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The Chitinolytic Activity of *Listeria monocytogenes* EGD Is Regulated by Carbohydrates but Also by the Virulence Regulator PrfA▽†

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Chitin, an insoluble polymer of *N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant carbohydrate polymers in marine and terrestrial environments. Chitin hydrolysis by *Listeria monocytogenes* depends on two chitinase-encoding genes, *chiA* and *chiB*, and the aim of this study was to investigate their regulation. Chitin induces the expression of both chitinases in late exponential growth phase, and *chiA* but not *chiB* is furthermore induced by the monomer GlcNAc. Furthermore, their expression is subjected to catabolite control. Chitinases expressed by bacterial pathogens have proven to be important not only for nutrient acquisition and environmental survival but also for infecting animals and humans. Interestingly, the central *L. monocytogenes* virulence gene regulator, PrfA, is required for the chitinolytic phenotype, as chitinase activity was significantly reduced in *prfA* mutant cells compared to its level in wild-type cells. In agreement with this, Northern blot analysis showed that the amounts of *chiA* and *chiB* transcripts upon induction by chitin were significantly lower in the *prfA* mutant than in the wild type. The chitinolytic activity and *chiA* and *chiB* expression were reduced in the absence of the *sigB* gene, indicating that σ^B is also important for the production of chitinases. The *chiA*, *chiB*, and *chiA chiB* mutants were not impaired for *in vitro* adhesion and invasion in epithelial cell lines, but the *chiA chiB* double mutant showed less survival ability in a chitin-enriched medium. The regulation of chitinolytic activity in *L. monocytogenes* is complex, and taken together, the results indicate that the biological role of this activity may not be limited to the external environment.

Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen primarily causing severe infections in elderly and immunocompromised individuals. Although *L. monocytogenes* is widely distributed in nature and though food and feed transmission is critical for human and animal infections, the transmission pathways and natural reservoirs are not well defined (44). Recently, we observed that *Listeria* spp., including *L. monocytogenes*, encode two chitinases, ChiA (Lmo1883) and ChiB (Lmo0105), that are able to hydrolyze chitin, a highly insoluble carbohydrate polymer widely distributed in nature (28).

Many bacteria from terrestrial and marine environments utilize chitin as a source of carbon and nitrogen by expressing chitinase activities, resulting in the formation of the dimer chitobiose $(GlcNAc)_2$ and the monomer *N*-acetylglucosamine (GlcNAc). The majority of bacterial chitinases belong to glycosyl hydrolase family 18, including the *Listeria* enzymes (50). Chitinolytic bacteria, e.g., species of the genera *Bacillus*, *Listeria*, *Serratia*, and *Vibrio*, often produce multiple chitinases, and the synergy between these is assumed to be necessary for effective chitin degradation (2, 38, 49, 57, 58). The expression of chitinases is regulated by substrates; however, there is great diversity among various chitinolytic bacteria regarding regulatory mechanisms. Substrates such as $(GlcNAc)_{2–6}$ and chitin induce chitinase gene expression, whereas GlcNAc can act both as a repressor and an inducer (4, 30, 31, 51, 54, 58). In addition, a number of regulatory parameters are known to play a role in the expression of chitinases in various Gram-negative bacteria, including a two-component regulatory system (*chiS*) (30), quorum-sensing signaling (12, 56), and the histone-like nucleoid-structuring proteins H-NS and StpA (13, 23). Also, environmental factors, such as temperature, pH, salinity, and the concentration of nitrogen, play a role in the expression of chitinases (4, 11).

The genetic regulation of chitinolytic activity is not as well understood for Gram-positive bacteria, but *in silico* and microarray analyses have identified some regulatory parameters in *L. monocytogenes.* Thus, it was found by microarray analysis that the stress-responsive alternative sigma factor σ^B and the motility gene repressor MogR are involved in the regulation of *chiA* expression (20, 46). Furthermore, *in silico* analysis of the *L. monocytogenes* genome revealed that *chiB* is preceded by a completely conserved σ^{54} promoter and, therefore, is putatively σ^{54} regulated (1).

