Genetic Dissection of DivIVA Functions in *Listeria monocytogenes*

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ABSTRACT DivIVA is a membrane binding protein that clusters at curved membrane regions, such as the cell poles and the membrane invaginations occurring during cell division. DivIVA proteins recruit many other proteins to these subcellular sites through direct protein-protein interactions. DivIVA-dependent functions are typically associated with cell growth and division, even though species-specific differences in the spectrum of DivIVA functions and their causative interaction partners exist. DivIVA from the Gram-positive human pathogen *Listeria monocytogenes* has at least three different functions. In this bacterium, DivIVA is required for precise positioning of the septum at midcell, it contributes to the secretion of autolysins required for the breakdown of peptidoglycan at the septum after the completion of cell division, and it is essential for flagellar motility. While the DivIVA interaction partners for control of division site selection are well established, the proteins connecting DivIVA with autolysin secretion or swarming motility are completely unknown. We set out to identify *divIVA* alleles in which these three DivIVA functions could be separated, since the question of the degree to which the three functions of *L. monocytogenes* DivIVA are interlinked could not be answered before. Here, we identify such alleles, and our results show that division site selection, autolysin secretion, and swarming represent three discrete pathways that are independently influenced by DivIVA. These findings provide the required basis for the identification of DivIVA interaction partners controlling autolysin secretion and swarming in the future.

IMPORTANCE DivIVA of the pathogenic bacterium *Listeria monocytogenes* is a central scaffold protein that influences at least three different cellular processes, namely, cell division, protein secretion, and bacterial motility. How DivIVA coordinates these rather unrelated processes is not known. We here identify variants of *L. monocytogenes* DivIVA, in which these functions are separated from each other. These results have important implications for the models explaining how DivIVA interacts with other proteins.

KEYWORDS rough colony phenotype, Min system, autolysin secretion, cell division, division site selection

DivIVA is a strongly conserved cell division protein found in most Gram-positive bacteria (1). It interacts with the membrane and clusters at curved membrane areas (2, 3). The cell poles and the invaginating septum during cell division are regions where the membrane is naturally bent, and consequently, DivIVA typically clusters at these sites (4–7). DivIVA serves as a central scaffold protein for the recruitment of other factors, and the list of recognized proteins, the localization of which depends on DivIVA, is constantly increasing (1). Among the DivIVA-dependent proteins are integral transmembrane proteins, as well as cytosolic proteins that are primarily involved in cell division (8–11), cell wall biosynthesis (7, 12, 13), or chromosome segregation (2, 14, 15).

DivIVA proteins generally consist of two domains, a strictly conserved N-terminal

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domain required for lipid binding and a lesser-conserved C-terminal domain (16), both forming parallel coiled-coil dimers. The N-terminal extensions of the two helices forming the coiled coil in the N-terminal domain cross each other and fold back onto the coiled-coil structure (16). Hydrophobic amino acids are exposed to the solvent at the apical end of this structure, and DivIVA interacts with membranes through the insertion of these side chains into the phospholipid bilayer (16, 17).

In addition to membrane binding, the lipid binding domain also mediates the interaction of DivIVA with binding partners, such as MinJ (18). In *Bacillus subtilis*, MinJ serves as a molecular bridge between DivIVA and MinD to ensure septal and polar recruitment of the division site selection proteins MinCD (10, 11). MinJ is a polytopic transmembrane protein and interacts with that domain of DivIVA, which already is in closest proximity to the membrane, the lipid binding domain (18). The C-terminal domain of DivIVA, which is oriented toward the cytoplasm, also associates with binding partners, such as the centromere-binding protein RacA (18), contributing to chromosome segregation in *B. subtilis* cells (19, 20). Considering that RacA is a soluble cytoplasmic protein, it therefore seems that DivIVA interacts with transmembrane proteins via its lipid binding domain and with cytoplasmic proteins through its C-terminal domain (18).

Further support for this compartment-specific mode of protein interactions with the two domains of DivIVA proteins was reported with GpsB, a paralogue of DivIVA in selected Gram-positive bacteria (21, 22). GpsB and DivIVA share their N-terminal lipid binding domains but differ in their C-terminal domains (21, 22). GpsB interacts with the bifunctional penicillin binding protein (PBP) A1, an integral transmembrane protein, and mutations preventing this interaction were mapped to the N-terminal domain of GpsB (22).

We are interested in DivIVA of *Listeria monocytogenes*, a firmicute bacterium that causes invasive disease in humans upon the ingestion of contaminated food. This bacterium has the remarkable ability to actively invade nonphagocytic human cell types, to multiply within their cytoplasm, and to spread from cell to cell (23, 24). The deletion of *divIVA* strongly impaired each of these three steps of the *L. monocytogenes* infection cycle (25). Secretion of the two autolysins p60 (CwhA) and NamA (MurA) through the SecA2-dependent secretion pathway is considerably reduced in an *L. monocytogenes* $\Delta divIVA$ mutant (25). Both autolysins are necessary for daughter cell segregation after the completion of cell division; thus, a $\Delta divIVA$ mutant grows as a long chain of unsegregated daughter cells (25). How DivIVA affects the activity of this secretion route remains unclear, but the recruitment of p60 and NamA preproteins to the divisional septum apparently requires DivIVA (25). Most likely, the failure of the $\Delta divIVA$ mutant to invade human cells, to multiply within them, and to spread from one cell to the other is due to steric hindrances associated with cell chain formation. Moreover, DivIVA is important for the swarming motility of *L. monocytogenes*, as the absence of *divIVA* leads to a complete loss of flagellar motility. This defect could not be cured through cocultivation with a nonmotile but autolysin-producing *L. monocytogenes* mutant. In such an experiment, autolysins provided in *trans* suppress the chain-forming phenotype but do not cure the swarming defect of the $\Delta divIVA$ mutant, suggesting that its swarming defect is not merely explained by cell chain formation (25). In addition to these functions, *L. monocytogenes* DivIVA is also required for precise positioning of the divisional septum in the middle of a dividing cell. This activity is dependent on the septal recruitment of the division-inhibitory MinCD proteins through DivIVA (26). In contrast to *B. subtilis*, where this interaction is mediated through MinJ (10, 11), DivIVA of *L. monocytogenes* seemingly interacts with MinD in a direct fashion, as suggested by bacterial two-hybrid experiments (26). Thus, not only do $\Delta divIVA$ mutant cells form cell chains, but also the individual cells in these chains are considerably elongated (26).

