Reports

PCR-based screening of targeted mutants for the fast and simultaneous identification of bacterial virulence factors

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Understanding the strategies used by pathogens to infect, survive, and proliferate in their hosts requires the identification of virulence factors. We developed PCR-based screening of targeted mutants to facilitate quick, simultaneous detection of multiple novel bacterial virulence genes. Based on direct PCR screening of pooled targeted mutants, this approach provides a fast and sensitive measure of virulence attenuation while significantly reducing the number of animals and time required. We demonstrate that the careful design of gene-specific primers allows the direct relative quantification of mixed mutants in infected mouse organs. Indeed, we show that the band intensity of the PCR product is directly related to the quantity of the corresponding strain in a pool of mutants. We applied the PCR-based screening of targeted mutants to the murine model of listeriosis and revealed new genes required for full pathogenicity of *Listeria monocytogenes*, a facultative human intracellular pathogen. PCR-based screening is a simple, useful, and fast technique to test pools of targeted bacterial mutants *in vivo*, without the requirements for a rigorous purification step, complicated PCR set-up, or special equipment. This approach can be adapted to other bacterial systems, constituting a significant advance in the field of infection biology.

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Virulence genes encode products necessary for the entry, survival, or persistence of pathogens within their hosts (1). The identification of virulence determinants is thus required to understand the mechanisms used by bacterial pathogens to establish infection. A number of different strategies have been applied to identify virulence-related genes. The construction of gene disruption mutants and their individual *in vivo* phenotype analysis is a common approach for the functional characterization of targeted genes (2,3). However, when applied to a large number of genes, this approach is time and animal consuming. Random mutagenesis strategies like signature-tagged mutagenesis (STM) (4) are

useful approaches for large-scale screenings of mutants *in vivo*, but cannot be used to target predefined genes. A multiple competitive index assay was previously developed (5), but it only allows the simultaneous analysis of a maximum of four strains and requires the additional integration of site-specific vectors that prevents future mutant complementation by integrative vectors. Other techniques were also recently applied to follow the *in vivo* behavior of diverse pathogens (6,7). Although these strategies allowed important advances in the understanding of infectious processes, all are complex and involve an important development step. PCR is a useful technique that can be directly applied to different

templates such as bacteria. This approach was previously adapted to screen pools of STM mutants (8) and then successively used for other STM screens (9,10). However, PCR-based screening has never been applied to pools of targeted mutants and was previously used only to discriminate between the presence and absence of a mutant in a pool without relative quantification.

Our aim was to develop a PCR-based screening of targeted mutants to (i) achieve a fast and simple characterization of predefined target genes, while reducing and refining the use of animals; (ii) allow the simultaneous analysis of selected bacterial mutants in a single group of mice by direct PCR on bacteria; and (iii) correlate the

Method summary:

To allow the fast and simultaneous detection of novel bacterial virulence genes, we developed PCR screening-based targeted mutagenesis. We show that the careful design of gene-specific primers allows the direct relative quantification of mixed mutants in infected mouse organs by direct PCR screening of pooled bacterial mutants. This approach provides a sensitive measure of virulence attenuation that requires less time and fewer animals than alternative procedures, and does not require rigorous DNA purification, complicated PCR set-up, or special equipment.

PCR product to the quantity of the corresponding strain in a pool. In addition, our purpose was to design the simplest, most useful, and fastest technique to test pools of targeted bacterial mutants *in vivo*, without rigorous purification steps, complicated PCR set-up, or special equipment.

Our general approach (Figure 1) consists of individually culturing bacterial mutants for selected genes. Individual cultures were then mixed in equal amounts to constitute the input pool, which was inoculated to a single group of mice. Afterward, bacteria were recovered from infected mouse organs (output pool). The relative abundance of each mutant in the output pool was calculated and compared with the input pool by direct conventional PCR using bacterial cells as the DNA template. The design of sequence-specific primers is critical for this approach. Indeed, the primers should have similar compositions and melting temperatures to simplify mutant comparison in a single PCR run. Primers were designed following these simple rules: (a) avoid cross homology with other sequences in the genome; (b) the primer length should be between 19 and 22 nucleotides; (c) GC content should be around 50%; (d) the melting temperature should be around 60°C; (e) a GC clamp should be introduced at the 3' end of primers but avoiding runs of more than three G/C; and (f) avoid complementary sequences within and between primers and avoid repeats.