Chitinases expressed by bacterial pathogens have proven to be important not only for nutrient acquisition and environmental survival but also for infecting humans and animals. Recently, it was shown that a *chiA* mutant of *Legionella pneumophila* has a reduced ability to persist in the mouse lung (10) and that a chitin-binding protein of *Vibrio cholerae* is involved in attachment to intestinal epithelia (25). Furthermore, the expression of chitinase genes of non-*cholerae Vibrio* species and of *chiA* in *L. monocytogenes* is upregulated in the rabbit and mouse intestine, respectively (4, 53). Like *Legionella* and pathogenic vibrios, *L. monocytogenes* is an environmental pathogen capable of switching from a saprophytic to a pathogenic lifestyle (10, 19, 53). In *L. monocytogenes*, this transfor-

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TABLE 1. PCR primers used in this study

Primer	Sequence (5′–3′)
chiA-fw	CTTTTGGCACTAGGT
chiA-re	CATCCACATTGGCTG
chiB-fw	CCACGCCTTGTTTAT
chiB-re	TTACGGTGATTGGTC
lmo106-fw	TTATTTCTGCGACAG
lmo106-re	CAGCTTTAGCGGAAC
lmo106-a	ATATAAAGTGTACCGAAATGGGACCGAAGT
lmo106-b	TCCACCAATATCGTATACAAAATACATTC CTCT
lmo106-c	TGTATACGATATTGGTGGACAAAATAAAAG GTAACT
lmo106-d	AAGCTAGATGCCACGGAAGAAGAAATTATT

mation appears to be mediated through complex regulatory pathways that modulate the expression of virulence factors in response to environmental conditions and include σ^B and central virulence regulatory factor PrfA (24, 26, 37, 47). PrfA positively regulates the expression of virulence genes that are essential for the intracellular survival of *L. monocytogenes*. The activity of PrfA is influenced by environmental conditions like temperature, pH, fermentable carbohydrates that are taken up by the phosphoenolpyruvate-dependent phosphotransferase (PTS) system, and bacterial growth phase (26, 33), ensuring that virulence factors are downregulated outside a host.

In this study, we examined regulatory aspects of the expression of *chiA* and *chiB* in *L. monocytogenes* EGD, and we show that the expression of *chiA* and *chiB* is subject to catabolite control and is induced by chitin in the late exponential growth phase. Furthermore, we find that the chitinolytic activity and the expression of *chiA* and *chiB* of *L. monocytogenes* are stimulated by PrfA and σ^B. These results are discussed in the context of the diverse habitats of *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Wild-type strain *L. monocytogenes* EGD and *prfA* mutant strains were obtained from Werner Goebel (Biozentrum, University of Würzburg, Germany). The isogenic EGD Δ*chiA* and Δ*chiB* deletion mutants and the *chiA chiB* double mutant (26) were obtained from our laboratory collection. The Δ*sigB* mutant was obtained from Lone Brøndsted within our department. An in-frame deletion in the coding region of *lmo0106* of *L. monocytogenes* EGD was constructed as described previously (27) by the gene splicing by overlap extension method (16) using primers lmo106-a, lmo106-b, lmo106-c, and lmo106-d (Table 1).

The bacteria were cultured on brain heart infusion agar (BHI agar; Oxoid) or BHI broth (Oxoid) that contained 2 g liter^{-1} glucose or in Luria broth (LB) containing tryptone (10.0 g liter^{-1}), yeast extract (5.0 g liter^{-1}), NaCl (10.0 g liter^{-1}), agar (15.0 g liter^{-1}), and phosphate buffer, pH 6.9 (Oxoid). The effect of chitin, glucose, or *N*-acetyl-D-glucosamine (GlcNAc) (catalog no. A8625; Sigma-Aldrich) on gene expression was examined by supplementing LB with acid-hydrolyzed chitin (2.5 g liter^{-1}) (catalog no. C9213; Sigma-Aldrich) or GlcNAc (2 g liter^{-1}) and/or glucose (2 g liter^{-1}) (Merck). Acid-hydrolyzed/colloidal chitin was prepared as described previously (17), omitting the filtration and freeze-drying steps. All cultures were incubated under shaken aerobic conditions at 30°C, except for one experiment that was done at 37°C. Samples for RNA extraction of bacteria in late exponential and early stationary phases were taken at optical densities at 600 nm (OD_{600}) of 0.3 and 0.7, respectively, for bacteria grown in LB and at OD_{600} of 0.6 and 1.5, respectively, for bacteria grown in LB supplemented with either glucose or GlcNAc and in BHI. The higher OD values in the latter cases were due to the higher final OD value of *Listeria* grown in LB supplemented with carbohydrates and in BHI compared to the OD value after growth in LB alone. The numbers of CFU ml^{-1} at the time of sampling were determined by plating serial dilutions in 0.9% saline on BHI agar plates.