To better understand how *L. monocytogenes* DivIVA orchestrates its multifarious functions, we aimed to identify *divIVA* alleles in which these functions are separated from each other. A domain-swapping approach between *L. monocytogenes* and

FIG 1 Expression of chimeric DivIVA proteins in *L. monocytogenes*. (A) Schematic illustration showing the DivIVA chimeras analyzed in this study. Gray and white shaded areas indicate regions corresponding to *L. monocytogenes* and *B. subtilis* DivIVA, respectively. LBD, lipid binding domain; CTD, C-terminal domain; TD, tetramerization domain. The table to the right summarizes the division (Div), autolysin secretion (Atl), and swarming (Mot) phenotypes of strains expressing the *divIVA* chimeras (+, wild type-like phenotype; -, $\Delta divIVA$ mutant-like phenotype; +/-, intermediate phenotype). (B) Western blot showing expression of chimeric DivIVA proteins in *L. monocytogenes*. Cellular proteins were isolated from strains EGD-e (wt), LMS2 ($\Delta divIVA$), LMS30 (+*Lm divIVA*), LMKK8 (+*Bs divIVA*), LMKK13 (+*Lm-104-Bs divIVA*), LMS157 (+*Lm-71-Bs divIVA*), LMKK12 (+*Lm-57-Bs divIVA*), LMKK14 (+*Bs-57-Lm divIVA*), and LMS149 (+*Bs-17-Lm-104-Bs divIVA*) grown in BHI broth containing 1 mM IPTG. Equal amounts of total cellular proteins were loaded, and DivIVA proteins were visualized by immunostaining using a polyclonal antiserum against *B. subtilis* DivIVA (top). A parallel Western blot detecting GpsB was performed to demonstrate equal loading (bottom).

B. subtilis DivIVA demonstrated the importance of the N-terminal domain for general DivIVA functionality but also identified *divIVA* alleles in which its functions in autolysin transport and division site placement could be uncoupled. Furthermore, we identified amino acid exchanges in the N-terminal domain of DivIVA that unlink swarming from autolysin secretion and division site selection. With this and further control experiments, we conclude that the three functions of DivIVA in SecA2-dependent transport, division site selection, and swarming are independent of each other.

RESULTS

***B. subtilis* DivIVA is not functional in *L. monocytogenes*.** Previous work has shown that DivIVA from *L. monocytogenes* (DivIVA^{Lm}) is not functional in *B. subtilis*, even though the two proteins share 65% sequence identity (18) and are almost of the same length (175 versus 164 amino acids, respectively) (Fig. 1A). Apparently, subtle sequence variations between DivIVA^{Lm} and DivIVA from *B. subtilis* (DivIVA^{Bs}) are sufficient to prevent the relevant *B. subtilis* interaction partners from binding to DivIVA^{Lm}.

In *L. monocytogenes*, DivIVA affects (i) SecA2-dependent autolysin secretion, required for daughter cell separation (25); (ii) MinCD-dependent control of division site positioning (26), necessary for cell length maintenance; and (iii) swarming (25). Thus, a *L. monocytogenes* $\Delta divIVA$ mutant forms long chains of unsegregated daughter cells with irregular length that do not swarm on semisolid agar plates. Presently, it is not known which of these three functions depend on which DivIVA domain, and it is even not clear whether they can be separated at all. To address this question, we first determined the complementation activity of DivIVA^{Bs} in an *L. monocytogenes* $\Delta divIVA$ mutant. For this purpose, an *L. monocytogenes* strain was constructed that lacks its endogenous *divIVA* gene but carries the *divIVA* gene of *B. subtilis* 168 under the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter at an ectopic site

(strain LMKK8). The expression of *divIVA*^{Bs} in this strain was confirmed by Western blotting (Fig. 1B). Strain LMKK8 formed long cell chains, similar to the *L. monocytogenes* Δ *divIVA* mutant (Fig. 2A and C), suggesting that SecA2-dependent autolysin secretion is not supported by DivIVA^{Bs}. Likewise, strain LMKK8 was not able to swarm on semisolid agar plates (Fig. 3), indicating that *divIVA*^{Bs} is nonfunctional in terms of swarming. Finally, LMKK8 cells (median cell length, 1.33 μ m) are as elongated as Δ *divIVA* mutant cells (median cell length, 1.38 μ m), whereas wild-type cells are shorter (median cell length, 1.28 μ m) (Fig. 2B). This shows that DivIVA^{Bs} also cannot take over MinCD control in *L. monocytogenes* cells. Taken together, these results indicate that DivIVA^{Bs} does not complement any of the three Δ *divIVA* phenotypes in *L. monocytogenes*.

Separation of DivIVA functions using chimeric DivIVA^{Lm/Bs} proteins. To identify regions in DivIVA needed for support of its three functions, a domain-swapping approach similar to the one we have previously used for the identification of protein interaction domains in DivIVA^{Bs} (18) was performed in *L. monocytogenes*. For this purpose, a set of chimeric *divIVA* genes, consisting of different parts of *divIVA*^{Bs} and *divIVA*^{Lm} (Fig. 1A), was expressed in the *L. monocytogenes* Δ *divIVA* mutant background, and their expression was analyzed by Western blotting (Fig. 1B). This showed that all the constructed chimeric DivIVA proteins were expressed, albeit to different degrees.

SecA2 activity and autolysin secretion (Atl phenotype) can be measured indirectly based on cell chain formation. Thus, the cellular morphology of strains expressing *divIVA* chimeras was analyzed by fluorescence microscopy of logarithmically growing cultures after membrane staining. Under these conditions, wild-type cells grow as individual rods or cell doublets, whereas the Δ *divIVA* mutant forms chains of cells with up to ~50 cells per chain (Fig. 2A and C). The formation of long cell chains similar to the Δ *divIVA* mutant was also observed with strains expressing the *divIVA*^{Lm-57-Bs} (LMKK12) and the *divIVA*^{Bs-17-Lm-104-Bs} (LMS149) chimeras. In contrast, strain LMKK13, expressing *divIVA*^{Lm-104-Bs}, formed wild-type-like rods, and strain LMS157 (*divIVA*^{Lm-71-Bs}) and strain LMKK14 (*divIVA*^{Bs-57-Lm}) showed an intermediate phenotype with partial daughter cell segregation (Fig. 2A and C). These data indicate that the first 104 N-terminal residues of DivIVA^{Lm} are important for full SecA2-dependent autolysin secretion.