Using the Gram-positive facultative intracellular pathogen *Listeria monocytogenes* (*Lm*) as an infection model, we showed that the careful design of gene-specific primers allowed the direct relative quantification of mixed selected mutants in infected mouse organs. In addition, we demonstrated that PCR-based screening of targeted mutants could be used not only with tagged insertion mutants, but also with deletion mutants. This approach provides a fast and sensitive measure of virulence attenuation while significantly reducing the time and number of animals required.

Materials and methods

Bacterial strains and media

Lm EGDe (ATCC BAA-679) was used. *Listeria* were grown in BHI and *Escherichia coli* in LB medium at 37°C with shaking. When required, the following antibiotics were used: Chloramphenicol 7 µg/mL for *Listeria* and 20 µg/mL for *E. coli*; Erythromycin 5 µg/mL for *Listeria* and 150 µg/mL for *E. coli*; Ampicillin 100 µg/mL for *E. coli*.

Figure 1. The strategy for PCR-based screening of targeted mutants. Individual cultures of bacterial mutants for selected genes are mixed in equal amounts to constitute the input pool, which is inoculated into a single group of mice. Afterward, bacteria are recovered from infected mouse organs. The relative abundance of each mutant in the output pool is calculated and compared with the input pool by direct conventional PCR using bacterial cells as the DNA template. In this example, two mutants are absent from the output pool and thus considered as attenuated in virulence.

Mutagenesis

Insertional mutagenesis was performed using the pAUL-A vector as described (11). Primers contained a 5'-end specific tag carrying the sequences previously described (8). Gene deletions were performed using pMAD vector as described (12,13). Insertions and deletions were verified by PCR and DNA sequencing.

Preparation of input pools

Individual overnight cultures of mutants were subcultured in 50 mL of BHI (or BHI supplemented with erythromycin for insertion mutants), grown until $OD_{600nm} = 0.6$, and mixed in equal volume. Equal CFU numbers of the individual cultures were confirmed by plating. The mixed culture was centrifuged at 4°C, washed in cold PBS, and re-suspended in 3 mL PBS.

In vivo infection

Oral infections were performed as described (14). 5×10^9 CFU in PBS with 150 mg/mL $CaCO_3$ were delivered intragastrically to six-week-old specific-pathogen-free female BALB/c mice, which were starved for 12 h (5 mice/group). At 72 h post-infection,

livers and spleens were recovered and CFU numbers were determined by plating organ homogenates.

Animal experiments were performed in strict accordance with the IBMC guidelines for laboratory animal husbandry. The protocol was approved by the department for animal health and protection of the Portuguese Agriculture Ministry (Permit Number: 0420/000/000/2007).

Mutant screening by PCR

PCR screening was performed using the primers shown in Table 1. For input pools, PCR reactions were performed using a 1/10 dilution of the pool suspension as the template. For output pools, mouse organs were recovered 72 h post-infection, homogenized, and plated on BHI. After overnight incubation, total colonies were re-suspended in 1 mL sterile PBS and PCR was performed using a 1/10 dilution as the template. PCR amplifications were performed according to a protocol that comprised an initial denaturation step at 94°C for 5 min, followed by 25 cycles of 94°C (30 s), 55°C (30 s), and 72°C (30 s), and a final step at 72°C for 5 min.

Figure 2. Generation of a pool of tagged insertion mutants and assessment of the PCR screening sensitivity and confidence. (A) PCR amplification products obtained using tag- and gene-specific primers on an input pool of tagged insertion mutants. We tested if the band intensity measure was independent of the amount of each strain mixed in equal quantity. Values are relative to positive control arbitrarily fixed to 100%. (B) Correlation between the proportion of a mutant in a pool and intensity of its corresponding PCR product. The quantity of the TM3 mutant was artificially decreased (from 1/6 to 1/6000) in the input pool while the other mutants were maintained at the proportion 1/6. We tested if the differences in band intensity were independent of the relative quantity of the TM3 mutant in the pool. Values are relative to TM3 (1/6) band intensity arbitrarily fixed to 100%. (C) Effect of the variation of the quantity of the TM3 mutant on the band intensity of TM+. We tested if the band intensity obtained for TM+ was independent of its fixed amount when mixed with increasing quantity of TM3. Values are relative to TM3 1/6 arbitrarily fixed to 100%. (D) Direct correlation between band intensity and CFUs in an input pool. PCR amplification products obtained from wild type (WT) and tagged insertion mutants in input pools, using tag- and gene-specific primers (upper panel). Values are relative to WT arbitrarily fixed to 100%. CFUs were obtained after differential plating of inputs on BHI and BHI+antibiotic (lower panel). (E) Relative quantity of WT and insertion mutants (TM- and TM2) in livers and spleens of a single group of five BALB/c mice 72 h after oral infection with a pool containing 5×10^9 total bacteria per mouse. The proportion of each mutant was estimated in the output pool by PCR screening and band quantification (upper panel). Values are relative to positive controls arbitrarily fixed to 100%. In parallel, serial dilution of organ homogenates were plated on BHI and BHI+antibiotic to determine CFUs (lower panel). Results are means of three independent quantifications. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

