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YspM, a Newly Identified Ysa Type III Secreted Protein of *Yersinia enterocolitica*[^2]

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*Yersinia enterocolitica* has three type three secretion systems, the flagellar, the plasmid Ysc type III secretion system (T3SS), and the chromosomal Ysa T3SS. The Ysc T3SS, through the proteins it secretes (Yops), prevents phagocytosis of *Y. enterocolitica* and is required for disease processes in the mouse host. Recent data demonstrate a role for the Ysa T3SS during initial colonization of the mouse via secretion of Ysps (*Yersinia* secreted proteins). This work characterizes the discovery of a newly identified Ysa type III secreted protein, YspM. Expression of *yspM* is regulated by temperature, NaCl concentration, and other known regulators of the *ysa* system. In addition, YspM is translocated into host cells via the Ysa T3SS. YspM is homologous to proteins classified as GDSL bacterial lipases, which possess a catalytic triad of amino acids (Ser, Asp, and His) located in three of five blocks of amino acid identity. Sequence analysis of the JB580v strain of *Y. enterocolitica* shows that, due to a premature stop codon, it no longer encodes the fifth block of amino acid identity containing the predicted catalytic histidine. However, seven other biotype 1B strains sequenced did possess the domain. A functional difference between the forms was revealed when YspM was expressed in Saccharomyces cerevisiae. Yeast growth was uninhibited when YspM from JB580v was expressed but greatly inhibited when YspM from Y295 (YspM<sub>Y295</sub>) was expressed. Site-directed mutagenesis of the histidine of YspM<sub>Y295</sub> ablated the toxic effects. These results indicate that YspM is secreted by the Ysa T3SS and that, possibly due to lipase activity, it targets eukaryotic cellular component(s).

*Yersinia enterocolitica* is a gram-negative, enteric pathogen that infects humans through ingestion of contaminated food or water. Infected individuals can develop several disease manifestations including self-limiting enteritis, diarrhea, lymphadenitis, and, in severe cases, septicemia leading to death (6, 22). Orally infected mice develop a similar disease and serve as a useful tool to study *Y. enterocolitica* pathogenesis (8, 16, 17). The mouse model has been used to demonstrate that upon infection, *Y. enterocolitica* transverses the stomach and colonizes the small intestine, where the bacteria invade and replicate within Peyer’s patches (2, 8, 28). The ability to survive within Peyer’s patches is due, in part, to the presence of a plasmid-encoded Ysc-Yop type III secretion system (T3SS) (39). Several of the secreted effector Yops prevent phagocytosis of *Y. enterocolitica* by macrophages and polymorphonuclear cells due to their immune modulatory functions (10). This is a process of critical importance, demonstrated by the fact that *Y. enterocolitica* bacteria lacking specific Yops do not colonize the Peyer’s patches, mesenteric lymph nodes, or spleens to levels seen with wild-type bacteria (39).

Biotype 1B strains of *Y. enterocolitica*, which is the most pathogenic of the species, possess a second T3SS, the chromosomally encoded Ysa (*Yersinia* secretion apparatus) T3SS (12, 15). The Ysa T3SS is responsible for secreting at least 15 Ysps, or Ysps secreted proteins, when cultures are grown at 26°C in the presence of 290 mM NaCl (23, 47). While the exact role of the Ysa T3SS plays in pathogenicity has yet to be determined, there is a decrease in virulence when a commonly used biotype 1B strain, JB580v, lacking a functional Ysa T3SS is used to infect mice. These Ysa T3SS mutant strains have a 10-fold attenuation, based on 50% lethal dose analysis, following oral inoculation and are deficient in their ability to colonize the terminal ileum of the mouse during the first 24 h of oral infection (15, 23). Several Ysps have been shown to have a role in the early colonization events of the gastrointestinal tract, as demonstrated by competitive index analysis following oral inoculation (23). Interestingly, strains of bacteria lacking some Ysps have a greater colonization defect than a *ysa* apparatus mutant alone. Two Ysps that had a defect in colonization of the mouse, YspP and YspK, have been further characterized to have enzymatic properties. YspK has demonstrated protein kinase activity, and YspP has demonstrated phosphatase activity (23).

Expression of the Ysa T3SS and secretion of its cognate Ysps are regulated by both environmental (high NaCl and lower temperatures) and genetic factors. Genetic factors involved in regulation of the *ysa* T3SS include the putative phororelay systems encoded by *ysrRS* and *rcsC-ysjN-rcsB* as well as the araC-like regulator *YsaE* and the chaperone *SycB* (42, 43). Genes encoding the *ysa* apparatus, the chaperone *sycB*, and four genes (*yspBCDA*) that encode proteins with homology to proteins involved in host cell pore formation are located in a single genetic locus, and expression is controlled by two promoters. The genetic organization of the locus reveals that the *ysaE* promoter drives transcription of a long transcript that encodes the structural genes that make up the *ysa* secretion apparatus, *sycB*, and *yspBCDA*. Upon expression, *YsaE* and *SycB* act in concert to further activate transcription of the

[^2]: Corresponding author. Present address: Department of Microbiology and Immunology, 116 Manning Drive, 804 Mary Ellen Jones CB# 7290, University of North Carolina, Chapel Hill, NC 27599. Phone: (919) 966-9956. Fax: (919) 962-8103. E-mail: vlmiller@med.unc.edu.
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y sicBCDA operon (43). YsrRS and RcsC-YojN-RcsB are believed to be responsible for sensing changes in the environment and then activating expression of the ysc operon. In support of this model, it has been shown that not only YsaE and SycB but also YsrRS and RcsB are required for secretion of Ysps by the Ysa T3SS (42, 43). Interestingly, with the exception of yspBCDA, the other Ysps are encoded elsewhere throughout the chromosome, and little is known about the regulation of their expression.

