Structural basis of $V_H$-mediated neutralization of the food-borne pathogen *Listeria monocytogenes*

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Listeria monocytogenes causes listeriosis, a potentially fatal food-borne disease. The condition is especially harmful to pregnant women. Listeria outbreaks can originate from diverse foods, highlighting the need for novel strategies to improve food safety. The first step in Listeria invasion is internalization of the bacteria, which is mediated by the interaction of the internalin family of virulence factors with host cell receptors. A crucial interaction for Listeria invasion of the placenta, and thus a target for therapeutic intervention, is between internalin B (InlB) and the receptor c-Met. Single-domain antibodies (VH$_H$H, also called nanobodies, or sdAbs) from camel heavy-chain antibodies are a novel solution for preventing Listeria infections. The VH$_H$H R303, R330, and R326 all bind InlB with high affinity; however, the molecular mechanism behind their mode of action was unknown. We demonstrate that despite a high degree of sequence and structural diversity, the VH$_H$H bind a single epitope on InlB. A combination of gentamicin protection assays and fluorescent microscopy establish that InlB-specific VH$_H$H inhibit Listeria invasion of HeLa cells. A high-resolution X-ray structure of VH$_H$H R303 in complex with InlB showed that the VH$_H$H binds at the c-Met interaction site on InlB, thereby acting as a competitive inhibitor preventing bacterial invasion. These results point to the potential of VH$_H$H as a novel class of therapeutics for the prevention of listeriosis.

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The atomic coordinates and structure factors (codes 6DBA, 6DBD, 6DBE, 6DBF, and 6DBG) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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tosis also requires the entire N-terminal internalin domain (residues 31–321, InlB249), including the IR region (16).

Although interruption of the InlB–c-Met interaction is an intriguing approach for preventing Listeria cellular invasion, one potential pitfall is that the protein is buried in the peptidoglycan layer. One innovative solution is to use single-domain antibodies (VHH), derived from the antigen-binding fragment of the heavy-chain antibodies found in camels (17). VHH are 10 times smaller (12–15 kDa) than conventional IgG antibodies (150 kDa) and may be able to penetrate the Listeria cell wall to bind InlB.

Previously, four VHH (R303, R326, R330, and R419) that bind the LRR domain of InlB with nanomolar affinity were isolated from a nonimmune phage display library (18, 19). As the InlB-LRR domain is crucial for interaction with c-Met, we hypothesized that these VHH could inhibit bacterial endocytosis and protect the cells from Listeria invasion. We demonstrate that InlB-specific VHH effectively neutralize Listeria invasion in vitro. Furthermore, high-resolution X-ray structures reveal that the mechanism behind VHH-mediated Listeria inhibition is competitive inhibition of the InlB–c-Met interaction.

## Results

### VHH bind overlapping epitopes on InlB

Previous work had identified several VHH (R303, R330, R419, and R326) from a phage display library that bound the LRR domain of InlB (residues 36–249; InlB249) (18). Using an indirect ELISA the relative affinity of the VHH for the LRR domain of InlB was compared (Fig. 1A). A variable concentration of immobilized InlB was detected using a fixed concentration of biotinylated VHH. Consistent with previously reported surface plasmon resonance results (18), VHH R303, R326, and R330 bound to immobilized InlB with a similar apparent affinity (Fig. 1A). However, no binding of VHH R419 to InlB249 was observed.

Because the VHH were originally generated by screening a phage display library against a truncated version of InlB with only the cap and the LRR domain (InlB249), we next investigated whether the VHH would also bind InlB if the IR domain was also present (InlB321) (Fig. 1B). R303 and R330 both bound to InlB321 with similar affinity; however, R326 bound with ~2-fold lower affinity to InlB321 when compared with R303 and R330 (Fig. 1B).

The VHH R303, R330, and R326 displayed variability in their CDR sequences and canonical CDR cluster classification (Table 1). Furthermore, based on nucleotide sequence alignments with antibody germ line segments, the VHH may be derived from different species of Camelid. This was expected, as the phage display library used to isolate these VHH is originated from the immune repertoire of three species of Camelid (18, 19). R303 is unquestionably from Camelus dromedaries, whereas R330, R326, and R419 are derived from either Llama glama or Vicugna pacos. Given this diversity, we next investigated the epitope specificity of the VHH.

