Supporting Information

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SI Text

Determination of the Relevant Threshold to Screen the Decrease of Mutants After in Vivo Challenge. To determine the relevant threshold of variation between the injected and recovered pools, we challenged a pool of 60 mutants in two rabbits and quantified these 60 tagged mutants in the injected pool and in the two recovered pools. The mean variation (the ratio of the quantity of injected mutants to the quantity of recovered mutants) was 1.13 ± 0.37 for the first rabbit and 1.01 ± 0.33 for the second. The maximal decrease was less than 2.5-fold; Fig. S2 presents the distribution of these variations. Therefore we decided to use a threefold decrease in the recovered mutants relative to injected mutants as the threshold in screening the mutant gene library.

Screening of 3,000 Unidentified, Randomly Selected Tagged Mutants in the Rabbit Ligated Ileal Loop Model. We carried out our initial mutant screening on random, unidentified mutants, considering that all mutants could be informative, whether the transposon target was a gene or an intergenic region. Indeed, intergenic regions can contain regulatory elements that also may affect bacterial adaptation. Moreover, studying mutants in intergenic regions is a way to target essential genes, because mutation in their promoter or transcriptional terminator can modify their transcription without completely knocking out function.

Forty-three pools of 70 tagged mutants were challenged in rabbit ligated ileal loops (one pool per loop, 5.10^7 cfu per loop) for a duration of 16 h. Because we anticipated that the mechanisms implicated in gut establishment are complex and multigenic, we applied a low-stringency threshold. The proportion of each mutant in the recovered pool was determined by quantitative PCR (qPCR) using the 70 different DNA tags. When the proportion of a mutant in the recovered pool was at least fivefold less or fivefold more than 1/70, we used qPCR to determine the proportion of this mutant in the injected pool. Each identified mutant was challenged again in a pool of 70 mutants, and mutants displaying at least a threefold decrease or increase in quantity between the input and output pools in the two independent screenings were selected. Several genes were identified in this screening, and all of them also were identified in the screening of gene mutants. Only two mutants in an intergenic region showed alterations in gut establishment. The first mutant was a transposon insertion in the LSEI_1600 gene transcription terminator. Because the LSEI_1600 gene already had been identified as a gut establishment gene (*SI Materials and Methods* and Table 1), the gut-sensitive phenotype of this intergenic mutant probably results from a decrease of LSEI_1600 mRNA stability. For the second mutant, the transposon was integrated in a 660-bp intergenic region between genes LSEI_0318 and LSEI_0319. This sequence does not match noncoding RNA in the Rfam database (1). This initial screening revealed that the intergenic region contributed very little to gut establishment; therefore we restricted our search to mutants in which transposons were inserted into genes.

SI Materials and Methods

Bacterial Strains and Culture Conditions. *Lactobacillus casei* CIP 107868 (ATCC 334) was grown statically in MRS medium (Difco) at 37 °C. *Escherichia coli* TG1 was grown aerobically in LB medium with shaking at 37 °C. The antibiotics used were 50 μg/mL ampicillin and 50 μg/mL erythromycin for *E. coli* and 5 μg/mL erythromycin and 5 μg/mL chloramphenicol for *L. casei*.

Genetic Manipulation. DNA manipulation, purification, ligation, restriction analysis, and gel electrophoresis were carried out as described by Sambrook et al. (2). PCR and restriction products were purified using a gel and PCR clean-up kit (Macherey Nagel). Plasmids were prepared from *E. coli* using the Nucleospin plasmid kit (Macherey Nagel). *L. casei* and *E. coli* were transformed by electroporation as described by Aukrust et al. (3) and Dower et al. (4), respectively, using a Gene Pulser and a Pulse Controller apparatus (Bio-Rad).

