of Lactococcus lactis: identification of intracellular stress signals. *Mol Microbiol* **35**, 517-528.

27. Wegmann, U., O’Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M. J., Kuipers, O. P., van Sinderen, D., and Kok, J. (2007) Complete genome sequence of the prototype lactic acid bacterium Lactococcus lactis subsp. cremoris MG1363. *J Bacteriol* **189**, 3256-3270.

28. Daveran-Mingot, M. L., Campo, N., Ritzenthaler, P., and Le Bourgeois, P. (1998) A natural large chromosomal inversion in Lactococcus lactis is mediated by homologous recombination between two insertion sequences. *J Bacteriol* **180**, 4834-4842.

29. Reichmann, N. T., Cassona, C. P., and Grundling, A. (2013) Revised mechanism of D-alanine incorporation into cell wall polymers in Gram-positive bacteria. *Microbiology* **159**, 1868-1877.

30. Mercier, C., Durrieu, C., Briandet, R., Domakova, E., Tremblay, J., Buist, G., and Kulakauskas, S. (2002) Positive role of peptidoglycan breaks in lactococcal biofilm formation. *Mol Microbiol* **46**, 235-243.

31. Martinez, B., Zomer, A. L., Rodriguez, A., Kok, J., and Kuipers, O. P. (2007) Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CcsSR. *Mol Microbiol* **64**, 473-486.

32. Martinussen, J., Schallert, J., Andersen, B., and Hammer, K. (2001) The pyrimidine operon pyrRP-car from Lactococcus lactis. *J Bacteriol* **183**, 2785-2794.

33. Chopinet, L., Formosa, C., Rols, M. P., Duval, R. E., and Dague, E. (2013) Imaging living cells surface and quantifying its properties at high resolution using AFM in QI mode. *Micron chap48*, 26-33.

34. Dufrene, Y. F. (2014) Atomic force microscopy in microbiology: new structural and functional insights into the microbial cell surface. *MBio* **5**, e01363-01314.

35. Formosa, C., Pillet, F., Schiavone, M., Duval, R. E., Ressier, L., and Dague, E. (2015) Generation of living cell arrays for atomic force microscopy studies. *Nat Protoc* **10**, 199-204.

36. Touhami, A., Nysten, B., and Dufrene, Y. F. (2003) Nanoscale mapping of the elasticity of microbial cells by atomic force microscopy. *Langmuir* **19**, 4539-4543.

37. Renye, J. A., Jr., and Somkuti, G. A. (2010) Nisin-induced expression of pediocin in dairy lactic acid bacteria. *J Appl Microbiol* **108**, 2142-2151.

38. Regulski, K., Courtin, P., Meyrand, M., Claes, I. J., Lebeer, S., Vanderleyden, J., Hols, P., Guillot, A., and Chapot-Chartier, M. P. (2012) Analysis of the peptidoglycan hydrolase complement of Lactobacillus casei and characterization of the major gamma-D-glutamyl-L-lysyl-endopeptidase. *PLoS One* **7**, e32301.

39. Rallu, F., Gruss, A., and Maguin, E. (1996) Lactococcus lactis and stress. *Antonie Van Leeuwenhoek* **70**, 243-251.

40. Jordan, S., Hutchings, M. I., and Mascher, T. (2008) Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiol Rev* **32**, 107-146.

41. Martinussen, J., Andersen, P. S., and Hammer, K. (1994) Nucleotide metabolism in Lactococcus lactis: salvage pathways of exogenous pyrimidines. *J Bacteriol* **176**, 1514-1516.

42. Hauryliuk, V., Atkinson, G. C., Murakami, K. S., Tenson, T., and Gerdes, K. (2015) Recent functional insights into the role of (p)pGpp in bacterial physiology. *Nat Rev Microbiol* **13**, 298-309.

