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Expression of chitinases in *Listeria monocytogenes* is influenced by *lmo0327* that encodes an internalin-like protein.

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Running Head: *lmo0327* affects expression of listerial chitinases

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Abstract: The chitinolytic system of *Listeria monocytogenes* thus far comprises two chitinases, ChiA and ChiB, and a lytic polysaccharide monoxygenase, Lmo2467. The role of the system in the bacterium appears to be pleiotropic, as besides mediating hydrolysis of chitin, the second most ubiquitous carbohydrate in nature, the chitinases have been deemed important for colonization of unicellular moulds, as well as mammalian hosts. In order to identify additional components of the chitinolytic system, we screened a transposon mutant library for mutants exhibiting impaired chitin hydrolysis. The screening yielded a mutant with a transposon insertion in a locus corresponding to *lmo0327* of the EGD-e strain. *lmo0327* encodes a large (1349 aa) cell-wall associated protein that has been proposed to possess murein hydrolase activity. Single inactivation of *lmo0327*, as well as of *lmo0325* that codes for a putative transcriptional regulator functionally related to *lmo0327*, led to an almost complete abolishment of chitinolytic activity. The effect could be traced at the transcriptional level, as both *chiA* and *chiB* transcripts were dramatically decreased in the *lmo0327* mutant. In accordance with that, we could barely detect ChiA and ChiB in the culture supernatants of the mutant strain. Our results provide new information regarding the function of the *lmo0325*- *lmo0327* locus in *L. monocytogenes* and link it to the expression of chitinolytic activity.

**Importance:** Many bacteria from terrestrial and marine environments express chitinase activities enabling them to utilize chitin as the sole source of carbon and nitrogen. Interestingly, several bacterial chitinases may also be involved in host pathogenesis. For example, in the important food borne pathogen *Listeria monocytogenes*, the chitinases ChiA and ChiB, and the lytic polysaccharide monoxygenase Lmo2467 are implicated in chitin assimilation, but also act as virulence factors during infection of mammalian hosts. Therefore, it is important to identify their regulators and induction cues in order to understand how the different roles of the chitinolytic system are controlled and mediated. Here we provide evidence for the importance of *lmo0327* and *lmo0325*,
encoding a putative internalin / autolysin and a putative transcriptional activator, respectively, in the efficient expression of chitinase activity in L. monocytogenes, and thereby provide new information regarding the function of the lmo0325-lmo0327 locus.

INTRODUCTION

Chitin is an insoluble aminopolysaccharide, comprised of repeated alternating N-acetylglucosamine (GlcNAc) units, and present in abundance in marine and soil environments (1). In these environments, chitin can constitute an important carbon and nitrogen nutrient source (2–4) and in accordance with that a number of bacteria have been found to be chitinivorous (4, 5). Central to the scavenging and catabolism of chitin are chitinases and associated chitin-binding proteins/lytic polysaccharide monooxygenases (LPMOs), which coordinately allow the bacteria to degrade the chitin to assimilative chitooligosaccharides, including the N,N'-diacetylchitobiose [(GlcNAc)_2] dimer and the GlcNAc monomer. Chitinases and LPMOs have recently been recognized as enzymes of high biotechnological potential (6–12). In addition, chitinases and LPMOs have been, rather surprisingly, implicated in the infectious processes of several chitinolytic pathogenic bacteria, where they act as virulence factors (reviewed in (13)). The two-fold significance of chitinolytic systems, has led to increased efforts to describe and characterize these systems, their individual elements, as well as their mode of regulation.

In Vibrios dozens of proteins have been found to be involved in chitin sensing, chemotaxis, degradation and assimilation (2, 14–18). In terms of degradation, these include extracellular enzymes, such as chitinases (16, 19) and the GlcNAc-binding protein GbpA (20), as well as periplasmic hydrolases that participate in the degradation of the oligosaccharides to the dimeric and monomeric subunits (21, 22). Components responsible for recognition of chitin and subsequent
induction of the chitinolytic system include a chitooligosaccharide-specific porin (23–25), encoded by \textit{chiP}, as well as ChiS, a hybrid sensor kinase that is proposed to respond to chitooligosaccharides introduced into the periplasmic space through the action of ChiP and other porins (14).

