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Yersinia enterocolitica Type III Secretion: *yscM1* and *yscM2* Regulate *yop* Gene Expression by a Posttranscriptional Mechanism That Targets the 5′ Untranslated Region of *yop* mRNA

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Pathogenic *Yersinia* spp. secrete Yops (*Yersinia* outer proteins) via the type III pathway. The expression of *yop* genes is regulated in response to environmental cues, which results in a cascade of type III secretion reactions. *yscM1* and *yscM2* negatively regulate the expression of *Yersinia enterocolitica yop* genes. It is demonstrated that *yopD* and *lcrH* are required for *yscM1* and *yscM2* function and that all four genes act synergistically at the same regulatory step. Further, SycH binding to the protein products of *yscM1* and *yscM2* can activate *yop* gene expression even without promoting type III transport of YscM1 and YscM2. Reverse transcription-PCR analysis of *yopQ* mRNA as well as *yopQ* and *yopE* gene fusion experiments with the *npt* (neomycin phosphotransferase) reporter suggest that *yscM1* and *yscM2* regulate expression at a posttranscriptional step. The 178-nucleotide 5′ untranslated region (UTR) of *yopQ* mRNA was sufficient to confer *yscM1* and *yscM2*-mediated regulation on the fused reporter, as was the 28-nucleotide UTR of *yopE*. The sequence 5′-AUAAA-3′ is located in the 5′ *yop* UTRs, and mutations that alter the sequence motif either reduced or abolished *yscM1*- and *yscM2*-mediated regulation. A model is proposed whereby YopD, LcrH, YscM1, YscM2, and SycH regulate *yop* expression in response to specific environmental cues and by a mechanism that may involve binding of some of these factors to a specific target sequence within the UTR of *yop* mRNAs.

Many gram-negative bacterial pathogens employ a virulence strategy known as type III secretion to establish infections in animal and plant hosts (24, 33). Three pathogenic *Yersinia* species, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, share a common tropism for lymphatic tissues in their mammalian hosts (13, 45). Yersiniae use the type III pathway for the secretion of virulence factors and for the targeting of Yops (*Yersinia* outer proteins) into the cytoplasm of host macrophages (36, 38, 50, 51). These mechanisms manipulate the host's innate immune response, thereby preventing bacterial phagocytosis and killing (17). *Y. enterocolitica* transports 14 proteins via the type III pathway: LcrV, YopB, YopD, YopE, YopH, YopM, YopN, YopO, YopP, YopQ, YopR, YopT, YscM1, and YscM2 (1, 19, 25, 26, 32, 35, 40, 41, 44, 49, 52). The genes encoding secretion substrates and type III machinery components are located on a 70-kb virulence plasmid (13).

Expression of *yop* genes and secretion of encoded polypeptides are tightly regulated as *Yersinia* responds to specific environmental cues with type III transport reactions (9, 10, 37). Bacterial host entry is accompanied by a temperature shift to 37°C, activating the transcription of type III genes via the regulatory factor LcrF (VirF) (12, 29, 30, 54, 57). *Y. enterocolitica* assembles the type III secretion apparatus when cued by an extracellular amino acid signal (37, 39). A third signal, host serum proteins, triggers the secretion of YopB, YopD, YopR, and LcrV into the extracellular milieu (36–38). Bacterial contact with macrophages or tissue culture cells results in the insertion of needle complexes, a surface extension of type III machines, into the eukaryotic plasma membrane (31). This mechanism is thought to generate the fourth signal, i.e., a drop in the environmental calcium concentration, also referred to as low-calcium response (Lcr) or the calcium signal, from 1.2 mM (extracellular host fluids) to about 100 nM (intracellular fluid) (37). The calcium signal activates type III transport of YopE, YopH, YopM, YopN, YopO, YopP, YopT, and YscM1 into the eukaryotic cytosol (also named type III targeting or translocation) (5, 7, 27, 35, 36, 38, 41, 42, 50, 51).

Only when *Y. enterocolitica* is activated by a calcium signal is YopQ polypeptide synthesized and secreted, a phenotype which greatly facilitates genetic analysis of *yop* gene expression (2). Several genes are responsible for the regulation of *yopQ* expression (2). Mutations in class I genes (*yopN*, *tyeA*, *sycN*, *yscB*, and *lcrG*) bypass the requirement for the calcium signal, causing expression and secretion of YopQ in the presence of calcium at 37°C (2, 9, 11, 37). Mutations in class II genes, *yopD* and *lcrH*, lead to a phenotype that allows for the synthesis but not for the secretion of YopQ in the presence of calcium at 37°C (2, 37). *yopD* encodes a secretion substrate for the type III pathway (26), while *lcrH* (*sycD*) encodes a regulatory protein in the bacterial cytoplasm (4). LcrH (SycD) binds to YopB and YopD and has been implicated in promoting the type III secretion of the two polypeptides (53). Mutations in class III genes, i.e., any of the 21 *ysc* genes that encode the type III machinery, abolish *yopQ* expression in the presence and absence of calcium (2).

The *lcrQ* gene specifies a 12-kDa type III secretion substrate and is needed to inhibit *yop* gene expression when the type III pathway of *Y. pseudotuberculosis* is not induced (43, 48). Overexpression of *lcrQ* abolishes *yop* expression even under condi-

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tions that induce type III secretion (43, 48). Unlike *Y. pseudotuberculosis* and *Y. pestis*, each of which harbors a single *lcrQ* gene, the *Y. enterocolitica* virulence plasmid encodes two *lcrQ* homologs, *yscM1* and *yscM2* (52). Knockout mutations in *yscM1* and *yscM2*, but not single deletions, cause mutant *Yersinia* strains to overexpress *yop* genes (52). Overexpression of each gene alone (*yscM1* or *yscM2*) is sufficient to repress the synthesis of Yops (7, 52). Thus, *yscM1* and *yscM2* must display overlapping and at least partially redundant functions (52). *yscM1* and *yscM2* are expressed in the presence of Ca^{2+} ($+Ca^{2+}$ conditions); however, their protein products are secreted only when calcium has been depleted from the culture medium (7, 43). A regulatory function of *yscM1* and *yscM2* for the expression and secretion of YopQ has hitherto not been examined.

Previous work on the regulation of *yop* expression suggested a mechanism whereby the protein product of *lcrQ* blocks the transcription of *yop* genes (7, 43, 52). Once the type III machinery is assembled and functional, LcrQ (YscM1 and YscM2) is depleted from the bacterial cytoplasm and transcription of *yop* genes is activated (43, 52). This model resembles the transcriptional control of flagellar biosynthesis, a type III pathway that is regulated by a sigma factor (sigma-28 or FliA) for RNA polymerase and by *flgM* (34). The *flgM* gene product functions as an anti-sigma factor and controls transcription by sequestration of FliA from RNA polymerase. As *Salmonella enterica* serovar Typhimurium completes the flagellar hook and basal body, FlgM is depleted from the cytoplasm by secretion, thereby allowing FliA-mediated transcription of flagellin genes (34). During tissue culture infection, *Y. pseudotuberculosis* LcrQ and *Y. enterocolitica* YscM1 are injected into the cytosol of eukaryotic cells (7). YscM1 and YscM2 both bind to the bacterial chaperone SycH, which is required for the effective type III transport of YopH and YscM1 (7). A *sycH* mutant is defective in both YopH and YscM1 targeting, and the overall expression of *yop* genes during infection is decreased (7). These and other observations suggest that SycH plays an important role in regulating *yop* expression (7, 56).

A requirement of class II genes for LcrQ function, *yopD* in *Y. pestis* and *lcrH* in *Y. pseudotuberculosis*, has been reported previously (48, 55). *Y. enterocolitica* YopD and LcrH have been suggested elsewhere to regulate expression of *yopQ* by a posttranscriptional mechanism that involves binding of YopD and LcrH to a −45 to +45 nucleotide sequence of *yopQ* mRNA (2). Δ(*yopD*), Δ(*lcrH*), and Δ(*yscM1 yscM2*), but not Δ(*sycH*), mutations allow mutant *Yersinia* to bypass the requirement for an amino acid signal to catalyze type III secretion during tissue culture infection (37). In this report we sought to determine whether *Y. enterocolitica yscM1* and *yscM2* operate at the same step of regulation as do *yopD* and *lcrH*. It is shown that *yopD* and *lcrH* are required for *yscM1* and *yscM2* function and that all four genes act synergistically at the same regulatory step. Further, SycH binding to the protein products of *yscM1* and *yscM2* can activate *yop* gene expression. Reverse transcription-PCR (RT-PCR) analysis of *yopQ* mRNA suggests that *yscM1* and *yscM2* have a minimal effect on transcriptional inhibition. Experiments that fused *yopQ* and *yopE* gene sequences to the *npt* (neomycin phosphotransferase) reporter suggest that *yscM1* and *yscM2* regulate expression at a posttranscriptional step. The 178-nucleotide 5′ untranslated region (UTR) of *yopQ* mRNA is sufficient to confer *yscM1*- and *yscM2*-mediated regulation on the fused reporter, as is the 28-nucleotide UTR of *yopE*. Mutations in nucleotide sequence 5′-AUAAA-3′, which is found in many 5′ *yop* UTRs, either reduced or abolished *yscM1*- and *yscM2*-mediated regulation, suggesting that the conserved sequence element targets transcripts for YopD-, LcrH-, YscM1-, YscM2-, and SycH-mediated regulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are summarized in Table 1. *Y. enterocolitica* strains EC3 Δ(*yopD lcrH*), EC4 Δ(*yopD yscM1 yscM2*), EC6 Δ(*yopD lcrH yscM1 yscM2*), and EC7 Δ(*lcrH yscM1 yscM2*) were constructed by allelic exchange by using the suicide plasmids pVLΔ*yopD*, which carries a frameshift-stop codon mutant allele of *yopD*, and pCT129, which carries a deletion mutant allele of *lcrH* (7, 15, 36, 38, 39). Strain EC3 was generated through mating of *Escherichia coli* S17-1 pVLΔ*yopD* with *Y. enterocolitica* CT133. Selection of merodiploids was performed on Luria-Bertani agar supplemented with nalidixic acid (35 μg/ml) and chloramphenicol (20 μg/ml) and incubation at 26°C. Double crossovers were selected by incubation on Luria-Bertani agar supplemented with nalidixic acid (35 μg/ml) and 5 mM sucrose. Mutants were screened by inducing type III secretion in tryptic soy broth (TSB) supplemented with 5 mM EGTA at 37°C. Protein was precipitated with trichloroacetic acid (TCA) and detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. *Yersinia* strains EC4, EC6, and EC7 were generated by mating *E. coli* S17-1 carrying pVLΔ*yopD* or pCT129 with *Y. enterocolitica* EC2 or EC4, respectively.