43. Turner, R. D., Vollmer, W., and Foster, S. J. (2014) Different walls for rods and balls: the diversity of peptidoglycan. *Mol Microbiol* **91**, 862-874.
45. Steen, A., Palumbo, E., Deghorain, M., Cocconcelli, P. S., Delcour, J., Kuipers, O. P., Kok, J., Buist, G., and Hols, P. (2005) Autolysis of Lactococcus lactis is increased upon D-alanine depletion of peptidoglycan and lipoteichoic acids. J Bacteriol 187, 114-124
46. de Jong, A., Hansen, M. E., Kuipers, O. P., Kilstorp, M., and Kok, J. (2013) The transcriptional and gene regulatory network of Lactococcus lactis MG1363 during growth in milk. PLoS One 8, e53085
47. Brouwer, R. W. W. (2014) Computational methods for the analysis of bacterial gene regulation, University of Groningen
48. Pinto, J. (2015) In principio erat Lactococcus lactis: towards a membrane protein overproducer host, University of Groningen
49. Brouwer, R. W. W. (2014) Computational methods for the analysis of bacterial gene regulation, University of Groningen
50. Vollmer, W., and Holtje, J. V. (2004) The architecture of the murein (peptidoglycan) in gram-negative bacteria: vertical scaffold or horizontal layer(s)? J Bacteriol 186, 5978-5987
51. Schleifer, K. H., and Kandler, O. (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36, 407-477
52. Shepherd, J., and Ibba, M. (2013) Direction of aminoacylated transfer RNAs into antibiotic synthesis and peptidoglycan-mediated antibiotic resistance. FEBS Lett 587, 2895-2904
53. Wilson, D. N., and Nierhaus, K. H. (2007) The weird and wonderful world of bacterial ribosome regulation. Crit Rev Biochem Mol Biol 42, 187-219
54. Gasson, M. J. (1983) Plasmid complements of Streptococcus lactis NCDO 712 and other lactic streptococci after protoplast-induced curing. J Bacteriol 154, 1-9
55. Law, J., Buist, G., Haandrakens, A., Kok, J., Venema, G., and Leenhouts, K. (1995) A system to generate chromosomal mutations in Lactococcus lactis which allows fast analysis of targeted genes. J Bacteriol 177, 7011-7018
56. Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Miera, I., Dabrowska, M., Venema, G., and Kok, J. (1996) A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol Gen Genet 253, 217-224
57. Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6, 343-345
58. Courtin, P., Miranda, G., Guillot, A., Wessner, F., Mezange, C., Domakova, E., Kulakauskas, S., and Chapot-Chartier, M. P. (2006) Peptidoglycan Structure Analysis of Lactococcus lactis Reveals the Presence of an L,D-Carboxypeptidase Involved in Peptidoglycan Maturation. J Bacteriol 188, 5293-5298
59. Regulski, K., Courtin, P., Kulakauskas, S., and Chapot-Chartier, M. P. (2013) A novel type of peptidoglycan-binding domain highly specific for amidated D-Asp cross-bridge, identified in Lactobacillus casei bacteriophage endolysins. J Biol Chem 288, 20416-20426
60. Glauner, B. (1988) Separation and quantification of muropeptides with high-performance liquid chromatography. *Anal Biochem* **172**, 451-464
61. van Hijum, S. A., de Jong, A., Baerends, R. J., Karsens, H. A., Kramer, N. E., Larsen, R., den Hengst, C. D., Albers, C. J., Kok, J., and Kuipers, O. P. (2005) A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data. *BMC Genomics* **6**, 77
62. Zomer, A. L., Buist, G., Larsen, R., Kok, J., and Kuipers, O. P. (2006) Time-resolved determination of the CcpA regulon of Lactococcus lactis spp. cremoris MG1363. *J. Bacteriol.*, JB.01013-01006
63. Hutter, J. L., and Bechhoefer, J. (1993) Calibration of atomic-force microscope tips. *Rev. Sci. Instrum.* **64**, 1868–1873
64. Kuipers, O. P., de Ruyter, P., Kleerebezem, M., and de Vos, W. M. (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology* **64**, 15-21
65. Duwat, P., Cochu, A., Ehrlich, S. D., and Gruss, A. (1997) Characterization of Lactococcus lactis UV-sensitive mutants obtained by IS1 transposition. *J Bacteriol* **179**, 4473-4479.
66. Boyer, H. W., and Roulland-Dussoix, D. (1969) A complementation analysis of the restriction and modification of DNA in Escherichia coli. *J Mol Biol* **41**, 459-472
67. Duwat, P., Cochu, A., Ehrlich, D., and Gruss, A. (1997) Characterization of Lactobacillus sake chromosome and insertional inactivation of the ptsI and lacI genes. *Appl Environ Microbiol* **63**, 2117-2123
68. Maguin, E., Duwat, P., Hege, T., Ehrlich, D., and Gruss, A. (1992) New thermosensitive plasmid for gram-positive bacteria. *J Bacteriol* **174**, 5633-5638
69. Bryan, E. M., Bae, T., Kleerebezem, M., and Dunny, G. M. (2000) Improved vectors for nisin-controlled expression in gram-positive bacteria. *Plasmid* **44**, 183-190

**FIGURE LEGENDS**

**FIGURE 1.** Scheme of the *L. lactis* MG1363 chromosomal locus that contains *guaA* and other genes involved in purine metabolism; black arrows indicate the oligonucleotides used for mapping (see exp. procedures).