\textbf{ChiP, ChiS, and respective ChiR response regulator-like components have been identified in other Gram-negative chitinolytic bacteria as well, such as \textit{Serratia marcescens} (26, 27), \textit{Escherichia coli} (28) and \textit{Salmonella Typhimurium} (29). Thus, it is possible that these sensing and regulatory elements are commonly used by chitinolytic Gram-negative bacteria in terms of chitin response.}

In contrast, the periplasmic localization of some of these elements, and the general role of the periplasmic space in the two-step (extracellular and periplasmic) degradation of chitin in Gram-negative bacteria, imply that the chitin response should be structurally different in their Gram-positive counterparts. Interestingly, a ChiSR two component system involved in chitin response has also been described in the Gram-positive \textit{Streptomyces} (30–32). To our knowledge, no analogues of ChiP have been described in Gram-positive bacteria so far. In general, little is known about the regulators of Gram-positive chitinolytic systems, although a number of Gram-positive chitinases and LPMOs have been identified and characterized (see for example (33–36)).

\textit{Listeria monocytogenes} is a Gram-positive food-borne pathogen that can cause listeriosis, a rare but potentially fatal disease. \textit{L. monocytogenes} is chitinolytic (37), and its system is one of the best-characterized Gram-positive systems so far. Chitin hydrolysis relies on two chitinases, ChiA and ChiB, as well as an LPMO (Lmo2467) (37–39). These proteins have pleiotropic roles, contributing also to colonization of eukaryotic unicellular and/or mammalian hosts (40–42). Expression of the two chitinases depends on the positive effects of the major \textit{L. monocytogenes} regulators \(\sigma^B\) and PrfA, as well as the \textit{agr} system (43, 44). At the same time, the small RNA LhrA exerts an inhibitory effect on the translation of the \textit{chiA} mRNA (45). Hfq and Lmo0106 appear to also play as yet undescribed roles in the regulation of chitinolytic activity (43, 45).
In this study, we employed a transposon screening system to identify novel components of the listerial chitinolytic system, or regulators of chitinase activity. Our screen yielded \textit{lmo0327} as a gene essential for efficient expression of the chitinases. We additionally investigated the related putative transcriptional regulator Lmo0325, and found \textit{lmo0325} to be also necessary for effective chitin hydrolysis.

**MATERIALS AND METHODS**

**Bacterial strains and standard growth conditions**

The \textit{L. monocytogenes} EGD wild type and its mutant derivative with a pAUL-A (Erm^R) insertion in \textit{lmo0327} were provided by Magdalena Popowska (46). The N53-1 strain was obtained from Lone Gram (47).

A mutant with a pAUL-A insertion in \textit{lmo0325} was constructed in the same way as the \textit{lmo0327} mutant, using the primer set 5’TTGGATTGATTTCGTCGAGCTATC3’ and 5’CCACTTCGCTTTCTTCATCCA3’ (46). The non-polar effects of the insertion on \textit{lmo0326} and \textit{lmo0327} were confirmed by RT-PCR, as described previously (46).

Bacteria were routinely grown aerobically in brain heart infusion (BHI, Oxoid) at 37°C, unless stated otherwise.

In order to draw growth curves of the wild-type and mutant strains, the strains were first grown aerobically in LB broth, lennox (LB, BD Difco) at 30°C and 200 rpm overnight. The next day, the cultures were diluted in LB supplemented with 0.05% glucose to an optical density at 600 nm (OD$_{600}$) of 0.05, and thereafter grown at 30°C and 190 rpm. Growth was monitored by periodically measuring OD$_{600}$ in an Eppendorf BioPhotometer Plus, as well as by determining the number of CFU/mL by spreading serial dilutions in 0.9% saline on BHI agar plates.
Colloidal chitin preparation

Colloidal chitin was prepared from chitin from shrimps shells (C9213, Sigma) as described previously (44).