Plasmid pEC52 was constructed through a three-way ligation of pDA330 vector backbone liberated with *Eco*RI and *Bam*HI to a *yopQ* promoter sequence containing additional bases (−178 to −148 positions) of the 5′ *yopQ* UTR generated with the primers YopQ1 (3) and YopQUTR7, 5′-AACATATGACT CCGTGACGTTGCTCATT-3′, yielding a fragment with 5′ *Eco*RI and 3′ *Nde*I ends and a fragment containing the 5′ UTR of neomycin phosphotransferase (*npt*) linked 5′ to the *npt* open reading frame (ORF) generated with the primers Npt1Nde, 5′-AACATATGATCAAGAGACAGGATGAGGAT-3′, and Npt3 (3), yielding a fragment with 5′ *Nde*I and 3′ *Bam*HI ends. pEC53 to pEC57 were generated through ligation of the pEC52 vector backbone liberated with *Eco*RI and *Nde*I combined with 5′ *Eco*RI-3′ *Nde*I *yopQ* promoter fragments containing 3′ truncations of the 5′ *yopQ* UTR generated through combination of the primer YopQ1 with the following primers: YopQUTR6 (pEC53), 5′-AACATATGAT GCATCGGAATATTTCAAG-3′; YopQUTR5 (pEC54), 5′-AACATATGAAA ATAGAATATCTACTCTCAATG-3′; YopQUTR4 (pEC55), 5′-AACATATG TGTATAATTAAACTCACTCCGTA-3′; YopQUTR3 (pEC56), 5′-AACATA TGACTACACTATTTAATAACCGTCA-3′; and YopQUTR2 (pEC57), 5′-AA CATATGAAAATTTACTTTATAAACTACACTAT-3′. pEC58 was generated from the primer YopQUTR1, 5′-AAGGTACCAGTGACTACTCCAAAATTT ACTT-3′, yielding a fragment with a 3′ *Kpn*I site. This fragment was ligated with a fragment generated from the primers Npt1Kpn and Npt3. Constructs containing 5′ truncations of the 5′ *yopQ* UTR were constructed through a three-way ligation of pDA330 vector backbone liberated with *Eco*RI and *Bam*HI to a *yopQ* promoter fragment generated with the primers YopQ1 and YopQUTR8N, 5′-AACATATGTTATTTATTTTAAAGCTACTGATGT-3′, yielding a 5′ *Eco*RI-3′ *Nde*I *yopQ* promoter fragment. Second fragments with 5′ *Nde*I and 3′ *Bam*HI ends were generated by using pEC58 as a template for PCRs that utilized a common (3′) primer, Npt3, in combination with the primers YopQUTR9 (5′-AACATATGGTGGAGAATACTTGAAATATTCC-3′), YopQUTR10 (5′-AACATATGGGGGAGCTCATTGAGAGTAG-3′), YopQUTR11 (5′-AACA TATGTTTATAAATTACGGAGTGAGTTTA-3′), YopQUTR12 (5′-AACAT ATGCACTCGTAGTGACGGTTATTA-3′), and YopQUTR13 (5′-AACATAT GGTTTATAAAGTAAATTTTGGAGTAG-3′) to yield pEC70 to pEC74, respectively. pEC80 was constructed through a three-way ligation of pDA183 vector backbone liberated with *Eco*RI and *Nde*I to a *yopQ* promoter sequence generated with the primers YopQ1 and YopQUTR8K, yielding a fragment with 5′ *Eco*RI and 3′ *Kpn*I ends, and a set of oligonucleotides, E1 (5′-CTTGTTTT AATAGCCAAGGGAATAAATAGTCCA-3′) and E2 (5′-TATGGACTATTT ATTCCCTTGGCTATTAAAACAAGGTAC-3′), that contain the *yopE* UTR between 5′ *Kpn*I and 3′ *Nde*I restriction sites. pEC138 to pEC145 were constructed through ligation of oligonucleotide pairs into pEC80 vector backbone liberated with *Kpn*I and *Nde*I. All sets of oligonucleotides were identical to E1 and E2 with the exception of the transversion substitutions at positions indicated in Table 1. pEC83 was generated through the ligation of pDA183 vector back-

TABLE 1. Bacterial strains and plasmids

Designation	Property	Reference
Y. enterocolitica strains		
W22703	O:9 serotype; pYVe227 Nal^r; wild-type isolate	14
CT133	Δ(*lcrH*) Nal^r	2
EC2	Δ(*yscM1 yscM2*) Nal^r	7
EC3	Δ(*yopD lcrH*) Nal^r	This study
EC4	Δ(*yopD yscM1 yscM2*) Nal^r	This study
EC5	Δ(*yscM2*) Nal^r	7
EC6	Δ(*yopD lcrH yscM1 yscM2*) Nal^r	This study
EC7	Δ(*lcrH yscM1 yscM2*) Nal^r	This study
MC3	Δ(*yopQ*) Nal^r	3
VTL1	Δ(*yopN*) Nal^r	36
VTL2	Δ(*yopD*) Nal^r	38
E. coli strains		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	28
S17-1	λ*pir*$^+$	16
Plasmids		
pCT129	pLC28 derivative, *lcrH* deletion construct	2
pDA183	*yopQ* pro-*Q*UTR-*npt* Cm^r	3
pDA184	*yopQ* pro-*Q*UTR-*yopQ* (1–15aa)-*npt* Cm^r	3
pDA208	*yopQ* pro-*Q*UTR-*yopQ* (1–10aa)-*npt* Cm^r	3
pDA209	*yopQ* pro-*Q*UTR-*yopQ*-*npt*UTR-*npt* Cm^r	3
pDA218	*yopQ* pro-*Q*UTR-*yopQ* Cm^r	3
pDA219	*tac* promoter fused to *yopQ*; Cm^r	3
pDA243	*yopQ* pro-*Q*UTR-*yopQ*-*npt* Cm^r	3
pDA259	*tac* promoter fused to *gst*; Cm^r	7
pDA325	*tac* promoter fused to *lcrH*; Cm^r	2
pDA330	*yopQ* pro-*npt*UTR-*npt* Cm^r	2
pEC52	*yopQ* pro-*Q*UTR($^-$178–$^-$148)-*npt*UTR-*npt* Cm^r	This study
pEC53	*yopQ* pro-*Q*UTR($^-$178–$^-$120)-*npt*UTR-*npt* Cm^r	This study
pEC54	*yopQ* pro-*Q*UTR($^-$178–$^-$88)-*npt*UTR-*npt* Cm^r	This study
pEC55	*yopQ* pro-*Q*UTR($^-$178–$^-$58)-*npt*UTR-*npt* Cm^r	This study
pEC56	*yopQ* pro-*Q*UTR($^-$178–$^-$28)-*npt*UTR-*npt* Cm^r	This study
pEC57	*yopQ* pro-*Q*UTR($^-$178–$^-$12)-*npt*UTR-*npt* Cm^r	This study
pEC58	*yopQ* pro-*Q*UTR($^-$178–$^-$1)-*npt*UTR-*npt* Cm^r	This study
pEC70	*yopQ* pro-*Q*UTR($^-$150–$^-$1)-*npt*UTR-*npt* Cm^r	This study
pEC71	*yopQ* pro-*Q*UTR($^-$120–$^-$1)-*npt*UTR-*npt* Cm^r	This study
pEC72	*yopQ* pro-*Q*UTR($^-$90–$^-$1)-*npt*UTR-*npt* Cm^r	This study
pEC73	*yopQ* pro-*Q*UTR($^-$60–$^-$1)-*npt*UTR-*npt* Cm^r	This study
pEC74	*yopQ* pro-*Q*UTR($^-$30–$^-$1)-*npt*UTR-*npt* Cm^r	This study
pEC80	*yopQ* pro-*yopE*UTR-*npt* Cm^r	This study
pEC83	*npt* pro-*npt*UTR-*npt* Cm^r	This study
pEC84	*npt* pro-*Q*UTR-*npt* Cm^r	This study
pEC102	*yopE* pro-*npt*UTR-*npt* Cm^r	This study
pEC112	*yopQ* pro-*npt*UTR(*yopE*UTR hyb)-*npt* Cm^r	This study
pEC138	*yopQ* pro-*yopE*UTR(A$^-$11C)-*npt* Cm^r	This study
pEC139	*yopQ* pro-*yopE*UTR(A$^-$10C)-*npt* Cm^r	This study
pEC140	*yopQ* pro-*yopE*UTR(T$^-$9G)-*npt* Cm^r	This study
pEC141	*yopQ* pro-*yopE*UTR(A$^-$8C)-*npt* Cm^r	This study
pEC142	*yopQ* pro-*yopE*UTR(A$^-$7C)-*npt* Cm^r	This study
pEC143	*yopQ* pro-*yopE*UTR(A$^-$6C)-*npt* Cm^r	This study
pEC144	*yopQ* pro-*yopE*UTR(T$^-$5G)-*npt* Cm^r	This study
pEC145	*yopQ* pro-*yopE*UTR Δ($^-$11–$^-$5)-*npt* Cm^r	This study
pEC148	*yopQ* pro-*npt*UTR(*yopQ*UTR hyb)-*npt* Cm^r	This study
pEC260	*tac* promoter fused to *gst*; Tet^r	7
pEC345	*tac* promoter fused to *yscM1*; Cm^r	7
pEC346	*tac* promoter fused to *gst-yscM1*; Cm^r	7
pEC347	*tac* promoter fused to *gst-yscM1*; Tet^r	7
pEC348	*tac* promoter fused to *yscM2*; Cm^r	7
pEC349	*tac* promoter fused to *gst-yscM2*; Cm^r	7
pEC350	*tac* promoter fused to *gst-yscM2*; Tet^r	7
pKR12	*tac* promoter fused to *yopD; lcrH* Cm^r	This study
pVLΔ*yopD*	pLC28 derivative; *yopD* deletion construct	38

