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Listeria monocytogenes σ^H Contributes to Expression of Competence Genes and Intracellular Growth

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ABSTRACT

The alternative sigma factor σ^H has two functions in Gram-positive bacteria: it regulates sporulation and the development of genetic competence. *Listeria monocytogenes* is a nonsporulating species in which competence has not yet been detected. Nevertheless, the main competence regulators and a series of orthologous genes that form the competence machinery are present in its genome; some of the competence genes play a role in optimal phagosomal escape. In this study, strains overexpressing σ^H and strains with a σ^H deletion were used to elucidate the contribution of σ^H to the expression of the competence machinery genes in *L. monocytogenes*. Gene expression analysis showed that σ^H is, indeed, involved in *comG* and *comE* regulation. Unexpectedly, we observed a unique regulation scheme in which σ^H and the transcription factor ComK were involved. Population-level analysis showed that even with the overexpression of both factors, only a fraction of the cells expressed the competence machinery genes. Although we could not detect competence, σ^H was crucial for phagosomal escape, which implies that this alternative sigma factor has specifically evolved to regulate the *L. monocytogenes* intracellular life cycle.

IMPORTANCE

***Listeria monocytogenes* can be an intracellular pathogen capable of causing serious infections in humans and animal species. Recently, the competence machinery genes were described as being necessary for optimal phagosomal escape, in which the transcription factor ComK plays an important role. On the other hand, our previous phylogenetic analysis suggested that the alternative sigma factor σ^H might play a role in the regulation of competence genes. The present study shows that some of the competence genes belong to the σ^H regulon and, importantly, that σ^H is essential for intracellular growth, implying a unique physiological role of σ^H among *Firmicutes*.**

Listeria monocytogenes is a Gram-positive foodborne pathogen with a multifaceted lifestyle: it can live harmlessly as a saprophyte in a diversity of environmental locations, but it can also be an intracellular pathogen capable of causing serious infections in humans and animal species. *L. monocytogenes* is a resilient microorganism: it cannot form spores, but it is able to endure several environmental and host stresses, such as low pH, low temperature, exposure to bile and fatty acids, high osmolarity, competition with intestinal flora, and intracellular nutrient and iron starvation (1, 2).

Alternative sigma factors constitute the principal strategy used to control the response to specific stress conditions, growth transitions, and morphological changes by the regulation of stress response and virulence genes, other regulators, and small RNAs. *L. monocytogenes* has four alternative sigma factors: σ^B, σ^H, σ^C, and σ^L (3). Transcriptomic and proteomic analyses have found an overlap between the regulons and cross connections between regulators, suggesting a complex mechanism for the fine-tuning of stress responses in *L. monocytogenes* (4–6). σ^B is the most extensively characterized and is considered to be the main stress-associated sigma factor. Null mutations of the remaining sigma factors have identified limited phenotypic consequences. σ^L is associated with growth under osmotic and low-temperature stress (7). σ^C, a member of the extracytoplasmic function (ECF) family of sigma factors, is present only in lineage II strains and seems to contribute to thermal resistance (8). σ^H protein levels have been reported to increase at low pH (3.5) (9), and a deletion mutant showed reduced growth under alkaline conditions and in minimal medium, as well as reduced virulence in a mouse model (2).

According to a phylogenetic analysis of σ^{70}-family sigma factors in members of the *Firmicutes*, *L. monocytogenes* σ^H shares a common ancestor with *Bacillus* σ^H, *Streptococcus* ComX, and *Staphylococcus* σ^H (10). This ComX/σ^H group of sigma factors is responsible for spore formation (*Bacillus*) or competence development (*Streptococcus* and *Staphylococcus*). *Listeria* σ^H shows a high similarity with sporulation sigma factors, including *Bacillus* σ^H, rather than with competence development factors.

L. monocytogenes is not able to form spores (11), and indeed, it lacks almost all of the sporulation-specific genes (12). On the other hand, its genome contains homologues for the competence machinery genes, although attempts to detect natural transformation have been unsuccessful (13). The main components of the competence machinery are the pseudopilus (ComG proteins), which brings the exogenous DNA to the membrane transport machinery; the DNA receptor (ComEA), which delivers the bound

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference or source
Strains		
L. monocytogenes		
EGDe	Parent strain	35
wt	Phage-cured derivative of EGDe	This study
+sigH	wt carrying pRIT5H-sigH; Cm^r	This study
ΔsigH	wt with *sigH* deletion	This study
ΔsigH+comK	Strain ΔsigH carrying pRIT5H-comK; Cm^r	This study
ΔcomK	wt with *comK* deletion	This study
ΔcomK+sigH	Strain ΔcomK carrying pRIT5H-sigH; Cm^r	This study
+sigH+comK	wt carrying pRIT5H-sigH and pMK3-comK; Cm^r Km^r	This study
ΔsigHΔcomK	wt with *sigH* and *comK* deletions	This study
wt/pJEBan2-PcomGA	wt carrying pJEBan2-PcomGA; Erm^r	This study
+sigH/pJEBan2-PcomGA	Strain +sigH carrying pJEBan2-PcomGA; Cm^r Erm^r	This study
+sigH+comK/pJEBan2-PcomGA	Strain +sigH+comK carrying pJEBan2-PcomGA; Cm^r Km^r Erm^r	This study
wt/pJEBan3	wt carrying pJEBan3; Erm^r	This study
ΔsigH/pJEBan3	Strain ΔsigH carrying pJEBan3; Erm^r	This study
ΔcomK/pJEBan3	Strain ΔcomK carrying pJEBan3; Erm^r	This study
ΔsigHΔcomK/pJEBan3	Strain ΔsigHΔcomK carrying pJEBan3; Erm^r	This study
wt/pMAD-tet	wt carrying pMAD-tet; Tet^r	This study
ΔsigH/pMAD-tet	Strain ΔsigH carrying pMAD-tet; Tet^r	This study
ΔsigH/pMAD-tet-sigH	Strain ΔsigH carrying pMAD-tet-sigH; Tet^r	This study
c-ΔsigH	Chromosomal complementation of Δ*sigH*	This study
Staphylococcus aureus		
RN	*sigH* translation initiation site mutant of RN4220	This study
RN+sigH/pJEBan2-PcomGA	RN carrying pRIT5H-sigH and pJEBan2-PcomGA; Cm^r Erm^r	This study
Plasmids		
pRIT5H	Shuttle vector; Cm^r	10
pRIT5H-comK	pRIT5H derivative overexpressing *comK*	This study
pRIT5H-sigH	pRIT5H derivative overexpressing *sigH*	This study
pMK3	Shuttle vector; Km^r	24
pMK3-comK	pMK3 derivative overexpressing *comK*	This study
pMAD-tet	pMAD derivative with temperature-sensitive replication *ori*; Tet^r	25
pMAD-tet-ΔsigH	pMAD-tet derivative, *sigH*-targeting vector	This study
pMAD-tet-ΔcomK	pMAD-tet derivative, *comK*-targeting vector	This study
pMAD-tet-sigH	pMAD-tet derivative carrying a 3-kbp *sigH* locus	This study
pJEBan2	Reporter plasmid; *Pdlt-cfp* Erm^r	29
pJEBan2-PcomGA	Reporter plasmid; *PcomGA-cfp* Erm^r	This study
pJEBan3	Reporter plasmid; *Pdlt-yfp* Erm^r	29

[a] Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Erm^r, erythromycin resistant; Tet^r, tetracycline resistant.