As the ysp genes are not located together on the Y. enterocolitica chromosome, it remains possible that there are additional, unidentified proteins secreted by the Ysa T3SS. Comparative phylogenetic analysis of Y. enterocolitica by Howard et al. identified an open reading frame (ORF), YE3614, with homology to a type III secreted protein of Salmonella (18).

Work in our laboratory also identified YE3614 as a potential type III secreted protein (unpublished data). In this work, we examined the ability of YE3614 to be secreted and translocated by the Ysa T3SS. We also investigated the regulation of expression of YE3614 compared to other genes of the ysa regulon. Finally, we examined the effects of expression of YE3614 in eukaryotic cells using the Saccharomyces cerevisiae model system.

MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains, yeast strains, and plasmids used in this study are presented in Table 1 and further described below. Unless indicated, bacterial cultures were grown overnight in Luria broth (LB) (170 mM NaCl) at 26°C for Y. enterocolitica or 37°C for Escherichia coli. For promotion of protein secretion via the Ysa T3SS, bacteria were grown overnight in (170 mM NaCl) at 26°C for Y. enterocolitica or 37°C for Escherichia coli.

TABLE 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td>Bacteria strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JB580v</td>
<td>Y. enterocolitica 8081v ΔyenR (r− m−); wild type</td>
<td>19</td>
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<tr>
<td>JB580c</td>
<td>JB580 cured of plasmid pYVe8081; lacks Ysc/Yop T3SS</td>
<td>43</td>
</tr>
<tr>
<td>Y295</td>
<td>Y. enterocolitica, biotype 1B, O:8</td>
<td>26</td>
</tr>
<tr>
<td>657-83</td>
<td>Y. enterocolitica, biotype 1B, O:20</td>
<td>4, 26</td>
</tr>
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<td>658-83</td>
<td>Y. enterocolitica, biotype 1B, O:21</td>
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<td>659-83</td>
<td>Y. enterocolitica, biotype 1B, O:18</td>
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<tr>
<td>634-83</td>
<td>Y. enterocolitica, biotype 1B, O:4,32</td>
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<td>9286-78</td>
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<td>3, 26</td>
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<tr>
<td>9287-78</td>
<td>Y. enterocolitica, biotype 1B, O:20</td>
<td>3</td>
</tr>
<tr>
<td>2440-87</td>
<td>Y. enterocolitica, biotype 1B, O:8</td>
<td>4, 26</td>
</tr>
<tr>
<td>YVM1374</td>
<td>JB580v ΔyacC</td>
<td>This study</td>
</tr>
<tr>
<td>YVM1178</td>
<td>JB580v ΔsycC</td>
<td>This study</td>
</tr>
<tr>
<td>YVM932</td>
<td>JB580v ΔysaE</td>
<td>This study</td>
</tr>
<tr>
<td>YVM981</td>
<td>JB580v ΔycB</td>
<td>43</td>
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<tr>
<td>YVM1006</td>
<td>JB580v ΔyssR</td>
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<td>43</td>
</tr>
<tr>
<td>YVM1236</td>
<td>JB580v ΔrcsB</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli 517-1 λpir</td>
<td>Tp’ Str’ recA thi pro hsdR hsdM+ RP4::2-Tc::Mu::Km Tn7 λpir lysogen</td>
<td>25</td>
</tr>
</tbody>
</table>

Yeast strains

SCW01 | S. cerevisiae MATα leu2Δα met15Δα ura3Δα | Gift of Vogel lab |
SCW06 | SCW01 containing pDEST52 | This study |
SCW07 | SCW01 containing pSW13 | This study |
SCW08 | SCW01 containing pSW16 | This study |
SCW11 | SCW01 containing pSW46 | This study |

Plasmids

pSIF003-R1 Used for PCR amplification of cyaA | 41 |

pENTR-SD-TOPO Kan’ cloning vector | Invitrogen |
pDEST52 GAL1 promoter, C-terminal V5 epitope tag, 2αm or ORA3 | Invitrogen |
pKW44 pWKS130 backbone; for construction of C-terminal cyaA translation fusions | This study |
pSW11 yspM<sub>8081</sub> cloned into pENTR-SD-TOPO | This study |
pSW12 yspM<sub>293</sub> cloned into pENTR-SD-TOPO | This study |
pSW13 GAL1 promoter driving expression of YspM<sub>8081</sub>V5 fusion protein | This study |
pSW16 GAL1 promoter driving expression of YspM<sub>8081</sub>V5 fusion protein | This study |
pSW29 yspM<sub>293</sub> (His<sup>40</sup>) cloned into pENTR-SD-TOPO | This study |
pSW41 yspE promoter and yspM<sub>8081</sub>; codons 1 to 150 cloned into pKW44 | This study |
pSW42 500-bp yspM<sub>8081</sub> promoter region cloned into pKW44 | This study |
pSW43 500-bp yspM<sub>8081</sub> promoter region and yspM<sub>8081</sub>; codons 1 to 150 cloned into pKW44 | This study |
pSW45 500-bp yspM<sub>293</sub> promoter region and yspM<sub>293</sub> codons 1 to 150 cloned into pKW44 | This study |
pSW46 GAL1 promoter driving expression of YspM<sub>293</sub> (His<sup>40</sup>)V5 fusion protein | This study |
pSW47 500-bp flanking regions of yscC ORF in pSR47S; for construction of ΔyacC strain | This study |
pKW43 500-bp flanking regions of yscC ORF in pSR47S; for construction of ΔyacC strain | This study |
pKW53 500-bp flanking regions of rcsB ORF in pSR47S; for construction of ΔrcsB strain | This study |
pKW53S 500-bp flanking regions of rcsB ORF in pSR47S; for construction of ΔrcsB strain | This study |
pSW42 8081v codons 1 to 150 cloned into pKW44 | This study |
pSW43 8081v codons 1 to 150 cloned into pKW44 | This study |
pSW44 8081v codons 1 to 150 cloned into pKW44 | This study |
pSW45 8081v codons 1 to 150 cloned into pKW44 | This study |

Acknowledgments

This work was supported by the Intramural Research Support Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and by a National Institutes of Health Research Career Award AI053777.