The possibility that the VHH bound distinct InlB epitopes was investigated using a competitive ELISA. A single fixed concentration of InlB249 or InlB321 was immobilized, and a mixture of biotinylated R303, R326, or R330 was added along with an 80-fold higher concentration of unlabeled R303 to act as an inhibitor (Fig. 1C).

Assuming the VHH bound to spatially distinct epitopes, the expectation was that R303 would not act as an inhibitor for the other VHH. On the other hand, if the VHH bound to overlapping epitopes, R303 should inhibit binding. In all cases, R303...
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**Table 1**

| V₄H | CDR-1 | Cluster | CDR-2 | Cluster | CDR-3 | Cluster |
|-----|-------|---------|-------|---------|-------|---------|
| R303 | AASGHTYSTYCMG | 13-6 | RINVGGSTTW | 10-3 | TLHRFCNTWSLGNTNLV | 16-1 |
| R326 | VTSRIGEILLVGG | 13-5 | SIDRANGTRT | 9-1 | GALSGVNPWA | 11-1 |
| R330 | AASGSSVTMGG | ND² | DISWNSSSTY | 10-2 | NADDLMDYD | 11-1 |
| R419 | AASGRTYSTYAMG | 13-4 | AINWSSGNTH | 10-2 | AAPKHTGDHY | 11-1 |

¹ CDR definitions and structural clusters defined in North et al. (22).
² Not determined; CDR loop could not be classified.

acted as an inhibitor to binding, suggesting that all three V₄H (R303, R330, and R326) bound overlapping epitopes (Fig. 1C).

**InlB-specific V₄H inhibit Listeria invasion of HeLa cells in vitro**

Interaction of InlB with the host cell c-Met receptor is essential for Listeria invasion of epithelial cells (11, 12). Interference of this interaction may provide a site for therapeutic intervention by preventing Listeria colonization and invasion. Gentamicin protection assays were employed to determine whether InlB-specific V₄H could inhibit Listeria invasion in vitro.

*L. monocytogenes* were treated with the four V₄H (R303, R330, R326, and R419) and allowed to invade HeLa cells in vitro. InlB was used as a positive control, as it has been previously shown to inhibit Listeria invasion of HeLa cells (10), and an irrelevant anti-GFP V₄H (20) was used as a negative control. Following protein treatment, gentamicin was added to eradicate noninternalized Listeria. HeLa cells were lysed, the internalized bacteria were counted, and the efficiency of Listeria invasion was calculated (Fig. 2).

As R303, R326, and R330 bound overlapping epitopes on InlB and InlB (Fig. 1), it was hypothesized that these three V₄H would perform similarly in the invasion assay. However, there were some differences in the ability of V₄H to inhibit Listeria invasion. R303 and R330 were both highly effective at inhibiting Listeria internalization of HeLa cells (94 ± 1.4 and 75 ± 1.9%, respectively; Fig. 2). However, R326 exhibited a reduced ability to inhibit Listeria invasion of HeLa cells (36 ± 5.5%). Given that R419 did not bind InlB (Fig. 1), it was not surprising that the V₄H resulted in a level of invasion inhibition similar to that of the irrelevant V₄H control (Fig. 2).

As a second line of evidence to evaluate V₄H neutralization of Listeria, a fluorescence microscopy–based invasion assay was conducted (21). A strain of constitutively expressing GFP Listeria was constructed, followed by biotinylation and subsequent invasion of the strain into HeLa cells. Listeria cells were treated with PBS (negative control), an irrelevant GFP-specific V₄H (negative control), InlB (positive control), and each of the InlB-specific V₄H. Following invasion, the cells were treated with streptavidin conjugated to DyLight550. If the GFP-expressing Listeria invaded the HeLa cells, they would not be detected by the red-labeled streptavidin; however, if the Listeria were impeded from cell invasion, they would be available for detection by the labeled streptavidin and would thus be stained red.

The negative controls, PBS and irrelevant V₄H, resulted in minimal red staining of the GFP-expressing Listeria, indicating that the strain had invaded the HeLa cells (Fig. 3). When treated with the InlB-positive control and with R303, R330, and R326, the majority of the Listeria cells were stained red, indicating that they remained extracellular to the HeLa cells and had been inhibited from invasion (Fig. 3). R419 did not inhibit Listeria invasion, consistent with the results of the ELISA and gentamicin protection assays (Figs. 1–3).