DNA to the channel (ComEC); and an ATP-binding protein (ComFA), which is also involved in transport across the membrane (14, 15). This machinery is required for natural genetic competence (a specific physiological state developed to undergo natural transformation), and it is broadly conserved among species, including noncompetent bacteria (15). A recent study reported a new function for the competence machinery genes during the intracellular growth of *L. monocytogenes*: the *comG* operon and *comEC* gene are necessary for efficient phagosomal escape (16).

Regulation of the expression of competence genes varies among species (17). In *Bacillus subtilis*, the main regulator is the transcription factor ComK (18), whereas *Streptococcus pneumoniae* (19, 20) and *Staphylococcus aureus* (21) use the sigma factor ComX/σ^H as the key regulator. In addition, staphylococcal ComK participates in the expression of competence genes (22). Thus, the ComX/σ^H alternative sigma factor or the transcriptional factor ComK plays a major role in the expression of competence genes in a species-specific manner.

L. monocytogenes has both *comK* and *sigH* genes in its genome. ComK was recently demonstrated to be the regulator of competence genes (16). In the present study, we aimed to clarify whether the alternative sigma factor σ^H is involved in the expression of competence genes in *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and growth media. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml) or erythromycin (150 μg/ml). *L. monocytogenes* strains were grown in brain heart infusion (BHI) medium. BHI plates were supplemented with chloramphenicol (7.5 μg/ml), tetracycline (10 μg/ml), erythromycin (10 μg/ml), kanamycin (20 μg/ml), or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 100 μg/ml) when necessary.

TABLE 2 Oligonucleotides used in this study

Name	Sequence (5′–3′)	Target	Reference or source
sigH-F	CTGAATTCGGAGGAATACAGGCAGTTATGGATAATTCAGTGA	*sigH*	This study
sigH-R	CTTAGGTCGACAATTGTTTTTTCATTCTAGC	*sigH*	This study
V05	GGATCGAATTCTCAAACATAGAGGAGTTG	*comK*	This study
V06	CTACTCTGCAGTAGCTGGCTTTTATTTA	*comK*	This study
V07	GTCATGAATTCAATTATATCCTC	*comG* promoter	This study
V09	CGACCTCAATCCGGTAGTTTTGAAAAAA	*comGA*	This study
V10	AGGGGATGGCATTTTTCTCCGCAGAAGG	*comGA*	This study
V11	CATGGATCCAAGAACCTCCTTACAA	*comG* promoter	This study
V12	ATGAAGGCAGCTATTTATATACGCG	*int*	This study
V13	GTAATTGTTTTGCTTCTTCTTC	*int*	This study
V14	CGGCGAATTCTTCTGCAAGCAATCTC	*comK* upstream region	This study
V15	TTCATTCCCGGGCTCTATGTTTGTAT	*comK* upstream region	This study
V28	ATCCCGGGCAGCTACGTAATAGTAGCTGGC	*comK* downstream region	This study
V29	TTAGATCTAGGTGGAGTAGCATCTGTTGG	*comK* downstream region	This study
V38	GGATACCAGATGGTAAAACAGATACAG	*comEA*	This study
V39	CTACTGTTTTTTCGCCAATACCTGAAACG	*comEA*	This study
V40	CGGGGATCCCAAGTTCAATTTGTACCTAAA	*sigH* upstream region	This study
V43	TAGCTGCAGCGGCTCCTTTACGACAGGAT	*sigH* upstream region	This study
V44	AGAGGATCCGATGGAACAAATTCTGCTTG	*sigH* upstream region	This study
V45	AAACTGCAGTGACTAGCTAAGAGCCAAATG	*sigH* downstream region	This study
V46	TCTGTCGACTCGGCTTCTCGTTTAAACTC	*sigH* downstream region	This study
V47	TTTGTCGACCCGGAGTAAGTATGAACTAC	*sigH* downstream region	This study
V53	GCCGTCGACTTCCTGAATAAATCTTTCA	*Pspa* (pRIT5H)	This study
V63	ATCTTCCCGGGAAGACTGTATCAAGAA	*comFA*	This study
V64	CACTTTTTCTCTGGTGTTGCTGTGATTA	*comFA*	This study
V67	CGAATTTTACAAGCTCTATCGTTCCCAT	*comEC*	This study
V68	TGTAGCAGCTCGTATGACTGGCGGA	*comEC*	This study
OP133	CACCTGGAGTAAACCAATTAGTACG	*rpoB*	28
OP134	TAGTGGGTTAAGCATGATATCAACA	*rpoB*	28

Phage excision. A prophage-cured derivative of the *L. monocytogenes* EGDe strain was isolated by bacteriophage induction using UV light (23). Cells (10 ml) were collected by centrifugation at 6,000 × *g* for 10 min, resuspended in 10 ml of phosphate-buffered saline (PBS), and transferred to a sterile petri dish. Next, they were exposed to UV light irradiation for 60, 90, or 120 s. After irradiation, serial dilutions were made, plated, and incubated at 37°C. Colonies from which phage was excised were detected by PCR using primers for the bacterial *comK* gene and phage *int* gene (Table 2).

Construction of overexpression strains. Overexpression strains were constructed using the pRIT5H high-copy-number plasmid (10). The *sigH* gene (*lmo0243*) was amplified using primers sigH-F and sigH-R. The forward primer included 18 bp of the *rpoD* gene (*lmo1454*) and contained its Shine-Dalgarno sequence and starting codon; the starting codon of *sigH* was thereby changed from GTG to ATG. The amplified fragment was inserted into the EcoRI-SalI sites of the pRIT5H plasmid. The resulting vector (pRIT5H-sigH) could not be cloned into *E. coli* JM109, probably due to the toxic effect of σ^H in *E. coli*. Therefore, it was directly cloned into *L. monocytogenes* EGDe.

The *comK* gene was amplified with primers V05 and V06 and introduced into the EcoRI-PstI sites of pRIT5H, generating the *comK* expression vector pRIT5H-comK. Another *comK* expression vector (pMK3-comK) was constructed by transferring the *Pspa-comK* fragment, amplified from pRIT5H-comK with primers V53 and V06, into the SalI-PstI site of pMK3 (24).

Construction of deletion mutant and complementation strains. Deletion strains were constructed by double homologous recombination using the pMAD-tet plasmid (25). A pair of fragments encompassing the *sigH* gene was amplified by PCR with primers V44-V43 and V45-V47. These were digested with the primer-attached PstI sites, ligated, and amplified by PCR with primers V44 and V47. The resultant fragment was introduced into the BamHI-SalI site of the pMAD-tet vector. Fragments surrounding the *comK* gene (amplified with primers V14-V15 and V28-V29) were sequentially inserted into the EcoRI-SmaI and SmaI-BglII sites of pMAD-tet.

Mutants were isolated using the protocol of Boneca et al. (26) with slight modifications. After electroporation, bacterial cells were plated onto BHI plates supplemented with tetracycline and X-Gal and cultured at 30°C for at least 48 h. Blue colonies were selected, transferred into BHI broth supplemented with tetracycline, and cultured at 42°C for 48 h. Dilutions were plated onto BHI–tetracycline–X-Gal plates and cultured overnight at 42°C. Light blue colonies were selected and subjected to at least three cycles of growth in drug-free BHI broth at 30°C for 12 h and at 42°C for 12 h. Serial dilutions were made, plated onto BHI–X-Gal agar, and incubated at 42°C. White colonies were selected, and successful gene deletion was checked by PCR.