Construction of a mariner-based transposon library

The mariner-based transposon delivery plasmid pMC38 was used for the generation of a random mutant bank of *L. monocytogenes* N53-1, as described by Cao et al. (48). Competent *L. monocytogenes* N53-1 cells were prepared and electroporation was performed as described by Kastbjerg *et al.* (49). Cells were spread on BHI agar with erythromycin (5 µg/mL). The *L. monocytogenes* N53-1 harboring pMC38 was grown overnight in BHI broth with erythromycin (5 µg/mL) and kanamycin (10 µg/mL) at 30°C with shaking. The cultures were diluted 1:200 in BHI broth with erythromycin (5 µg/mL) and grown for 1 h at 30°C with shaking, and then shifted to 40°C to force transposon integration for around 6 h until the OD$_{600}$ was between 0.3 and 0.5. To select for chromosomal integration of the transposon, the cultures were grown at 40°C on BHI agar plates supplemented with erythromycin (5 µg/mL) for 3 to 4 days.

Southern hybridization

To evaluate the randomness of transposition we arbitrarily picked 18 erythromycin-resistant colonies from our library. Genomic DNA was purified from the 18 isolates and from wild-type *L. monocytogenes* N53-1 using the Fast DNA Kit (MP Biomedicals, 116540-400) as described by Holch *et al.* (50). DNA was precipitated with ethanol, and 4 µg DNA were digested with 20 U HindIII (Promega) for 3 h at 37°C. A 400 bp-long fragment of the *ermC* of the transposon was
amplified as a DNA probe, as described by Cao et al. (48). Labeling of the fragment and DNA hybridization were performed according to the protocol supplied with the Biotin DecaLabelTM DNA Labeling Kit (Fermentas, K0652) and the Biotin chromogenic detection kit (Fermentas, K0662).

Transposon screening

Cells from the freezing stock of the N53-1 mariner-based transposon library were either streaked on BHI agar plates containing 5 μg/mL erythromycin or diluted in 0.9% saline and then plated on the plates. After overnight growth at 37°C, individual clones were picked with toothpicks and spotted on assay plates (LB agar plates containing 6 mg/mL acid-hydrolyzed colloidal chitin, as well as 5 μg/mL erythromycin). The clones were compared based on the size of the clearing zone produced after approximately five days of incubation at 30°C. Clones that seemed to exhibit an altered pattern of hydrolysis compared to the average were selected for further testing. This consisted of growing them overnight at 30°C under aerobic conditions (200 rpm) in BHI supplemented with 5 μg/mL erythromycin, and spotting 10 μL of overnight culture on assay plates. The size of the clearing zone was recorded after 4-6 days of growth at 30°C. Clones that produced zones larger or narrower than average were kept in the form of freezing stocks containing 15% glycerol.

Identification of the site of transposon insertion

In order to identify the site of transposon insertion, we used the method of Cao et al. (48), consisting of two rounds of arbitrary PCR aimed at amplifying the DNA sequences flanking the transposon, and of sequencing of the resulting products (Macrogen, Europe). However, in contrast
to Cao et al. (48), DreamTaq Green DNA polymerase (Thermo Scientific) was used for the
amplifications.

Sequencing of the *lmo0327* locus of strain N53-1

Chromosomal DNA from strain N53-1 was extracted with the aid of the DNeasy Blood& Tissue Kit
(Qiagen), according to the manufacturer’s instructions. Sequencing was carried out by Macrogen,
Europe.

Determining chitinolytic activity

The wild-type and the *lmo0325* and *lmo0327* mutant strains were grown overnight in BHI, which in
the case of the mutants had been supplemented with 5 μg/μL erythromycin. The next day, they were
spotted on LB-chitin agar plates without erythromycin. After a five-day incubation period at 30°C,
the size of the produced clearing zones was compared between the wild-type and the mutant.

Sample collection for total RNA extraction

In order to collect samples for RNA extraction, the cells were grown aerobically in LB at 30°C and
200 rpm overnight. The next day, the cultures were diluted in LB supplemented with 0.05% glucose
to an OD$_{600}$ of 0.05, and then grown at 30°C and 190 rpm. After 1 h of growth half of the culture of
each strain was induced by addition of colloidal chitin to a final concentration of 1.7 g/L. The cells
were then grown until late-exponential phase and samples for RNA were collected at OD$_{600}$ 0.7 for
the wild-type EGD and 0.65 for the *lmo0327* mutant from both induced and uninduced cells.
Stationary phase samples were collected approximately 1 h after growth had been arrested, as
indicated by OD$_{600}$ measurements (approximately after 6 h of growth for the wild-type and 9-10 h of growth for the mutant; see Fig. S1).