DivIVA controls the activity of the Min system; thus, the inactivation of DivIVA causes cell elongation in addition to cell chaining. As a result, the cell length distribution of a Δ *divIVA* mutant is shifted toward longer cell lengths (22, 26). Measurement of cell lengths in strains expressing *divIVA* chimeras showed that the DivIVA^{Lm-104-Bs} and the DivIVA^{Lm-71-Bs} chimeras supported a wild-type-like cell length distribution. However, when the *L. monocytogenes* part of the chimeric proteins was further reduced down to those present in the DivIVA^{Lm-57-Bs} or the DivIVA^{Bs-17-Lm-104-Bs} chimera, the fraction of elongated cells increased (Fig. 2A and B). Remarkably, a cell length profile even shorter than in the wild type was observed with cells expressing the DivIVA^{Bs-57-Lm} chimera. This indicates that DivIVA regions 1 to 71 or 57 to 175 are important during cell division.

Swarming analysis of the same set of strains revealed that only strains expressing the *divIVA*^{Lm-104-Bs}, the *divIVA*^{Lm-71-Bs}, or the *divIVA*^{Lm-57-Bs} chimera were able to swarm (Fig. 3). These three chimeric proteins still contained the N-terminal domain of DivIVA^{Lm} that comprises amino acid positions 1 to 57. In contrast, strains either expressing the *divIVA*^{Bs-17-Lm-104-Bs} or the *divIVA*^{Bs-57-Lm} chimera, in which the N-terminal domain of DivIVA^{Bs} was fused to the C-terminal domain of DivIVA^{Lm}, were unable to swarm (Fig. 3). Taken together, these results indicate that different regions in DivIVA^{Lm} are required to sustain normal daughter cell segregation, cell division, and swarming.

The interaction of DivIVA and MinD is independent of MinJ. The importance of an N-terminal region in DivIVA (amino acids 1 to 71) for normal cell division of *L. monocytogenes* is in good agreement with data obtained in *B. subtilis* showing that the N-terminal domain of DivIVA^{Bs} interacts with MinJ (18). However, our observation that the *divIVA*^{Bs-57-Lm} chimera also supports the normal cell division of *L. monocytogenes*

FIG 2 Effect of chimeric DivIVA proteins on cell length and chaining of *L. monocytogenes*. (A) Fluorescence micrographs showing Nile red-stained cells. Strains EGD-e (wt), LMS2 (ΔdivIVA), LMS30 (+Lm
(Continued on next page)

FIG 3 Swarming motility of *L. monocytogenes* strains expressing chimeric DivIVA proteins. Strains EGD-e (wt), LMS2 ($\Delta divIVA$), LMS30 (+Lm *divIVA*), LMKK8 (+Bs *divIVA*), LMKK13 (+Lm-104-Bs *divIVA*), LMS157 (+Lm-71-Bs *divIVA*), LMKK12 (+Lm-57-Bs *divIVA*), LMKK14 (+Bs-57-Lm *divIVA*), and LMS149 (+Bs-17-Lm-104-Bs *divIVA*) were stab inoculated into LB soft agar (containing 1 mM IPTG) and cultivated for 24 h at 30°C. The diameters of their swarming halos were determined in four independent experiments and plotted in the diagram shown below. Significance levels are indicated: ***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.01$.

seems to conflict with this model, as it suggests that either MinJ or MinD may also interact with the C-terminal DivIVA domain. An interaction of MinJ with the C-terminal DivIVA domain was not detected in previous *B. subtilis* experiments (18), but an interaction of MinD with the C-terminal DivIVA domain would be in accordance with results that claimed a direct interaction of MinD with DivIVA without the help of MinJ (26).

To confirm that MinD interacts with DivIVA in a MinJ-independent way, we performed pulldown experiments in *L. monocytogenes* cells expressing MinD-His₁₀ as bait

FIG 2 Legend (Continued)

divIVA), LMKK8 (+Bs *divIVA*), LMKK13 (+Lm-104-Bs *divIVA*), LMS157 (+Lm-71-Bs *divIVA*), LMKK12 (+Lm-57-Bs *divIVA*), LMKK14 (+Bs-57-Lm *divIVA*), and LMS149 (+Bs-17-Lm-104-Bs *divIVA*) were grown in BHI broth containing 1 mM IPTG at 37°C to mid-logarithmic growth phase and analyzed by microscopy. Div and Atl phenotypes are indicated. (B) Cell lengths of 300 cells per strain were measured, and the length distribution is illustrated in a box plot (<http://shiny.chemgrid.org/boxplotr/>). Center lines show the medians; box limits indicate the 25th and 75th percentiles, as determined by the R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; and outliers are represented by dots. $n = 300$ sample points. Median cell lengths are given above the diagram. (C) Lengths of cell chains of ~200 cells per strain were measured and illustrated in the same kind of box plot as in panel B.

FIG 4 DivIVA binding of MinD is MinJ independent. Western blots showing pulldown eluates obtained with MinD-His as the bait protein in wild-type (LMJR86) and Δ minJ mutant backgrounds (LMJR85). Strains expressing MinJ-His (LMJR84, positive control) and SecA2-His (LMKK3, negative control) were included. DivIVA was detected using a polyclonal antiserum raised against *B. subtilis* DivIVA (left blot), and His-tagged proteins were detected using an anti-His antibody (right blot). EGD-e (wt) was included as another negative control. Please note that MinJ-His (45.5 kDa) runs at an aberrant position after cross-linking and heat treatment (26).

and either containing the *minJ* gene or not. Cells of strains LMJR86 (*minD-his₁₀*) and LMJR85 (*minD-his₁₀ ΔminJ*) were grown in brain heart infusion (BHI) broth, and proteins were cross-linked with formaldehyde. MinD-His₁₀ protein complexes were affinity purified and de-cross-linked by heat treatment. As previously shown (26), DivIVA can be detected by Western blotting in the pulldown eluates obtained with MinD-His₁₀ as the bait protein in cells with a wild-type background (Fig. 4). Likewise, DivIVA was pulled down with MinD-His₁₀ from lysates of LMJR85 cells that do not contain *minJ*. Control experiments confirmed that the MinD-His₁₀ protein was pulled down whether MinJ was present or not (Fig. 4). Furthermore, DivIVA was pulled down with its known interaction partner MinJ-His and not detected in the absence of any bait protein. SecA2-His, as another negative control, only pulled down traces of DivIVA material (Fig. 4). This result shows that the interaction of MinD with DivIVA does not require MinJ in *L. monocytogenes*.