To proceed to signature-tagged mutagenesis (STM), 70 DNA tags previously used for *Salmonella typhimurium* STM (5), composed of invariable arms with EcoRI and HindIII restriction sites surrounding a variable region about 40-bp long, were cloned individually into the EcoRI site of pVI110 to generate 70 differently tagged transposable vectors (Fig. S1). Tag sequence was determined by sequencing (GATC Biotech) using primers PVI110TAG1 (5′-GATCAATTCTTGAAGACGAAAG-3′) and PVI110TAG2 (5′-CTGCAAGGCGATTAAGTTGG-3′) that anneal 100 nt upstream and downstream of the tag insertion site, respectively.

The tagged mutant library in *L. casei* was obtained using the P_{junc}-TpaseIS$_{1223}$ system as recently described (6). Briefly, *L. casei* first was transformed with an unstable plasmid (pVI129) carrying the IS$_{1223}$ transposase gene and then was transformed independently with the 70 tagged transposable vectors generated in this study (the integrative pVI110 plasmid carrying the transposon and an erythromycin-resistance cassette). Mutants obtained for each tagged transposable vector were ordered in plates.

Sequencing the Transposon Insertion Sites and Assembling the Library of ORF Mutants. Genomic DNA was extracted individually from each mutant using the NucleoSpin 96 Tissue kit (Macherey Nagel) with some modifications adapted for *Lactobacillus*. Each DNA extract using the primer IRL (5′-GTTGGGAGCTCTCCCATATG-3′) was sequenced to identify transposon insertion sites, and the obtained sequences were BLASTed against the *L. casei* genome. Thereafter, mutants were sorted by the localization of the transposon. The mutants in which the transposon was integrated into a gene for which a mutant had not already been found were reassembled in pools. Because all mutants in the pools were tagged differently, the number of mutants in each pool ranged from 25 to 70. When there were fewer than 40 mutants in a pool, intergenic region mutants were added to attain the 40 mutants needed to maintain sufficient competition between mutants.

Inoculation of Rabbit Ligated Ileal Loops. Mutants were grown individually in 96-well microplates, assembled in pools, washed with PBS, and resuspended in PBS to a final OD_{600} of 0.1. They were challenged in rabbit ligated ileal loops as previously described (7, 8) with the following modifications. Between 10 and 12 ileal loops were made in each rabbit. In each loop, 0.5 mL of bacterial suspension was injected (corresponding to a total bacterial quantity of 5×10^7 cfu per loop). Challenges were carried out over 16 h. Rabbit were killed, and each loop was removed integrally by severing the spacers just outside the ligatures and was transferred into cold PBS. The whole intestinal loop containing the bacteria then was homogenized by bead-beating using 2.8-mm ceramic beads in a Precellys 24 apparatus. The resulting homogenate (about $5 \geq 10^7$ living *L. casei* bacteria per loop) was diluted and spread on agar plates to obtain isolated colonies. Each pool was screened in two different rabbits, and the position of injected pools was randomized. This in vivo model protocol was approved

by the Comité Regional d'Ethique pour l'Experimentation Animale in Paris 1 (protocol no. 20070004).

Mutant Quantification After in Vivo Challenge. Genomic DNA was isolated from injected and recovered pools. For each injected pool, 5 mL of cell culture was used. For each recovered pool, 5,000–10,000 colonies were recovered by washing plates with TE buffer (5 mM Tris, 2 mM EDTA). Then the same DNA isolation procedure was applied. Bacterial suspensions were washed with TE buffer and centrifuged. Bacterial pellets were incubated for 1 h at 65 °C in 500 μL lysis buffer (TE buffer, 1% SDS, 0.4 mg/mL proteinase K). The obtained cell suspensions were mixed with 0.7 mL phenol:chloroform:isoamyl alchool at a ratio of 25:24:1 and with 0.1-μm glass beads and were disrupted by bead-beating using one treatment of 40 s at a speed of 6.5 in a FastPrep reciprocating device (Qbiogene). DNAs were isolated from the aqueous phase by two phenol-chloroform extractions, followed by DNA precipitation in ethanol and washing in 70% ethanol. DNA pellets were dissolved in 200 μL TE buffer.