References

in LB lacking NaCl at 26°C and subcultured into LB plus 290 mM NaCl. For examination of protein secretion via the Ysc T3SS, bacteria were grown overnight in LB at 26°C and subcultured into LB containing 20 mM Na$_2$C$_2$O$_4$ and 20 mM MgCl$_2$ grown at 37°C. Antibiotics were used as needed at the following concentrations: carbenicillin, 50 μg/ml; kanamycin, 100 μg/ml; and nalidixic acid, 20 μg/ml.

*S. cerevisiae* was grown in YPD (yeast extract, peptone, and dextrose) medium at 30°C unless otherwise indicated. For protein induction studies, *S. cerevisiae* was grown at 30°C in synthetic complete medium [500 ml of medium contains 2.5g (NH$_4$)$_2$SO$_4$, 0.85 g of yeast nitrogen base, and 1g of dropout mix minus uracil for plasmid maintenance (US Biological)] containing either 2% glucose or 2% galactose.

The plasmid (pSW47) to construct the in-frame $\Delta$yscC null strain used in this study was generated as follows. Primers P135 and P136 were used to amplify an approximately 500-bp region upstream and including the start codon of *yscC* from JB580v. Primers P137 and P138 were used to amplify an approximately 500-bp region downstream and including the stop codon of *yscC*. The two fragments were digested with BamHI and ligated together. This product was then used in a PCR with primers P135 and P138, and the resulting product was digested with SalI and NotI and cloned into the same sites of pRS426, resulting in pSW47.

Plasmid pKW43 was used to construct the in-frame $\Delta$yscC null strain was generated in a similar manner using primers ysaC-delA and ysaC-delB to amplify the fragment 500 bp upstream of *ysaC*, and primers ysaC-delC and ysaC-delD were used to amplify the 500-bp region downstream. Plasmid pKW53 was used to generate the in-frame $\Delta$yscC null strain used in this study was generated as follows. Primers P135 and P136 were used to amplify an approximately 500-bp region upstream and including the start codon of *yscC* from JB580v. Primers P137 and P138 were used to amplify an approximately 500-bp region downstream and including the stop codon of *yscC*. The two fragments were digested with BamHI and ligated together. This product was then used in a PCR with primers P135 and P138, and the resulting product was digested with SalI and NotI and cloned into the same sites of pRS426, resulting in pSW47.

Table 2. Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
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<td>P1</td>
<td>AAG GAA AAA AGC GGC CGC TAT ACT CAG TAT CAG GTG TTC</td>
</tr>
<tr>
<td>P2</td>
<td>GCT CTA GAA CCA AGG GAT ACA ATG GGC AAA TC</td>
</tr>
<tr>
<td>P3</td>
<td>CCC GCT CTA GTC TAG ACA TAA TTT CCT CTT GAT T</td>
</tr>
<tr>
<td>P27</td>
<td>CGG CTC GAA ATG AGT ATT AAT TTT AAC CAC AAC</td>
</tr>
<tr>
<td>P63</td>
<td>CAG TGT TGG TTT ACT GAA TTT TTC GG</td>
</tr>
<tr>
<td>P68</td>
<td>CAC GAT GGG AAG TAT TAA TTT TTA CCA CAA C</td>
</tr>
<tr>
<td>P69</td>
<td>GGA ACA TAT CCA GAT ATG ATA GGC GTG TTC</td>
</tr>
<tr>
<td>P70</td>
<td>GCT CTA GAC ATG ACT ATT TCT CTT GTC</td>
</tr>
<tr>
<td>P81</td>
<td>TCT TTT AAT GAC AAT TCA AAT CCA TCC CAA GAA TGG CAT GCC</td>
</tr>
<tr>
<td>P82</td>
<td>GGC ATG CAA TTC TGG GGA TGG ATT GTA ATT CTT AAC AAA AGA</td>
</tr>
<tr>
<td>P124</td>
<td>ATG AGA ATG CGG CCG CGA ATT CCC CAA CTT TGG</td>
</tr>
<tr>
<td>P125</td>
<td>GGG AAA TCC ATA TGT ATT TAT TCC CTT GGC</td>
</tr>
<tr>
<td>P126</td>
<td>GAC AGC AAT AAC ATG CCG ATG CTC GAT</td>
</tr>
<tr>
<td>P127</td>
<td>CGT CTA GAA CCA AGG GAT ACA ATG GGC AAA TC</td>
</tr>
<tr>
<td>P128</td>
<td>GCT CTA GAC ATG ACT ATT TCT CTT GTC</td>
</tr>
<tr>
<td>P135</td>
<td>ACG CGT CGA CAT GCA AAA ATT CCT ACT AAA AAA CTT GGC</td>
</tr>
<tr>
<td>P136</td>
<td>CGC GGA TCC CGG AAA AGC CAT ACT ATT TAA ATC CC</td>
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<tr>
<td>P137</td>
<td>CGC GGA TCC AAG CGT GGC GTA TGG TGA</td>
</tr>
<tr>
<td>P138</td>
<td>ATA AGA ATG CGG CGG TCG TAA AGA AAT AAC TCT AAT TCC</td>
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<td>P139</td>
<td>ATG AGA ATG CGG CCG CGA ATT CCC CAA CTT TGA CAC</td>
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<tr>
<td>P140</td>
<td>GGC ATG CAA TTC TTG GGA TGG ATT TGA ATT GTC ATT AAA AGA</td>
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<td>P141</td>
<td>CAC CAT GGG AAA TAT TAA TTT AAA CCA CAA</td>
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<td>P142</td>
<td>GGA ACA TAT CCA GAT ATG ATA GGC GTG TTC</td>
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<td>P154</td>
<td>GGT GAG GTC AGG AAA TCA ACC C</td>
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<td>P155</td>
<td>GGC TCG AGT TGA TGG TGA CTG ATA ACC C</td>
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<tr>
<td>P156</td>
<td>GGC GAT CTC ACA TTA AGG TTT TGT ATG GTT AGG</td>
</tr>
<tr>
<td>P157</td>
<td>GGC GAT CTC AAT AAC ACC TGC GAC TAC TGC TGA TGC ACC AGA TGA TGC GAC</td>
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<tr>
<td>P158</td>
<td>GGC TCG ACC CCT TGA TGC ACC AGA TGA TGC GAC</td>
</tr>
<tr>
<td>P159</td>
<td>GAT TGG TCA CGC ACC TCT CCA ACC ACC TGC GAC</td>
</tr>
<tr>
<td>P160</td>
<td>CGG TCA CGC GGG GAC CGG GCA TAA ACG ACC TCG</td>
</tr>
<tr>
<td>P161</td>
<td>CCG GGA TCC GCT CAT GAC TCT TCC GAA GCT AAC CG</td>
</tr>
<tr>
<td>P162</td>
<td>ATA AGA ATG CGG CGG TCG TAA ACG TGC GTG</td>
</tr>
</tbody>
</table>