bone liberated with *Eco*RI and *Nde*I combined with a 5′ *Eco*RI-3′ *Nde*I *npt* promoter fragment containing the *npt* UTR generated from NptProm5′Eco, 5′-AAGAATTCGCGCAAGGGCTGCTAAAG-3′, and Npt3. pEC84 was generated through three-way ligation of pDA183 vector backbone liberated with *Eco*RI and *Nde*I to *npt* promoter sequence generated from primers NptProm5′Eco and NptProm-UTR3′Bgl, 5′-AAAGATCTTGATCCCCTGCGCCATCAG-3′, yielding a 5′ *Eco*RI and 3′ *Bgl*II fragment, and *yopQ* UTR generated with primers YopQUTR15, 5′-AAAGATCTTCATATAAACAATGAGCAACGTC-3′, and YopQUTR16, 5′-AACATATGAGTGACTACTCCAAAATTTACTT-3′, yielding a 5′ *Bgl*II and 3′ *Nde*I fragment. pEC102 was generated through ligation of pDA330 vector backbone liberated with *Eco*RI and *Kpn*I to a *yopE* promoter sequence generated from primers YopE1 and YopEproREVKpn, 5′-AAGGTACCAGGTTATCTTAGTGGGAAAATAG-3′, yielding a 5′ *Eco*RI and 3′ *Kpn*I fragment. pEC112 and pEC148 were constructed through ligation of a pDA330 backbone liberated with *Kpn*I and *Bam*HI combined with 5′ *Kpn*I-3′ *Bam*HI 5′ *npt* UTR mutations and an *npt* ORF generated through combination of Npt3 with the following primers: pEC112, Npt(QUTR)hyb, 5′-AAGGTACC TGACTGACTGATTATAAAACAGGATGAGGATCGTTTCG-3′, and pEC148, Npt(EUTR)hyb2, 5′-AAGGTACCTGACTGACTGATCAAGAGACAGGAT GAGGAATAAATCGCATGATTGAACAAGATGG-3′. pKR12 was generated by three-way ligation of pEC345 (6) vector backbone liberated with *Nde*I and *Bam*HI to a *yopD* coding sequence generated through primers YopDstart, 5′-AACATATGACATATAAATATCAAGACAGACAG-3′, and YopDstop, 5′-AAGGATCCGTCAGACAACACCAAAAGCGG-3′, yielding a 5′ *Nde*I and 3′ *Bam*HI fragment with a 5′ *Bgl*II and 3′ *Bam*HI fragment generated from primers LcrHSDBgl, 5′-AAAGATCTAGGTAATTATGCAACAAGAGAC-3′, and LcrHstop, 5′-AAGGATCCTCATGGGTTATCACCGCACT-3′, containing the *lcrH* gene with its native ribosome binding sequence. Ligations were transformed into *E. coli* DH5α and screened by restriction analysis. Plasmid constructs were sequenced with fluorescently labeled dideoxy chain termination PCRs (University of Chicago CRC DNA Sequencing Facility). All DNA cloning manipulations were performed with pCR2.1 (Invitrogen) in *E. coli* DH5α. Plasmid constructs were electroporated into yersiniae, and transformants were selected on TSB agar supplemented with nalidixic acid (35 μg/ml) and chloramphenicol (20 μg/ml) and grown at 26°C (8). *Yersinia* strains containing the expression vectors pEC260, pEC347, and pEC350 were also cultured with a supplement of tetracycline (5 μg/ml).

***Yersinia* secretion.** Yersiniae were grown to stationary phase overnight in TSB supplemented with nalidixic acid (35 μg/ml) (and chloramphenicol [20 μg/ml] where needed) and diluted 1:50 into 30 ml of fresh TSB medium supplemented with 5 mM $CaCl_2$ or EGTA ($\pm Ca^{2+}$) and antibiotic in 125-ml glass Erlenmeyer flasks. Cultures were incubated on a rotary shaker for 2 h at 26°C and induced at 37°C for 3 h. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 1 mM immediately upon temperature shift. Cultures were centrifuged at 15,000 × *g* for 15 min, and the supernatant was separated from the cell pellet. Proteins in both fractions were precipitated on ice with 10% TCA, centrifuged at 15,000 × *g* for 15 min at 4°C, washed in acetone, and suspended in sample buffer. All samples were analyzed by SDS–15% PAGE. Following electrotransfer to a polyvinylidene difluoride membrane, proteins were examined by immunoblotting with purified rabbit polyclonal antisera raised against *Yersinia* antigens and glutathione *S*-transferase (Schneewind laboratory), anti-NptII (U.S. Biological), and anti-Cat (Sigma). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G was used to generate chemiluminescent signals that were visualized on a Fluorchem 8800 Imaging System (Alpha Innotech).

Expression experiments. Yersiniae transformed with both reporter and expression constructs were grown to stationary phase overnight in TSB supplemented with chloramphenicol (20 μg/ml) and tetracycline (5 μg/ml) and diluted 1:50 into 4 ml of fresh TSB supplemented with 5 mM EGTA ($-Ca^{2+}$) and antibiotics in 10-ml borosilicate glass tubes. Cultures were incubated on a wheel for 2.5 h at 26°C and induced at 37°C for 1.5 h. Expression constructs were induced at time of temperature shift with IPTG, added to 1 mM. Readings of optical density at 600 nm (OD_{600}) were taken for standardization, and 1-ml aliquots of cultures were precipitated on ice with 10% TCA. Samples were centrifuged at 15,000 × *g* for 15 min at 4°C, washed with acetone, solubilized in sample buffer, and loaded on SDS-polyacrylamide gels based on correction for OD_{600}. Samples were analyzed by immunoblotting. Timed promoter experiments were performed in 125-ml glass Erlenmeyer flasks containing 50 ml of TSB supplemented with 5 mM $CaCl_2$ with a 1:50 dilution of stationary-phase culture and appropriate antibiotics. Cultures were immediately incubated at 37°C for the time course. Two 1-ml aliquots were collected at each time point for measurement of OD_{600} and TCA precipitation. Precipitated proteins were processed for analysis as described above, and all quantification of immunoreactive signals was performed with Fluorchem software (Alpha Innotech).

RT-PCR analysis. *Yersinia* cultures were prepared as explained under "*Yersinia* secretion" above except that the culture volumes were increased to 500 ml. Cultures were centrifuged at 15,000 × *g* for 15 min, and the supernatant was separated from the cell pellet. The cell pellet was resuspended in 30 ml of lysis buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl). Aliquots containing proteins

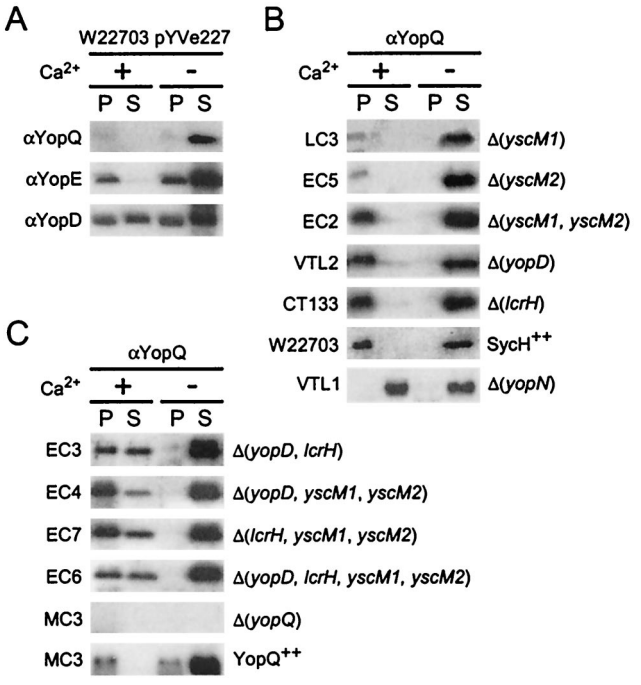

FIG. 1. Regulation of *yop* expression in *Y. enterocolitica*. *Y. enterocolitica* strains were cultured in TSB supplemented with 5 mM $CaCl_2$ ($+Ca^{2+}$) or 5 mM EGTA ($-Ca^{2+}$) for 2 h at 26°C and then induced for type III secretion at 37°C for 3 h. Cultures were centrifuged to separate the bacterial pellet (P) from the culture supernatant (S). Proteins in both fractions were precipitated with TCA and analyzed by SDS-PAGE and immunoblotting. (A) Protein of *Y. enterocolitica* strain W22703 (wild type) was analyzed by immunoblotting for the synthesis and secretion of YopQ, YopE, and YopD. (B and C) Synthesis and secretion of YopQ in various *Y. enterocolitica* strains were analyzed by immunoblotting with antisera raised against purified YopQ. Plasmid-encoded genes were overexpressed ($^{++}$) by adding 1 mM IPTG when shifting the temperature of bacterial cultures to induce the type III virulon.