Survival during long-term starvation. To determine survival during long-term starvation, wild-type and mutant bacteria were grown in LB supplemented with glucose (0.5 g liter^{-1}) and with or without chitin (2.5 g liter^{-1}) at 30°C. The numbers of CFU ml^{-1} were determined after serial dilutions in 0.9% saline on BHI agar plates.

Examination of chitinase activity. Examination of the chitinase activities of *L. monocytogenes* cultures was done as previously described (28), except that acid-hydrolyzed/colloidal chitin (see above) was used as the substrate. The plates were incubated under aerobic conditions at 30°C and scored for hydrolytic ability (clearing zones) for up to 10 days.

RNA extraction and Northern hybridization. Cells of *L. monocytogenes* wild-type and mutant strains were grown to mid-logarithmic growth phase and late exponential phase in LB or in LB supplemented with one or more of the following additional carbohydrates: chitin, glucose, or GlcNAc in a concentration of 2.5, 2, or 2 g liter^{-1}, respectively. The bacterial cells were lysed using a FastPrep FP120 instrument (BIO101; ThermoSavant) for 45 s at speed setting 6.0. Total RNA was extracted from the cells using an RNeasy mini kit (Qiagen, Denmark) according to the manufacturer's directions. Analysis of transcripts was done as previously described (27). Hybridization probes were generated by PCR from chromosomal DNA of *L. monocytogenes* EGD using primers specific for the *chiB* gene (chiB-fw and chiB-re), the *chiA* gene (chiA-fw and chiA-re), and the *lmo0106* gene (lmo106-fw and lmo106-re) (Table 1). RNA extracted from at least two independent experiments was analyzed.

Adhesion and invasion. Invasion was studied in the murine macrophage cell line J774A.1 (ATCC TIB-67), the mouse fibroblast cell line L929 (European Collection of Animal Cell Cultures [ECACC] no. 85011425), and in enterocyte-like Caco-2 cells (ATCC HTB-37; Promochem). Adhesion was only studied in Caco-2 cells. J774A.1 and L929 were propagated in Dulbecco's modified Eagle's medium (DMEM, catalog no. BE12-604F; Lonza) supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (FBS) (catalog no. DE14-830; Lonza) and 25 μg/ml gentamicin (catalog no. 15750-037; Gibco). Caco-2 cells were cultured in MEM enriched with Glutamax and HEPES, supplemented with 20% heat-inactivated FBS, 0.1 mM nonessential amino acids, and 0.5 ml of gentamicin (50 mg/ml). All chemicals were from Invitrogen. Cells were incubated with 5% CO_2 at 37°C. All cell assay experiments were carried out in duplicate in two independent trials with the gentamicin protection assay as previously described (18). Briefly, the monolayers of cells were infected with approximately 1×10^7 CFU ml^{-1} of bacteria, and after 1 h of infection, the monolayers were washed. For determination of adhesion, 0.1% Triton X-100 was added to loosen and lyse the Caco-2 cells. For invasion, the washed cells were overlaid with medium containing 25 μg ml^{-1} gentamicin to kill extracellular bacteria before the cells, after washing, were lysed with 0.1% Triton X-100. The numbers of adhered and intracellular bacteria were determined by plating appropriate dilutions on BHI agar plates. The adhesion and intracellular indices were calculated as the number of adhered or invaded bacteria divided by the number of bacteria in the inoculum times 100%.

RESULTS

Chitin induces the expression of chitinase genes *chiA* and *chiB*, but only *chiA* is induced by the monomer GlcNAc. In *L. monocytogenes*, chitin hydrolysis depends on two genes encoding chitinases, *chiB* (*lmo0105*) and *chiA* (*lmo1883*) (28). Examination of the *L. monocytogenes* EGD-e genome sequence revealed putative transcriptional terminators downstream of both *chiA* and *chiB*, and a recent experimental analysis of the *L. monocytogenes* operon structure showed that *chiA* and *chiB* are expressed as monocistronic transcripts (53).