The *sigH*-complemented strain ΔsigH/pMAD-tet-sigH was generated as follows. A 3-kbp fragment containing the *sigH* gene was amplified by PCR using primers V40 and V46 from the genomic DNA of the wild-type (wt) strain. The fragment was ligated into the BamHI-SalI site of the pMAD-tet vector to generate pMAD-tet-sigH. The ligation was directly introduced into the Δ*sigH* strain by electroporation. The existence of the *sigH* locus in strain ΔsigH/pMAD-tet-sigH was confirmed by PCR. As a vector control, pMAD-tet was introduced into the Δ*sigH* strain (ΔsigH/pMAD-tet). Cells carrying pMAD-tet derivatives were grown at 30°C.

The *sigH*-cured strain (strain c-ΔsigH) was constructed from strain ΔsigH/pMAD-tet-sigH by the same protocol described above. The precise recovery of the *sigH* locus was confirmed by PCR.

Growth curves. Overnight cultures were diluted 1:200, and 200 μl was transferred to a 96-well sterile polystyrene microtiter plate (Corning). The optical density at 600 nm was measured, using a multimode plate reader

(EnSpire; PerkinElmer), every 30 min for 24 h with shaking (180 rpm) at 37°C.

Semiquantitative reverse transcription-PCR (RT-PCR). A stationary-phase culture (10 ml) was centrifuged at 5,000 rpm for 10 min at 4°C. Pellets were frozen with liquid nitrogen and stored at −80°C until RNA purification. RNA was purified according to a previously published method (27) with some modifications. Cells were resuspended in 460 μl of cold resuspension solution (10% glucose, 12.5 mM Tris, pH 7.6, 65 mM EDTA) and transferred into a 2 ml screw-cap microtube containing 500 μl of acid phenol solution, pH 4.5 (Sigma), and 0.4 g of glass beads (acid washed; diameter, ≤106 μm; Sigma). Cells were disrupted by use of a FastPrep-24 instrument (MP Biomedicals) for 30 s at 6.0 m/s twice with a 1-min interval between disruptions. The tubes were then centrifuged at 4°C for 5 min at 13,000 rpm, and the supernatant was transferred to a new tube. One milliliter of TRI Reagent (Sigma) and 100 μl of chloroform-isoamyl alcohol (24:1) were added, followed by vortexing and centrifugation at 13,000 rpm for 5 min at 4°C. The aqueous phase was transferred to a new tube and subjected to a second chloroform-isoamyl alcohol extraction. RNA was precipitated by the addition of 500 μl of isopropanol, incubation for 15 min at −30°C, and centrifugation for 15 min at 13,000 rpm and 4°C. The pellet was washed with 1 ml of 70% ethanol, dried, and resuspended in 100 μl of Milli-Q water. The purified RNA was DNase treated using a Turbo DNA-free kit (Invitrogen) following the manufacturer's rigorous DNase treatment protocol.

For cDNA synthesis, 1 μg of total RNA was mixed with 5× first-strand buffer (final concentration, 1×; Clontech) and random primers (0.03 μg/μl; Invitrogen). This mixture was exposed to 65°C for 5 min and to ice for 5 min. Then, dithiothreitol (10 mM), deoxynucleoside triphosphate mix (1.7 mM), RNaseOUT (0.4 U/μl; Invitrogen), and PowerScript reverse transcriptase (2 U/μl; Clontech) were added. The mixture (30 μl) was incubated at 42°C for 70 min and 95°C for 10 min. The absence of genomic DNA in the RNA preparations was verified by adding control tubes with all the components except the reverse transcriptase. PCRs were performed using 2 μl of cDNA for *comK*, *sigH*, *comGA*, *comFA*, *comEA*, *comEC*, and *rpoB* (the primers are listed in Table 2).

Cyan fluorescent protein (CFP) reporter assay. A reporter plasmid was constructed using pJEBan2 (29). The *comG* promoter region was amplified (with primers V07 and V11) and cloned between the EcoRI and BamHI sites of the pJEBan2 plasmid (directly upstream of the *cfp* gene). Overnight cultures of strains carrying the reporter vector were diluted in drug-free medium and incubated for 8 h. Fluorescence microscopy was carried out with an Olympus FSX100 microscope. The bacteria in five distinct fields were counted in each of three independent experiments.

Site-directed mutagenesis of *sigH* in *S. aureus*. Potential translation initiation codons (TTG) of *sigH* in *S. aureus* RN4220 were changed to Ochre stop codons (TAA) by using pMADt492 as described previously (21).

Intracellular survival. Intracellular survival in cells of the mouse macrophage cell line RAW 264.7 (American Type Culture Collection) and the human epithelial cell line HeLa was tested. Cells were cultured in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), L-glutamine (2 mM), and 10% heat-inactivated fetal bovine serum (FBS; Equitech-Bio) at 37°C with 5% CO_2.

Cells were seeded into 24-well plates (1×10^6 cells/ml, 1 ml per well). Following overnight incubation, the medium was replaced with 1 ml of RPMI 1640 supplemented with L-glutamine (2 mM) and 5% FBS and the cells were incubated for 30 min. Macrophages were infected with RPMI 1640 suspensions of *L. monocytogenes* at a multiplicity of infection (MOI) of 1. At 30 min postinfection, the macrophages were washed twice with PBS, and fresh medium containing gentamicin (5 μg/ml) was added. HeLa cells were infected at an MOI of 50 and were washed at 1 h postinfection (hpi). At each time point, the cells were washed twice with PBS and lysed with cold PBS with Triton X-100 (0.05%). Cell lysates were diluted with BHI, plated, and cultured for 24 h. Data were normalized using an intracellular growth coefficient (IGC), which was calculated as follows: $(IB_{t=n} - IB_{t=0})/IB_{t=0}$, where $IB_{t=n}$ is intracellular bacterial numbers (numbers of CFU per well) at a specific time point ($t = n$), and $IB_{t=0}$ is the intracellular bacterial numbers (numbers of CFU per well) at 30 min postinfection (for RAW 264.7 cells) or 1 h postinfection (for HeLa cells) ($t = 0$) (30).

Phagosomal escape. For phagosomal escape assays, *L. monocytogenes* strains were tagged with the reporter plasmid pJEBan3 (29). RAW 264.7 cells were plated onto 18-mm glass coverslips in 12-well plates (2×10^6 cells/ml, 1 ml per well) and were infected at an MOI of 1 as described above. At 3 hpi, the cells were washed twice with PBS and fixed with paraformaldehyde (4%) for 10 min at room temperature. They were then washed twice with PBS, permeabilized with 0.1% Triton X-100 for 5 min at room temperature, and washed twice with PBS, and actin was labeled with Alexa Fluor 594-phalloidin (5 U/ml; Molecular Probes, Invitrogen) with 1% bovine serum albumin for 20 min at room temperature. After labeling, the coverslips were washed twice with PBS and mounted with ProLong Diamond antifade reagent with DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes, Invitrogen). Fluorescence microscopy was carried out with an Olympus FSX100 microscope, and 150 to 160 intracellular bacterial cells were examined in each experiment.