**FIGURE 2.** Comparison of lysozyme resistance of *L. lactis* WT MG1363 and its mutants VES2824, in which a large region that included the *guaA* gene was deleted, and VES 4883, in which only the *guaA* gene was deleted. Serially diluted cultures were grown on M17G agar plates supplemented with lysozyme and guanine.

**FIGURE 3.** Comparison of lysozyme resistance of the ΔpyrB, ΔguaA, dltD, and ΔponA mutants and the parental strain MG1363. VES2824 is the spontaneous lysozyme-resistant mutant. The plate test was performed in M17G medium supplemented with 100 µg/ml of uracil and lysozyme.

**FIGURE 4.** Electron transmission micrographs (A) and evaluation of CW thickness (B) of *L. lactis* control strain MG1363 and its isogenic mutant VES6497 (ΔpyrB). Arrows indicate the measured interval. Scale bar = 50 nm.

**FIGURE 5.** Imaging and probing of the nanomechanical properties of living *L. lactis* cells. Height images of cells of (A) *L. lactis* MG1363, (B) MG1363 + uracil (100 µg/ml), (C) ΔpyrB mutant, and (D) ΔpyrB mutant + uracil (100 µg/ml), which were trapped in micro-structured PDMS stamps. (E, F, G, and H) Rigidity images corresponding to height images shown in panels A, B, C, and D, respectively. (I) Representative indentation curves obtained for MG1363 (gray line), MG1363 + uracil (light gray line), ΔpyrB mutant (yellow line), and ΔpyrB mutant + uracil (dark yellow line). Black empty circles show in each case the fit with the Hertz model. (J) Histogram showing the Young’s modulus values for both strains, with or without uracil. In each case, Young’s modulus values were measured on 12 cells (n = 12288 curves), and Young’s modulus medians were calculated from fits in a
Gaussian model. The three asterisks show significant differences between the rigidity of strain MG1363 and that of the ΔpyrB mutant at a p-value < 0.0001 (unpaired t-test).

**FIGURE 6.** A. Growth and complementation of the ΔpyrB mutant. B. Complementation of growth of the ΔpyrB mutant by uracil.

**FIGURE 7.** A. Growth of MG1363 (WT) and a ΔpyrB mutant that carried a plasmid encoding *Lb. casei* BL23 PGH Lc-P40. B. Growth of WT and a ΔpyrB mutant that carried a mutation in the ponA gene. Bacteria were grown in M17G medium without uracil.

**FIGURE 8.** Growth of MG1363 (WT) and ΔpyrB mutants carrying (A) the pgdA gene of *L. lactis* on a multicopy plasmid or (B) deletion of the acmA gene in M17G medium supplemented with uracil.

**FIGURE 9.** Schematic representation of incorporation of D-Asp in *L. lactis* PG. Representative D-Asp in PG stem peptides are presented as squares: black - L-Ala, green - D-Glu, violet - L-Lys, red - L- and D-Asp, light red D-Asn. N-acetyl-glucosamine is presented as blue hexagon, N-acetyl-muramic acid – as light blue hexagon.