Total RNA extraction and Northern blot analysis

Total RNA extraction and Northern blot analysis were carried out as described previously for the Northern blot analysis of $agrA$ transcripts (44). The radioactive probes were generated by PCR amplification using the primers described by Larsen et al. (43) Differences in the amounts of transcripts were considered to be significant only if they exceeded 2-fold.

Protein precipitation of bacterial supernatants

In order to collect samples of chitin-induced cells, the cells were initially grown aerobically in LB at 30°C and 200 rpm. Following overnight incubation, the cultures were diluted in LB supplemented with 0.05% glucose to an OD$_{600}$ of 0.05, and then grown at 30°C and 190 rpm. After approximately 4 hours of growth, 10 mL of the culture was induced by transfer to flasks containing 0.1 g of colloidal chitin. The induced and uninduced cells were left to grow overnight at 30°C and 190 rpm.

The next day, the OD$_{600}$ of the uninduced cells was recorded. 9 mL of induced cells was spun down at 6000 rpm at 0°C for 7 min. The supernatants were transferred to new tubes and the proteins were precipitated with trichloroacetic acid (Sigma-Aldrich) and normalized as described by Paspaliari et al. (44).

Western blot analysis
For Western blot analysis, 2 μL of samples was separated in a 4 to 12% NuPAGE Bis-Tris gel (Invitrogen) running MOPS buffer. Thereafter the separated samples were transferred to a polyvinylidene difluoride (PVDF) membrane with the iBlot dry blotting system (Invitrogen). Western blot analysis was carried out with the aid of the Western Breeze Chemiluminescent Kit-Anti-Rabbit (Invitrogen), according to the manufacturer’s instructions. The anti-ChiA and anti-ChiB antibodies used for the analysis were obtained from Paspaliari et al. (44).

**SDS-PAGE analysis of extracellular proteins bound to chitin beads**

As for the western blot, the cells were initially grown aerobically in LB at 30°C and 200 rpm overnight. The next day, the cultures were diluted in 23 mL of LB supplemented with 0.05% glucose to an OD₆₀₀ of 0.05, and thereafter grown at 30°C and 190 rpm. After approximately 1 h of growth the cells were induced by addition of colloidal chitin (10 g/L) and left to grow overnight. The overnight-grown cells were separated from the supernatant by centrifugation. The supernatant was sterile-filtered using a 0.2 micron sterile filter (Millipore), and incubated overnight with 75 μL of chitin magnetic beads (New England Biolabs) at 30°C and 200 rpm to allow binding of the chitinases to the beads. The next day the beads were separated with the aid of a magnet and washed twice in 1 mL 50 mM Tris pH 8.0. The washed beads were then resuspended in 20 μL of SDS-PAGE loading buffer (Invitrogen) and boiled for 10 min at 99°C to release the proteins. The proteins were separated on a 10% NuPAGE Bis-Tris gel (Invitrogen) and stained with SYPRO Ruby (Invitrogen), according to the manufacturer’s instructions.

**Bioinformatic analyses and Accession number**

All bioinformatic analyses were carried out using the CLC Main Workbench software (CLC Bio).
The sequence of the gene in which the transposon had been inserted in the N53-1 strain was deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and given the Accession number MF314113.

RESULTS

Transposon screen of *L. monocytogenes* N53-1 for mutants with altered chitinase activity

In order to identify regulators and proteins important for the chitinolytic activity of *L. monocytogenes* we screened a mariner-based transposon library for mutants with enhanced or impaired ability to hydrolyze chitin. The strain N53-1 was chosen for the screening, as we have found it to be a strain of relatively high chitinolytic potential, a trait that can facilitate the identification of mutants. The phenotypic evaluation was based on the size of the clearing zone produced after growth on erythromycin-selective LB-chitin agar plates.

A total of approximately 3650 clones were screened. Among these, one mutant exhibited a dramatic decrease in chitinolytic activity (resembling ΔchiA mutants in the EGD strain), and was selected for further study (Fig. 1).