The plasmids used to detect protein production in yeast were generated using the Gateway system from Invitrogen. Briefly, primers P68 and P69 were used to amplify the ~900-bp coding region of *YE3614* from JB580v. This product was cloned into pENTR SD-TOPO (Invitrogen) per the manufacturer’s protocol, generating pSW11. Next, pSW11 and pDEST52 (Invitrogen) were combined using the enzyme Gateway LR Clonase II (Invitrogen), according to the manufacturer’s instructions, resulting in pSW13, a C-terminal V5-tagged expression vector for YspM from strain JB580v (*YspM*$_{JB580v}$). pSW16 was generated in the same fashion using primers P70 and P67 by first cloning the *yscC* ORF from clinical isolate Y295 into pENTR SD-TOPO (pSW12) and recombining pSW12 with pDEST52 to generate the C-terminal V5-tagged fusion protein. Briefly, the H304N site-directed mutant in *YspM*$_{Y295}$ was generated as follows: primers P70 and P82, which contained the *H304N* mutation in pSW12, were used to amplify the first ~700 bp of *yscC* from pSW12 that contained the histidine at position 304 replaced with an asparagine. Next, primers P81 and P67, which contained the *H304N* mutation in pWB1, were used to generate a ~200-bp product downstream of and including the *H304N* mutation. These two products contained ~35 bp of overlap at the 5’ end and were combined and used in a PCR along with primers P70 and P67. The resulting product was cloned into pENTR SD-TOPO to generate pSW29. The C-terminal V5-tagged yeast expression vector for YspM in the Y295 strain carrying the H304N mutation (*YspM*$_{Y295H304N}$), pSW46, was generated using techniques described above.
In-frame deletion construction. The yscC in-frame deletion (YVM1374) was constructed as follows. A total of 500 µl of E. coli S17-1-λ pir carrying pSW47 was mixed with 500 µl of JBS80v, plated onto LB agar, and incubated overnight at 26°C. The resulting lawn of cells was scraped and added to 1 ml of phosphate-buffered saline and plated at various dilutions onto LB agar containing nalidixic acid to select against E. coli and on kanamycin to select for plasmid integration. The resulting colonies were plated onto LB agar containing nalidixic acid and 5% sucrose to select for colonies that had undergone recombination and lost the plasmid. The resulting colonies were checked for kanamycin sensitivity and confirmed for the presence of the yscC deletion by PCR. Absence of Yop secretion was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis for separation of trichloroacetic-acid-precipitated supernatants followed by Coomassie staining (S. E. Witowski and V. L. Miller, unpublished results). The ΔyscB in-frame deletion (YVM1178) and the ΔyeBC (YVM1236) in-frame deletion were constructed in a similar fashion.

Protein secretion preparations, SDS-PAGE, and Western blot analysis. Secreted proteins were collected as described previously (47). Briefly, for detection of secretion via the Ysa T3SS, saturated cultures grown overnight in LB without NaCl at 26°C (noninducing conditions) were back-diluted to an optical density at 600 nm (OD600) of 0.2 into LB containing 290 mM NaCl (inducing conditions) and grown for 6 h at 26°C. For detection of secretion via the Ysc T3SS, saturated cultures grown overnight in LB medium at 26°C were back-diluted to an OD600 of 0.2 into LB containing 20 mM Na2C2O4 and 20 mM MgCl2 (inducing conditions) and grown for 6 h at 37°C. Bacterial cells were removed from 5 ml of culture by centrifugation for 1 min at 13,000 rpm; this process was repeated. The supernatant was then passed through a 0.22-µm-pore-size syringe filter. Cold trichloroacetic acid was added to a final concentration of 10% (vol/vol) and incubated at 4°C overnight. Samples were centrifuged for 10 min at 13,000 rpm at 4°C. Supernatants were discarded, and the pellet was washed with cold acetone and resuspended in 1 ml Tris, pH 9.0. Samples were boiled for 5 min in 1× sample buffer; OD600 equivalents were loaded onto SDS-polyacrylamide gels (31). Proteins were transferred to a nitrocellulose membrane for Western analysis. Blots were blocked for 1 h at room temperature in 1× Tris-buffered saline and 0.1% Tween-20 (TBST) containing 5% milk. Primary antibody against CysA (purchased from List Biologicals and a gift from the laboratory of Erik Hewlett) was added at a dilution of 1:5,000 and incubated at room temperature for 2 h. Membranes were washed several times with 1× TBST and goat anti-mouse immunoglobulin G-horseradish peroxidase (Sigma) secondary antibody diluted 1:15,000 in TBST containing 5% milk and incubated for 30 min at room temperature. Membranes were washed again in TBST, and proteins were detected by chemiluminescence (ECL, Amersham).