from each fraction were precipitated on ice with 10% TCA, centrifuged at 15,000 × *g* for 15 min at 4°C, washed in acetone, and suspended in sample buffer. Protein fractions were analyzed by SDS-PAGE and immunoblotting. The pellet fraction was next subjected to lysis with a French pressure cell (16,000 lb/in^2). One-hundred-microliter aliquots were used for total RNA isolation with the SV Total RNA isolation kit (Promega) according to the manufacturer's protocol. Eluted RNA samples were digested with 2 U of DNase (Promega) for 1 h at 37°C. Samples were extracted with phenol-chloroform, ethanol precipitated, and suspended in 90 μl of nuclease-free H_2O. $OD_{260/280}$ measurements were taken to calculate the concentration of each sample and for standardization. RT reactions (25-μl reaction mixtures) were performed with 0.6 μg of total RNA in the presence or absence of reverse transcriptase (Promega) and primer YopQcod2 (100 pmol), 5′-AAGGATCCTCATCCCATAATACATTTTTGAT-3′, according to protocol. Each reaction mixture was diluted to 100 μl in H_2O, and 20 μl of the sample was used as template for PCRs (100-μl reaction mixtures) with primers 5′ Q2Bgl5 (20 pmol), 5′-AAAGATCTTGGAGTAGTCACTATGTTTATT-3′, and 3′ YopQcod2 (20 pmol) (40 cycles, 1-min extension at 72°C) (Qiagen). Virulence plasmid DNA (pYVe227) was used as a positive-control template for PCR. The 549-bp *yopQ* ORF was analyzed by 2% agarose gel electrophoresis and compared to a molecular size standard, a 1-kb DNA ladder (Invitrogen). The gel was stained with ethidium bromide and visualized on a UV light table.

RESULTS

***yscM1*, *yscM2*, *yopD*, and *lcrH* act synergistically to regulate *yopQ* expression.** *Y. enterocolitica* W22703 (wild type) was grown at 37°C in TSB supplemented with either 5 mM calcium chloride ($+Ca^{2+}$) or 5 mM EGTA ($-Ca^{2+}$). Cultures were centrifuged, and the extracellular medium was separated (supernatant) from the bacterial sediment (pellet). Protein in both fractions was precipitated with TCA and analyzed by SDS-PAGE and immunoblotting (Fig. 1A). Wild-type yersiniae secreted YopD both in the presence and in the absence of calcium. Although YopE was synthesized in the presence of calcium, yersiniae did not secrete the polypeptide unless calcium was chelated from the growth medium. YopQ synthesis and secretion occurred only in the absence of calcium.

Knockout mutations in class II genes (*yopD* and *lcrH*) allowed *yopQ* expression in the presence of calcium; however, the mutant yersiniae did not secrete YopQ polypeptide under these conditions. Knockout mutations in *yscM1* and *yscM2* alone resulted in an intermediate phenotype, as only small amounts of YopQ were detected in bacteria grown in the presence of calcium. A strain carrying deletions in both genes, the Δ(*yscM1 yscM2*) strain, displayed a class II phenotype with *yopQ* expression but no secretion in the presence of calcium. The knockout mutation of *sycH* did not affect the calcium regulation of *yopQ* expression or YopQ secretion (data not shown). In contrast, overexpression of *sycH* from the IPTG-

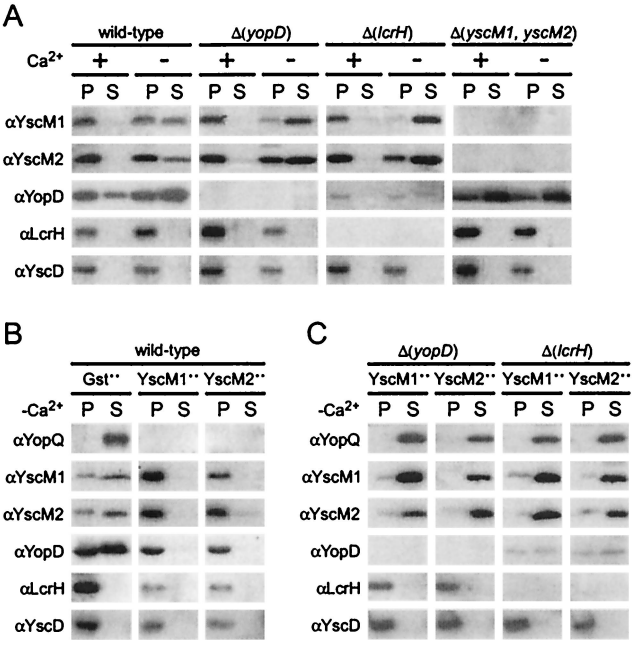

FIG. 2. *yopD* and *lcrH* are required for the function of YscM1 and YscM2. *Y. enterocolitica* strains were analyzed for type III secretion as described in the legend to Fig. 1. (A) *Y. enterocolitica* strains W22703 (wild type), VTL2 [Δ(*yopD*)], CT133 [Δ(*lcrH*)], and EC2 [Δ(*yscM1 yscM2*)] were analyzed by immunoblotting with antisera raised against purified YscM1, YscM2, YopD, LcrH, and YscD. The type III machinery component YscD is located within bacteria. (B) *Y. enterocolitica* W22703 (wild type) harboring pDA259 (encoding Gst), pEC345 (encoding YscM1), or pEC348 (encoding YscM2) were cultured in $-Ca^{2+}$. Plasmid-borne genes were overexpressed ($^{++}$) by adding 1 mM IPTG when shifting the temperature of bacterial cultures to induce the type III virulon. (C) *Yersinia* strains VTL2 [Δ(*yopD*)] and CT133 [Δ(*lcrH*)] were transformed with pEC345 and pEC348 and analyzed by immunoblotting.

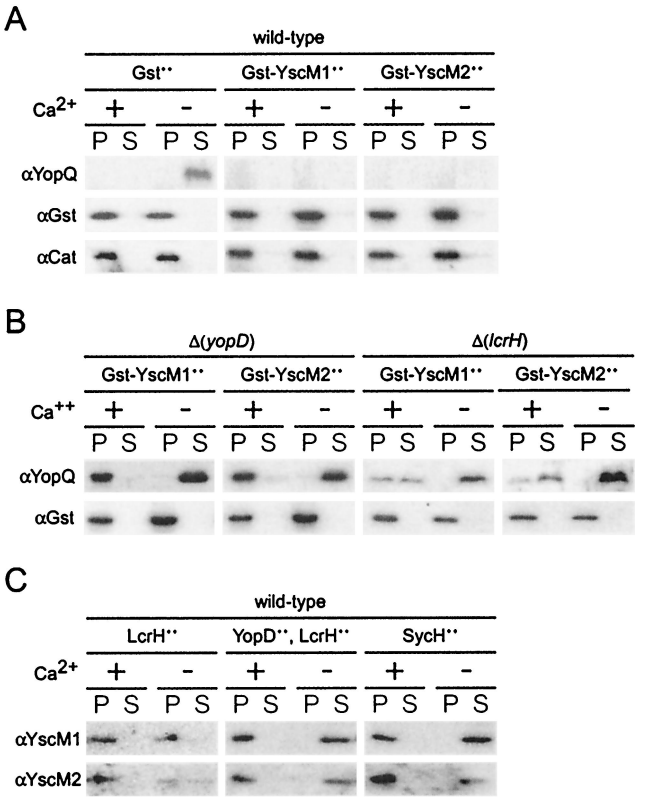

FIG. 3. Nonsecretable YscM1 and YscM2 also require *yopD* and *lcrH* for function. *Y. enterocolitica* strains were analyzed for type III secretion as described in the legend to Fig. 1. *Y. enterocolitica* strains W22703 (wild type) (A and C) and VTL2 [Δ(*yopD*)] and CT133 [Δ(*lcrH*)] (B) were analyzed by immunoblotting with antisera raised against purified YopQ, Gst, YscM1, YscM2, and Cat. Plasmids pEC260 (encoding Gst), pEC347 (encoding Gst-YscM1), pEC350 (encoding Gst-YscM2), pDA325 (encoding LcrH), pKR12 (encoding LcrH and YopD), and pEC441 (encoding SycH) were transformed into *Yersinia* strains, and expression was induced by the addition of 1 mM IPTG when shifting the temperature of bacterial cultures to induce the type III virulon.

inducible *tac* promoter resulted in a class II phenotype (Fig. 1B).

We tested whether the phenotype of *yopQ* expression without YopQ secretion in the presence of calcium was altered in mutant strains lacking multiple class II genes. The combined deletions of Δ(*yopD*) and Δ(*lcrH*); Δ(*yopD*) and Δ(*yscM1 yscM2*); Δ(*lcrH*) and Δ(*yscM1 yscM2*); or Δ(*yopD*), Δ(*lcrH*), and Δ(*yscM1 yscM2*) allowed for the expression of *yopQ* in the presence of calcium. To our surprise, some secretion of YopQ could be observed when the mutant yersiniae were grown in the presence of calcium. This phenotype was distinct, as overexpression of *yopQ* from an inducible promoter in the Δ(*yopQ*) strain did not result in secretion of YopQ in the presence of calcium (Fig. 1C). It should also be noted that this phenotype was distinct from the secretion of YopQ by class I mutant strains, as Δ(*yopN*) mutants quantitatively transport YopQ into the extracellular medium in the presence of calcium (Fig. 1B).