Identification of the site of transposon insertion

Given that the genome sequence available for strain N53-1 is not closed (GenBank accession number HE999705.1; (50)), we mapped the site of transposon insertion in relation to the genome of the well-annotated EGD-e strain instead (GenBank accession number NC-00321.1). The two strains are of the same serotype and their genomes have been found to be relatively similar (50).
With respect to the genome of EGD-e, we found the transposon insertion to be between nucleotide 2463875 and nucleotide 3876 of the 4047 bp-long lmo0327 (Fig. 2). lmo0327 encodes a cell-surface protein with proposed murein hydrolase activity (46). The protein has been classified as an internalin, as it carries the characteristic leucine-rich (LRR) repeats of the internalin family (51). Besides the repeats, the protein also harbors 14 cell wall surface repeats, as well as an LPXTG cell wall anchor domain. The position of the transposon insertion lies close to the C-terminus, between the end of the cell wall surface anchor repeats and the LPXTG cell wall anchor domain. Although this insertion does not necessarily preclude the expression of the N-terminal part of the protein (46), it can be assumed to prevent the correct sorting of the protein and its anchoring to the cell wall. This should in turn be expected to abolish the activity of the protein.

The transposon insertion should not have caused any polar effects, as the coding region of lmo0327 is flanked by terminators and a previous study has shown that insertional inactivation of the gene does not inhibit transcription of any of its upstream or downstream adjacent genes (46).

As the strain that was used for the transposon analysis was N53-1, we also tried to map the insertion site on the genome of this strain (genome accession number HE999705.1 (50)). Surprisingly, the corresponding locus differed from that of EGD-e. Namely, in the case of N53-1, the insertion appeared to be in an internalin J gene (NCBI locus tag: BN419_0374), which in turn contained extensive deletions and a premature stop codon when compared to the lmo0327 locus in EGD-e. Careful examination, however, revealed that these differences partly arose from the incomplete nature of the sequence provided for strain N53-1, leading to an erroneous annotation of the gene as internalin J, which in EGD-e is encoded by lmo2821 (53). To confirm that, we sequenced the corresponding locus of N53-1. Compared to the EGD-e lmo0327, we found it to contain a single 414 bp (138 aa)-long in-frame deletion, confirmed also by PCR (results not shown). Alignment of the nucleotide and protein sequences between the two strains can be found in Fig. S2 and S3.
deletion corresponds to the area of the cell-wall anchor repeats, and likely does not influence the
functionality of the protein.

Inactivation of \textit{lmo0327} results in reduced chitinolytic activity

In order to confirm the phenotype, we performed the chitinolytic activity assay on the EGD strain
and its corresponding \textit{lmo0327} mutant, which carries an insertion that inactivates the Lmo0327
protein (46). Overnight cultures of the wild-type and the mutant strain were spotted on colloidal
chitin agar plates. After a five-day incubation period at 30°C, the mutant displayed no visible
clearing zone surrounding the colony, and slight hydrolysis could only be discerned upon colony
removal (Fig. 3A). In contrast, the wild-type strain produced a clearly-defined clearing zone (Fig.
3A). After prolonged incubation, a narrow clearing zone was also visible for the mutant (results not
shown).

It should be noted here that all assays for the \textit{lmo327} mutant were conducted in the absence of
erythromycin, to allow for direct comparison with the wild-type EGD. As the assay was done under
non-selective conditions, we compared the stability of the pAUL-A plasmid insertions in the
absence of antibiotic selection under the conditions relevant for the assay. Although we recorded no
loss of antibiotic resistance, the chitinolytic phenotype was slightly less pronounced than when the
cells had been previously grown with erythromycin (results not shown). This suggests that the
insertion was fairly stable, but does not exclude that the actual differences may be even greater than
those observed here.