**Structures of V₄H R303, R326, and R330**

X-ray structures of V₄H R303, R326, and R330 were determined at resolutions ranging from 1.3 to 1.8 Å (Table 2). Consistent with the divergent amino acid sequences of the V₄H CDRs (Table 1), the X-ray structures revealed variability in the antigen-binding sites. The CDRs were assigned using the definitions reported by North et al. (22). The CDR loop conformations were assigned from the X-ray structures using the PyIgClassify CDR loop database (23) (Table 1).
R303 was solved to a resolution of 1.3 Å, and the structure contains two molecules in the asymmetric unit arranged in a head-to-tail fashion. R303 had the longest CDR-3 of the three V₄H with a length of 16 amino acids (Table 1 and Fig. 4A). A noncanonical disulfide bond was formed between CDR-1 and CDR-3 (residues 33–102) that linked the long 16-residue CDR-3 loop against the framework region of the antibody (Fig. 4A). CDR-3 formed a short helical segment (residues 102–107) in proximity to the noncanonical disulfide bond. The fixing of CDR-3 against the framework region resulted in a large solvent-accessible surface area (1970 Å²) available for antigen recognition. The CDR-1 loop bisects the antibody paratope, creating two relatively flat interaction surfaces on either side of the loop. The paratope region between CDR-1 and CDR-3 showed a positively charged electrostatic surface, with a wide pocket-like structure forming (Fig. 4A).

The structure of V₄H R326 was solved to a resolution of 1.8 Å and contained a tetramer in the asymmetric unit. Unlike R303, R326 had no disulfide bond connecting CDR-3 to CDR-1. Structurally, R326 was distinct from the other two V₄H with the three CDR loops protruding from the framework region, forming a convex paratope structure (Fig. 4B). The paratope was a large solvent-accessible surface area (1650 Å²) with a positively charged electrostatic-accessible surface (Fig. 4B).

The structure of V₄H R330 was solved to a resolution of 1.6 Å and contained a dimer in the asymmetric unit. Similar to R326, the paratope of R330 was a wide, roughly convex shape with a positively charged solvent-accessible surface area of 2050 Å² (Fig. 4C). Interestingly, the structure of CDR-1 of R330 did not fall into one of the previously characterized structural clusters identified by North et al. (22) (Table 1). CDR-1 also was disordered at the apex of the loop (residue 28 in chain A; residues 29 and 30 in chain B).

**Structure of R303–InlB₂₄₉ and R303–InlB₃₂₁**

To determine the molecular mechanism behind V₄H neutralization of *Listeria* invasion, the structures of V₄H R303 in complex with the LRR domain of InlB (InlB₂₄₉) and the longer InlB fragment of the LRR domain linked to the IR region (InlB₃₂₁) were both determined to a resolution of ~1.5 Å. The two complex structures crystallized in different space groups (Table 2). R303 in complex with InlB₂₄₉ crystallized as a monomer, whereas R303 with InlB₃₂₁ was a dimer in the asymmetric unit.

The overall binding interactions between R303 and InlB₂₄₉ and InlB₃₂₁ were identical (Fig. 5A), indicating that the IR domain of InlB₃₂₁ played no role in binding. This finding is consistent with the observation that R303 binds to both proteins (InlB₂₄₉ and InlB₃₂₁) with similar affinity (Fig. 1, A and B).

The entire interaction between R303 and InlB occurs on an electronegative cavity on the concave face of the InlB-LRR domain, resulting in an approximate buried surface area of 1400 Å². The bulk of the binding interactions are mediated by CDR-3 and CDR-2, with CDR-1 displaying only limited contact with InlB (Fig. 5B).