Mutations in the N-terminal domain separate *divIVA* functions in swarming and division. Previously published work on the DivIVA homolog GpsB has identified a strongly conserved groove on the surface of the N-terminal GpsB domain. This groove is formed by amino acids Y27, D33, D37, and I40 and contributes to the interaction of GpsB with the cytosolic N terminus of the major bifunctional penicillin binding protein (PBP) A1 (22). This protein motif is invariably present in most, if not all, GpsB homologs, but it is less conserved in DivIVA proteins (22). DivIVA of *L. monocytogenes* contains three of these four amino acids, but the D33 residue of *L. monocytogenes* GpsB is replaced by asparagine (residues Y20, N26, D30, and I33 in DivIVA). In order to analyze the importance of these residues for *divIVA* function, we constructed *divIVA* alleles in which the Y20, D30, or I33 residue was mutated. These alleles were then introduced into the chromosome of the Δ *divIVA* mutant in a way that the *divIVA* open reading frame is restored at its original position in the chromosome but now expresses mutated *divIVA* variants (see inset in Fig. 7A). Replacement of Y20 with a phenylalanine residue (strain LMS196) did not affect DivIVA function, since this strain was fully motile (Fig. 5A), formed smooth colonies (data not shown), and grew as short rods with normal cell lengths (Fig. 5B). The exchange of D30 with alanine (strain LMS198) led to the loss of swarming motility (Fig. 5A), but autolysin secretion and cell division were unaffected, as deduced from the absence of cell chaining and normal cell length distribution (Fig. 5B),

FIG 5 *divIVA* mutations separating division and swarming from autolysin secretion. (A) Swarming motility of *L. monocytogenes* strains EGD-e (wt), LMS2 ($\Delta divIVA$), LMS196 (*divIVAY20F*), LMS198 (*divIVAD30A*), and LMS197 (*divIVA133A*) on semisolid LB agar plates. Swarming halo diameters were measured three times, and average values and standard deviations are shown in the diagram below. Significant differences are indicated by asterisks ($P < 0.01$). (B) Morphology of the same set of strains during exponential growth in BHI broth at 37°C. Micrographs show cells stained with Nile red. Div and Atl phenotypes are indicated. Please note that strain LMS197 (*divIVA133A*) has the filamentous Div⁻ phenotype but does not form chains. Cell lengths of 300 cells per strain were measured, and the results are presented in the diagram below, at the bottom of which median cell lengths are shown. (C) Effect of the *divIVA133A* mutation on GFP-MinD localization. Strains LMKK30 and LMS227 were grown in BHI broth (containing 1 mM IPTG) at 30°C to mid-logarithmic growth phase and analyzed by fluorescence microscopy (right). Phase-contrast images are included for orientation (left). Scale bar is 5 μ m.

respectively. Thus, factors linking DivIVA with swarming motility likely no longer bind to DivIVAD30A, whereas the MinJ and MinD proteins still do. A partially inverse effect was observed with the I33A mutation (strain LMS197). Cells carrying this mutation were still able to swarm (~50% of wild-type halo diameter) (Fig. 5A) but displayed a clear Div

FIG 6 Swarming motility is MinCDJ independent in *L. monocytogenes*. Swarming of *L. monocytogenes* strains EGD-e (wt), LMS2 ($\Delta divIVA$), LMS120 ($\Delta minJ$), LMS148 ($\Delta minC$), LMKK35 ($\Delta minCD$), and LMKK54 ($\Delta minCDJ$) on LB soft agar plates. Swarming halo diameters were measured three times, and the average values and standard deviations are shown in the diagram shown below. Significance levels are indicated: *, $P < 0.01$.

phenotype, typically characterized by cell elongation (Fig. 5B) (26). The Div phenotype depends on the recruitment of MinCD by DivIVA to the division septum (26). In good agreement with the above-mentioned observation, septal recruitment of green fluorescent protein (GFP)-MinD was distorted, and GFP-MinD was present at lateral membrane regions instead of the division septum in *divIVA*/33A mutant cells (Fig. 5C). None of the three *divIVA* mutants led to the formation of rough colonies (data not shown) or to cell chaining (Fig. 5B), suggesting that residues Y20, D30, and I33 are not important for control of the SecA2-dependent autolysin secretion. These results suggest that a stretch in DivIVA that is equivalent to the PBP A1 interaction groove of GpsB contributes to binding of MinD and factors affecting flagellar motility. Thus, a common region in the N terminus of DivIVA and GpsB proteins may be used for interaction with binding partners.

Swarming motility is MinCDJ independent in *L. monocytogenes*. In the undomesticated *B. subtilis* strain 3610, MinJ is required for swarming motility without affecting flagellum formation (11). Likewise, an *L. monocytogenes* $\Delta divIVA$ mutant is unable to swim, even though it produces flagella (25). These facts, together with the observation that the I33A exchange in DivIVA affected cell division and motility, suggested that DivIVA could affect the swarming motility of *L. monocytogenes* through the Min system. This hypothesis was tested in a simple swarming assay, comparing the swarming motility of the $\Delta divIVA$ mutant with that of mutants lacking the *min* genes. As can be seen in Fig. 6, the lack of neither *minC* (strain LMS148), *minJ* (LMS120), *minCD* (LMKK35), nor *minCDJ* (LMKK54) impaired the swarming motility of *L. monocytogenes*, whereas the $\Delta divIVA$ mutant was completely unable to swarm under this condition. This result demonstrates that the effect DivIVA exerts on swarming motility of *L. monocytogenes* does not involve the MinCDJ proteins.