***yopD* and *lcrH* are required for *yscM1* and *yscM2* function.** Three of the four class II gene products are transported by the *Yersinia* type III pathway. We tested whether knockout mutations in *yopD*, *lcrH*, *yscM1*, and *yscM2* affected the expression or the type III transport of class II gene products. Wild-type yersiniae secreted YscM1 and YscM2 in the absence but not in the presence of environmental calcium ions (Fig. 2A). As expected, YopD was secreted both in the presence and in the absence of calcium, whereas LcrH was not secreted (37). Knockout mutations of *yopD* or *lcrH* reduced neither *Yersinia* secretion nor the concentration of YscM1 and YscM2. Consistent with previous reports, the deletion of *lcrH* (*sycD*) caused a significant reduction in the bacterial concentration of YopD (53). Δ(*yscM1 yscM2*) mutant yersiniae synthesized greater amounts of YopD and LcrH than did the wild-type strain (Fig. 2A). It seems that the phenotype of Δ(*yopD*) and Δ(*yscM1 yscM2*) mutant yersiniae cannot be explained as the altered stability or the increased secretion of class II gene products. It is conceivable, however, that the regulatory role of the *lcrH* gene product involves stabilizing YopD in the cytoplasm of yersiniae.

Previous work showed that the overexpression of LcrQ (YscM1 or YscM2) blocked the expression of *yop* genes as well as the type III transport of *yop* gene products (7, 43). We wondered whether knockout mutations in *yopD* and *lcrH* affected the ability of YscM1 and YscM2 to prevent type III

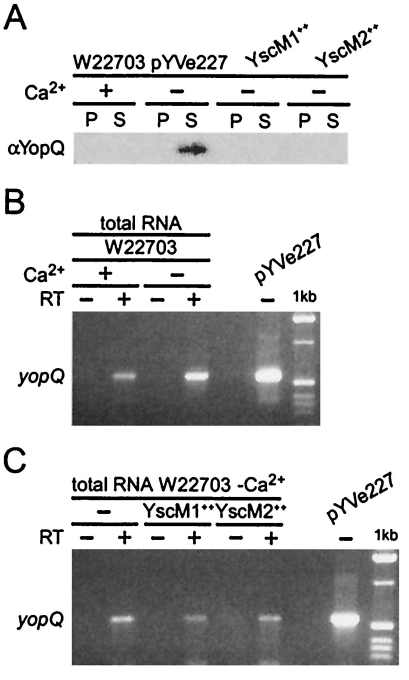

FIG. 4. Transcription of *yopQ* mRNA is not blocked by YscM1 and YscM2 overexpression. (A) *Y. enterocolitica* W22703 (wild type) and strains harboring plasmids pEC345 (encoding YscM1) and pEC348 (encoding YscM2) were analyzed for type III secretion as described in the legend to Fig. 1. Each fraction was analyzed by immunoblotting with antisera raised against purified YopQ. (B and C) Total RNA was isolated from the bacterial sediment (pellet), and RT-PCR analysis of *yopQ* mRNA was performed (+) or reverse transcriptase was omitted from the reaction mixture (−) prior to PCR amplification. The 549-bp *yopQ* ORF was analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized on a UV light table. The size of the amplicon was compared to a molecular size standard (1 kb). Virulence plasmid DNA (pYVe227) was used as a positive control for the PCR amplification step.

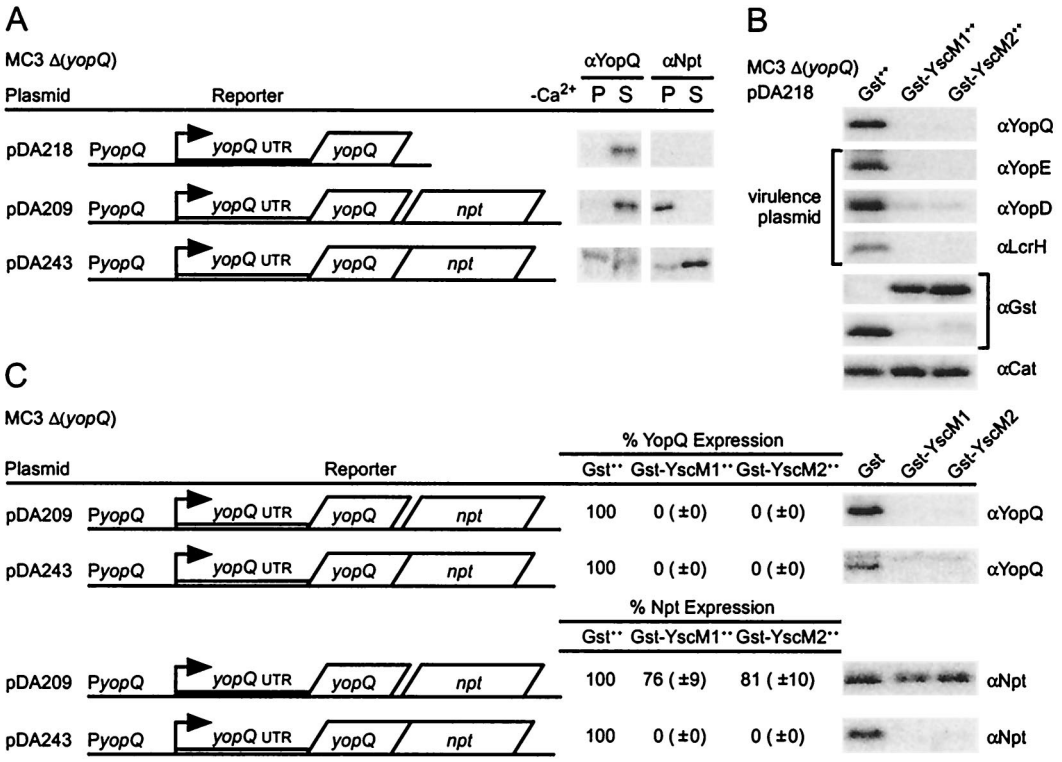

FIG. 5. Gst-YscM1 and Gst-YscM2 block the expression of plasmid-encoded YopQ at a posttranscriptional step. (A) *Y. enterocolitica* MC3 [Δ(*yopQ*)] harboring plasmid pDA218, pDA209, or pDA243 was cultured in $-Ca^{2+}$ and analyzed for synthesis and secretion of YopQ as described in the legend to Fig. 1 with YopQ- and Npt-specific antisera. (B) *Y. enterocolitica* MC3 [Δ(*yopQ*)] harboring pDA218 was transformed with pEC260 (encoding Gst), pEC347 (encoding Gst-YscM1), or pEC350 (encoding Gst-YscM2). The expression of pEC260-, pEC347-, or pEC350-carried genes was induced by the addition of 1 mM IPTG when shifting the temperature of bacterial cultures to induce the type III virulon. Protein precipitated from total cultures was analyzed by immunoblotting for the synthesis of YopQ, Gst, or Cat (plasmid encoded) and YopE, YopD, or LcrH (virulence plasmid encoded) with specific antisera. (C) *Y. enterocolitica* MC3 [Δ(*yopQ*)] harboring plasmid pDA209 or pDA243 was cultured in $-Ca^{2+}$ and analyzed for synthesis of YopQ and Npt. *Y. enterocolitica* MC3 [Δ(*yopQ*)] harboring pDA209 or pDA243 was transformed with pEC260 (encoding Gst), pEC347 (encoding Gst-YscM1), or pEC350 (encoding Gst-YscM2). The expression of pEC260-, pEC347-, or pEC350-carried genes was induced as described for panel B. Quantification of immunoreactive signals is reported as the percent amount of the Npt signal compared to the signal for bacteria overexpressing Gst.

secretion. Overexpression of *yscM1* or *yscM2* in wild-type yersiniae abolished the secretion of YscM1, YscM2, and YopD (Fig. 2B). A block in YopQ secretion could not be assessed, as overexpression of *yscM1* or *yscM2* prevented the expression of *yopQ*. Overexpression of *yscM1* or *yscM2* in Δ(*yopD*) and Δ(*lcrH*) mutant yersiniae affected neither the expression nor the secretion of YscM1, YscM2, and YopQ (Fig. 3C). Thus, *yopD* and *lcrH* are absolutely required for YscM1- and YscM2-mediated repression of *yop* gene expression.

As Δ(*yopD*) and Δ(*lcrH*) mutant yersiniae secreted YscM1 and YscM2, the class II phenotype of these variants may be caused by the depletion of YscM1 and YscM2 from the bacterial cytoplasm. If so, overexpression of Gst-YscM1 or Gst-YscM2, two nonsecretable hybrids that regulate the expression of *yop* genes, should complement the class II phenotype of Δ(*yopD*) and Δ(*lcrH*) mutant strains (7). As expected, Gst-YscM1 and Gst-YscM2 blocked the synthesis of YopQ in wild-type yersiniae (Fig. 3A). Overexpression of Gst-YscM1 and Gst-YscM2 in Δ(*yopD*) mutant yersiniae did not complement the class II phenotype, as YopQ synthesis in the presence of calcium and YopQ secretion in the absence of calcium still occurred (Fig. 3B). This result was consistent with similar studies of LcrQ in Δ(*yopD*) *Y. pestis* (55). A similar result was observed when Gst-YscM1 and Gst-YscM2 were analyzed in Δ(*lcrH*) mutants (Fig. 3B); however, overexpression of Gst-YscM1 and Gst-YscM2 caused the secretion of YopQ in the presence of calcium, a phenotype distinct from the non-*gst*-fused forms of *yscM1* and *yscM2*. In sum, *yopD*, *lcrH*, and *yscM1* or *yscM2* is each required for the control of *yop* gene expression at a unique step. Further, *yscM1* or *yscM2* appears to fulfill overlapping but nonredundant functions and can be inactivated by the binding of the chaperone SycH to YscM1 and YscM2.