Consistent with the picomolar affinity of R303 for the InlB-LRR domain (18), there were extensive polar and nonpolar contacts between the antibody and InlB. Interactions originating from CDR-3 on R303 are of central importance and form the majority of the binding interactions (Fig. 5). There were a series of salt bridges that likely contribute significantly to the high-affinity binding of the V₄H. The salt bridges are formed between Arg-100vhh on CDR-3 of R303 and Glu-194inl and Glu-236inl on InlB (where the superscript “vhh” denotes residues on V₄H R303 and the superscript “inl” denotes residues on InlB) (Fig. 5B). This central arginine residue on R303 also forms a hydrogen bond to Tyr-214inl. Additional polar interactions include 12 hydrogen bonds between the antibody and InlB. On CDR-3, Asn-103vhh–hydrogen-bonds to Ser-168inl, Asp-189inl, and Thr-190inl. The adjacent residue on CDR-3, Thr-104vhh, hydrogen-bonds to the hydroxyl side chain of Tyr-170inl (Fig. 5B). On CDR-2, Ser-56vhh and Ser-57vhh form hydrogen bonds to Asp-233inl (Fig. 5B). In addition to the polar contacts, there are aromatic stacking interactions, with the side chain of Phe-104vhh on CDR-3 inserting between Tyr-214inl and Tyr-170inl (Fig. 5B).

**Discussion**

**Specificity of V₄H isolated from a nonimmune library**

The anti-InlB V₄H (R303, R330, and R326) used in this study were isolated from a preimmune phage display library from the naive immune repertoires of camels, alpacas, and llamas (18). Each of the isolated V₄H was unique in terms of primary sequence diversity and CDR canonical structure (Table 1). Furthermore, based on alignment with germ line gene segments, the V₄H originate from different species of Camelid (R303 (camel), R326 (llama or alpaca), and R330 (llama or alpaca)). However, despite this structural and sequence diversity, the
specificity of the V₄H converged onto a single epitope (Fig. 1C). This epitope was centralized to a negatively charged cavity on the concave face of the LRR domain of InlB (Fig. 5A).

The specific structural features of the InlB antigen and the particular binding properties associated with V₄H in general may be responsible for the observed V₄H specificity. It has been
observed previously that V\textsubscript{H} often bind concave features on protein antigens due to the convex shape of the paratope formed on the three CDR loops (24, 25). Given this preference, the V\textsubscript{H} specificity toward the InlB-LRR electronegative cavity may be the result of the protein only having this one concave surface feature.

**V\textsubscript{H} properties facilitate neutralization of Listeria**

The biophysical and binding properties of V\textsubscript{H} are distinct compared with traditional monoclonal antibodies. V\textsubscript{H} are small and stable, and their convex shape allows V\textsubscript{H} to bind protein cavities, which are frequently inaccessible to traditional monoclonal antibodies (17). This combination of properties provides several advantages that may have contributed to the effectiveness of V\textsubscript{H} R303, R330, and R326 for the in vitro neutralization of *L. monocytogenes* (Figs. 2 and 3). In particular, the small size and preferential binding of V\textsubscript{H} toward protein cavities may explain the success of V\textsubscript{H} at *Listeria* neutralization compared with traditional antibody formats.

Several mouse anti-InlB antibodies displayed variable effectiveness at inhibiting *Listeria* invasion of Vera cells, suggesting that specific epitopes must be recognized for neutralization to occur (10). In some cases, InlB epitopes may be inaccessible; an InlB-specific ScFv was only able to bind InlB following enzymatic digestion of the bacterial cell wall, suggesting that the epitopes were buried in the cell wall (26). As V\textsubscript{H} R303, R330, and R326 are all able to neutralize *Listeria* invasion (Figs. 2 and 3), it can be inferred that the immunodominant epitope must be accessible to the V\textsubscript{H}. The small size of the V\textsubscript{H} may facilitate penetration of the bacterial peptidoglycan layer to access the protein–protein interaction surface on InlB. This further highlights the specific advantages of using V\textsubscript{H} in targeting difficult-to-access cell surface epitopes.

**InlB-specific V\textsubscript{H} inhibit Listeria invasion through competitive inhibition**

The neutralization of *Listeria* invasion by V\textsubscript{H} R303, R330, and R326 could potentially be mediated by two different mechanisms. The V\textsubscript{H} could bind InlB and inhibit its interaction with c-Met simply through steric effects, or the V\textsubscript{H} could competitively inhibit the native interaction of InlB with c-Met. The X-ray structure of R303 in complex with InlB (Fig. 5) permits an analysis of the molecular mechanism behind the antibacterial activity of the V\textsubscript{H}.