Contribution of DivIVA functions to *in vitro* virulence. The *L. monocytogenes* $\Delta divIVA$ mutant is heavily impaired in its ability to invade nonphagocytic human cells, to multiply within them, and to spread from one cell to another in various *in vitro* virulence models (25). We assumed that this severe degree of virulence attenuation could be explained by the combination of cell chaining, immotility, and distorted cell division rather than by negative effects on specific virulence factors required for host cell invasion, intracellular multiplication, or cell-to-cell spread (25). The amino acid exchanges in the N-terminal domain of DivIVA described above provided the first opportunity to answer the question as to what degree these three different defects contribute to virulence attenuation of the $\Delta divIVA$ mutant, as none of these affected growth in liquid culture (Fig. 7A). Intracellular multiplication was tested in J774.A1 mouse ascites macrophages. *L. monocytogenes* wild-type bacteria are rapidly phagocytosed by these macrophages, escape from their phagosomes, and then multiply within their cytoplasm. In contrast, the intracellular growth of the $\Delta divIVA$ mutant stopped 2 h postinfection, and then the number of intracellular bacteria steadily decreased, indicating efficient killing of $\Delta divIVA$ mutant cells by the macrophages (Fig. 7B). This suggests that the $\Delta divIVA$ mutant is unable to survive under the harsh conditions of their phagosomes. Interestingly, strains carrying the Y20F (Div⁺ Atl⁺ Mot⁺) and I33A (Div⁻ Atl⁺ Mot^{+/-} ["+/-" indicates an intermediate phenotype]) exchanges in DivIVA grew like the wild type in this assay, whereas the D30A (Div⁺ Atl⁺ Mot⁻) mutant multiplied somewhat slower (Fig. 7). These data show that virulence attenuation of the *L. monocytogenes* $\Delta divIVA$ mutant does not result from defects in cell division and is only to a minor extent explained by the motility defect associated with the $\Delta divIVA$ mutation. Instead, the virulence defect of the $\Delta divIVA$ mutant is likely attributed to its chain-forming mode of growth. This conclusion was further substantiated by an experiment measuring the intracellular growth of the Div⁻ Atl⁻ Mot^{+/-} strain LMKK12, expressing the *divIVA*^{Lm-57-Bs} allele, in J774 cells. This strain, forming chains but showing residual motility, was completely unable to proliferate inside macrophages (Fig. 7C).

DISCUSSION

DivIVA is a pivotal protein for the recruitment of several functionally distinct proteins to the cell poles and the septum. Despite their diversity among the different bacteria, some DivIVA interaction partners can be found in several species, such as the ParB/Spo0J proteins, required for chromosome segregation. Binding of DivIVA to these proteins was reported for *B. subtilis*, *Streptococcus pneumoniae*, *Corynebacterium glutamicum*, *Mycobacterium tuberculosis*, and *Streptomyces coelicolor* (6, 14, 27). Seven different interaction partners of DivIVA are known in *B. subtilis* (MinJ, RacA, Spo0J, SpoII_E, SecA, Maf, and ComN) (2, 10, 11, 27–31), and DivIVA of *S. pneumoniae* supposedly interacts with up to 14 distinct binding partners, among which are most divisome components and the two cell wall hydrolases LytB and PcsB (6). Given this multitude of structurally different interaction partners that even are active in different cellular compartments (i.e., the membrane and the cytoplasm), competition for a commonly used docking site in DivIVA seems unlikely. However, if separated binding sites for interaction with different protein partners exist in DivIVA, it must be possible to find mutations that affect the binding of one interaction partner without impairing the binding of others (18). In fact, the binding sites for MinJ and RacA are nonoverlapping in *B. subtilis* DivIVA, whose lipid binding domain interacts with the transmembrane protein MinJ (Fig. 8) and the C-terminal domain with cytosolic RacA (18).

Here, we present the first set of DivIVA variants separating its different functions in the dangerous human pathogen *L. monocytogenes*. First of all, we show that the N-terminal 104 amino acids of *L. monocytogenes* DivIVA contain the region essential for SecA2-dependent autolysin secretion (Fig. 8). As soon as this section in the respective chimeras is further shortened down to amino acids 1 to 71 or 1 to 57 or when the N-terminal domain of DivIVA^{Lm} was replaced with that of DivIVA^{Bs}, the formation of cell chains was observed, indicating less efficient autolysin transport. Thus, DivIVA residues

FIG 7 Effect of selected *divIVA* mutants on intracellular growth in mouse macrophages. (A) Growth of *L. monocytogenes* strains EGD-e (wt), LMS2 (Δ divIVA), LMS196 (divIVAY20F), LMS198 (divIVAD30A), and LMS197 (divIVA^{I33A}) in BHI broth. Inset: Western blot showing DivIVA protein levels during growth under identical conditions in the same set of strains. (B) Intracellular growth of the same set of strains in J774 mouse macrophages. Significance levels are indicated: ***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.01$. (C) Effect of the *divIVA*^{Lm-57-Bs} allele (Div⁻ Atl⁻ Mot^{+/-}, strain LMKK12) on intracellular growth in J774 mouse macrophages (cultivated in the presence of 1 mM IPTG for induction of *divIVA* alleles). *L. monocytogenes* strains EGD-e (wt), LMS2 (Δ divIVA), and LMS30 (+Lm *divIVA*) were included for comparison. Intracellular growth was recorded based on CFU determination. Average values and standard deviations in panels A to C were calculated from experiments performed in triplicate. Significance levels are indicated: ***, $P < 0.0001$; **, $P < 0.001$.

inside and outside the lipid binding domain of DivIVA are crucial for autolysin secretion. The protein that links DivIVA with this process is presently still unknown, and, due to the participation of both DivIVA domains, we still cannot decide whether transmembrane or soluble proteins should rather be considered as candidates for this. Also, none of the three tested amino acid exchanges in the N-terminal DivIVA domain resulted in the formation of cell chains. Thus, it seems that DivIVA regions other than those supporting the interaction of GpsB with PBP A1 contribute to the interaction of DivIVA with this unknown factor.