Translocation assays. The following protocol was adapted from Sory et al. (37). Chinese hamster ovary (CHO) cells were passaged in F12 medium containing 10% fetal bovine serum, and cells were grown at 37°C in the presence of 5% CO2. Cells were seeded into 24-well tissue culture plates at a density of 4 × 105 cells/well 24 h prior to the start of the translocation assay. Thirty minutes before translocation, CHO cells at a multiplicity of infection of 100:1 and incubated at 37°C in 5% CO2 for 2 h. Cells were washed and covered with F12 medium containing 10% fetal bovine serum and lysed under denaturing conditions (50 mM HCl and boiling for 5 min). The resulting lysate was neutralized with NaOH, and cyclic AMP (cAMP) was extracted with ethanol. Lysate was then centrifuged at 13,000 rpm for 5 min, and the supernatant was removed and dried in a Speed Vac; intracellular cAMP levels were assayed by a commercially available enzyme immunoassay system (Biotrak, Amersham).

RNA extraction and Northern blot analysis. Total cellular RNA was extracted from cell pellets that had been stored at −80°C using Trizol-LS reagent (Invitrogen). Trizol was added to the frozen cell pellet to a density of 10 OD units/ml, and RNA was extracted per the manufacturer’s instructions. Total RNA was treated with DNase using DNA free, following the manufacturer’s protocol (Ambion). For Northern blotting, 10 µg of RNA was separated in a 1% formaldehyde agarose gel and transferred to nitrocellulose membranes by capillary action (31). For dot blots, 5 µg of RNA was mixed with 50 µl of denaturing buffer (50% formamide, 7% formaldehyde, 1× SSC [1× SSC is 0.15 M NaCl plus 0.005 M sodium citrate]) and transferred to nitrocellulose membranes by vacuum suction (31). The yscM gene-specific probe was generated using primers P27 and KW154 and labeled with [α-32P]dATP by random-primer labeling per the manufacturer’s protocol (Roche). Next, the radiolabeled PCR probe was hybridized to blots in 50% formamide buffer. To control for equivalent loading, the 16S RNA was probed. An oligonucleotide probe (KW157) was labeled with [α-32P]dATP by T4 polynucleotide kinase. Hybridization and washes were conducted in a sodium pyrophosphate buffer (hybridization buffer containing 0.1% sodium pyrophosphate, 4× SSC, 5% Denhardt’s reagent, 0.1% SDS, and 100 µg/ml salmon sperm DNA; wash buffer containing 0.1% sodium pyrophosphate, 4× SSC, and 0.1% SDS) (S. Strand, personal communication). The yopH gene-specific signal was normalized by dividing it by the 16S signal. Triplicate values were averaged and normalized to the peak of expression (for time course analysis) or to the wild-type levels (for analysis of regulators). Specific mRNA signals were quantified using an FLA-5000 phosphorimager and ImageGauge, version 4.2.2, software (Fuji Film Medical Systems, Stamford, CT).

Analysis of YopM toxicity in S. cerevisiae. Yeast cells containing pDEST52 (strain SCW06), pSW13 (SCW07), pSW16 (SCW08), or pSW46 (SCW11) were grown overnight at 30°C in synthetic complete medium lacking uracil with 2% glucose. Yeast was subcultured to an OD600 of 0.2 into synthetic complete medium lacking uracil with 2% galactose and grown at 30°C. Samples were taken every hour, and OD600 measurements were taken over the course of growth. To examine expression of tagged protein, Western blot analysis to detect the V5 epitope was performed. The following protocol was adapted from Sato et al. (33). Briefly, at the end of the induction growth curve, OD equivalents of yeast were collected on one centrifugation, washed one time with water, immediately frozen by a dry ice-ethanol bath, and stored at −80°C. Cell pellets were thawed and resuspended in 100 µl of breaking buffer (40 mM Tris, pH 7.0, 8 M urea, 5% SDS, 0.1 M EDTA, 10% glycerol, 0.4 mg/ml bromophenol blue, 140 mM β-mercaptoethanol, and protease inhibitor cocktail [Roche Diagnostics]). The mixture was boiled for 5 min and vortexed with an equal volume of glass beads for 1 min before SDS-PAGE separation of proteins and Western blot analysis using primary mouse anti-V5 antibody (Sigma) diluted 1:2,000 in TBST and goat anti-mouse immunoglobulin G-horseradish peroxidase (Sigma) secondary antibody diluted 1:12,000 in TBST.

RESULTS

Identification of a potential Y. enterocolitica type III secreted protein. Matsumoto and Young identified 15 proteins (Ysps) that are secreted via the Ysa T3SS. Interestingly, most of these proteins are encoded in locations distant from the ysa locus in the Y. enterocolitica JB850v genome (23). A genetic screen, unrelated to type III secretion, conducted in our laboratory brought to our attention a potential 16th Ysa type III secreted protein, YE3614, encoded on the Y. enterocolitica JB850v chromosome (P. Revell and V. L. Miller, unpublished results). The results of this initial screen were complex, and thus YE3614 was not followed up on in that context. However, the similarity of YE3614 to a type III secreted protein became of interest to us and was studied further. The hypothesis that YE3614 could be a type III secreted protein was based on protein sequence analysis. One of the closest homologs of YE3614 is the SPI2 (Salmonella pathogenicity island 2) secreted effector, SseJ, from Salmonella enterica. Additionally, like the Ysa T3SS, YE3614 is present only in biotype 1B strains of Y. enterocolitica (18). Furthermore, a YE3614 homolog is not found in Yersinia pestis and Yersinia pseudotuberculosis, which also lack a Ysa T3SS homolog. Based on this knowledge, we hypothesized that YE3614 would most likely be a Ysa type III secreted protein rather than a Yse type III secreted protein. Examination of the chromosomal region surrounding YE3614 reveals insight into the evolution of this region of the chromosome (Fig. 1A). Thomson et al. have identified a region that is hypervariable among Y. enterocolitica biotypes, spanning from YE3450 to
YE3644, which they have termed the plasticity zone (38). The authors speculate that the plasticity zone did not arise from a single acquisition but, rather, from multiple independent insertion events. Additionally, surrounding YE3614 is a region containing remnants of an ancestral enteric flagellar cluster termed flgII. (YE3610, YE3610A, YE3611, and YE3614A) (38); many of these ORFs appear to be pseudogenes. Interestingly, YE3614 does not have homology to a flagellar protein. However, downstream of YE3614 is a region termed IS330. This region is classified as a highly degenerative copy of the Y. enterocolitica IS10-like transposase. Therefore, it is possible that YE3614 was inserted into the genome in an event distinct from the acquisition of the flgII. Two pieces of evidence lead us to believe that YE3614 and YE3614A are not in an operon. First, they are separated by 228 bp. Second, the GC content of the genes within the flgII remnant, YE3610, YE3610A, YE3611, and YE3614A, is 55%, 41%, 41%, and 38.7%, respectively, while the GC content for YE3614 is only 31%. This information, combined with the data collected by Thomson et al., supports the hypothesis that YE3614 was acquired more recently via a horizontal transfer event (38).