An \textit{lmo0325} mutant shows the same phenotype as the \textit{lmo0327} insertion mutant
In the EGD-e genome, *lmo0327* is preceded by *lmo0326* and *0325*. Both of these genes are predicted to encode HTH-type Rgg transcriptional regulators (XRE family) that bear similarity to each other (46). Additionally, they have been suggested to both be essential for the function of Lmo0327, as their inactivation leads to the loss of an autolytic activity attributed to Lmo0327 (46). In gel analyses of the surface proteins of EGD, inactivation of *lmo0326* resulted in the disappearance of a protein band, which was hypothesized to correspond to Lmo0327 (46). This raises the possibility that Lmo0326 is a positive regulator of Lmo0327. No similar analysis has been conducted for *lmo0325*. However, on the String database (string-db.org) for prediction of protein interactions it is suggested to interact with *lmo0327* based on genomic neighborhood and co-occurrence across firmicute species.

These indications prompted us to investigate whether *lmo0325* might also be important for the chitinolytic phenotype. Indeed, upon comparison of an *lmo0325* mutant with the wild-type strain and the *lmo0327* mutant, we discovered that inactivation of *lmo0325* gave rise to a phenotype indistinguishable from that of the *lmo0327* mutant (Fig. 3B). This should not be due to polar effects on *lmo0327* resulting from the inactivation, as we found the transcription of both *lmo0326* and *lmo0327* to be unaffected by the insertion into *lmo0325* (data not shown).

**The amounts of chitinase transcripts are decreased in the *lmo0327* mutant**

In order to investigate whether the reduced chitinolytic activity observed for the mutants was due to a decrease in chitinase production itself, we carried out a northern blot analysis to compare the levels of chitinase transcripts between the wild-type EGD and the *lmo0327* mutant (Fig. 4). From the northern blot analysis it can be seen that the transcript levels of the chitinases in the mutant were in general lower than for the wild-type under all conditions that produced detectable...
wild-type transcripts, and were in general very close or below the detection limit of the assay. The differences were the greatest in stationary phase under the presence of chitin, namely at the condition of maximum induction of the chitinases, and in particular in the case of chiA.

Our results, therefore, indicate that inactivation of lmo0327 significantly diminishes transcription of both chitinase genes.

ChiA and ChiB production is decreased in the lmo0327 mutant

We additionally compared the extracellular levels of ChiA and ChiB in culture supernatants grown overnight in the presence of chitin. We employed two different types of analysis.

First, we compared by western blot analysis the levels of overnight-secreted ChiA and ChiB between the wild-type and the lmo0327 mutant strain (Figure 5A). For the amount of sample used for the analysis we could detect no signal for either ChiA or ChiB in the culture supernatants of the mutant. In contrast, we detected strong signals in the wild-type supernatants. This confirms the results from the northern blot and the phenotypic analysis, namely that production of both chitinases is greatly impaired upon inactivation of lmo0327.

It should be noted here, that the difference observed in the western blot might be affected by the fact that a fraction of the chitinases remained bound to the chitin substrate after overnight incubation and was thereby not present in the supernatant visualized in the western blot. However, we have previously shown that this limitation should not greatly influence the results, as, firstly, there is still significant amount of chitin remaining in the solution both in the mutant, as well as in the wild-type cultures, and, secondly, the amount of chitin-bound chitinases is relatively small compared to the amount of chitinases in the supernatant (44).
Still, binding to chitin may be the reason why we could not detect any chitinase signal for the mutant strain at all, although small amounts of chitinases should still be produced, according to the phenotypic assay. To confirm the presence of ChiA and ChiB in the culture supernatants of the mutant, we incubated the supernatants from a larger culture volume together with chitin beads and analyzed the proteins bound to them by SDS-PAGE. This analysis revealed that both ChiA and ChiB are indeed produced at low levels by the *lm0327* mutant, although this might also be related to partial instability of the insertion (Fig. 5B). Additionally, despite not being a truly quantitative assay, the assay confirmed the difference in the levels of secreted chitinases between the wild-type and the mutant that was observed in the western blot.

**DISCUSSION**

The biotechnological potential of bacterial chitinases (6–12), as well as their recognition as virulence factors of bacterial pathogens (13), has sparked an interest in deciphering the mode of function and regulation of bacterial chitinolytic systems. Although the structure and regulation of Gram-negative chitinolytic systems is being progressively elucidated, little is known about the regulation of the respective Gram-positive ones.