C-Met is a receptor tyrosine kinase whose ectodomain consists of six domains: Sema, Psi, and four Ig-like domains (Ig1–4) (27). The natural ligand for c-Met is the hepatocyte growth factor/scatter factor (HGF/SF). In healthy cells, the c-Met–HGF/SF interaction mediates cell signaling related to embryogenesis and tissue regeneration, and deregulation of c-Met is also important in carcinogenesis (28). Interestingly, whereas *L. monocytogenes* hijacks c-Met as a vehicle for bacterial entry, the interaction of InlB with c-Met does not mimic the natural HGF/SF ligand, as the two proteins bind c-Met at distinct sites (9, 27).

InlB–c-Met receptor binding and subsequent cell signaling events that ultimately result in bacterial internalization are mediated by different domain–domain interactions. A fragment comprising the cap region and LRR domain of InlB (InlB\textsubscript{241}) is the minimum unit for c-Met receptor binding (9). The binding of the InlB\textsubscript{241} fragment to the Ig1 domain of c-Met occurs at the electronegative cavity on the concave face of the InlB-LRR domain (Fig. 6A) (27). However, c-Met receptor activation and cell invasion by *L. monocytogenes* require a larger fragment of InlB, consisting of the cap region, LRR domain, and interrepeat (InlB\textsubscript{321}) (16). The secondary, weaker interaction of the InlB-IR domain with the c-Met Sema domain (Fig. 6A) is required for receptor activation and not binding (27).

V\textsubscript{H} R303 binds InlB directly at the c-Met receptor-binding site: the electronegative cavity on the concave face of InlB...
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(Figs. 5A and 6A). Overlap of the structure of R303–InlB321, with that of the c-Met ectodomain in complex with InlB321 (27) demonstrated that R303 would directly occupy the same physical space as the c-Met Ig1 domain, mimicking the interaction with the Ig1 domain (Fig. 6B).

The binding of the c-Met Ig1 domain to InlB is mediated by many of the same residues involved in the R303–InlB interaction. There are five residues on InlB that are important for c-Met receptor binding: Asp-128inl, Glu-150inl, Tyr-170inl, Tyr-214inl, and Trp-124inl (27). Of these five InlB residues, four are either interacting with R303 directly through hydrogen bond interactions (Tyr-170inl and Tyr-214inl; Figs. 5B and 6C) or are buried upon complex formation (Glu-150inl and Trp-124inl, Fig. 6C).

The high-affinity binding of R303 to the c-Met receptor-binding site on InlB provides a clear molecular mechanism for the neutralization of L. monocytogenes by the V₄H used in this study. By mimicking the interactions of c-Met, the natural ligand of InlB, the V₄H are acting as high-affinity competitive inhibitors, neutralizing bacterial invasion.

Therapeutic potential of Listeria-specific V₄H

Listeria infections are a particular challenge facing pregnant women. Maternal infection is frequently asymptomatic or displays nonspecific symptoms, making diagnosis a serious challenge during prenatal care (29, 30). Even in cases with diagnosis, antibiotic treatment is not always successful, presumably due to the intracellular nature of the pathogen (31).

Prevention of Listeria infection is currently the most effective strategy for safeguarding women from the disease during pregnancy. Typically, pregnant women are advised to avoid consumption of foods at high risk of Listeria contamination. However, a series of deadly Listeria outbreaks in fresh produce, fruit, and other foods traditionally at low risk of Listeria contamination, highlight the need for alternative and novel approaches to safeguarding the food supply (32–34).

A prophylactic strategy of blocking Listeria entry into nonphagocytic cells by inhibiting the interaction of InlB with the c-Met receptor is a potential venue of Listeria treatment or prophylactic. A recent report using the c-Met inhibitor tansipimycin as a Listeria antibiotic suggests that this approach may represent a viable therapeutic strategy (35).

The ability of InlB-specific V₄H to neutralize Listeria invasion in vitro points to a therapeutic potential for the prevention or treatment of listeriosis. There have been several recent reports of using V₄H as anti-bacterial agents against a variety of bacterial pathogens, including Clostridium difficile, Bacillus anthracis, Shigella, botulism, and Bordetella pertussis (36–40). In each of these cases, the anti-bacterial strategy was to employ the high-affinity binding of V₄H to neutralize secreted bacterial toxins. The use of InlB-specific V₄H represents a novel approach to combating bacterial disease using V₄H. The dependence of the internalin–host cell receptor interaction in Listeria pathogenesis provides a novel mechanism of V₄H-mediated therapeutic intervention by inhibiting host cell invasion (Figs. 2 and 3). Although further in vivo studies are required to validate the therapeutic potential of V₄H for the treatment and prevention of listeriosis, the results presented here highlight the future potential of V₄H as anti-bacterial agents.