These conditions were determined to allow a semi-quantitative measure of relative differences in template between samples by endpoint PCR, ensuring that saturation had not been reached. To ensure that the reaction was within the exponential phase of amplification, samples were amplified with increasing numbers of cycles and template concentrations. For insertional mutants, each tag-specific primer was used in combination with a primer specific to the gene in which the tag was inserted to generate ≈ 400 bp products. For deletion mutants, pairs of gene-specific primers were designed to amplify a ≈ 400 bp fragment mapping the scar resulting from the deletion of each gene. PCR products were analyzed on 1% agarose gel. Band quantification was performed using QuantityOne software (Bio-Rad, Hercules, CA, USA). The relative band intensity was calculated using the formula: $\% \text{band intensity} = ((S_o/S_i)/(C_o/C_i)) \times 100$. (S_o = Sample band intensity Output; S_i = Sample band intensity Input; C_o = Positive control band intensity Output; C_i = Positive control band intensity Input). Results represent means of at least three independent experiments.

Statistics

Student's *t*-test was used and *P*-values were considered as: $P > 0.05$ = Not significant; $P < 0.05$ = significant(*); $P < 0.01$ = Very significant(**); $P < 0.001$ = Extremely significant(***)

Results and discussion

Generation of a pool of tagged insertion mutants

We first tested the feasibility of our approach using tagged insertion mutants. To create an input pool of targeted tagged insertion mutants, a collection of six unique 21-mers was synthesized (pTMF primers, Table 1) and used to tag selected genes by insertional mutagenesis. The tags were previously designed to have an invariable region for PCR optimization and a variable region for specific amplification while preserving the same melting temperature (8). A tagged mutant for a major *Lm* virulence factor was constructed (TM-) and used as strongly attenuated mutant (negative control). A tagged mutant for a gene previously shown to be irrelevant to virulence (TM+) was used as a non-attenuated strain (positive control). In addition, four other genes with unknown function in virulence were mutated by insertional mutagenesis to complete the input pool (TM1-TM4). To have every mutant equally represented in the input pool, individual cultures were grown until exponential phase and mixed

in equal quantities as verified by CFU determination. This strategy overcomes possible growth effects of specific mutants in mixed cultures that would result in their underrepresentation in the pool. The equal representation of each mutant in the input pool was analyzed by PCR using tag-specific primers in combination with target gene-specific primers designed as described above (Table 1). After PCR and band quantification, the relative band intensity was calculated, verifying the equal abundance of each mutant in the pool (Figure 2A). In addition, the absence of cross-amplification from different tagged mutants was confirmed by sequencing the unique DNA amplification product observed for each tag.

Determination of the sensitivity and reliance of the PCR screening

To further confirm that the PCR band intensity can be directly related to the quantity of the corresponding mutant in a pool and to determine the detection limit of PCR screening, we assessed the sensitivity of our method. The quantity of one tagged mutant (TM3) was artificially decreased (from 1/6 to 1/6000) in the pool while maintaining other mutants at the 1/6 proportion. After PCR, we observed a dose-dependent decrease in the intensity of the band corresponding to TM3. Each 10-fold dilution resulted in a $\approx 30\%$ reduction of the corresponding band intensity (Figure 2B). In addition, the variation of the proportion of TM3 did not affect the band intensity corresponding to another mutant (TM+) in the pool (Figure 2C).

Results indicate that the band intensity can be directly related to the amount of the corresponding strain and showed that the variation of the quantity of one mutant did not affect the relative quantities of other strains in the pool. However, quantifications based on PCR as performed here are only semiquantitative. Also, results obtained using this approach are an indication of a mutant being attenuated rather than an exact quantification of its growth attenuation.