*L. monocytogenes* is one of the best-studied Gram-positive bacteria in respect to its chitinolytic system, and a number of regulators of chitinase activity have already been identified in the bacterium (43, 45, 54). Despite these advances, the regulatory system of the listerial chitinases is expected to be more complex, including yet uncharacterized components. As an example, a conserved σ\(^{54}\) promoter box has been found in the promoter of *chiB*, pointing to σ\(^L\) being a *chiB* regulator (55).
In order to identify new regulatory components affecting chitinase activity, we carried out a mariner-based transposon screen on the strain N53-1, which exhibits strong chitinolytic activity. Our screening yielded one mutant severely impaired in chitin hydrolysis. The transposon insertion was mapped in a gene corresponding to the lmo0327 locus in EGD-e, which is predicted to encode a protein of the internalin family with putative murein hydrolase activity (46, 51).

To confirm the implication of the gene in chitinase activity, we subsequently tested an EGD strain with insertional inactivation of lmo0327. Chitinolytic examination of this mutant on agar plates confirmed the phenotype exhibited by the transposon, as the mutant was only marginally positive for chitin hydrolysis. A similar phenotype was observed when the neighboring gene lmo0325 was inactivated.

The fact that inactivation of lmo0327 affected chitinase expression in both the N53-1 and EGD strains suggests that this effect is not strain-specific.

To investigate whether the inactivation affects a specific or both chitinases we analyzed the chiA and chiB transcripts of the lmo0327 mutant and the wild-type grown with and without chitin. Under these conditions chitin is necessary for induction of chitinase transcription (43). Our analysis revealed that although chitin could still induce a low-level transcription at stationary phase, both chitinase transcripts, and chiA in particular, were dramatically reduced in the mutant, and close to the detection limit of the assay. This suggests that lmo327 affects chitinase expression at the transcriptional level. In agreement with that, western blot analysis detected neither ChiA nor ChiB in culture supernatants in the absence of lmo0327.

Incubation of a larger volume of lmo0327 mutant culture supernatants with chitin beads ultimately revealed the presence of both chitinases in the supernatant, albeit at very low levels. This might
indicate that chitinase production is not totally abolished in the mutant. Alternatively, it could be an artifact resulting from instability of the pAUL-A insertion plasmid in a subpopulation of the cells.

When interpreting these results the growth properties of the insertional mutant compared to the wild-type EGD-e should be taken into account. Namely, we recorded a deceleration in the growth rate of the mutant during the transition between late exponential and stationary phase (Fig. S1). This difference might be related to the previously reported inhibition of cell separation exhibited by *lmo0327* mutant cells in exponential phase (46). In any case, the difference is not expected to greatly influence the results presented here.

The exact role of Lmo0327 in relation to the chitinolytic system, as well as its mechanism of action, are not easy to predict, as the function of the protein remains largely unknown. Its classification as an internalin does not provide much help in this case, as internalins have been proposed to be functionally diverse (51, 56). However, the presence of the LRR-repeats might indicate a site for protein-protein interactions (52, 57). A role in virulence has been proposed for the protein, as it has been found to be important for intracellular replication in epithelial cells with an additional moderate role in adhesion/invasion (58). In agreement with that, the protein has been detected on the cell wall of intracellularly growing bacteria (59).

On the other hand, the protein had also previously been hypothesized not to contribute to virulence, as *lmo0327* is expressed at higher levels at temperatures relevant for environmental rather than host-associated growth (56, 60). *lmo0327* in general follows the temperature-expression patterns of σ^B^-dependent internalins (56). However, it has so far not been found to be σ^B^- or prfA-dependent (61–63), despite the presence of an imperfect prfA box in its promoter region (64). The absence of regulation by PrfA in rich media should not exclude the possibility that *lmo0327* might be PrfA-dependent under specific conditions only, as is for the example the case for the *Listeria* chitinases (43).
Interestingly, murein hydrolase activity has been suggested for Lmo0327, due to zymographic activity of extracts of cells expressing a gene fragment comprised of lmo0325, lmo0326 and the N-terminal part of lmo0327 (46). All of these three genes, but not lmo0324 that was additionally tested, are important for this activity. However, as the other two genes are transcriptional regulators, the activity has been attributed to Lmo0327. In support of this, a band of autolytic activity corresponding to the size of Lmo0327 is detected in cell wall extracts of the wild-type EGD, but is absent in mutant cells with inactivated lmo0327 (46). In addition, mutants lacking lmo0327, and lmo0326 (its putative positive regulator) show impaired ability to separate in exponential phase, as well as a slower rate of murein turnover (46). All this supports that Lmo0327 is an autolysin. However, more experimental data are needed to provide concrete proof, especially in view of the fact that Lmo0327 does not contain any identified domain associated with peptidoglycan hydrolysis and does not exhibit similarity with autolysins on basic homology searches.