To identify inducers of *chiA* and *chiB* expression in *L. monocytogenes* EGD, we grew the bacteria in the absence and presence of chitin and GlcNAc and measured the expression of *chiA* and *chiB* by Northern blot analysis. We have previously suggested that chitinolytic activity in *L. monocytogenes* is subjected to catabolite repression mediated by easily fermentable carbohydrates (28). Therefore, the identification of inducible substrates was performed in Luria broth, which is a poor growth medium for *L. monocytogenes* due to low concentrations of fermentable carbohydrates (3, 45). When chitin was

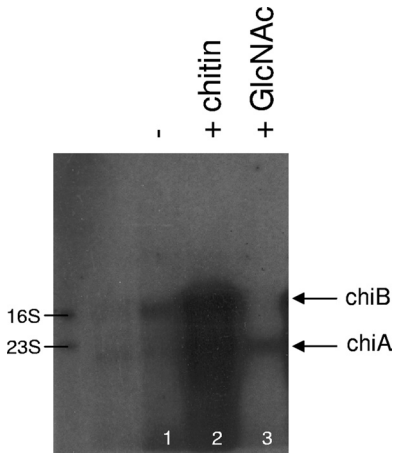

FIG. 1. The expression of *chiA* and *chiB* of *Listeria monocytogenes* EGD is induced by chitin, whereas only *chiA* is induced by GlcNAc. Northern blot analysis of *chiA* and *chiB* transcripts. RNA was isolated from wild-type cells grown in LB (−; lane 1) or in LB supplemented with 0.25% (wt/vol) chitin (lane 2) or with 0.2% GlcNAc (lane 3) to early stationary phase. The positions of the 16S and 23S rRNA bands are indicated. The arrows indicate the *chiA* and *chiB* transcripts. The results presented are representative of three independent experiments.

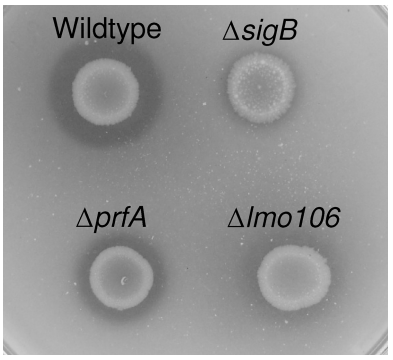

FIG. 2. The chitinolytic activity of *Listeria monocytogenes* depends on σ^B, PrfA, and Lmo0106. Wild-type and *prfA*, *sigB*, and *lmo0106* mutant strains were spotted on chitin agar plates, and the plates were incubated for 5 days at 30°C. The photograph presented is representative of 3 independent experiments.

added to the growth medium of *L. monocytogenes* EGD, the amounts of both *chiA* and *chiB* mRNA were dramatically increased (Fig. 1). Probing with *chiA*- and *chiB*-specific probes identified major bands of approximately 1,100 and 2,300 bp, respectively, indicating that *chiA* and *chiB* are expressed as monocistronic transcripts, in agreement with the findings of Toledo-Arana et al. (53) (Fig. 1). When GlcNAc was added to the growth medium, a weak increase in the amount of *chiA* transcripts was detected (Fig. 1). Thus, GlcNAc is sufficient to induce the expression of *chiA* in *L. monocytogenes*. Interestingly, we were unable to detect any transcripts of *chiB* in medium with GlcNAc alone, indicating a variation in the inducing capacity of this compound (Fig. 1).

***lmo0106* is not a transcriptional regulator of *chiA* and *chiB*.** The *chiB* gene is located upstream of *lmo0106* that encodes a gene annotated as a transcriptional regulator/sugar kinase. In order to investigate whether Lmo0106 modulates chitinolytic activity, we constructed an in-frame deletion of *lmo0106* and observed that the chitinolytic activity of the resulting mutant was markedly reduced compared to that in the wild type when examined on chitin agar plates (Fig. 2). To examine whether Lmo0106 is a transcriptional regulator of *chiA* or *chiB*, we compared the amount of *chiA* and *chiB* mRNA produced in the wild type and the *lmo0106* deletion mutant in chitinase-inducing growth medium. The expression of *chiA* and *chiB* was similar in both strains after growth in LB supplemented with either chitin or GlcNAc (Fig. 3). Thus, Lmo0106 is not a transcriptional regulator of *chiA* and *chiB*, and we propose a posttranscriptional role in modulating chitin hydrolysis.