Both SseJ and YE3614 are classified as bacterial GDSL lipases based on protein sequence. Members of this family are underlined. Arrowheads indicate catalytic residues (Ser, Asp, and His), black boxes indicate identical amino acids, and gray boxes indicate similar amino acids.

YE3644 is secreted into culture supernatants. (A) pSW43 (JB580v yspM ORF and promoter) was transformed into the wild-type (WT; JB580v), ∆ysaC (YVM1178), and ∆ysC (YVM1374) strains. Bacteria were grown under Ysa T3SS-inducing conditions. Samples were harvested after 6 h, and samples were prepared for Western blotting using an antibody against CyaA. (B) pSW41 (JB580v yspM ORF and yspE promoter) was transformed into the strains listed above. Bacteria were then grown under Ysc T3SS-inducing conditions (37°C and absence of Ca2+), and samples were prepared as described in panel A. C) pSW45 (Y295 yspM ORF and promoter) was transformed into the strains listed above and grown under Ysa T3SS-inducing conditions, and supernatants and cells were analyzed as described in panel A.

YE3614 is secreted by the Ysa T3SS. The homology of YE3614 to a SPI2-secreted effector suggested that YE3614 might be secreted by Y. enterocolitica. While we hypothesized that YE3614 was a Ysa type III secreted protein, it was necessary to determine if YE3614 was indeed secreted and, if so, if it was dependent on the Ysa or the Ysc T3SS. To test this, a CyaA reporter fusion was constructed containing the predicted promoter and the first 150 amino acids of YE3614 from S. enterica. The five blocks of amino acid identity found in GDSL lipases are underlined. Arrowheads indicate catalytic residues (Ser, Asp, and His), black boxes indicate identical amino acids, and gray boxes indicate similar amino acids.
secreted by Ysc T3SS as well as the Ysa T3SS, and the presence of the YspM protein is capable of being translocated into CHO cells by the Ysa T3SS. pSW43 (JB580v yspM ORF and promoter) was transformed into the wild-type (WT; JB580v), pYVe8081v-cured (JB580c), and ΔyscC (YVM1374) strains. Strains were used to infect CHO cells in triplicate at 26°C for 2 h. Cytosolic levels of cAMP were measured as described in Materials and Methods and compared to uninfected CHO cells and CHO cells infected with JB580v carrying the negative control plasmid, pSW42 (JB580v yspM promoter). Results of a representative assay are shown.

bacterial cells and supernatants were harvested. Protein secretion was seen in bacteria lacking a Ysc T3SS but not in a strain lacking a Ysa T3SS (Fig. 2A).

To determine if YspM was also secreted by the Ysc T3SS, wild-type, ΔyscC, and ΔysaC strains of bacteria carrying pSW43 were grown under Ysc-inducing conditions, at 37°C in the absence of CaCl₂, and bacterial cells and culture supernatants were examined for the presence of CyaA signal by Western blotting. Under these conditions, no CyaA signal was present in either cells or supernatants in any strain examined (data not shown). Together, these data support the model that YspM is a type III secreted protein and is natively secreted by the Ysa T3SS but not the Ysc T3SS. However, the lack of secretion of YspM by the Ysc T3SS may be due to lack of expression under Ysc-inducing conditions.

To test the possibility that the lack of YspM secretion by the Ysc T3SS is due to a lack of protein production and not the specificity of the apparatus itself, expression of the YspM-CyaA fusion protein was placed under the control of the yopE promoter (pSW41), a promoter that is active under Ysc inducing conditions. This promoter was chosen as it is a promoter native to Y. enterocolitica that is strongly expressed under Ysc T3SS-inducing conditions. When pSW41 was transformed into JB580v and bacteria were grown under Ysc-inducing conditions, CyaA signal was detected in the culture supernatants (Fig. 2B). To determine if this secretion was via the Ysa or the Ysc T3SS, pSW41 was transformed into the ΔysaC and the ΔyscC strains. CyaA signal was detected in culture supernatants of the ΔysaC strain but not in the ΔyscC strain (Fig. 3B). This demonstrates that the YspM protein is capable of being secreted by Ysc T3SS as well as the Ysa T3SS, and the presence or absence of protein secretion is dependent on protein production rather than specificity of the apparatus.