Experimental procedures

Expression and purification of V₄H

The plasmids (pSJF2H) for V₄H R303 and R330 were a generous gift of Dr. Roger MacKenzie (National Research Council, Ottawa, Canada). Genes for V₄H R419 and R326 were codon-optimized and synthesized as double-stranded gene blocks (GenScript, Piscataway, NJ). R326 and R419 were cloned into the plasmid pET22b using the restriction enzyme sites NcoI and XhoI.

Plasmids for R303 and R330 were transformed into Escherichia coli TG1, whereas R419 and R326 were transformed into E. coli BL21 (DE3) for protein expression. All of the InlB-specific V₄H were extracted from the periplasm using an osmotic shock procedure and purified using Ni-NTA chromatography and size-exclusion chromatography, as described previously (41). The control anti-GFP V₄H was expressed and purified as described previously (20).

Expression and purification of InlB

The sequences for InlB-LRR (InlB249) and InlB-LRR-IR (InlB321) were codon-optimized for E. coli expression and synthesized as gene blocks (GenScript). InlB249 was cloned into pET-15-TEV-NESG, and InlB 321 was cloned into pET28a. Both plasmids were transformed into E. coli BL21 (DE3) for expression.

Cultures were grown overnight (30 °C, 225 rpm) in 2× YT medium with ampicillin (100 μg/ml). The overnight culture was transferred to 6 × 1 liter of 2× YT-amp and incubated (30 °C, 225 rpm) until A₆00 of 0.5. The culture was then induced with isopropyl β-D-thiogalactopyranoside (0.4 mM) and incubated overnight (20 °C, 225 rpm). Bacteria were harvested by centrifugation (6 °C, 5000 × g, 10 min). The pellet was suspended in TBS buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and lysed using sonication. The cytoplasmic fraction (supernatant) was isolated by centrifugation (6 °C, 10,000 rpm, 30 min). Both proteins were purified by Ni-NTA affinity and size-exclusion chromatography.

Indirect and competitive ELISA

For the indirect ELISA, a 96-well plate was coated with serial dilutions of InlB (5–0.02 ng/μl) in PBS overnight at 4 °C. The wells were blocked for 1 h with BSA (3% in PBS). Biotinylated V₄H (R303, R330, R326, and R419) were used as a primary antibody (15 μg/ml, 1 h). The plate was washed three times with PBS-Tween (0.05% Tween 20) followed by the addition of streptavidin horseradish peroxidase (Fisher) (1:50,000 dilution in 3% BSA 1 h). Finally, detection was carried out by the addition of 3,3,5,5-tetramethyl benzidine (15 min). The reaction was stopped by the addition of 0.18 μl H₂SO₄, and the absorbance was measured at 450 nm using a plate reader (BioTek Instruments Inc., Winooski, VT).

A similar procedure was carried out for the competitive ELISA except that InlB was immobilized at a fixed concentra-
culture medium (HyClone) containing 2.05 mM L-glutamine, 10% FBS, and penicillin/streptomycin and incubated at 37 °C with 5% CO₂. For infection, log phase *L. monocytogenes* (A₆₀₀ = 0.3–0.5) were grown at 37 °C in 2× YT medium agitated at 225 rpm.

Treatment solutions of InlB₂₄⁹ and V₄₄H diluted to 100 μg/ml in unsupplemented RPMI 1640 were added to a 24-well cell plate containing 1×10⁵ HeLa cells/well and incubated for 30 min at 37 °C, 5% CO₂. Log phase *L. monocytogenes* (MOI of 50:1) were then added to the wells, and the plate was centrifuged (1000 rpm for 3 min) and incubated at 37 °C with 5% CO₂ for 1 h. Infected cells were washed twice with PBS to remove nonadherent bacteria. To kill extracellular bacteria, RPMI 1640 containing 100 μg/ml gentamicin was added and incubated for 60 min (37 °C, 5% CO₂). To enumerate intracellular bacteria, wells were washed once with PBS and then lysed with 1% Triton X (Sigma) in PBS at the appropriate times. Recovered intracellular bacteria were quantified by plating serial dilutions on LB agar plates and enumerating colony counts.