Although overexpression of *sycH* (pEC441) increased the secretion of YscM1 and YscM2 in the absence of calcium, it did not induce the secretion of YscM1 and YscM2 in the presence of calcium (7). Overexpression of *sycH* did induce the expression of *yopQ* in the presence of calcium (Fig. 1B), suggesting that the binding of SycH to YscM1 and YscM2 may be sufficient to inactivate the regulatory properties of class II gene products (Fig. 3C). This mechanism of induction is not essential for the regulation of *yopQ* in the presence of calcium, as Δ(*sycH*) mutants express *yopQ* in a manner that is indistinguishable from that of wild-type strains. As a control, overexpression of both *yopD* and *lcrH* did not affect the secretion of YscM1 and YscM2 (Fig. 3C). Surprisingly, the overexpression

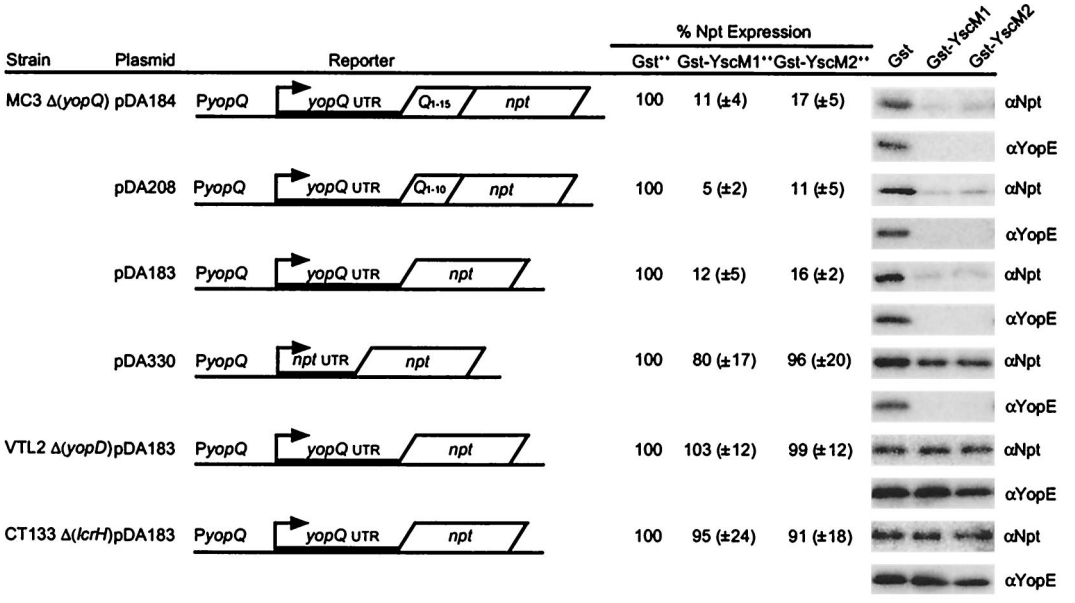

FIG. 6. The 5′ UTR of *yopQ* mRNA is the target of Gst-YscM1- and Gst-YscM2-mediated repression. *Y. enterocolitica* strains MC3 [Δ(*yopQ*)], VTL2 [Δ(*yopD*)], and CT133 [Δ(*lcrH*)] harboring plasmid pDA183, pDA184, pDA208, or pDA330 were cultured in $-Ca^{2+}$ and analyzed for synthesis of YopE and Npt. *Y. enterocolitica* strains were transformed with pEC260 (encoding Gst), pEC347 (encoding Gst-YscM1), or pEC350 (encoding Gst-YscM2). The expression of pEC260-, pEC347-, or pEC350-carried genes was induced by the addition of 1 mM IPTG when shifting the temperature of bacterial cultures to induce the type III virulon. Quantification of immunoreactive signals is reported as the percent amount of the Npt signal compared to the signal for bacteria overexpressing Gst.

of LcrH alone reduced the secretion of YscM1 and YscM2 in the absence of calcium (Fig. 3C). These results are consistent with the assignment of a regulatory role to *lcrH*, independent of YopB and YopD stabilization, that coordinates the regulatory circuit with the type III machinery (21–23).

YscM1 and YscM2 regulate *yopQ* expression at a posttranscriptional step. Wild-type *Y. enterocolitica* W22703 does not express *yopQ* when cultures are grown at elevated temperatures in the presence of calcium. Previous data suggested that LcrQ (YscM1-YscM2) overexpression blocks the expression of various *yop* genes at a transcriptional step (43, 52). We tested whether the *yopQ* mRNA transcript was present in cells grown under these conditions. *Y. enterocolitica* W22703 (wild type) was grown at 37°C in TSB supplemented with either 5 mM calcium chloride ($+Ca^{2+}$) or 5 mM EGTA ($-Ca^{2+}$). *Y. enterocolitica* W22703 (wild type) harboring low-copy-number plasmids expressing *yscM1* (pEC345) or *yscM2* (pEC348) under control of the IPTG-inducible *tac* promoter was grown at 37°C in TSB in the absence of calcium. Cultures were centrifuged, and the extracellular medium was separated (supernatant) from the bacterial sediment (pellet). Protein in both fractions was precipitated with TCA and analyzed by SDS-PAGE and immunoblotting. YopQ was synthesized and secreted in the wild-type strain only in the absence of calcium. Overexpression of *yscM1* or *yscM2* completely blocked the synthesis of *yopQ* in the absence of calcium (Fig. 4A). Total RNA was isolated from the pellet fraction of the cultures, and quantitative RT-PCR analysis was performed on each of the strains. Amplification of the *yopQ* coding sequence demonstrated the presence of *yopQ* mRNA transcript in *Y. enterocolitica* W22703 in both the presence and absence of calcium (Fig. 4B). Overexpression of YscM1 or YscM2 had only a slight effect on the concentration of *yopQ* mRNA transcript (Fig. 4C). pYVe227 virulence plasmid DNA containing *yopQ* was used as a control for PCR amplifications. Together these results suggest that YscM1 and YscM2 block the expression of *yopQ* at a posttranscriptional step.

To analyze the regulatory properties of YscM1 and YscM2, plasmid-carried gene fusions were employed that tethered *yopQ* to neomycin phosphotransferase (*npt*), a gene that is not a member of the *Yersinia* virulence regulon (virulon) or type III pathway and that is therefore not regulated by *yopD*, *lcrH*, *yscM1*, and *yscM2* (2). *Y. enterocolitica* MC3 harbors a knockout mutation of *yopQ* [Δ(*yopQ*)]. Plasmid pDA218 carries the wild-type *yopQ* gene. When pDA218 is transformed into strain MC3, the plasmid restores *yopQ* expression and YopQ secretion (2). pDA209 carries a fusion of *yopQ* to a promoterless *npt* gene. Upon induction of the type III pathway, a single transcript specifying two polypeptides, YopQ and Npt, is generated. Finally, pDA243 carries a translational fusion between *yopQ* and *npt*. The resulting hybrid, YopQ-Npt, is transported by the type III pathway and synthesized in a manner that is subject to regulation by the *Yersinia* virulon (Fig. 5A) (3). Overexpression of *gst-yscM1* and *gst-yscM2*, but not of *gst* alone, abolished the expression of *yopQ* encoded by pDA218 (Fig. 5B). This result indicates that plasmid-expressed *yopQ* is regulated in a manner that is indistinguishable from that of virulence plasmid-carried *yopQ*. Overexpression of *gst-yscM1* and *gst-yscM2* also abolished the expression of *yopQ* carried by pDA209, whereas the expression of *npt*, located at the 3′ end of the pDA209 transcript, was not significantly reduced (Fig. 5C). As a control, the translational hybrid *yopQ-npt* was regulated in a manner resembling that of wild-type *yopQ*. These results suggest that, similarly to LcrH and YopD (2), YscM1

and YscM2 also regulate *yopQ* expression at a posttranscriptional step, as the expression of fused *npt* is coregulated in translational but not in transcriptional hybrids.

YscM1- and YscM2-mediated regulation requires the 5′ UTR of *yop* mRNA. We sought to identify *yopQ* nucleotide sequences that were sufficient to confer *yopD*-, *lcrH*-, *yscM1*-, and *yscM2*-mediated regulation on *npt*. *yopQ* was truncated at its 3′ end, and DNA fragments were fused to *npt* in a manner that allowed translation of *yopQ-npt* hybrids. Plasmids were transformed into *Y. enterocolitica* MC3, and class II-mediated *yopQ-npt* regulation was measured by overexpressing Gst-YscM1 or Gst-YscM2. *yopQ-npt* expression was calibrated by measuring the concentration of YopQ-Npt in *Yersinia* cultures expressing Gst alone (100%). Deletion of *yopQ* codons 16 to 193 diminished the repressive effect of class II genes by a factor of about 5 to 10, as 11 and 17% of $YopQ_{1-15}$-Npt were detected in extracts with overexpressed Gst-YscM1 and Gst-YscM2, respectively (Fig. 6). These values must be compared with 0% of $YopQ_{1-193}$-Npt in *Yersinia* harboring Gst-YscM1 or Gst-YscM2 (Fig. 5C). Further 3′ truncations of *yopQ* coding sequence did not affect class II regulation, as $yopQ_{1-10}$-*npt* and $yopQ_1$-*npt* displayed similar concentrations of YopQ-Npt in *Yersinia* expressing Gst-YscM1 and Gst-YscM2. It should be noted that the $yopQ_{1-10}$-*npt* hybrid (pDA208) contains sufficient information for the polypeptide to be secreted by the type III pathway, whereas the $yopQ_1$-*npt* hybrid (pDA183) does not (data not shown) (3, 46). The 178-nucleotide 5′ *yopQ* UTR (3) appears sufficient to confer class II-mediated repression when appended to transcripts that are not part of the *yop* virulon (pDA183 in Fig. 6). As a control, plasmid-carried *npt* encompassing the *yopQ* promoter and 5′ *npt* UTR (pDA330) (2) was not subject to Gst-YscM1- and Gst-YscM2-mediated regulation (Fig. 6). Deletion of *yopD* or *lcrH* abolished the regulation of 5′ *yopQ* UTR fusions to *npt*, indicating that 5′ *yopQ* UTR fusions repressed reporter synthesis via class II genes.