PCR-based screening of targeted mutants: detection of new virulence genes

To refine the *in vivo* identification of bacterial virulence factors, we developed an approach for the simultaneous analysis of multiple targeted mutants (Figure 1). The pool of insertion mutants described above was used for *in vivo* challenge of mice. The relative quantity of each mutant in mouse liver and spleen was assessed after oral infection. Three days post-infection, a time point that—under these experimental conditions—corresponded to the peak of the infection, organs were recovered, homoge-

Table 1. Primers used for PCR screening.

Primer name	Sequence (5'→3')
pTM1F	gtaccgcgcttaagcattgtg
pTM1R	gttgtgtaggtgcctgttgcg
pTM2F	gtaccgcgcttaataacgtgg
pTM2R	gtctctaaaaccgcgtcttcac
pTM3F	gtaccgcgcttaaaagctcgc
pTM3R	actgccagcaattgccggac
pTM4F	gtaccgcgcttaaatagcctg
pTM4R	caccagtacgacctgatgcg
pTM-F	gtaccgcgcttaatacagacg
pTM-R	ccgcaaatagagccaagcttcc
pTM+F	gtaccgcgcttaactagtagg
pTM+R	ggatgcaaatcccattgtccg
pDM1F	gtcagccagcactcattgg
pDM1R	ggatgtcccaacagaaatagc
pDM2F	aacgtgttggtgcgtacttcgg
pDM2R	cggcatcatgtaacttcattcc
pDM3F	ctcagggtgaagaacatcgc
pDM3R	ctagatttaccactagacgac
pDM-F	gcgtgactttcttcaacagc
pDM-R	gctggaacaatcacacttgc
pDM+F	gacaattcagcgagaacc
pDM+R	cacttggtgatagcaatttagc

nized, and plated on BHI. Total colonies were re-suspended in PBS to constitute the output pool. The proportion of each tagged mutant in the output pool was estimated by PCR screening and band intensity quantification. The relative quantity of each mutant corresponded to the ratio of band intensities obtained from the output vs. input pools, relative to the positive control. The formula used (see Materials and methods) allowed the correction of intensity variations between mutant bands resulting from slight differences in oligonucleotide efficiencies. As expected, both in livers and spleens, TM- was nearly undetected as compared with TM+ (Figure 3A). These results validate our approach and confirm that PCR screening easily identifies virulence mutants. Interestingly, we also observed that, after oral inoculation, TM2 appeared underrepresented (decrease in band intensity $>30\%$) in both organs (Figure 3A). These results indicate a new gene implicated in *Listeria* pathogenicity and show the potential of the PCR-based screening of targeted mutants.

To definitively demonstrate the direct correlation between PCR detection and colony forming units (CFUs) of a specific mutant within a pool, we performed mixed infections using pools composed of the wild

Figure 3. PCR-based screening of targeted mutants: detection of new virulence genes. Relative quantity of each insertion (A) and deletion (B) mutant in livers and spleens of a single group of five BALB/c mice 72 h after oral infection with a pool containing 5×10^9 total bacteria per mouse. The proportion of each mutant was estimated in the output pool by PCR screening and band quantification and compared with the input pool. TM- and DM- were used as attenuated (negative) controls, and TM+ and DM+ were used as non-attenuated (positive) controls. Values are relative to positive controls arbitrarily fixed to 100%. Results are means of three independent experiments. (C) Bacterial counts of wild type (WT), DM+, DM2, TM2, and DM1 strains in the liver and spleen of BALB/c mice 72h after oral inoculation with individual mutants (5×10^9 bacteria per mouse). Values relative to WT arbitrarily fixed to 100%. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

type (WT) strain and an insertion mutant carrying an antibiotic resistance for differential selection. Two different pools were produced: (i) a pool containing the WT strain and an insertion mutant for a major *Lm* virulence factor highly attenuated in virulence (TM-) and (ii) a pool containing the WT strain and an insertion mutant moderately attenuated in virulence (TM2). The presence of equal quantities of both strains in each pool was verified by PCR and by plating on BHI and BHI with antibiotics. Results obtained by both techniques confirmed the equal quantity of each strain in input pools (Figure 2D). These two input pools were used for oral inoculation of BALB/c mice. Three days post-infection organs were recovered, homogenized, and plated on BHI. Total colonies were re-suspended in PBS to constitute the output pool. The proportion of each tagged mutant in the output pool was estimated by PCR screening and band intensity quantification. In parallel, serial dilution of organ homogenates were plated on BHI and BHI with antibiotics to determine CFUs. Results clearly confirmed a direct correlation between the intensity of the bands observed by PCR screening and the real number of bacteria in infected mouse organs (Figure 2E).