***chiA* and *chiB* expression is growth-phase dependent and subject to catabolite repression.** We examined the expression of the chitinase genes *chiA* and *chiB* in response to growth phase by Northern blot analysis. *L. monocytogenes* cells were grown to late exponential ($1.4 \times 10^8 \pm 0.4 \times 10^8$ CFU ml^{-1} [mean ± standard deviation]) and early stationary ($3.9 \times 10^8 \pm 0.5 \times 10^8$ CFU ml^{-1}) growth phase, respectively, in growth medium with and without chitin. The induction of *chiA* and *chiB* by chitin was clearly stronger at early stationary phase than at late exponential growth phase (Fig. 4). This was not a pH effect caused by the fermentative activity of *L. monocytogenes*, as the pH of the growth medium was similar at both growth phases (data not shown). *chiB* was not expressed in the absence of chitin at either growth phase, whereas there seems to be detectable transcription of *chiA* in the absence of chitin by cells in late exponential phase (Fig. 4). Previously, we found that the chitinolytic activity of *L. monocytogenes* EGD is repressed by glucose, suggesting that chitinolytic activity is subjected to catabolite repression (28). This conclusion was confirmed in the present study, as the amounts of *chiA* and *chiB* transcripts were reduced when 0.2% glucose was included in the chitin-containing growth medium (Fig. 4). We also examined the expression of *chiA* and *chiB* after growth in BHI (without chitin) at 37°C, since these growth conditions are generally used for culturing *L. monocytogenes*, and found that the amounts of transcripts were below the level of detection in early stationary phase (see Fig. S1 in the supplemental material). In contrast, *chiA* and *chiB* transcripts were detected at 37°C in LB supplemented with chitin but at reduced levels

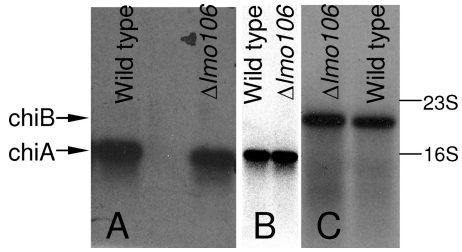

FIG. 3. The expression of *chiA* (A, B) and *chiB* (C) of *Listeria monocytogenes* is not affected by *lmo0106*. Northern blot analysis of *chiA* and *chiB* transcripts. RNA was isolated from wild-type and *lmo0106* mutant strains grown in LB supplemented with 0.2% GlcNAc (A) or grown in LB with 0.25% (wt/vol) chitin (B, C) to late exponential phase. The positions of the 16S and 23S rRNA bands are indicated. The arrows indicate the *chiA* and *chiB* transcripts. The results presented are representative of two independent experiments.

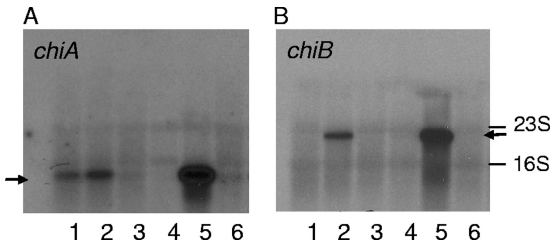

FIG. 4. The expression of *chiA* and *chiB* of *Listeria monocytogenes* is growth phase dependent and repressed by glucose. Northern blot analysis of *chiA* (A) and *chiB* (B) transcripts. RNA was isolated from wild-type cells grown in LB without chitin (lanes 1 and 4), with 2.5 g liter^{-1} (wt/vol) chitin (lanes 2 and 5), or with 2.5 g liter^{-1} chitin and 2 g liter^{-1} glucose (lanes 3 and 6) to an OD_{600} of 0.3 (lanes 1 to 3) or an OD_{600} of 0.7 (lanes 4 to 6). The positions of the 16S and 23S rRNA bands are indicated. The arrows indicate the *chiA* and *chiB* transcripts. The results presented are representative of two independent experiments.

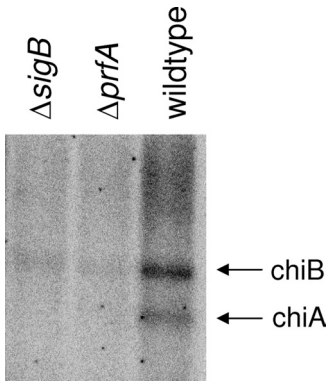

FIG. 5. *prfA* and *sigB* are important for the expression of *chiA* and *chiB* in *Listeria monocytogenes.* Northern blot analysis of *chiA* and *chiB* transcripts. RNA was isolated from wild-type and *sigB* and *prfA* mutant cells grown in LB supplemented with 0.25% (wt/vol) chitin to late log phase (4×10^8 CFU ml^{-1}). The arrows indicate the *chiA* and *chiB* transcripts. The results presented are representative of three independent experiments.

compared to the levels at 30°C (see Fig. S1 in the supplemental material).