**TABLES**

**TABLE 1.** Strains and plasmids used in this study and their relevant genetic properties.

| Strains                | Relevant genotype                                      | Reference |
|------------------------|-------------------------------------------------------|-----------|
| *L. lactis*            |                                                       |           |
| MG1363                 | plasmid-free strain                                    | (54)      |
| NZ9000                 | MG1363 pepN::nisRK                                     | (64)      |
| VES1842                | ponA mutant obtained by pVE1837 insertion in MG1363   | (30)      |
| MG1363acmAΔ1           | MG1363 derivative carrying a deletion in acmA          | (14)      |
| VEL1378                | MG1363 dltD::ISS1                                      | (65)      |
| VES2824                | MG1363 carrying deletion 33893 bp chromosomal fragment containing guaA gene | This work |
| VES3787                | MG1363 carrying pVES3787 (pgdA<sup>+</sup>)            | (7)       |
| VES4075                | MG1363 derivative carrying pVE3916                     | (7)       |
| VES4883                | MG1363 carrying deletion of guaA                       | This work |
| VES5160                | VEL1378 (dltD) carrying deletion of guaA               | This work |
| VES6497                | MG1363 carrying deletion of pyrB                       | This work |
| VES6530                | VES4883 (guaA) carrying deletions of pyrB              | This work |
| VES6831                | VES6497 (pyrB) carrying deletion acmAΔ1                | This work |
| Strain | Description | Source |
|--------|-------------|--------|
| VES6949 | VES6497 (pyrB) carrying mutation ponA | This work |
| VES6953 | VES6497 (pyrB) carrying pMSP3545:pyrB<sup>+</sup> (nis in-inducible pyrB) | This work |
| VES6955 | VES6497 (pyrB) carrying pLc-40 | This work |
| VES6957 | VES6497 (pyrB) carrying pMSP3545 | This work |
| VES6959 | VES6497 (pyrB) carrying pVE3916 | This work |
| VES6968 | MG1363 carrying pLc-P40 | This work |
| VES6996 | VES6497 (pyrB) carrying pVES3787 (pgdA<sup>+</sup>) | This work |

**E. coli**

| Strain | Description | Source |
|--------|-------------|--------|
| JIM4646 | TG1 with chromosomal copy of the repA gene | P. Renault, Jouy-en-Josas (66) |
| HB101  | F<sup>+</sup> mcrB mrr hsdS20 recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 glnV44 | laboratory collection |
| TG1    | F<sup>+</sup> traD36 lac<sup>F</sup> ΔlacZM15 proAB<sup>+</sup> / supE Δ(hsdM-mcrB)5 thi Δ(lac-proAB) | laboratory collection |

**Plasmids**

| Plasmid | Description | Source |
|---------|-------------|--------|
| pRV300  | Erythromycin resistant pBluescript derivative | (67) |
| pVE6007 | Replication-thermosensitive derivative of broad-host-range replicon pWV01 | (68) |
| pMSP3545| shuttle vector carrying the nisRK genes and PnisA promoter | (69) |
| pORI280 | repA-negative lacZ<sup>+</sup> derivative of pWV01 | (55,56) |
| pVE3916 | derivative of broad-host-range replicon pWV01 | T. Rochat and P. Langella (7) |
| pVES3787| pVE3916 derivative carrying pgdA gene | (7) |
| pVE1837 | pRV300 carrying 210 bp internal fragment of ponA | (30) |
| pLc-P40 | pMSP3545 carrying lcabl_00230 gene under nisin-inducible promoter | K. Regulski and M.-P. Chapot-Chartier, unpublished |
TABLE 2. Genes of VES4883 ($\Delta$guaA) and of lysozyme-treated MG1363 that were up- and down-regulated compared with those in MG1363 (WT). Only genes that were related to CW, involved in purine and pyrimidine metabolism, or whose expression was affected by more than 1.5-fold are indicated.