TEM. For transmission electron microscopy (TEM), RAW 264.7 macrophages (2×10^6 cells/ml) were infected at an MOI of 10 as described above. At 3 hpi they were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, harvested by scraping, and stored at 4°C until use. Samples were then processed by the electron microscopy facility at Tsukuba University according to the facility's protocols. Thin sections were observed with a JEOL JEM-1400 electron microscope.

RESULTS

Canonical σ^H recognition sequences are present upstream of the *comG* and *comE* operons. Among Gram-positive bacteria, two master regulators control the expression of competence-related genes: the transcription factor ComK (in *B. subtilis*) (18) and the alternative sigma factor σ^H (in *S. pneumoniae* [19, 20], *S. aureus* [21], and *Lactobacillus sakei* [31]). Rabinovich et al. reported the presence of a ComK recognition site (the ComK box) upstream of the competence machinery operons (*comG*, *comE*, and *comF*) in *L. monocytogenes* (16). However, the involvement of σ^H in the expression of these genes has not been explored. Using the *L. monocytogenes* and *B. subtilis* σ^H promoter consensus sequences (4, 32), a recognition site for σ^H was identified in the upstream region of the *comG* and *comE* operons but not in that of the *comF* operon (Fig. 1). Although transcriptome analysis did not detect any competence genes in its regulon (4, 6), this observation implies that σ^H may be involved in the expression of *comG* and *comE*.

σ^H can express *comE* and *comG* operons. The *L. monocytogenes* EGDe strain has a prophage-disrupted *comK* gene. This prophage has been reported to induce the expression of competence machinery genes by its excision from *comK* during intracellular growth (16). To eliminate the influence of the *comK*-disrupting phage, we cured the EGDe strain and used this as the wild-type (wt) strain for subsequent strain constructions and comparisons. To clarify the contribution of σ^H, strains overexpressing σ^H and strains with a σ^H deletion were constructed. No significant difference in bacterial growth was observed between the strains (Fig. 2).

The expression of the regulators (*sigH* and *comK*) and the competence machinery genes in cells grown to the stationary phase was determined by semiquantitative RT-PCR (Fig. 3). The first gene in each operon (*comGA*, *comEA*, and *comFA*) was used as the representative gene.

The wt strain (phage-cured EGDe) expressed detectable levels

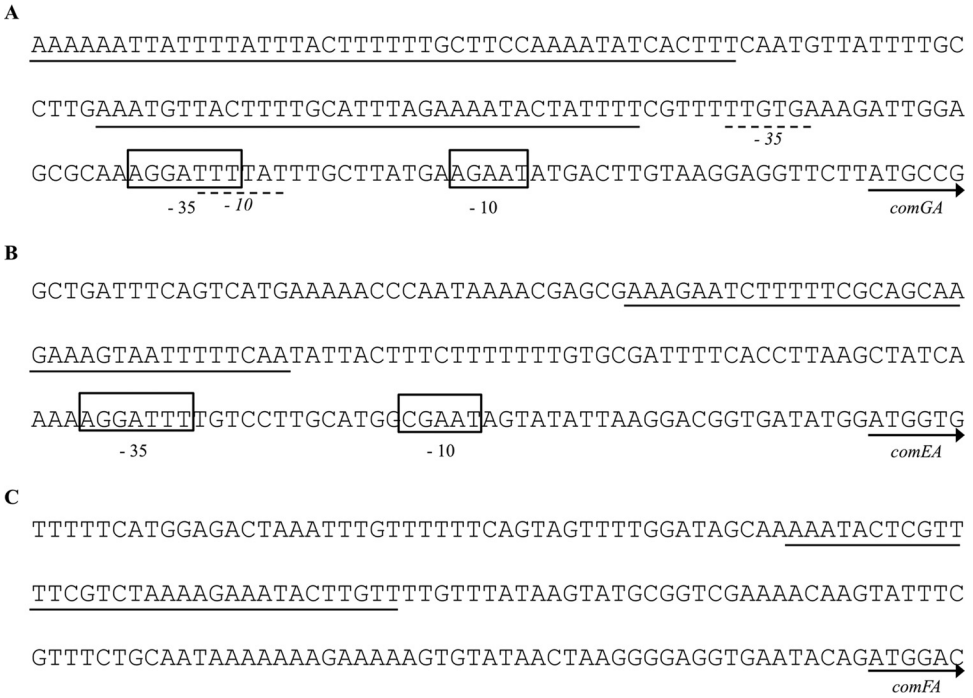

FIG 1 Consensus sequences recognized by ComK and σ^H in *L. monocytogenes*. (A) *comG* operon; (B) *comE* operon; (C) *comF* operon. Arrows, open reading frame; underlines, ComK box (16); boxes, σ^H promoter consensus sequence (in *B. subtilis*, AGGAWWT-12 to 14 residues-RGAAT, where W is A or T and R is A or G [32]; in *L. monocytogenes*, AGG . . . GAA [4]); dashed line, suggested σ^A (*rpoD*) promoter consensus sequence conserved among *Listeria* spp. (16). We could not find a conserved region similar to the σ^A-type consensus sequence in *comE* promoter regions.

of mRNAs from *comGA*, *comEA*, and *comFA*; this strain also expressed *sigH* mRNA, but *comK* expression was undetectable. The *comK* deletion mutant (strain ΔcomK) showed the same pattern as the wt strain. The deletion of the *sigH* gene (in strain ΔsigH) reduced the expression of *comGA* and *comEA* to undetectable levels, while it had no effect on *comFA* expression. Double deletion mutant ΔsigHΔcomK did not express *comGA* or *comEA*, but *comFA* could still be detected in this mutant. To test whether *comK* expression could induce the expression of competence genes in the absence of *sigH*, a plasmid (pRIT5H-comK) was introduced into strain ΔsigH, resulting in strain ΔsigH+comK. *comK* expression induced *comGA* expression but not *comEA* expression. In contrast, when both regulators were overexpressed (resulting in

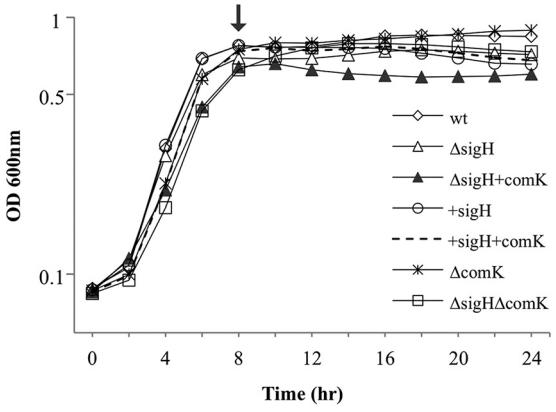

FIG 2 Growth of the *L. monocytogenes* strains (in BHI at 180 rpm and 37°C) used in this study. Arrow, sampling point; wt, phage-cured EGDe strain; ΔcomK, a *comK* deletion mutant; ΔsigH, a *sigH* deletion mutant; ΔsigH+comK, a strain overexpressing *comK* in the *sigH* deletion background, +sigH, the wt strain overexpressing *sigH*; +sigH+comK, the wt strain overexpressing *sigH* and *comK*; ΔsigHΔcomK, a *sigH* and *comK* double deletion mutant. OD 600nm, optical density at 600 nm.