ChiA and ChiB contribute to long-term survival in chitin-containing medium. Chitin has previously been shown to support the survival of *L. monocytogenes* in minimal medium with glucose (39). We therefore determined whether ChiA and ChiB are important for the survival of *L. monocytogenes* during starvation in chitin-containing medium. Wild-type and mutant strains were inoculated into LB supplemented with 0.5 g liter^{-1} glucose and with or without 2.5 g liter^{-1} chitin and incubated at 30°C for up to 6 weeks. No differences between wild-type and chitinase mutant strains were detected during the first 11 days of starvation (Table 2). However, after prolonged starvation, the number of CFU ml^{-1} of the *chiA chiB* double mutant decreased slightly more than the number of CFU ml^{-1} of the wild type, and the number was approximately 5 to 10 times lower than the number of the wild type (Table 2 and data not shown). The numbers of the *chiA* and the *chiB* mutants were between the numbers of the wild type and of the *chiA chiB* double mutant (Table 2).

***prfA* and σ^B are important for the expression of *chiA* and *chiB*, but the chitinases are not important for infection of epithelial cell lines.** In some pathogens, chitinases may be important for virulence, as shown for *L. pneumophila* (10). Therefore, we set out to determine whether PrfA was involved in the chitinolytic activity of *L. monocytogenes* EGD. Comparison of the chitinase activity of the EGD wild type with that of a *prfA* mutant showed that the mutant had reduced chitinolytic activity on chitin agar plates (Fig. 2). In agreement with this, we found by Northern blot analysis that the amounts of *chiA* and *chiB* transcripts were significantly smaller upon induction by chitin in the *prfA* mutant than in the wild type (Fig. 5). The alternative sigma factor σ^B contributes to virulence in *L. monocytogenes* and coregulates a subset of virulence genes with positive regulatory factor A (35, 37). Therefore, we determined the role of σ^B in the expression of *chiA* and *chiB* and in the chitinolytic activity of *L. monocytogenes.* The results showed that the expression of *chiA* and *chiB* (Fig. 5) and chitinolytic activity (Fig. 2) were reduced in the absence of σ^B, indicating that σ^B is important for the production of chitinases. Thus, in addition to the issue of the presence of carbohydrates, virulence-associated parameters affect the expression of the chitinolytic phenotype of *L. monocytogenes.* Therefore, we investigated the role of the chitinases in virulence *in vitro* by examining adhesion and invasion of epithelial cells. The results showed that there were no differences between the wild type and the *chiA*, the *chiB*, and the *chiA chiB* mutants in their ability to adhere or invade to Caco-2 cells (data not shown). Likewise, the chitinase mutants were not impaired in invasion of the murine macrophage cell line J774A.1 or the mouse fibroblast cell line L929 (data not shown).

TABLE 2. Survival of *Listeria monocytogenes* EGD during starvation in growth medium with 0.5 g liter^{-1} glucose and 2.5 g liter^{-1} chitin

Day	No. in viable population ($\log_{10}$ CFU ml^{-1}) of[a]:			
	Wild type	*chiA* mutant	*chiB* mutant	*chiA chiB* mutant
0	8.9	8.9	9.0	9.0
11	8.6	8.6	8.6	8.5
18	8.6	8.1	8.3	7.5
26	7.9	7.3	7.2	6.8

[a] Values are the means of duplicate samples. Numbers were determined after plating on BHI agar plates. The experiment was repeated three times with similar results.

DISCUSSION

Many bacterial species possess the ability to catabolize chitin, and not surprisingly, a variety of parameters regulate the expression of this phenotype. One important parameter concerns the presence of the substrate (chitin) and/or its degradation products, GlcNAc and/or $(GlcNAc)_2$, as well as the presence of other potential carbohydrate substrates. *V. cholerae* represents a particularly well studied organism in this respect. Thus, in the N16961 strain, the expression of three out of five annotated chitinase genes was upregulated by chitin and chitin oligosaccharides [$(GlcNAc)_{2–6}$]. A fourth gene was induced by both GlcNAc and chitin oligosaccharides, whereas the expression of the fifth gene was unaffected (31). However,

species and strain variation is seen, as chitinase genes are constitutively expressed in *V. cholerae* strains during growth in M9 minimal medium, whereas they are induced by chitin and GlcNAc in non-*cholerae Vibrio* strains (4). GlcNAc also induce chitinase production by a *Pseudoalteromonas* strain (51). In contrast, this compound acts as a repressor of chitinase gene expression in *Serratia marcescens* (54). Also, in *Bacillus pabuli*, $(GlcNAc)_{2-4}$ induces the production of chitinases, whereas no chitinases could be detected in the presence of GlcNAc (14).