| Category                          | Locus   | Gene   | $guaA$ | WT/lys | Function                                                                 |
|-----------------------------------|---------|--------|--------|--------|--------------------------------------------------------------------------|
| PG synthesis                      | llmg_0511 | ponA   | 4.0    |        | penicillin-binding protein 1A                                           |
|                                  | llmg_2316 | murC   | -2.4   |        | UDP-N-acetylmuramate-L-alanine ligase                                     |
|                                  | llmg_1329 | murB   | -4.9   |        | UDP-N-acetylmuramate dehydrogenase                                       |
|                                  | llmg_2165 | acmB   | 2.8    |        | N-acetylmuramoyl-L-alanine amidase                                       |
| Teichoic acid alanylation         | llmg_1220 | dltB   | 3.4    |        | basic membrane protein                                                   |
|                                  | llmg_1219 | dltA   | 3.3    |        | D-alanine-D-alanyl carrier protein ligase                                |
|                                  | llmg_1222 | dltD   | 2.6    |        | D-alanine transfer protein                                               |
| CesSR regulon                    | llmg_1649 | cesS   | 1.9    |        | TCS sensor histidine kinase CesS                                          |
|                                  | llmg_1648 | cesR   | 1.9    |        | TCS response regulator CesR                                              |
|                                  | llmg_2164 | llmg_2164 | 3.3  |        | predicted membrane protein                                               |
|                                  | llmg_0165 | llmg_0165 | 1.8  |        | predicted membrane protein                                               |
|                                  | llmg_0169 | llmg_0169 | 3.0  |        | predicted membrane protein                                               |
|                                  | llmg_1155 | spxB   | 3.1    |        | transcriptional regulator SpxB                                             |
|                                  | llmg_1102 | llmg_1102 | 1.5  |        | predicted membrane protein                                               |
|                                  | llmg_1103 | llmg_1103 | 1.9  |        | conserved hypothetical protein                                            |
| Pyrimidine metabolism            | llmg_0890 | pyrR   | -16.1  |        | pyrimidine operon regulator PyrR                                          |
|                                  | llmg_0891 | pyrP   | -9.1   |        | uracil permease                                                          |
|                                  | llmg_0893 | pyrB   | -9.6   |        | aspartate carbamoyltransferase                                           |
|                                  | llmg_0894 | carA   | -16.6  |        | carbamoyl phosphate synthase                                              |
|                                  | llmg_0952 | pyrDA  | 6.2    |        | dihydroorotate dehydrogenase                                             |
|                                  | llmg_1105 | pyrK   | -10.6  |        | dihydroorotate dehydrogenase                                             |
|                                  | llmg_1106 | pyrDB  | -12.3  |        | dihydroorotate dehydrogenase                                             |
|                                  | llmg_1107 | pyrF   | -20.8  |        | orotidine 5'-phosphate decarboxylase                                     |
|                                  | llmg_1508 | pyrC   | -30.3  |        | dihydroorotase                                                           |
|                                  | llmg_1509 | pyrE   | -15.3  |        | orotate phosphoribosyltransferase                                         |
| Purine metabolism                | llmg_0230 | guaB   | 9.5    |        | inositol-5-monophosphate dehydrogenase                                  |
|                                  | llmg_0993 | hprT   | -6.0   |        | hypoxanthine phosphoribosyltransferase                                   |
|                                  | llmg_1008 | guaA   | 192.5  |        | GMP synthase                                                            |
|                                  | llmg_1412 | guaC   | -17.9  |        | GMP reductase                                                           |
|                                  | llmg_2201 | purA   | -4.4   | 1.9    | adenylsuccinate synthetase                                               |
**TABLE 3.** Cross-linking index of PG and relative quantities of disaccharide building subunits in *L. lactis* MG1363 (WT), VES6497 (ΔpyrB), and VES4883 (ΔguaA).

|                         | WT (OD₆₀₀ 0.5) | WT (OD₆₀₀ 1.2) | pyrB (OD₆₀₀ 0.2) | guaA (OD₆₀₀ 0.2) |
|-------------------------|----------------|----------------|------------------|------------------|
| PG cross-linking index  | 31.7           | 34.4           | 34.7             | 34.1             |
| Sum* of disaccharide peptides without Asp/Asn (%) | 4.0            | 3.5            | 1.9              | 2.1              |
| Sum* of disaccharide peptides with Asp (%)        | 29.9           | 19.9           | 24.1             | 19.3             |
| Sum* of disaccharide peptides with Asn (%)         | 66.1           | 76.5           | 74               | 78.5             |
| Ratio Asn/Asp          | 2.2            | 3.8            | 3.1              | 4.1              |

* The sum was calculated by considering disaccharide peptide building subunits constituting monomers, dimers, and trimers.
Figure 2

VES2824
MG1363
VES4883

M17G
Lys 1 mg/ml
Lys 2 mg/ml
Lys 2 mg/ml
gua 20 µg/ml
Figure 3

| Strain  | Gene(s)     |
|---------|-------------|
| VES2824 |             |
| MG1363  |             |
| WT      |             |
| VES4883 | guaA        |
| VES6497 | pyrB        |
| VES4202 | dltD        |
| VES1842 | ponA        |
| VES6530 | guaA pyrB   |
| VES5160 | guaA dltD   |
| VES6518 | dltD pyrB   |

M17G U lys 0.75 mg/ml
Figure 4

(A) Images showing the thickness comparison between WT, pyrB, WT+U, and pyrB+U. The arrow indicates the direction of measurement. The scale bar represents 50 nm.

(B) Bar chart showing the thickness measurements in nanometers. The error bars indicate the variability in the measurements.

MG1363, MG1363+U, pyrB, pyrB+U
Figure 5

MG1363  MG1363 + U  pyrB  pyrB + U

I

J

**Figure 5**

- MG1363
- MG1363 + U
- pyrB
- pyrB + U

**I**

**J**

**Force (nN)**

**Young modulus (kPa)**

**Indentation (nm)**
Figure 7
Figure 8
Figure 9