We wished to determine whether class II-mediated regulation at the 5′ *yopQ* UTR required a *yop* promoter. We observed that the *npt* promoter is constitutively expressed in yersiniae (E. D. Cambronne, unpublished data). The *npt* reporter gene of pEC83 and pEC84 was expressed from the *npt* promoter. pEC84-encoded transcripts, but not pEC83-encoded transcripts, harbor the 5′ *yopQ* UTR (Fig. 7A). The expression of pEC83- and pEC84-carried *npt* in *Y. enterocolitica* strains W22703 (wild type) and EC6 [Δ(*yopD lcrH yscM1 yscM2*)] was measured 0, 60, and 120 min after inoculating cultures containing 5 mM calcium chloride and incubation at 37°C (Fig. 7B). Expression of pEC84-carried *npt*, but not of pEC83-carried *npt*, was decreased in wild-type yersiniae compared to the class II mutant strain EC6. These data indicated that the *yopQ* promoter was not required for posttranscriptional repression of *yop* genes by YopD, LcrH, YscM1, and YscM2 (Fig. 7C).

To test whether the 5′ UTR of other *yop* genes was also the target of class II gene-mediated regulation, the effect of Gst-YscM1 and Gst-YscM2 on the expression of *yopE* was analyzed (Fig. 8A). Gst-YscM1 and Gst-YscM2 abolished the expression of wild-type *yopE*. Plasmid pEC102 carried a fusion of the *npt* reporter gene to the *yopE* promoter. The expression of *npt* was reduced twofold by Gst-YscM1 and Gst-YscM2. However, fusion of the 28-nucleotide 5′ *yopE* UTR (20) to *npt* resulted in a greater-than-fivefold repression of *npt* in the presence of Gst-YscM1 or Gst-YscM2 (Fig. 8A). It therefore appears that the 5′ UTR of *yopE* may also be the target of class II-mediated gene regulation. A comparison of reported 5′ *yop* UTR nucleotide sequences revealed the presence of 5′-AUAAA-3′ in *yopQ*, *yopE*, and *yopH* (3, 6, 20) as well as in positions 5′ to the translational start sites of other *yop* genes (Fig. 8B). This sequence element was present four times in the 5′ *yopQ* UTR and once in the 5′ *yopE* UTR. Furthermore, the 5′ *npt* UTR (47) did not harbor this sequence element. Although the precise function of the 5′-AUAAA-3′ element is still unclear, the data are consistent with YopD, LcrH, YscM1, and YscM2 recognizing a feature of *yop* mRNAs to control gene expression at a posttranscriptional step (Fig. 8B).

Because of the relatively short 28-nucleotide 5′ *yopE* UTR and single-copy 5′-AUAAA-3′, we chose pEC80 as a substrate for mutagenesis that introduced single nucleotide transversion substitutions in the predicted sequence motif, including positions both −1 and +1 relative to the 5′-AUAAA-3′ sequence (Fig. 9A). As the 5′ UTR of *yop* genes is AU rich, cytidyl was chosen to replace adenyl nucleotides and guanyl was chosen to

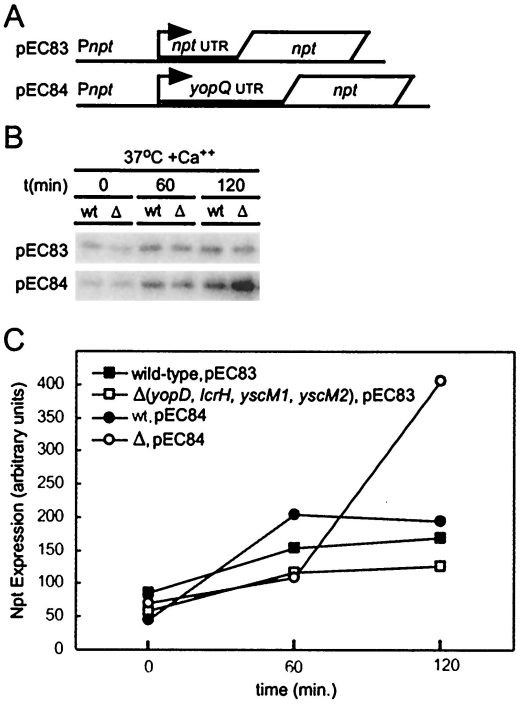

FIG. 7. The *yopQ* promoter is not required for class II-mediated posttranscriptional regulation of *yopQ*. *Y. enterocolitica* strains W22703 (wild type) and EC6 [Δ(*yopD lcrH yscM1 yscM2*)] were transformed with pEC83 or pEC84 (A). Both plasmids encode an *npt* reporter gene under the control of the *npt* promoter and either with or without 5′ *yopQ* UTR sequences. *Y. enterocolitica* strains were cultured in TSB supplemented with 5 mM $CaCl_2$ ($+Ca^{2+}$), and the type III virulon was induced at 37°C for 0, 60, and 120 min. Secretion was quenched by precipitating protein from total cultures followed by immunoblot analysis for the synthesis of Npt with specific antisera. Data generated in panel B were quantified and analyzed for panel C with chemiluminescent signals that were visualized on a Fluorchem 8800 Imaging System (Alpha Innotech). wt, wild type.

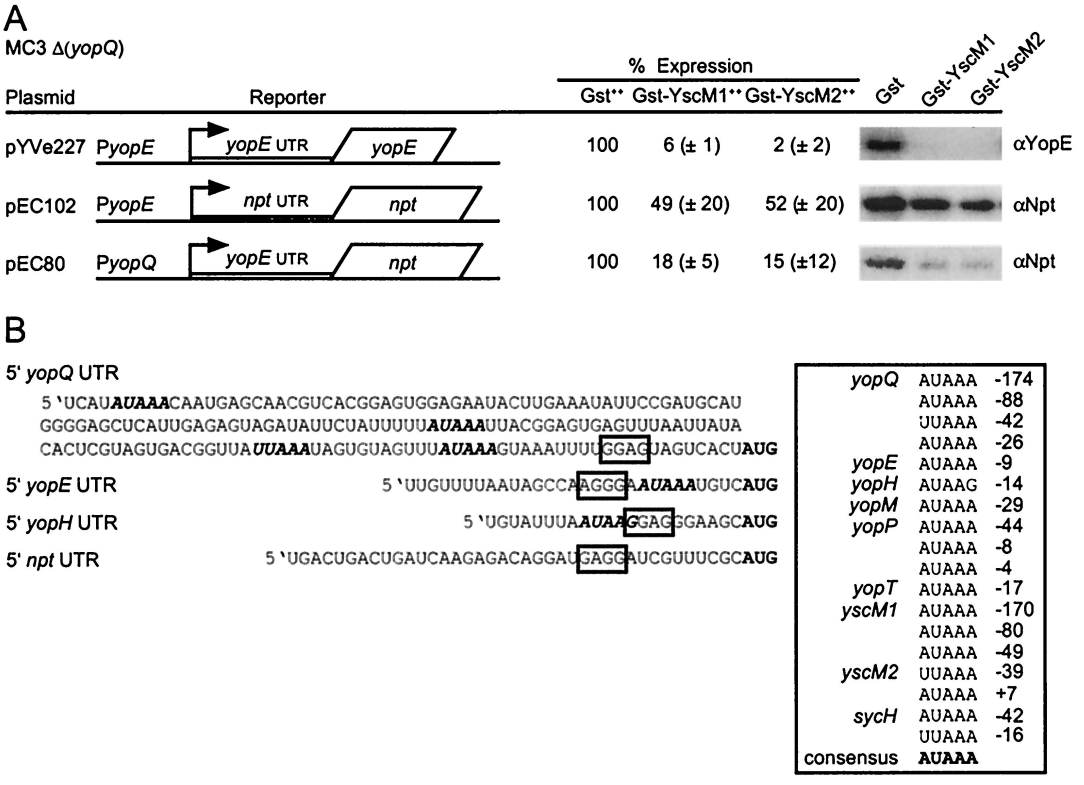

FIG. 8. The 5′ UTR of *yopE* mRNA is the target of Gst-YscM1- and Gst-YscM2-mediated repression. (A) *Y. enterocolitica* strain MC3 [Δ(*yopQ*)] harboring plasmid pEC80 or pEC102 was cultured in $-Ca^{2+}$ and analyzed for synthesis of YopE and Npt. *Y. enterocolitica* strains were transformed with pEC260 (encoding Gst), pEC347 (encoding Gst-YscM1), or pEC350 (encoding Gst-YscM2). The expression of pEC260-, pEC347-, or pEC350-carried genes was induced by the addition of 1 mM IPTG when shifting the temperature of bacterial cultures to induce the type III virulon. Quantification of immunoreactive signals is reported as the percent amount of the Npt signal compared to the signal for bacteria overexpressing Gst. (B) A conserved nucleotide sequence was identified in the 5′ UTR of *yopQ*, *yopE*, *yopH*, *yopM*, *yopP*, *yopT*, *yscM1*, *yscM2*, and *sycH* transcripts. Conserved sequences of individual genetic loci with nucleotide positions (relative to the translational AUG start codon) are indicated in the box with a consensus sequence listed at the bottom. Small boxes represent predicted ribosome binding sites.

replace uridyl nucleotides. Gst-YscM1 and Gst-YscM2 expression in each strain harboring the mutant reporter constructs failed to block expression of the *npt* reporter to the levels of the wild-type sequence control (pEC80). These results indicate that the sequence 5′-AAUAAAU-3′ is necessary for class II gene-mediated regulation (Fig. 9C). Replacement of 5′-AAU AAAU-3′ (pEC80) with 5′-CCGCCCG-3′ (pEC145) abolished expression of the reporter altogether even in the Gst-expressing control strain (Fig. 9A). It appears that the position of the AU-rich sequence between the Shine-Dalgarno binding site for 16S rRNA and the AUG start codon is also essential for gene expression in *Yersinia*.