A shorter N-terminal fragment, comprising only amino acids 1 to 71, needs to be present in the DivIVA chimera for full support of flagellar motility (Fig. 8), while partial

FIG 8 Protein interaction domains in *L. monocytogenes* DivIVA. Three cartoons summarizing the current view of how DivIVA interacts with its binding partners. Left, data from *B. subtilis* suggest that the N-terminal DivIVA domain binds MinJ (18). Middle, regions in the N-terminal and the C-terminal domains of *L. monocytogenes* DivIVA are crucial for MinD binding (this study). Right, residues 1 to 104 (N-terminal domain with a short section of the C-terminal domain) and 1 to 71 (N-terminal domain with the adjacent linker) of *L. monocytogenes* DivIVA are required for SecA2-dependent autolysin secretion and for flagellar motility (this study), respectively.

swarming was still observed in cells expressing the DivIVA^{Lm-57-Bs} chimera. Furthermore, point mutations in the N-terminal domain of DivIVA (D30A and I33A) were sufficient to interfere with swarming motility. Thus, we assume that the protein connecting DivIVA with the flagellum interacts with the lipid binding domain and that it is likely to be a transmembrane protein.

Remarkably, the N- and the C-terminal domains of *L. monocytogenes* DivIVA supported normal cell division (Fig. 8) in the chimera experiments independent of each other. This result is counterintuitive in the first instance but would be in agreement with previous work that showed that MinD and MinJ, the two proteins transmitting the spatial input of DivIVA to the cell division machinery, interact directly with DivIVA (26). In *B. subtilis*, the lipid binding domain of DivIVA interacts with the integral membrane protein MinJ (18), through which the contact to MinD is made. MinD is a soluble protein but also binds the membrane through an amphipathic helix (32). MinD is a ParA-like ATPase (33), and ParA of *Mycobacterium smegmatis* interacts with regions in the N- and C-terminal domains of DivIVA (34). It thus seems likely that *L. monocytogenes* MinD also might make contacts with the C-terminal domain of DivIVA (Fig. 8). More sophisticated experiments would be needed to test this hypothesis.

With the D30A exchange in *L. monocytogenes* DivIVA, we have identified a mutation that completely blocked swarming but affected neither autolysin secretion nor cell division. Likewise, the I33A mutation affected division but not autolysin transport. The D30A mutation now unequivocally shows that the swarming defect of the $\Delta divIVA$ mutant is not simply explained by the long cell chains it forms, simply because *divIVAD30A* cells do not form chains but still they are nonmotile. This conclusion is further substantiated by the observation that cells expressing the *divIVA*^{Lm-57-Bs} chimera could swarm, at least partially, even though they formed cell chains. All this is in good agreement with previous experiments, which already demonstrated that splitting of $\Delta divIVA$ cell chains did not restore motility (25). Apparently, growth as a cell chain does not prevent swarming *per se*. This concept is still challenged by the observation that a $\Delta secA2$ mutant is also unable to swarm (data not shown), but this might be for different reasons.

Finally, our results clarify that the chain-forming growth of the $\Delta divIVA$ mutant, which is due to deficient SecA2-dependent secretion of autolysins, must be the main reason for its virulence attenuation, at least in the infection model tested so far (25). Remarkably, the chosen macrophages even started to clear the infection with $\Delta divIVA$ mutant bacteria. This can be taken as further support for the assumption that DivIVA might be a useful drug target to treat *L. monocytogenes* infections in the future.

Taking these results together, we show that the three activities of DivIVA in cell division, SecA2-dependent transport, and swarming motility can be separated from each other. The chimeras and mutants of *divIVA* that we identified in this study might be useful as starting points in suppressor mutagenesis screens to identify those proteins that connect DivIVA with the flagellum and the SecA2-dependent protein secretion pathway.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Plasmids		
pIMK3	<i>P_{help}-lacO lacI neo</i>	36
pMAD	<i>bla erm bgaB</i>	38
pJR55	<i>P_{help}-minD-his₁₀ neo</i>	26
pSH2	<i>bla aprE5' spc lacI P_{spac}-divIVA^{Bs} aprE3'</i>	16
pSH19	<i>bla amyE3' spc P_{xyf}-divIVA^{Bs} amyE5'</i>	18
pSH186	<i>bla erm bgaB ΔdivIVA (lmo2020)</i>	25
pSH260	<i>bla amyE3' spc P_{xyf}-divIVA^{Lm-71-Bs} amyE5'</i>	18
pSH221	<i>P_{help}-lacO-divIVA^{Lm} lacI neo</i>	25
pKK16	<i>P_{help}-lacO-divIVA^{Bs} lacI neo</i>	This work
pKK17	<i>P_{help}-lacO-divIVA^{Lm-104-Bs} lacI neo</i>	This work
pKK18	<i>P_{help}-lacO-divIVA^{Lm-57-Bs} lacI neo</i>	This work
pKK21	<i>P_{help}-lacO-divIVA^{Bs-57-Lm} lacI neo</i>	This work
pKK90	<i>bla erm bgaB divIVA^{Lm}</i>	This work
pSH388	<i>P_{help}-lacO-divIVA^{Bs-17-Lm-104-Bs} lacI neo</i>	This work
pSH394	<i>P_{help}-lacO-divIVA^{Lm-71-Bs} lacI neo</i>	This work
pSH472	<i>bla erm bgaB divIVAD30A</i>	This work
pSH473	<i>bla erm bgaB divIVAY20F</i>	This work
pSH474	<i>bla erm bgaB divIVAI33A</i>	This work
<i>L. monocytogenes</i> strains		
EGD-e	Wild-type serovar 1/2a strain	Lab collection
LMJR84	<i>attB::P_{help}-minJ-his₁₀ neo</i>	26
LMJR86	<i>attB::P_{help}-minD-his₁₀ neo</i>	26
LMKK3	<i>attB::P_{help}-secA2-his₁₀ neo</i>	26
LMKK30	<i>attB::P_{help}-gfpA206K-minD neo</i>	26
LMKK35	<i>ΔminCD</i>	26
LMKK54	<i>ΔminCDJ</i>	26
LMS2	<i>ΔdivIVA</i>	25
LMS30	<i>ΔdivIVA attB::P_{help}-lacO-divIVA^{Lm} lacI neo</i>	25
LMS120	<i>ΔminJ</i>	26
LMS148	<i>ΔminC</i>	26
LMJR85	<i>ΔminJ attB::P_{help}-minD-his₁₀ neo</i>	This work
LMKK8	<i>ΔdivIVA attB::P_{help}-lacO-divIVA^{Bs} lacI neo</i>	This work
LMKK12	<i>ΔdivIVA attB::P_{help}-lacO-divIVA^{Lm-57-Bs} lacI neo</i>	This work
LMKK13	<i>ΔdivIVA attB::P_{help}-lacO-divIVA^{Lm-104-Bs} lacI neo</i>	This work
LMKK14	<i>ΔdivIVA attB::P_{help}-lacO-divIVA^{Bs-57-Lm} lacI neo</i>	This work
LMS149	<i>ΔdivIVA attB::P_{help}-lacO-divIVA^{Bs-17-Lm-104-Bs} lacI neo</i>	This work
LMS157	<i>ΔdivIVA attB::P_{help}-lacO-divIVA^{Lm-71-Bs} lacI neo</i>	This work
LMS196	<i>divIVAY20F</i>	This work
LMS197	<i>divIVAI33A</i>	This work
LMS198	<i>divIVAD30A</i>	This work
LMS227	<i>divIVAI33A attB::P_{help}-gfpA206K-minD neo</i>	This work