PCR-based screening of targeted deletion mutants

Insertion mutants are faster to obtain than deletion mutants but can have disadvantages (such as polar effects). Therefore, we tested the feasibility of our approach using targeted deletion mutants and pairs of gene-specific primers designed to have similar melting temperatures. The pool of deletion mutants included a negative control strain (DM-) carrying a deletion mutation for a major *Lm* virulence factor, and a positive control strain (DM+) carrying a deletion of a gene not required for virulence. The input pool, containing deletion mutants for three additional genes with unknown function in virulence (DM1-DM3), was produced and verified as described above. The relative presence of each strain in the liver and spleen of infected mice was monitored 72h after oral infection. The proportion of each mutant was estimated in the output pool compared with the input pool after PCR screening and calculation of relative band intensities. DM- was, as expected, undetectable in both organs (Figure 3B). For DM1, we also observed a dramatic reduction in band intensity in all conditions, indicating that DM1 is also highly attenuated (Figure 3B). In addition, DM2 showed a relative decrease of more than 30% in both organs (Figure 3B). Together, these results demonstrate the feasibility

and potential of the PCR-based screening of targeted mutants using deletion mutants for the identification of genes implicated in virulence.

Confirmation of virulence defects by individual mutant testing

To validate our approach, virulence-attenuated mutants identified by PCR-based screening were individually tested *in vivo*. BALB/c mice were infected with *Lm* WT, DM+, TM2, DM1, and DM2. The number of bacteria in mouse organs was monitored 72h after individual oral inoculation. TM2, DM1, and DM2 showed virulence attenuation in mouse organs (Figure 3C) that largely corroborate virulence reductions observed by PCR-based screening (Figure 3A-3B). Results obtained by individual testing and by PCR-based screening are comparable not only in terms of quality (attenuated or not in mice), but also regarding the attenuation level. The individual characterization of mutants confirmed their virulence phenotype, validating the PCR-based screening of targeted mutants as a powerful method for the rapid identification of genes required for full pathogenesis.

As a proof of concept, we used pools of up to six strains. Theoretically, the number of mutants is only limited by the ability to produce the expected and specific PCR fragment. However, oligonucleotides should be carefully designed to avoid cross-amplification, which could be difficult for highly complex templates. Besides PCR-screening associated limitations, other issues need to be considered. The animal model might limit the complexity of the inoculum and increasing pool complexity will reduce the respective quantity of each mutant. Thus, in a complex pool with several attenuated mutants, the inoculum could be insufficiently virulent, resulting in the elimination of all of the bacteria by the host. Moreover, the use of highly complex pools may exacerbate technical limitations. For example, a secreted molecule may complement a virulence defect of another strain in the pool or the route of infection may be hijacked by a defective competing strain.

The possibility of using a small number of animals to screen large numbers of mutants is important economically and ethically (15). The individual analysis of the virulence potential of five mutants currently requires the use of at least 30 mice. To test the same number of mutants, our approach only involves five mice, representing a reduction of more than 80% in animals, time, and financial resources. In addition, intra-animal experiments minimize inherent inter-animal variations and improve the identification of

mutants with reduced competitive fitness within the host.

The advantage of the PCR-based screening of targeted mutants over conventional random mutagenesis techniques (4) is the ability to target specific genes. In addition, we showed that this technique could be used not only with insertion but also with deletion mutants, usually preferred in order to avoid insertional mutagenesis pitfalls. Even if it was not the original purpose of this study, it would be interesting to apply the method to insertion and deletion mutants for the same gene and analyze results in separate assays to rule out any differences between the two approaches. This would also support the validity of the assay in reproducibly identifying attenuations. Nonetheless, we are aware of the technical limitations of our approach. In particular, the use of purified bacterial genomic DNA instead of direct PCR on bacteria, as well as analysis by quantitative PCR, would improve the accuracy and sensibility of the method. However, our purpose was to design the simplest technique to test pools of bacterial mutants *in vivo*. We demonstrated here that PCR-based screening, without a rigorous purification step, complicated PCR set-up, or special equipment, is able to allow an easy and simultaneous detection of mutants impaired in virulence.