Replicate wells were included in which total and surface-adherent *Listeria* were enumerated by harvesting the supernatant immediately after incubation of bacteria with HeLa cells (total) or collecting the Triton X-100 lysate before treatment with gentamicin (adherent). Each experiment was done in duplicates, and duplicates were performed at least three times independently.

**Fluorescence microscopy**

GFP-expressing *L. monocytogenes* were created as described previously (42). HeLa cells were cultured in 1× RPMI 1640 culture medium (HyClone) containing 2.05 mM L-glutamine, 10% FBS, and penicillin/streptomycin and incubated at 37 °C with 5% CO₂. HeLa cells were seeded at a density of 4×10⁵ cells/ml onto a microscope coverglass placed in each well of a 24-well plate. GFP-*Listeria* was grown overnight in BH broth containing antibiotics, and the concentration was measured at A₆₀₀. The bacteria were washed three times with sterile 1× PBS (pH 7.4) and labeled with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific). After washing excess biotin by washing three times with 1% BSA, the bacteria were incubated with 100 μg/ml nanobodies at 37 °C for 30 min. HeLa cells were stained with 1 μl of 10 μg/ml 4’,6-diamidino-2-phenylindole and infected with biotinylated GFP-*Listeria* at an MOI of 50:1. After centrifuging for 15 min at 300 rpm, the plate was incubated for 1 h at 37 °C with 5% CO₂ followed by three washes with unsupplemented RPMI 1640. Biotinylated GFP-*Listeria* were detected by the addition of 2.5 μl of 1 mg/ml Streptavidin-Dylight550 (Thermo Scientific) to each well, and the plate was incubated for 30 min at 37 °C with 5% CO₂. The wells were washed with RPMI 1640, and the coverslips were fixed with 4% p-formaldehyde for 30 min at 4 °C. After washing the wells three times with 1× PBS (pH 7.4), the coverslips were removed from the plate, and Fluoromount-G (SouthernBio-tech) was added to mount them onto slides. The slides were analyzed in a Leica DMI3000 B fluorescence microscope at ×63 magnification.

**Crystallization of V₄₄H and R303–InlB complexes**

The crystallization and preliminary X-ray diffraction for R303 were reported previously (41). For complex formation, R303 and InlB were incubated (1:1.2 (w/w), 30 min, 25 °C) and purified by gel filtration chromatography (Bio-Rad NGC quest system using Enrich Sec70 column). The complexes (R303–InlB₂₄⁹ and R303–InlB₂₃₂) and purified V₄₄H (R330 and R326) were dialyzed against 10 mM HEPES, pH 7.4, and concentrated to 10 mg/ml.

Crystallization trials were carried out in Intelli 96-well sitting-drop plates using a Gryphon crystallization robot (Art Robbins Instruments). Sitting-crystal drops were set up using 1 μl of protein and 1 μl of reservoir solution. The proteins were screened using the PEgs, PEG II, and PACT crystallization suites (Qiagen Inc.). Crystal optimizations were carried out in 24-well Limbro plates (Hampton Research) using hanging-drop vapor diffusion and variable drop sizes. Optimal crystal conditions for V₄₄H R330 were 0.1 M HEPES, pH 7.5, 25% PEG 3350. For V₄₄H R326, the optimal crystal conditions were 0.2 M ammonium sulfate, 0.1 M sodium acetate, 22% PEG 3350. Crystals of R303–InlB₂₄⁹ grew in 0.2 M disodium tartrate, 20% PEG 3350. Finally, crystals of R303–InlB₂₃₂ grew in 0.1 M sodium citrate tribasic dihydrate, pH 5.0, 34% Jeffamine ED-2001.

**Data collection and X-ray structure determination**

Crystals were dipped in cryoprotectant (mother liquor supplemented with 25% glycerol) and flash-frozen in liquid nitrogen. X-ray data were collected at the Canadian Light Source on beamline 08ID-1 (43). Diffraction data were processed using Xia2 (44). All structures were solved by molecular replacement using Phaser as implemented in Phenix (45). For molecular replacement, the previously solved structures of R303 (41) and InlB₂₄⁹ (46) and InlB₂₃₂ (27) were used as search models. The structure was automatically built and refined using Phenix. Manual fitting of σA-weighted Fo−Fc electron density maps was carried out using Coot (47). The final model and refinement statistics are given in Table 2.

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