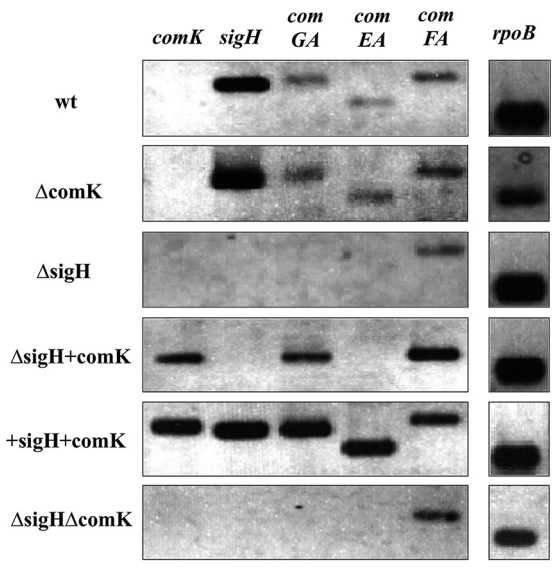

FIG 3 Semiquantitative RT-PCR of the *comK*, *sigH*, *comGA*, *comEA*, *comFA*, and *rpoB* genes. wt, phage-cured strain EGDe; ΔcomK, a *comK* deletion strain; ΔsigH, a *sigH* deletion strain; ΔsigH+comK, a strain overexpressing *comK* in the *sigH* deletion background; +sigH+comK, the wt strain overexpressing *sigH* and *comK*; ΔsigHΔcomK; a *sigH* and *comK* double deletion mutant. The sampling point is shown in Fig. 2. A representative result from 3 independent experiments is shown.

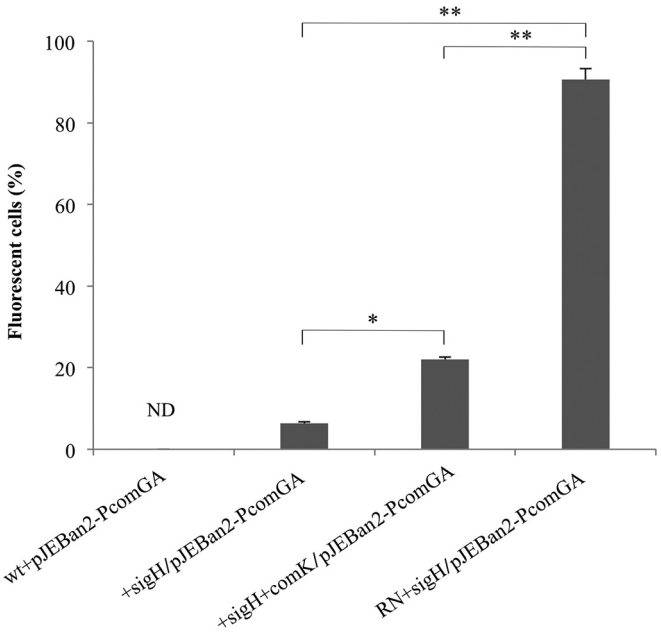

FIG 4 Cells expressing CFP under the control of the *comGA* promoter. wt, phage-cured strain EGDe; +sigH, the wt strain overexpressing *sigH*; +sigH+comK, the wt strain overexpressing *sigH* and *comK*; RN+sigH, *S. aureus* RN overexpressing *L. monocytogenes sigH*. Data represent the averages with SDs from 3 independent experiments. Significant differences are indicated. *, $P = 0.002$; **, $P = 0.00001$. ND, none detected. The sampling point is shown in Fig. 2.

strain +sigH+comK), mRNAs of both *comGA* and *comEA* were induced. Complementation of the ΔsigH strain restored the mRNA signal for the *comGA* and *comEA* genes (see Fig. S1A in the supplemental material).

These observations indicate the following: (i) σ^H and ComK are involved in *comGA* transcription, but σ^H is not essential for ComK-dependent induction; (ii) σ^H is essential for the expression of *comEA*, even when *comK* is overexpressed; and (iii) σ^H has no effect on *comFA* expression, while ComK has a positive effect. Thus, the alternative sigma factor σ^H can induce the transcription of the competence machinery operons, but its essentiality is different between *comG* and *comE*.

Competence genes are transcribed in a fraction of the population. We observed that σ^H can activate the transcription of competence genes in *L. monocytogenes*. In other Gram-positive species, the percentage of the population that expresses these genes and develops competence varies greatly. In *B. subtilis* and *S. aureus*, only a fraction of the cells can express competence genes (10 to 20% and 2%, respectively) (21, 33). In contrast, in *S. pneumoniae*, all the cells can become competent (34). To evaluate the activation of *com* genes at a population level, a reporter assay was performed. The promoter region of *comGA* was cloned upstream of the CFP-coding sequence in a reporter plasmid. The wt strain (with endogenous expression of the regulators) did not produce any fluorescent signal during growth (data obtained at 8 h are shown in Fig. 4; data obtained at 12, 16, and 20 h are not shown); it is likely that the mRNA levels detected in the semiquantitative RT-PCR (Fig. 3) were below the detection limit of this assay. Overexpression of σ^H showed that 6% of the cells expressed the *comG* operon; *comK* expression was not detected in this strain (data not shown). When ComK was also overexpressed, this percentage increased to 20%. Thus, the *comGA* promoter was active in a minor proportion of the population (Fig. 4). These observations suggest that the competence genes in *L. monocytogenes* are tightly regulated, resulting in subpopulation-specific expression, similar to the findings for *B. subtilis* and *S. aureus*.

The mechanisms regulating ComK and ComX/σ^H are different between species, and we wanted to test whether the subpopulation-specific expression of the *comG* promoter by *Listeria* σ^H could be observed in a different background. We were not able to use *E. coli* due to the toxic effect of σ^H. We overexpressed *L. monocytogenes sigH* in a *S. aureus* background (strain RN+sigH). The *S. aureus* strain employed (strain RN) carries mutations in the translation initiation codon of its own *sigH*. In the *S. aureus* background, we observed that 90% of the population expressed CFP (Fig. 4); this observation shows that σ^H can function as the sigma factor for the *comGA* promoter in almost all the population and implies the existence of a *Listeria*-specific regulation of σ^H.

σ^H is essential for intracellular growth in phagocytic and nonphagocytic cells. Attempts to detect natural transformation in *L. monocytogenes* have failed so far (13). Since *L. monocytogenes* competence genes are homologous to those of *B. subtilis* (35), all these efforts have been focused only on ComK regulation. Here, we tested the strain overexpressing both regulators (strain +sigH+comK) for transformation. The *S. aureus* protocol (21) was used, but we could not detect any transformants (data not shown).

Another role for the competence genes in *L. monocytogenes* is to facilitate optimal phagosomal escape (16). To test the role of σ^H during intracellular growth, RAW 264.7 macrophages were infected with *sigH* and *comK* single and double deletion mutant strains at a multiplicity of infection of 1. Strain ΔsigH was phagocytosed at significantly higher levels than the wt strain ($P = 0.0005$), but its intracellular growth was impaired; the number of CFU of strain ΔsigH did not increase throughout the time course tested. In accordance with findings described in a previous report (16), we observed an intracellular growth defect in strain ΔcomK as a delayed increase in its number of CFU. The double mutant could not grow; like strain ΔsigH, the numbers of CFU decreased over time (Fig. 5A). The overexpression of one regulator in the absence of the other could not restore normal intracellular growth (Fig. 5B). Intracellular growth was also tested in HeLa cells at a multiplicity of infection of 50. Strains ΔsigH and ΔsigHΔcomK showed impaired growth, while strain ΔcomK showed normal growth (Fig. 6). *sigH* complementation restored intracellular growth in RAW 264.7 and HeLa cells (see Fig. S1B to D in the supplemental material). Taken together, these observations lead us to believe that σ^H and ComK play different roles during intracellular growth.