YspM is translocated into host cells via the Ysa T3SS. To determine if YspM could be translocated into mammalian cells, the CyaA reporter fusion used to detect secretion, plasmid pSW43, was also used to examine translocation. Upon translocation of CyaA into the host cell cytosol, calmodulin present there can bind CyaA and activate its ability to catalyze the conversion of ATP to cAMP, which can subsequently be measured by enzyme-linked immunosorbent assay (37). Thus, an increase in cAMP levels corresponds with a protein that is translocated by the bacterium into the host cell. CHO cells were infected for 2 h at 26°C with the wild type, a strain lacking the pYVe8081 virulence plasmid that contains the entire ysa locus and thus is deficient for secretion via the Ysc T3SS (JB580c), and ΔysaC, all containing pSW43. Under these conditions there was a significant increase in intracellular cAMP levels in both wild-type and JB580c strains but not in the ΔysaC mutant (Fig. 3). No increase in cAMP levels was detected in the negative control strain carrying a plasmid with the predicted yspM promoter alone driving cyaA expression, pSW42. This indicates that YspM is translocated into CHO cells under these conditions, and this event requires a functional Ysa T3SS. When these assays were repeated under Ysc T3SS-inducing conditions, no rise in cAMP levels was detected (data not shown).

Expression of yspM is regulated similarly to the ysa operon. As yspM is encoded outside of the ysa locus, we sought to determine if the factors that control transcription of the ysa locus also affect expression of yspM. Northern blot analysis demonstrated that yspM was maximally expressed 2 h into the growth curve when bacteria were grown at 26°C in the presence of 290 mM NaCl. There was a 3.3-fold induction of yspM expression in the presence of NaCl as opposed to the absence of NaCl (Fig. 4A). Next, we looked for the presence of yspM transcript in mutants of known ysa regulators: ΔysaE (strain YVM932), ΔysaB (YVM981), ΔysrS (YVM969), ΔysrR (YVM1006), and ΔacsB (YVM1236). For these experiments wild-type and mutant
strains of bacteria were grown at 26°C in the presence of 290 mM NaCl, and RNA was extracted at 2 h as this is when maximal yspM transcript is present in wild-type cells. Expression of yspM was 16% of the wild-type level in ΔysaE, 18% in ΔyscC, 27% in ΔysrS, 60% in ΔysrR, and 33% in ΔyscB (Fig. 4B). Together, these data indicate that yspM expression is controlled by the same factors as the ysa apparatus; however, the mechanism of this regulation remains to be determined.

YspM from *Y. enterocolitica* JBS80v lacks a catalytic domain required for yeast growth inhibition. As many type III secreted proteins have been shown to exert an effect on eukaryotic cells, we sought to determine if YspM also had any effect. The use of an *S. cerevisiae* system as a tool to assess protein functionality has been described for other type III secreted effectors (21, 33, 35, 36). To determine if YspM was toxic for eukaryotic cell growth, a YspM fusion protein under the control of the *GAL1* promoter was constructed with a V5 tag at the C terminus (plasmid pSW13). This plasmid was then used to transform the effects of YspM on the yeast *S. cerevisiae*. The *GAL1* promoter is repressed when yeast cultures are grown in glucose and activated when grown in galactose, thus allowing for control of expression of potentially toxic proteins. When grown under noninducing conditions (2% glucose), yeast containing pSW13 (strain SCW07) had growth rates similar to the vector-only control, which does not encode a tagged protein (SCW06) (data not shown). When yeast was grown under inducing conditions (2% galactose), growth rates of strains SCW06 and SCW07 were similar (data not shown). Protein production was confirmed for SCW07 using Western blot analysis against the V5 tag (data not shown). Knowing that YspM from *Y. enterocolitica* JBS80v lacked the conserved histidine domain common to GDSL lipases, these data raised the possibility that absence of toxicity toward eukaryotic cells may be due to the lack of the conserved His catalytic residue.

After this result was observed, the sequence of yspM from *Y. enterocolitica* JBS80v was analyzed further, and it was discovered that the catalytic His residue-containing domain is present in a different reading frame but not translated due to a frameshift mutation (Fig. 5). As it was discovered that yspM is present in other biotype 1B strains, we sequenced the corresponding gene from eight additional biotype 1B clinical isolates to determine the prevalence of this frameshift mutation. Seven of these strains had sequences of yspM that encoded proteins containing all three catalytic residue-containing domains found in GDSL lipases; however, one strain, 634-83, was truncated within the first half of the protein (Fig. 5A). Strain Y295 was chosen for further analysis as this is also an O:8 serotype strain, as is JBS80v. Comparison of yspM from JBS80v (yspM *JBS80v*) and yspM from Y295 (yspM *Y295*) revealed the location of the frameshift mutation responsible for the truncation of yspM *JBS80v* (Fig. 5B). To determine if YspM *Y295* was also secreted by the Ysa T3SS, a CyaA reporter fusion was constructed containing 500 bp of the promoter region and the first 150 amino acids of YspM *Y295* (pSW45). This construct was transformed into a wild-type, ΔyscC, and ΔysaE strains. CyaA signal was detectable by Western blot analysis in the wild type and ΔyscC only when cultures were grown under Ysa-inducing conditions, demonstrating that the yspM promoter from strain Y295 is active in our laboratory strain (JBS80v) and that this protein can be secreted by the Ysa T3SS (Fig. 2C).

**DISCUSSION**

The Ysa T3SS was previously shown to secrete 15 different proteins into culture supernatants (23). Here, we identify a new protein from *Y. enterocolitica* that is natively secreting. The yspM ORF is expressed under the same conditions as the *ysa* locus and requires for full expression the known *ysa* regulators, YsaE, YscB, YsrRS, and YscB. In addition to being secreted by the Ysa T3SS, YspM *JBS80v* is translocated into host cells by the Ysa T3SS when cultures are grown under Ysa-inducing conditions. When YspM is expressed from a promoter active under Ysc secretion conditions, YspM can be secreted by the bacterium via the Ysc T3SS. Other studies in *Y. enterocolitica* have shown that YopE, YopN, and YopP are secreted by the Ysa and the Ysc T3SS, and YpLA, natively a flagellar type III secreted protein, can be secreted by both the Ysc and Ysa T3SS (45–47). As it is still unclear what signals within the host activate the various T3SSs, these data suggest that during infection (at least for a subset of type III secreted proteins), if YspM is expressed, then it potentially could be secreted by any T3SS. This theory is further supported by work showing that deletion of some individual *ysp* genes had a greater impact on virulence than deletion of the
FIG. 5. Sequence alignment of YspM from nine biotype 1B isolates of Y. enterocolitica. (A) Amino acid alignment of YspM from these strains demonstrates the presence of the fifth conserved domain found in GDSL lipases (underlined) in all but two isolates, JB80v and 634-83, and the location of the predicted catalytic histidine (arrowhead). The alignment also reveals the presence of various amino acid changes among the different YspM isolates examined. Unique amino acids are in black boxes, and similar amino acids are in gray boxes. (B) The DNA sequences of YspMJB80v and YspM634-83 reveal the insertion of an adenosine (bold) which shifts the reading frame to encode a stop codon (italicized). The reading frame of each sequence is noted.