The autolytic activity suggested for Lmo0327 raises the possibility of direct involvement of Lmo0327 in chitin hydrolysis, given the structural similarity between chitin and murein. The identification of the domain responsible for murein hydrolysis should aid in the examination of this possibility. However, it is more likely that chitin hydrolysis is solely affected by the effect exerted by lmo0327 on chitinase transcription and thereby expression. It would be tempting to imagine that based on its surface location, presence of the LRR-repeats and the transcriptional downstream effects, Lmo0327 could be a sensor, playing similar roles as ChiS and inducing chitinase transcription. However, this scenario is rendered unlikely by the absence of an established sensor-related domain in Lmo0327. It could also be that the effect of lmo0327 on chitinase production is indirect. More experimental data are necessary in order to formulate further hypotheses.

In conclusion, we provide here evidence that lmo0327 and lmo0325, encoding a putative internalin / autolysin and a putative transcriptional activator, respectively, are necessary for efficient expression.
of chitinase activity in *L. monocytogenes*. The identification of yet another completely novel element affecting chitinolytic system suggests that the system is even more complicated than previously anticipated and probably entails a great deal of interplay between individual regulatory factors, to ensure the proper and timely expression of the chitinases.

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Figure 1. Identification of a transposon insertion mutant with impaired chitinolytic activity in strain N53-1. For screening purposes, the chitinolytic activity of random transposon mutants was evaluated by spotting overnight cultures of the mutants on LB-colloidal chitin agar plates containing erythromycin and incubating them for several days at 30 °C. The overwhelming majority of the mutants exhibited strong chitinolytic activity, without great variance (left). In contrast, the chitinolytic activity of one of the mutants was greatly reduced (right).

Figure 2: Structure of lmo0327/Lmo0327 with the arrow pointing at the position of the transposon insertion. The sequence of lmo327 was obtained from ListiList (genolist.pasteur.fr/ListiList). Domain annotation was done based on the Pfam (pfam.sanger.ac.uk) and Uniprot (www.uniprot.org) databases, with the exception of the domains shown with an asterisk, whose annotation was based on the structures reported by Popowska and Markiewicz (46) and Bierne and Cossart (52), respectively.

Figure 3. The chitinolytic activity of L. monocytogenes EGD on colloidal chitin agar plates depends on lmo0327 (A) and lmo0325 (B). The results are representative of at least three independent experiments. Small empty parts of the plate of panel A and of the plate of panel B have been removed from the images, as indicated by the separating lines.

Figure 4. Transcription of chiA and chiB depends on lmo0327. Northen blot analysis of chiA and chiB transcripts in the wild-type EGD and the lmo0327 mutant. Samples were taken at mid-
exponential and stationary phase in LB supplemented with 0.05% glucose and 1.7 g/L colloidal chitin. The number above each band reflects the fold difference in relation to the respective transcript level of the first lane, i.e. the wild-type exponential phase sample grown without chitin. The 16S and 23S loading controls, visualized on the gel prior to the transfer to the membrane, can be seen below each band. In the Figure, the controls have been positioned under the corresponding bands of the Northern blot hybridization, in order to facilitate the interpretation of the results. The results are representative of three biological replicates harvested in the course of two independent experiments.

Figure 5. ChiA and ChiB levels in the supernatant depend on lmo0327. A. Western blot analysis of overnight cultures of the wild-type and the lmo0327 insertion mutant. B. SDS-PAGE analysis of ChiA and ChiB bound to chitin beads, after incubation of the beads with overnight-culture supernatants of the wild-type and the lmo0327 insertion mutant. The results are representative of at least two independent experiments.
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