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists all bacterial strains used in this study. *L. monocytogenes* was routinely cultivated in brain heart infusion (BHI) broth or on BHI agar plates at 37°C. If required, antibiotics and supplements were added to the growth media at the following concentrations: erythromycin, 5 μg · ml⁻¹; kanamycin, 50 μg · ml⁻¹; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 100 μg · ml⁻¹; and IPTG, 1 mM. *Escherichia coli* TOP10 was used as the standard host for all cloning procedures (35).

General methods, manipulation of DNA, and oligonucleotide primers. *E. coli* transformation and isolation of plasmid DNA were performed according to standard protocols (35). Electroporation of *L. monocytogenes* was done as described previously (36). Enzymatic modification of plasmid DNA was carried out as described by the instructions given by the manufacturers. QuikChange mutagenesis was employed for restriction-free modification of plasmids (37). The DNA sequences of the oligonucleotide primers are listed in Table 2.

Construction of strains expressing chimeric and mutated DivIVA proteins. Plasmid pKK16 was constructed to allow for the expression of *B. subtilis* divIVA (divIVA^{Bs}) in *L. monocytogenes* cells. For this purpose, the divIVA^{Bs} gene was amplified from plasmid pSH2 using the primers KK20/KK21. The resulting DNA fragment was then used as a megaprimer in a second PCR to replace the divIVA^{Lm} open reading frame of plasmid pSH221 with the divIVA^{Bs} gene. For the construction of plasmid pKK17, expressing the chimeric divIVA^{Lm-104-Bs} gene, the C-terminal region of divIVA^{Bs} was amplified from plasmid pSH19 using the primer pair SHW266/KK21 and used as a megaprimer in a second PCR with pSH221 as the template.

TABLE 2 Oligonucleotides used in this study

Name	Sequence (5'→3')
KK20	TTATAACAATAGTCGTATGGAGGTGCTAGATATGCCATTAAACGCCAAATGATATT
KK21	GATCGTCGACTCTAGAGGATCCCCGCGCTCGAGTTATTCCTTTCTCAATACAGC
KK113	CAATAGTCGTATGGAGGTGCTAGATATGCCATTATCGCCGCTGGATAT
KK114	CTTACTTGTCTCTAATAATCGTTTTAACGTTCTTCAGATTCAGCTG
SHW180	CGAAGTAAATGACTTCCTCGATC
SHW208	TAGAGTAATTCTGTAAAGGTCC
SHW266	GCAGAAAAAATGCTGACCGAATTATCAACGAATCGTTATCAAAATC
SHW353	CTTTAAATAATAGTGAAGAACGTATCGGACACTTTGCCAATATTG
SHW354	GTCGTATGGAGGTGCTAGATATGCCATTAAACGCCAAATGATATTC
SHW355	GTTTCTTCAATGTTTGTAATAATGACCGATTCTTCATCAAGCTCATTG
SHW506	CTTCGTCTCGTCATAACCTCTAAACTTTTTGTAAACGTCTTGTGTG
SHW508	ACGCCCCGCATAAAATGCCAGGAATTGGG
SHW530	GCGTCGACTTCATACTCAAGG
SHW734	TTAGAGGTTTTGACGAAGACGAAGTAAATGAC
SHW735	TCTTCGTCAAACCTCTAAAACACGGGTAAAC
SHW736	ACTTCCTCGCACAAATCATTAAAGATTATGAACAAG
SHW737	ATGATTTGTGCGAGGAAGTCATTTACTTCGTC
SHW738	ATCAAATCGCAAAAGATTATGAACAAGTTATCAAAG
SHW739	TAATCTTTTGCATTGATCGAGGAAGTCATTAC

Plasmid pKK18 was constructed by generating a DNA fragment comprising the C-terminal *divIVA*^{Bs} domain plus additional downstream plasmid backbone sequences using the oligonucleotides SHW208/SHW353 and pKK16 as the template. This DNA fragment was then used as a primer in a second PCR on plasmid pSH221 as the template in order to create the *divIVA*^{Lm-57-Bs} allele. For the construction of plasmid pKK21, expressing the *divIVA*^{Bs-57-Lm} allele, a PCR product comprising the N-terminal domain of *divIVA*^{Bs} was generated with the primer pair SHW354/SHW355 on plasmid pKK16. This fragment was then used as the primer in a second PCR with plasmid pSH221 as the template. Plasmid pSH388 was constructed by the generation of a PCR product with the primers SHW506/SHW508 and pKK16 as the template as the first step. This PCR product was then used as a primer in a second PCR with plasmid pKK17 as the template to create the *divIVA*^{Bs-17-Lm-104-Bs} allele. Plasmid pSH394 was generated using a similar strategy. Here, a PCR fragment was generated using the primer pair SHW180/SHW530 with plasmid pSH260 as the template. This DNA fragment was then used as a primer in a second PCR with plasmid pKK17 as the template to obtain the desired *divIVA*^{Lm-71-Bs} allele.

Plasmids carrying *divIVA* genes for ectopic expression were introduced into *L. monocytogenes* EGD-e by electroporation, and kanamycin-resistant clones were selected. Plasmid insertion at the *attB* site of the *tRNA*^{Arg} locus was verified by PCR. Subsequently, the chromosomal copies of *divIVA* were removed using plasmid pSH186. For this purpose, pSH186 was transformed into the respective *L. monocytogenes* recipient strains, and the *divIVA* gene was removed according to a protocol described previously (38). All gene deletions were verified by PCR.