entire Ysa apparatus (23). One possible explanation for this apparently anomalous observation is that when the Ysa T3SS is not present, these YspS are being secreted by alternative means during infection of the host, such as the Ysc T3SS.

Based on sequence homology, YspM is characterized as a bacterial lipase. These proteins have the ability to cleave fatty acid chains from the glycerol backbone and may also have cholesterol acyltransferase activity (40). Much work has been done to characterize a close homolog, SseJ, an SPI2 secreted effector from S. enterica, and to demonstrate its role in Salmonella-containing vacuole maintenance within infected cells and to show that the catalytic domains of the protein are required...
lysates of SCW07, SCW08, and SCW11 using anti-V5 antibody. Representative assays are shown. (C) Western blot analysis on cellular

FIG. 6. YspM Y295 is toxic for yeast growth. Yeast carrying plasmids pDEST-52 (strain SCW06), pSW13 (YspMY295-V5, strain SCW07), pSW16 (YspMY295-V5, strain SCW08), and pSW46 (YspMY295H304N-V5, strain SCW11) were analyzed for growth under induction conditions (A) and noninduction conditions (B). Samples were taken over the course of the growth curve and OD_{600} readings were measured. Representative assays are shown. (C) Western blot analysis on cellular lysates of SCW07, SCW08, and SCW11 using anti-V5 antibody. Samples were prepared as described in Materials and Methods.

for this activity. The SPI2 T3SS is crucial to the ability of Salmonella to replicate within host cells and to achieve full virulence within the mouse (reviewed in references 11, 14, and 9). The similarity between SesJ and YspM would lead one to think that YspM might also play a role in the survivability of Y. enterocolitica within infected host cells. While Y. enterocolitica is typically thought of as an extracellular pathogen, there is some evidence to support the idea that the bacterium can replicate intracellularly. Two studies have shown an outgrowth of Y. enterocolitica within infected host cells. In unactivated J774.J macrophages, there was a 30-fold increase in the number of bacteria obtained 25 h postinfection (29). Also, another study reported a 110-fold increase in bacteria recovered from infected J774 macrophages at 24 h postinfection (7). While the role of intracellular survival remains poorly understood in the context of Y. enterocolitica pathogenesis, it is possible that YspM, similar to SesJ, may play a role in that process.

While there is little sequence similarity, another possibility is that YspM acts like the phospholipase ExoU, a type III secreted protein from Pseudomonas aeruginosa. ExoU is an important virulence factor in the mouse model of infection and a potent cytotoxin causing multiple effects on a variety of cell types, including yeast, and these toxic effects are due, in part, to the phospholipase activity of ExoU (1, 33; also reviewed in reference 34). Like Y. enterocolitica, P. aeruginosa is an extracellular pathogen that exerts its effects on eukaryotic cells, in part, through a T3SS. It has been shown that ExoU requires the eukaryotic Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1) as a cofactor (32). Efforts to date in our laboratory have failed to demonstrate lipase activity for purified recombinant YspM (S. E. Witowski and V. L. Miller, unpublished results). Perhaps this is due to the requirement of a specific eukaryotic cofactor, similar to ExoU. Continued analysis of the enzymatic properties of YspM may help to determine if this protein functions to aid in either intracellular survival, like SesJ, or extracellular survival, like ExoU.

Analysis of yspM in strain JB580v of Y. enterocolitica reveals the presence of a naturally occurring mutation. This mutation truncates the protein such that the catalytic His-containing domain is no longer encoded by yspM. It was determined that this mutation was not acquired by passage of JB580v through our laboratory as a strain of JB580v from the laboratory of B. Wren also contained the same point mutation (Witowski and Miller, unpublished). When yspM from eight other clinical isolates was sequenced, seven of these strains did not have the mutation, and the yspM sequence did encode the His-containing domain. The eighth strain sequenced (634-83) contained a point mutation that truncated the protein within the first half of the protein. The strains sequenced were all biotype 1B strains and comprised several different serotypes. When a representative isolate, YspMY295, was assayed in yeast cells, its expression was toxic for yeast growth. Mutational analysis revealed the predicted catalytic His to be required for this toxicity, suggesting that YspM has potent biological activity and that this activity is dependent on the His-containing domain found in all GDSL bacterial lipases. Further investigation of the mechanism of YspM lethality in yeast may help elucidate a role for this protein in Y. enterocolitica pathogenesis.

Howard et al. in their comparative phylogenomic study of the evolution of Y. enterocolitica also found that yspM was present only in the eight highly pathogenic strains they examined (18). The information gathered in their work provides excellent insight into the evolution of the pathogenicity of Y. enterocolitica. Their discovery that only 20.8% of the total genome of all strains examined (including strains of high and low degrees of pathogenicity and nonpathogenic strains) was conserved as core genes indicates that the majority of the genome is distinct between the various strains (18). These data, combined with the location of a transposase-like element upstream of yspM, indicates that the gene was possibly acquired via horizontal transfer. The discovery of the inactive form of yspM in a strain of Y. enterocolitica (JB580v) that is still highly pathogenic to both people and mice is intriguing. These observations raise questions about when these mutations arose as
as about the interplay of YspM with other Ysa type III secreted effectors and their role in pathogenesis.

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