We show here that the two chitinase genes in *L. monocytogenes*, *chiA* and *chiB*, were induced by different substrates, as only *chiA* was induced by the monomer GlcNAc, whereas the expression of *chiB* depended on chitin oligosaccharides present in the chitin suspension. This indicates different roles for the two chitinases. We speculate that GlcNAc and $(GlcNAc)_2$ may be "indicator" molecules to signal the presence of chitin for *L. monocytogenes*, as hypothesized for marine bacteria (21), as chitin must be present in *L. monocytogenes* environmental reservoirs such as terrestrial and marine environments in more or less degraded forms (15). It is also of interest to note that *chiA* appears to be constitutively expressed in small amounts when *L. monocytogenes* is present in low numbers. Such a steady low-level activity of a presumably secreted protein generates a gradient of soluble chitin degradation products, allowing the chitinolytic organism to home in on available insoluble chitin in the surroundings as outlined for *Vibrio furnissii* (21). It remains to be demonstrated that *L. monocytogenes* possesses a chemotactic ability toward chitin.

We observed that the expression of the chitinase genes was repressed in the presence of easily fermentable carbohydrates, similar to other chitinolytic bacteria, such as *Bacillus* spp. and *Streptomyces lividans* (14, 36). A microarray study found that *lmo0106* and, to a lesser degree, *lmo1883* (*chiA*) are upregulated in two mutants defective in carbon catabolite control, the *hprK* and *ccpA* insertion mutants, indicating that *lmo0106* presumably is repressed by CcpA/HPr(Ser-P) in the presence of glucose (32). Taken together, these results indicate that one biological role for the chitinolytic phenotype of *L. monocytogenes* most likely is as a carbon and energy source in the natural environment. This conclusion is supported by the observations that chitin enhances the survival of *L. monocytogenes* Scott A in a defined minimal medium (39) and that the *chiA chiB* double mutant does not survive as well as the wild type and the *chiA* and *chiB* mutants in medium containing chitin (this study).

The *lmo0106* gene encodes a putative product with homology to the ROK protein family (52). The ROK family members are divided into two groups, of which one group carries an N-terminal extension and is composed of transcriptional regulators, whereas the other group is the sugar kinases that only harbor a C-terminal sugar-binding domain (52). When we inactivated *lmo0106*, we observed reduced chitinolytic activity, although *chiA* and *chiB* transcription was not affected. This result agrees with Lmo0106 belonging to the sugar kinase subfamily of ROK and thus, Lmo0106 affects chitinolytic activity at the posttranscriptional level. Interestingly, *lmo0106* is conserved in genome-sequenced strains of *L. monocytogenes* and *Listeria welshimeri* but not in the genome-sequenced chitinolytic *Listeria innocua* Clip 11262 strain (www.ncbi.nlm.nih.gov/).

It is also of interest to note that microarray studies have revealed that the *chiA* gene is upregulated by the alternative sigma factor σ^B that regulates stress response and virulence functions of *L. monocytogenes* (20). The growth phase-dependent expression of *chiA* and *chiB*, with higher levels of expression in early stationary phase than in late exponential phase, may perhaps be explained by an increased production of σ^B upon entry into stationary phase (3). We examined the chitinolytic activity of a mutant lacking σ^B and found that it displayed very low chitinolytic activity, showing that under the growth conditions on the chitin agar plates, σ^B is also important for chitinase production. Northern blot analysis confirmed that *chiA* and *chiB* expression was reduced in the absence of σ^B, while they were highly expressed in wild-type cells. Chitinolytic activity may also be controlled by quorum sensing, as shown for, e.g., *Chromobacterium violaceum* (6) and *Serratia proteamaculans* (7), but further studies are required to clarify this issue.

The expression of *chiB* is presumably regulated by σ^{54}, since a conserved σ^{54} promoter was identified upstream of *chiB* (1). The alternative sigma factor σ^{54}, encoded by *rpoN*, is involved in nitrogen and carbon utilization in various Gram-negative and Gram-positive bacteria, including *L. monocytogenes* (1, 48). However, the role of σ^{54} for *chiB* expression has not yet been verified *in vitro*, as microarray analysis of the wild type and mutant cells lacking σ^{54} revealed no difference in *chiB* expression in late exponential growth phase in BHI at 42°C, possibly because an unknown cognate activator was not active (1). Accordingly, we were unable to detect any expression of *chiA* or *chiB* in BHI without chitin at 37°C.