It was reported that the *comG* operon and the *comEC* gene (but not *comEA* or *comEB*) are required for optimal intracellular growth in macrophages (16). According to our results, *comG* operon expression can be induced by σ^H or ComK, while *comE* operon expression seems to be dependent on σ^H. To test whether the observed intracellular growth deficiency in strain ΔsigH is caused by the diminished transcription of *comEC*, we checked its transcription by semiquantitative RT-PCR. Unexpectedly, our results showed that *comEC* can be expressed in the absence of σ^H and can be activated by ComK (Fig. 7). Thus, the impaired intracellu-

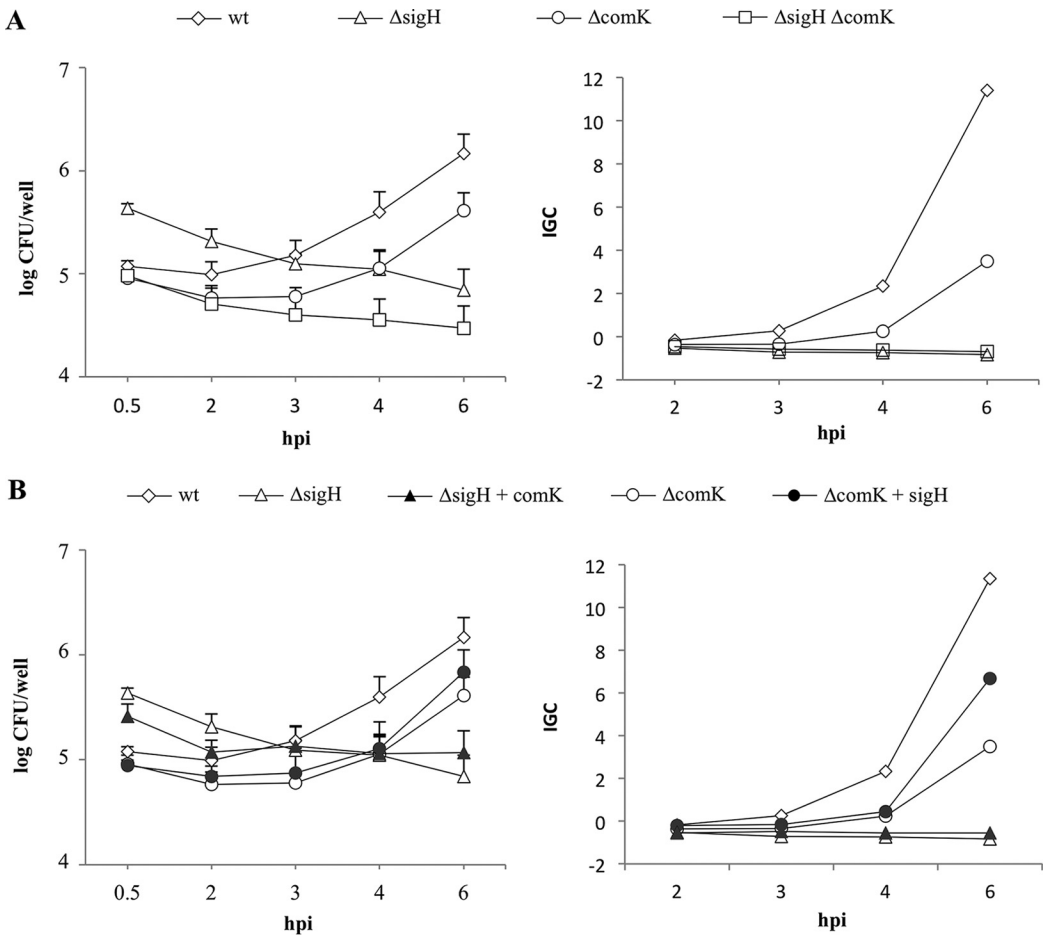

FIG 5 Intracellular growth in RAW 264.7 macrophages. The numbers of CFU (left) were normalized using an IGC (right). (A) wt, phage-cured strain EGDe; ΔsigH, a *sigH* deletion strain; ΔcomK, a *comK* deletion strain; ΔsigHΔcomK, a *sigH* and *comK* double deletion mutant; (B) wt, phage-cured strain EGDe; ΔsigH, a *sigH* deletion strain; ΔsigH+comK, a strain overexpressing *comK* in the *sigH* deletion background; ΔcomK, a *comK* deletion strain; ΔcomK+sigH, a strain overexpressing *sigH* in the *comK* deletion background. The means from 3 independent experiments are shown.

lar growth of strain ΔsigH was not simply attributable to the lack of the essential *comEC* expression.

In summary, σ^H has an essential role in intracellular growth in phagocytic and nonphagocytic cells. The lack of σ^H cannot be compensated for by ComK (which can induce the essential *comG* operon and the *comEC* gene) during macrophage infection. Importantly, the intracellular growth ability in both macrophages and HeLa cells was completely diminished in strain ΔsigH, while the intracellular growth of strain ΔcomK was merely delayed in macrophages and was normal in HeLa cells. These data suggest

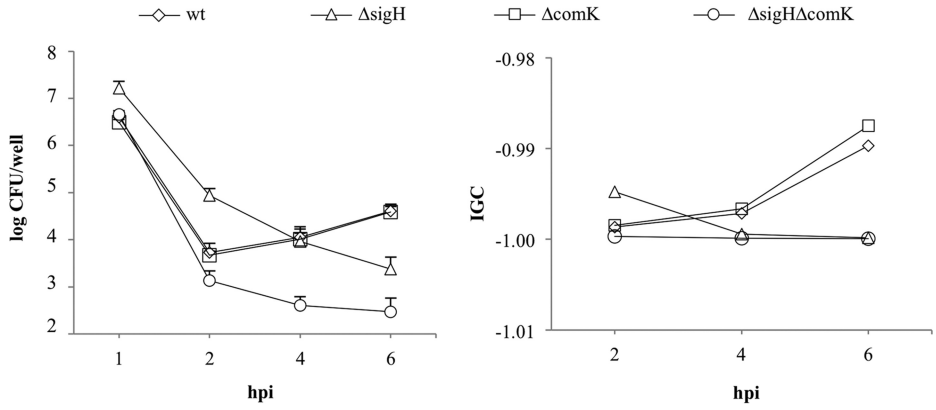

FIG 6 Intracellular growth in HeLa cells. The numbers of CFU (left) were normalized using an IGC (right). wt, phage-cured strain EGDe; ΔsigH, a *sigH* deletion strain; ΔcomK, a *comK* deletion strain; ΔsigHΔcomK, a *sigH* and *comK* double deletion mutant. The means from 3 independent experiments are shown.