qPCR was used to measure the proportion of each mutant in the pools. Based on the tag sequences (Table S3), primers used for qPCR were designed using the Primer3 software (Table S4). The first primer corresponded to an invariable pVI110 region and was common to the 70 primer couples. The second primers were designed on DNA tags to generate a 105- to 125-bp amplicon when associated with the first primer (Fig. S2). qPCR amplifications were carried out on a Bio-Rad iCycler. Each 20-μL reaction contained diluted isolated DNA, 0.25 μM of each primer, and 10 μL iQ SYBR Green Supermix (Bio-Rad). The QPVITAGR primer was associated with a tag-specific primer (Table S4) to create a specific primer couple for the enumeration of each tag. The 70 tagged transposable vectors were pooled at equimolar concentration, and serial dilutions of this mix were used as the matrix for qPCR with each primer couple to check the correlation with the initial DNA concentration. Four sets of six randomly selected tagged transposable vectors were diluted in different known concentrations and subjected to real-time quantification. For these 24 tagged transposable vectors, relative quantification by real-time PCR correlated with the concentration, confirming that amplifications were tag-specific. Primer couples CRTL3F-CTRL3R and CTRL4F-CTRL4R, designed on the tagged-pVI110 invariable region, were used for each mutant pool for global enumeration of all tags. Tags were quantified using comparative quantification (Cq) methods. The mean Cq values obtained for the CRTL3F-CTRL3R and CTRL4F-CTRL4R amplicons were used to determine the ratio of each tag to the complete set of tags in the injected and recovered pools. The decreased rate for each mutant corresponds to the ratio of the corresponding tag in the injected pool divided by the ratio in the recovered pool.

Bioinformatics. Gene annotations were found using the Integrated Microbial Genomes site (9) based on the analysis by Makarova et al. (10). Gene function predictions were made using the Kyoto Encyclopedia of Genes and Genomes (11) and Biocyc (12) databases.

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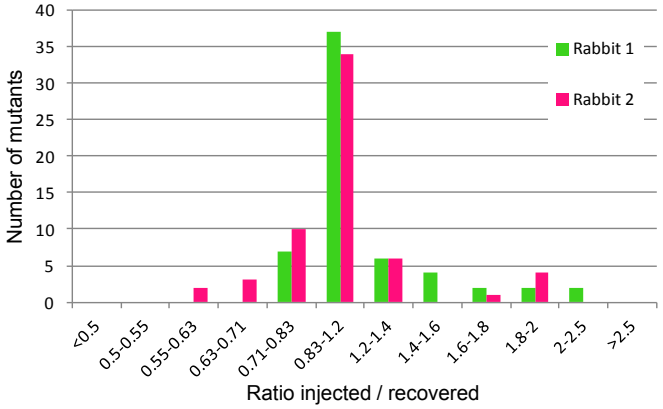

Fig. S1. Distribution of variation in the ratio of the relative quantity of each mutant in the injected pool vs. the recovered pool. Analysis was made from one pool of 60 mutants injected in two rabbits.

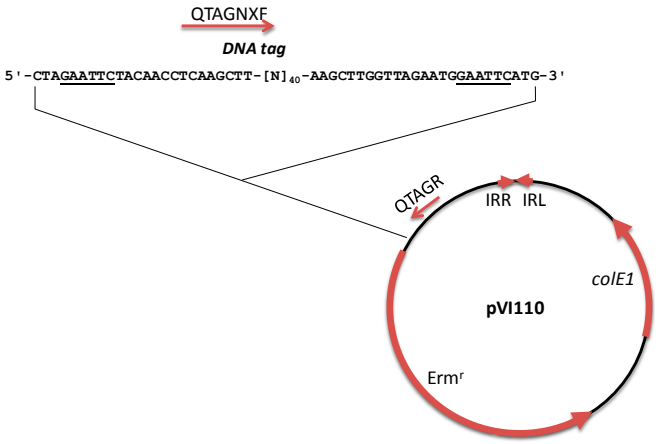

Fig. S2. Construction of tagged pVI110.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)