Construction of chromosomal *divIVA* mutants. For the construction of plasmid pKK90, the *divIVA* gene from *L. monocytogenes* EGD-e was amplified from chromosomal DNA using the oligonucleotides KK113/KK114, and the resulting PCR fragment was then used as the primer in a second PCR to introduce the *divIVA* gene into plasmid pSH186. Plasmids pSH472, pSH473, and pSH474 were then constructed by QuikChange mutagenesis using pKK90 as the template and the primer pairs SHW736/SHW737, SHW734/SHW735, and SHW738/SHW739, respectively. For the introduction of the mutated *divIVA* genes into the native site of the *L. monocytogenes* chromosome, plasmids pSH472, pSH473, and pSH474 were transformed into the *L. monocytogenes* $\Delta divIVA$ mutant LMS2. Allelic exchange was performed according to the homologous recombination protocol described by others (38). All replacements were confirmed by PCR.

Isolation of proteins and Western blotting. *L. monocytogenes* strains were grown in BHI broth and harvested by centrifugation at an optical density at 600 nm (OD₆₀₀) of 1.0. Cells were washed with ZAP buffer (10 mM Tris-HCl [pH 7.5] and 200 mM NaCl), resuspended in 1 ml of ZAP buffer also containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted by sonication. Cell debris was removed by centrifugation (1 min, 13,000 rpm in a tabletop centrifuge). The resulting supernatant was considered the total cellular protein extract. Protein concentration was determined by an adaptation of the Bradford protein assay using Roti-Nanoquant solution (Carl Roth), according to the manufacturer's instructions. Protein extracts were separated by standard SDS-polyacrylamide gel electrophoresis and transferred onto positively charged polyvinylidene difluoride (PVDF) membranes using a semidry transfer unit. Proteins of interest were immunostained using polyclonal rabbit antisera raised against *B. subtilis* DivIVA (39) or *L. monocytogenes* GpsB (22) or a monoclonal mouse antibody recognizing the polyhistidine epitope (GE Healthcare) as the primary antibody and anti-rabbit or anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Sigma-Aldrich) as the secondary antibodies. The peroxidase conjugates were detected on the PVDF membrane using the ECL chemiluminescence detection system (Thermo Scientific).

In vivo formaldehyde cross-linking and pulldown. Cross-linking was performed according to a previously published *B. subtilis* protocol (26, 40). Briefly, *L. monocytogenes* strains expressing His-tagged

bait proteins were grown in 500 ml of BHI broth at 37°C until an OD₆₀₀ of 1.0 was reached and then treated with formaldehyde (final concentration, 1%) for 30 min. Cross-linking was quenched by addition of 50 mM glycine (final concentration) for 5 min. Cells were harvested by centrifugation, resuspended in 2 ml of UT buffer (0.1 M HEPES, 0.5 M NaCl, 50 mM imidazole, 8 M urea, 1% Triton X-100, 1 mM PMSF, 1 mM dithiothreitol [DTT]), and disrupted by sonication. Cell debris was removed, and the cleared lysates were incubated with 200 µl of MagneHis solution (Promega, USA) overnight. The MagneHis particles were washed five times with 1.5 ml of UT buffer before protein complexes were eluted with the elution buffer (0.1 M Tris-HCl [pH 7.5], 0.5 M imidazole, 1% SDS, 10 mM DTT). Eluates were concentrated 5-fold using centrifugal microconcentrators. Samples were mixed with SDS-PAGE loading dye, and cross-linking was reversed by heating at 95°C for 1 h. De-cross-linked samples were separated by SDS-PAGE and analyzed by Western blotting.

Microscopy. Samples (0.4 µl) from logarithmically growing cultures were spotted onto microscope slides covered with a thin film of agarose (1.5% in distilled water), air dried, and covered with a coverslip. For staining of membranes, 1 µl of Nile red (100 µg · ml⁻¹ in dimethyl sulfoxide [DMSO]) was added to 100 µl of culture, and the mixture was shaken for 10 min before the cells were used for microscopy. Images were taken with a Nikon Eclipse Ti microscope coupled to a Nikon DS-MBWC charge-coupled-device (CCD) camera and processed using the NIS-Elements Advanced Research software package (Nikon).

Swarming assay. LB soft agar plates containing 0.3% agar were stab inoculated with the respective *L. monocytogenes* strains. Differences in motility were observed as swarming halos of various diameters after 1 day of incubation at 30°C. Statistical significance of the differences in halo diameter was determined using the unpaired *t* test.

Intracellular growth assay. Intracellular growth in macrophages was analyzed using J774.A1 mouse ascites macrophages (ATCC TIB-67), as described earlier (41). Briefly, 3 × 10⁵ J774.A1 mouse macrophages (ATCC) were seeded into the wells of a 24-well plate and cultivated in 1 ml of high-glucose Dulbecco's modified Eagle's medium (DMEM; 4.5 g/liter glucose, 110 mg/liter sodium pyruvate, 584 mg/liter L-glutamine) supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% carbon dioxide atmosphere 1 day before the infection experiment. *L. monocytogenes* strains were grown overnight in BHI (containing 1 mM IPTG where necessary) broth at 37°C and adjusted to an OD₆₀₀ of 0.2, followed by further dilution (1:100) in DMEM not containing FCS. Fifty-microliter aliquots of this resuspension (~1 × 10⁵ bacterial cells) were used to infect the J774 cells during an incubation step of 1 h at 37°C. The wells were then washed with phosphate-buffered saline (PBS), and all extracellular bacteria were killed during another 1 h of incubation in DMEM (without FCS) containing 40 µg/ml gentamicin. After one more PBS washing step, the wells were covered with fresh DMEM (without FCS) containing 10 µg/ml gentamicin. Sampling was performed by lysing the cells in 1 ml of ice-cold PBS containing 0.1% Triton X-100 at selected time points. Serial dilutions were plated on BHI agar plates and incubated overnight at 37°C to count the recovered bacterial colonies. Statistical significance was determined using the unpaired *t* test.

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