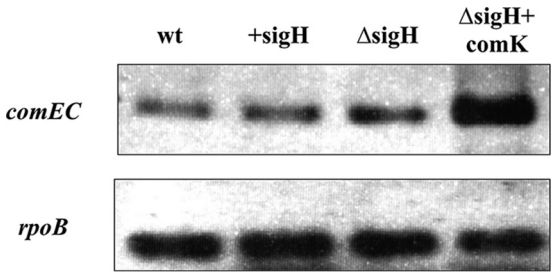

FIG 7 Semiquantitative RT-PCR of the *comEC* gene. wt, phage-cured strain EGDe; +sigH, the wt strain overexpressing *sigH*; ΔsigH, a *sigH* deletion strain; ΔsigH+comK, a strain overexpressing *comK* in the *sigH* deletion background. The sampling point is shown in Fig. 2.

that σ^H is responsible for certain essential processes during the *L. monocytogenes* intracellular life cycle.

σ^H is required for phagosomal escape. *L. monocytogenes* can infect phagocytic and nonphagocytic cells, where its successful intracellular life cycle relies on a crucial first step, i.e., phagosomal/vacuole escape (36, 37). In order to survive, bacteria need to escape vacuole acidification and fusion with lysosomes; phagosome/vacuole lysis occurs quickly after invasion. After escaping to the cytosol, *L. monocytogenes* grows and eventually infects the next cell by using actin-based motility. To determine the stage in which strain ΔsigH is impaired, we performed a phagosomal escape assay in RAW 264.7 macrophages and observed cytosolic bacteria by their association with actin filaments. At 3 hpi, most of the wt strain had escaped the phagosome (69%), whereas only 6% of strain ΔsigH was associated with actin. As expected, strain ΔcomK showed reduced phagosomal escape (28%) (Fig. 8). This result suggests that the intracellular growth deficiency of strain ΔsigH is due to a failure of phagosomal escape. However, this observation might be caused by a downregulation of the *actA* gene (responsible for actin polymerization [38]) and not by a deficiency in phagosomal escape. To test this, infected macrophages were observed using transmission electron microscopy (TEM) at 3 hpi; 50 to 70 bacterial cells were counted. While 66% of the cells of the wt strains and 45% of the cells of strain ΔcomK had escaped the phagosome, only 6% of the cells of strain ΔsigH were in the cytoplasm (Fig. 9), and thus, σ^H is a factor essential for phagosomal escape.

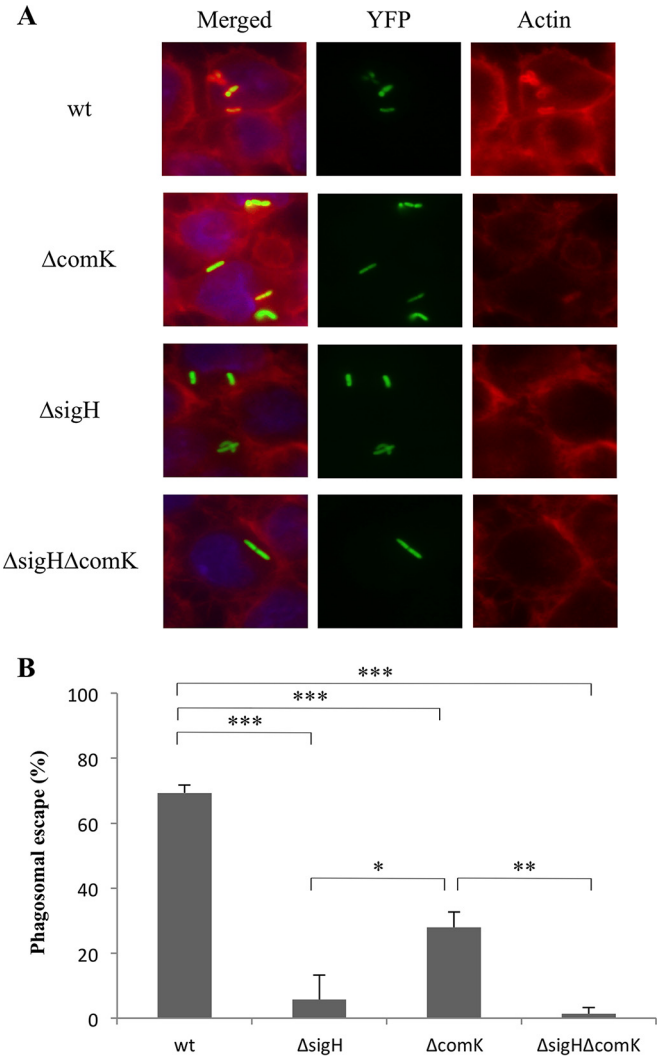

FIG 8 Phagosomal escape at 3 hpi in RAW 264.7 macrophages. (A) Representative images from fluorescence microscopy observations. YFP, yellow fluorescent protein. (B) Average values of phagosomal escape percentages with SDs from 4 independent experiments. Significant differences are indicated. *, $P = 0.01$; **, $P < 0.0001$; ***, $P < 0.00001$. wt, phage-cured strain EGDe; ΔsigH, a *sigH* deletion strain; ΔcomK, a *comK* deletion strain; ΔsigHΔcomK, a *sigH* and *comK* double deletion mutant.

DISCUSSION

Unique regulation of expression of competence genes in *L. monocytogenes*. We report, for the first time, the involvement of the alternative sigma factor σ^H in the expression of competence genes in *L. monocytogenes*. Together with the previous finding that the transcriptional factor ComK induces their expression (16), the regulation of competence machinery expression in *L. monocytogenes* has unique characteristics.

The transcription of the *comE* operon is shown to be complex. *comEA* transcription requires σ^H, while ComK is dispensable. However, the situation is the opposite in the case of *comEC*: σ^H is unnecessary and ComK induces its transcription. These observations suggest that the *comE* genes are not simply under the control of the *comEA* promoter activity alone. The *comE* operon has a ComK box upstream of the *comEA* gene (16), but we could not find any box close to *comEC*. In *B. subtilis*, ComK binds to the promoter region of *comG*, thus causing the upstream DNA to bend around it and allowing ComK to interact with the holoenzyme and stabilizing the RNA polymerase-promoter complex (39). The σ^H-independent expression of *comEC* might involve a similar mechanism; ComK binds upstream of *comEA* and bends to induce *comEC* expression with an RNA polymerase holoenzyme that contains another sigma factor.

With respect to the *comGA* promoter, the percentage of active cells increased when σ^H and ComK were overexpressed (Fig. 4), possibly integrating two distinct signaling inputs. The additive effect of ComK and σ^H was reported in a transcriptome analysis of *S. aureus*, but the regulons of σ^H and ComK in this species overlap completely (22).

comF transcription does not require σ^H but seems to be enhanced by ComK; *comFA* transcription could be detected even in the absence of both regulators. This observation indicates that the RNA polymerase holoenzyme that transcribes the *comF* operon