We used mutants for a major *Listeria* virulence factor to validate the efficiency of the *in vivo* selection and showed that highly attenuated mutants are easily detected by PCR-based screening. PCR-based screening allowed the detection of mutants with only one log decrease in virulence, which corresponds to the minimal decrease generally considered as a virulence attenuation (14). In addition, we observed a good correlation between the level of virulence attenuation measured by PCR-based screening and individual *in vivo* infection, thus definitively establishing the value of our strategy.

We chose to recover mouse organs 72h post infection at the peak of the infection. Since virulence factors can be expressed and active at different time points of the infection, in different organs, and following different routes of inoculation (16), the technique described here can be applied to bacterial mixtures extracted from various mouse organs, over the time course of the infection, and following diverse routes of inoculation.

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Competing interests

The authors declare no competing interests.

References

1. Wassenaar, T.M. and W. Gaastra. 2001. Bacterial virulence: can we draw the line? *FEMS Microbiol. Lett.* 201:1-7.
2. Cabanes, D., O. Dussurget, P. Dehoux, and P. Cossart. 2004. Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence. *Mol. Microbiol.* 51:1601-1614.
3. Cabanes, D., S. Sousa, A. Cebria, M. Lecuit, F. Garcia-del Portillo, and P. Cossart. 2005. Gp96 is a receptor for a novel *Listeria monocytogenes* virulence factor, Vip, a surface protein. *EMBO J.* 24:2827-2838.
4. Hensel, M., J.E. Shea, C. Gleeson, M.D. Jones, E. Dalton, and D.W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. *Science* 269:400-403.
5. Monk, I.R., P.G. Casey, M. Cronin, C.G. Gahan, and C. Hill. 2008. Development of multiple strain competitive index assays for *Listeria monocytogenes* using pIMC; a new site-specific integrative vector. *BMC Microbiol.* 8:96.
6. Blumenthal, A., C. Trujillo, S. Ehrh, and D. Schnappinger. 2010. Simultaneous analysis of multiple *Mycobacterium tuberculosis* knockdown mutants in vitro and in vivo. *PLoS One* 5:e15667.
7. Melton-Witt, J.A., S.M. Rafelski, D.A. Portnoy, and A.I. Bakardjiev. 2011. Oral infection with signature-tagged *Listeria monocytogenes* reveals organ-specific growth and dissemination routes in guinea pigs. *Infect. Immun.* 80:730-732.
8. Lehoux, D.E., F. Sanschagrín, and R.C. Levesque. 1999. Defined oligonucleotide tag pools and PCR screening in signature-tagged mutagenesis of essential genes from bacteria. *Biotechniques* 26:473-478, 480.
9. Winstanley, C., M.G. Langille, J.L. Fothergill, I. Kukavica-Ibrulj, C. Paradis-Bleau, F. Sanschagrín, N.R. Thomson, G.L. Winsor, et al. 2009. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*. *Genome Res.* 19:12-23.
10. Porvin, E., D.E. Lehoux, I. Kukavica-Ibrulj, K.L. Richard, F. Sanschagrín, G.W. Lau, and R.C. Levesque. 2003. In vivo functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets. *Environ. Microbiol.* 5:1294-1308.
11. Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans. 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. *J. Bacteriol.* 174:568-574.
12. Reis, O., S. Sousa, A. Camejo, V. Villiers, E. Gouin, P. Cossart, and D. Cabanes. 2010. LapB, a novel *Listeria monocytogenes* LPXTG surface adhesin, required for entry into eukaryotic cells and virulence. *J. Infect. Dis.* 202:551-562.
13. Arnaud, M., A. Chastanet, and M. Debarbouille. 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl. Environ. Microbiol.* 70:6887-6891.
14. Cabanes, D., M. Lecuit, and P. Cossart. 2008. Animal models of *Listeria* infection. *Curr. Protoc. Microbiol. Chapter 9:Unit9B* 1.
15. Nature-Immunology-Editorial. 2010. Reduce, refine, replace. *Nat. Immunol.* 11:971.
16. Camejo, A., C. Buchrieser, E. Couve, F. Carvalho, O. Reis, P. Ferreira, S. Sousa, P. Cossart, and D. Cabanes. 2009. In vivo transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. *PLoS Pathog.* 5:e1000449.

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