In addition to their roles in nutrient acquisition and environmental survival, chitinases may contribute to human and animal infections, as shown for *L. pneumophila* (10). To investigate the role of ChiA and ChiB of *L. monocytogenes* in infection, we analyzed the ability of the *chiA*, the *chiB*, and the *chiA chiB* mutants to adhere to and invade epithelial cells. We found that the absence of the chitinases did not influence this ability. Further studies are needed to elucidate whether the chitinases are important for virulence *in vivo*. Thus, the *chiA* mutant of *L. pneumophila*, which is defective in persistence in the lungs of mice, was not impaired in *in vitro* intracellular infection (10). Our observation that the deletion of *prfA*, encoding the major *L. monocytogenes* virulence gene regulator, reduced chitinolytic activity indicates that the biological roles of chitinases are not limited to the external environment, as the activation of *prfA* in general indicates the activation of a host-associated virulence phenotype (26). This notion is supported by the results of a recent microarray analysis showing that *chiA* and *chiB* are upregulated in the mouse intestine compared to their expression in BHI and that the induction of *chiA* is σ^B dependent (53). As the mammalian host does not contain chitin, the inducing signal in the host is currently unknown, but it remains a possibility that the absence of catabolite repression plays a role.

An alternative explanation for the induction of *chiA* and *chiB* in the mouse intestine would be that they represent bifunctional enzymes with an additional, as-yet-unidentified substrate other than chitin, as suggested for ChiA of *L. pneumo-*

phila (10). Thus, these enzymes may have other functions in the host, as suggested for ChiA of *Escherichia coli* (13). Alternative substrates could be postulated to include, e.g., N-linked glycans associated with host proteins, as shown for *Streptococcus pyogenes* and *Enterococcus faecalis* (8, 9), or peptidoglycan from the intestinal microflora. We have, however, shown that for at least ChiA, the latter possibility is not valid, as this enzyme lacks lysozymic activity (29). Further research is needed to illuminate the range of potential substrates for the *L. monocytogenes* chitinases. How PrfA regulates chitinolytic activity is at present unknown, but it is properly not directly linked to the expression of chitinase genes as no PrfA box was identified in the promoter region of *chiA* or *chiB.* In addition to the genes preceded by a PrfA box, expression profiles done by Milohanic et al. (34) of *L. monocytogenes* EGD and its isogenic *prfA* mutant identified 70 genes differentially transcribed during exponential growth in BHI. These include genes involved in general stress response and the transport and metabolism of carbohydrates. Among these is an operon encoding components of the mannose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) and genes encoding ABC transporters (34). PTS and ABC transporters are involved in the transport and metabolism of GlcNAc and $(GlcNAc)_2$ in both Gram-negative and Gram-positive bacteria, and this uptake can be important for the production of chitinases (22, 41, 42, 54). In *S. marcescens*, for example, $(GlcNAc)_2$ is taken up by the PTS system and mutants defective in $(GlcNAc)_2$ transport are unable to express chitinases (54). Likewise, in *Streptomyces coelicolor*, a major chitin degrader in soil, $(GlcNAc)_2$ uptake, mediated by ABC transporters, is essential for chitinase production (43). We speculate that the reduced chitinolytic activity in the absence of PrfA is partly caused by a reduced uptake of GlcNAc or $(GlcNAc)_2$. However, other factors linked to carbohydrate uptake and metabolism are presumably involved, and further studies are needed to elucidate how PrfA influences chitinolytic activity.

Recent findings suggest that the mechanism of virulence reflects an adaptive mechanism originating in the environment (25, 40, 55). Thus, toxin-coregulated pili involved in *V. cholerae* intestinal colonization have a role in biofilm formation on chitin-containing surfaces (40), and a chitin-binding protein mediates adherence to epithelial cells *in vitro* (25). The present study enhances the understanding of the regulation of the expression of the *L. monocytogenes* chitinolytic system. This system is regulated by the substrates chitin and GlcNAc, which are presumed to be of importance in the environment. Our finding that PrfA and σ^B have a role in chitinase expression shows that factors and pathways of *L. monocytogenes* involved in its pathogenicity in humans and animals may also have roles in its growth in the external environment, as outlined for *V. cholerae* (55), or that ChiA and/or ChiB potentially play a role in infection. This topic deserves further research into the issue of potential host-related targets of the chitinases, especially for ChiA, as well as the demonstration that they are actual virulence factors by the use of *in vivo* models. In fact, this was very recently shown, as *chiA* and *chiB* mutant cells have a reduced capacity to grow in the liver and spleen after intravenous infection of mice (5).

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