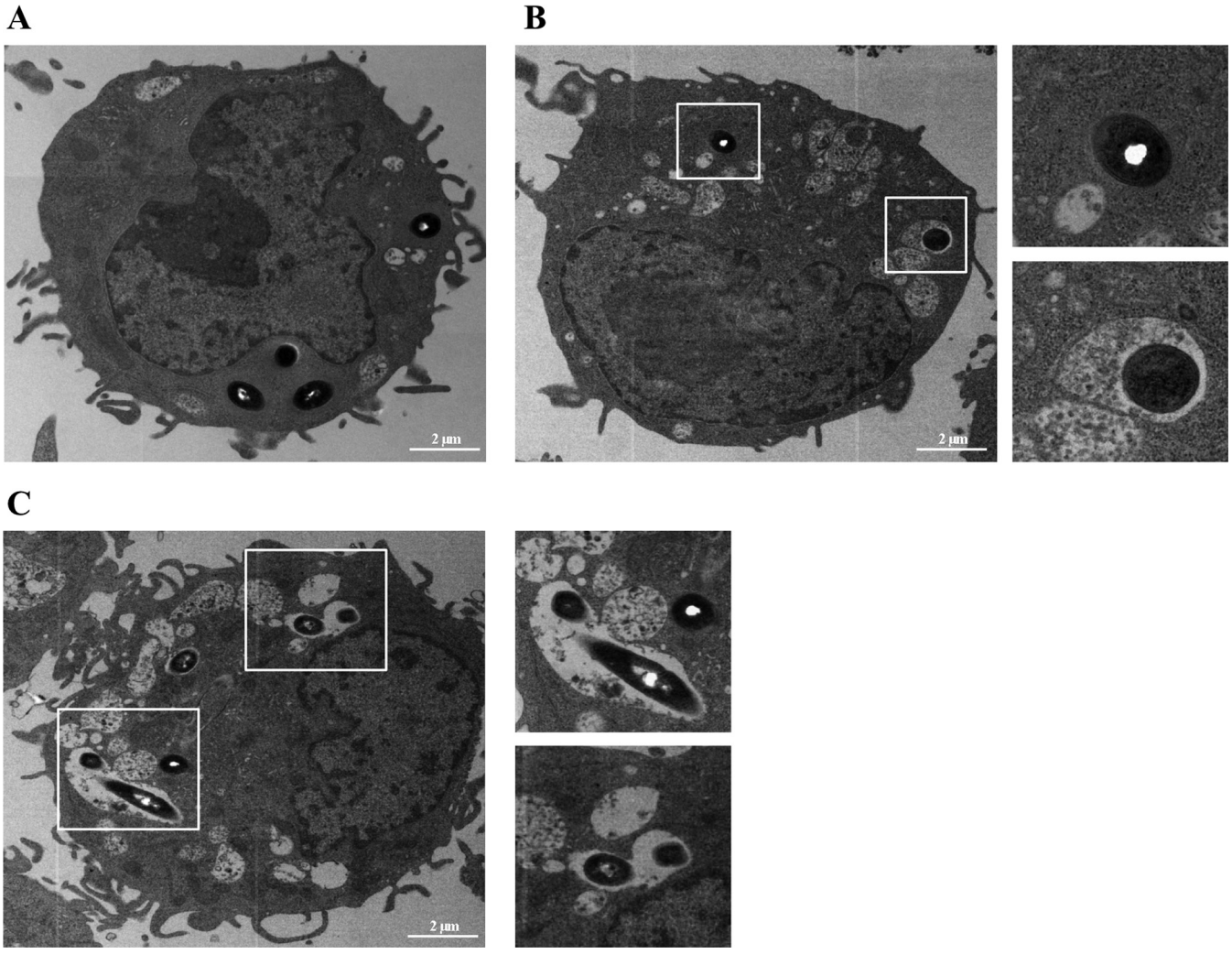

FIG 9 Representative TEM images of *L. monocytogenes* in RAW 264.7 macrophages. Macrophages were infected at an MOI of 10 and fixed at 3 hpi. (A) wt (phage-cured strain EGDe); (B) strain ΔcomK (a *comK* deletion mutant); (C) strain ΔsigH (a *sigH* deletion mutant). In panels B and C, the boxed areas in the panels on the left are enlarged in the two panels on the right.

contains another sigma factor, most likely the housekeeping σ^A (*rpoD*), because consensus sequences for σ^B (40) and σ^L (4) could not be found (σ^C controls few genes [4, 6], and there is no reported consensus sequence). Therefore, *comF* expression in *L. monocytogenes* resembles *B. subtilis* regulation, in which *comF* expression is regulated by σ^A and enhanced by ComK (18, 41).

Thus, the expression of the competence machinery genes in *L. monocytogenes* involves both regulators, σ^H and ComK, and their roles are different even within an operon depending on each *com* gene.

σ^H negative regulation. In *L. monocytogenes*, overexpression of σ^H and ComK could induce the expression of *PcomGA-cfp* in only 20% of the population. However, we also observed that σ^H could induce its expression in the entire population when it was expressed in a different species (*S. aureus*). The low percentage of activation observed in the *L. monocytogenes* background (even with the overexpression of both regulators) suggests the existence of a strong negative regulation in the majority of the population. One candidate is the negative competence regulator MecA, which negatively regulates ComK in *B. subtilis* (42) and σ^H in *Streptococcus mutans* and *Streptococcus thermophilus* (43, 44). Further studies on σ^H negative regulation are necessary to gain insight into the specific conditions that trigger the expression of competence genes.

Competence/transformation development. We succeeded in activating the transcription of competence machinery genes in a fraction of the population by overexpressing ComK and σ^H, but our attempt to detect transformants did not succeed. This does not exclude the possibility that *L. monocytogenes* has the ability to develop natural competence for DNA transformation. The competence state is a time-limited response to a specific environmental condition and to organism-specific processes (45). In *S. aureus*, for example, the transformation of σ^H-expressing cells further requires specific growth conditions (21). In the present study, we bypassed some signals by overexpressing ComK and SigH, but whether further additional layers of signaling or internal regulation are present remains to be evaluated.

σ^H function. In the present study, we have shown that the σ^H factor is required for phagosomal escape during intracellular infection. The *comK* mutant just delays its growth, suggesting that

competence genes are not essential for phagosomal escape. In contrast, SigH mutant growth was completely abolished (Fig. 5), even though it had the ability to express ComG and ComEC factors when ComK was expressed (Fig. 3 and 7, strain ΔsigH+comK). Therefore, we suppose that the mechanism of phagosomal escape controlled by σ^H is due to not merely the expression of the competence genes but also certain essential factors broadly required for the intracellular life cycle.

Leaving aside the main stress sigma factor (σ^B), σ^H positively regulates the largest number of genes and overlaps with other regulons, particularly σ^B (4, 6). It is expected that the function of σ^H in *L. monocytogenes* is complex and not directly linked to one specific process. The reported σ^H regulon does not include any of the genes involved in phagosome/vacuole escape (*prfA* [46], *hly* [47, 48], and *plcA* and *plcB* [49]), and indeed, we could not find a σ^H promoter consensus sequence in these genes. A recent report showed that a σ^H in-frame deletion mutant is affected during growth in minimal medium and shows a low but significant reduction in virulence in a mouse model, leading to the conclusion that σ^H has a role in the acquisition or utilization of nutrients (2). The impaired intracellular growth of our *sigH* mutant might be partly attributed to this broad physiological role. Alternatively, σ^H might have an indirect effect on the expression of the master regulator, *prfA*.

Among Gram-positive bacteria, σ^H has evolved to regulate two different stress-related processes: sporulation and competence development. Here, we showed that *L. monocytogenes* σ^H can induce the transcription of competence genes. In addition, σ^H was crucial for intracellular growth in phagocytic and nonphagocytic cells. Thus, the role of σ^H has further diverged in *L. monocytogenes* along with its intracellular life cycle.

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