To examine whether the conserved sequence element is sufficient to impose *yscM1*- and *yscM2*-mediated regulation on unrelated transcripts, 5′-AUAAA-3′ was inserted into the *npt* UTR at −10, i.e., following the Shine-Dalgarno box as in *yopE*, or at −27, i.e., one of the motif locations within the 5′ *yopQ* UTR (Fig. 9). Positional numbers refer to nucleotide positions upstream (−) or downstream (+) of the AUG start codon. Gst-YscM1 and Gst-YscM2 expression in each strain harboring the hybrid UTR constructs was measured as expression of the *npt* reporter. The −10 insertion in pEC148 prevented *npt* expression even in the Gst-expressing control strain, again demonstrating that nucleotide sequence substitutions between the Shine-Dalgarno box and the AUG have dramatic effects on gene expression. The introduction of 5′-AUAAA-3′ at −27 in pEC112 imposed a twofold regulation on the *npt* reporter due to overexpression of Gst-YscM1 or Gst-YscM2 (Fig. 9D). These results suggest that introduction of the 5′-AUAAA-3′ element in the 5′ UTR of *npt* genes can lead to the acquisition of class II gene-mediated regulation.

We asked whether the conserved sequence elements contained in the 5′ *yopQ* UTR are also necessary to confer class II gene-mediated regulation. Successive truncations of *yopQ* UTR sequences in increments of ~30 nucleotides were introduced in both the 5′ and 3′ directions (Fig. 10A). These fragments were fused at their respective 3′ ends to the 5′ end of the 5′ *npt* UTR and *npt* ORF. A 3′-end truncation was also constructed that simply removed the sequence containing the predicted ribosome binding site (−12 to −1) (pEC57). Plasmids were transformed into *Y. enterocolitica* MC3 Δ(*yopQ*) carrying plasmids for the overexpression of *gst-yscM1*, *gst-yscM2*, and *gst*. *npt* expression was calibrated by measuring the concentration of Npt in *Yersinia* cultures expressing Gst alone (100%). Appending the 178-nucleotide 5′ *yopQ* UTR to the *npt* UTR repressed the expression of *npt* by about fourfold (Gst-YscM1 or Gst-YscM2) compared to the Gst control (Fig. 10B). Almost all of the truncations of 5′ *yopQ* UTR remained sufficient

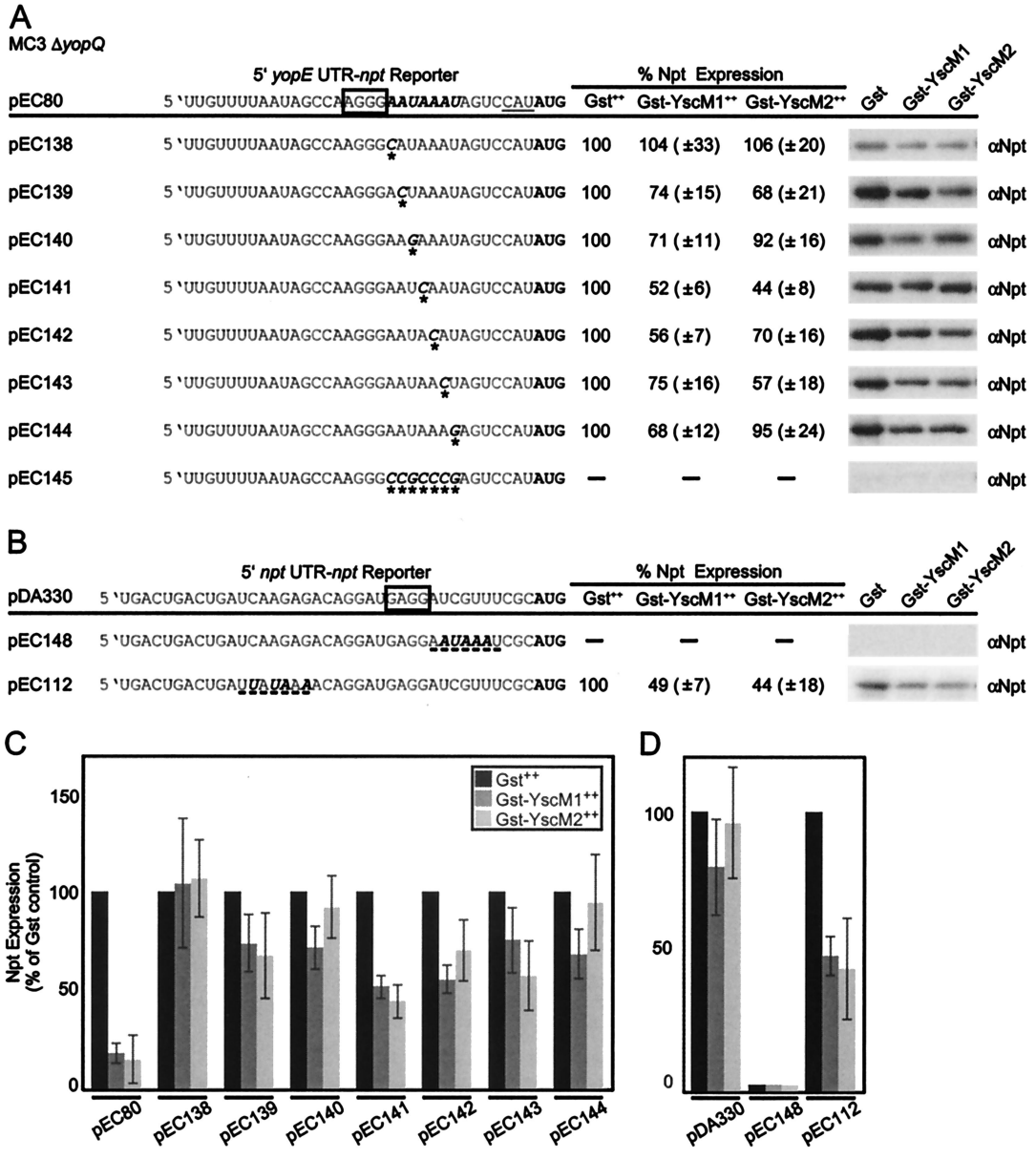

FIG. 9. The sequence 5′-AAUAAAU-3′ in the 5′ UTR of *yopE* mRNA is necessary for Gst-YscM1- and Gst-YscM2-mediated repression. (A and B) *Y. enterocolitica* strain MC3 [Δ(*yopQ*)] harboring plasmids pEC112, pEC138 to pEC145, and pEC148 was cultured in $-Ca^{2+}$ and analyzed for synthesis of Npt. *Y. enterocolitica* strains were transformed with pEC260 (encoding Gst), pEC347 (encoding Gst-YscM1), or pEC350 (encoding Gst-YscM2). The expression of pEC260-, pEC347-, or pEC350-carried genes was induced by the addition of 1 mM IPTG when shifting the temperature of bacterial cultures to induce the type III virulon. Quantification of immunoreactive signals is reported as the percent amount of the Npt signal compared to the signal for bacteria overexpressing Gst. Asterisks indicate nucleotide positions in each construct where transversion substitutions were introduced. Dashed lines indicate sites of positional introduction of conserved sequence in the 5′ *npt* UTR of the 5′ *yopE* UTR (pEC148) and the −28 5′ *yopQ* UTR (pEC112) with substituted nucleotides indicated in boldface italics. Wild-type 5′ *yopE* UTR (pEC80) and 5′ *npt* UTR (pDA330) are included for reference. The underlined sequence CAU in pEC80 represents insertion of nucleotides at the *Nde*I fusion site. Small boxes represent predicted ribosome (16S rRNA) binding sites (Shine-Dalgarno box). (C and D) Graphical representation of the data generated in panels A and B, respectively, with quantified data from pEC80 and pDA330 included for reference. All chemiluminescent signals were visualized, quantified, and analyzed on a Fluorchem 8800 Imaging System (Alpha Innotech).

to cause three- to fourfold regulation of expression of *npt* (Fig. 10B). One exception was construct pEC71 (−120 to −1), which failed to be synthesized even in the control strain expressing Gst, a phenotype that we do not yet understand. Truncating the *yopQ* UTR to 30 nucleotides at the 3′ end reduced repression to a twofold effect. Thus, the presence of multiple copies of 5′-AUAAA-3′ in the *yopQ* UTR has additive effects on the repression of *yop* genes; however, the presence of a single copy of 5′-AUAAA-3′ in the UTR appears sufficient to elicit a twofold YscM1- and YscM2-mediated repression. These observations also explain the differences in *yopQ* and *yopE* expression in the presence of calcium. As *yopE*

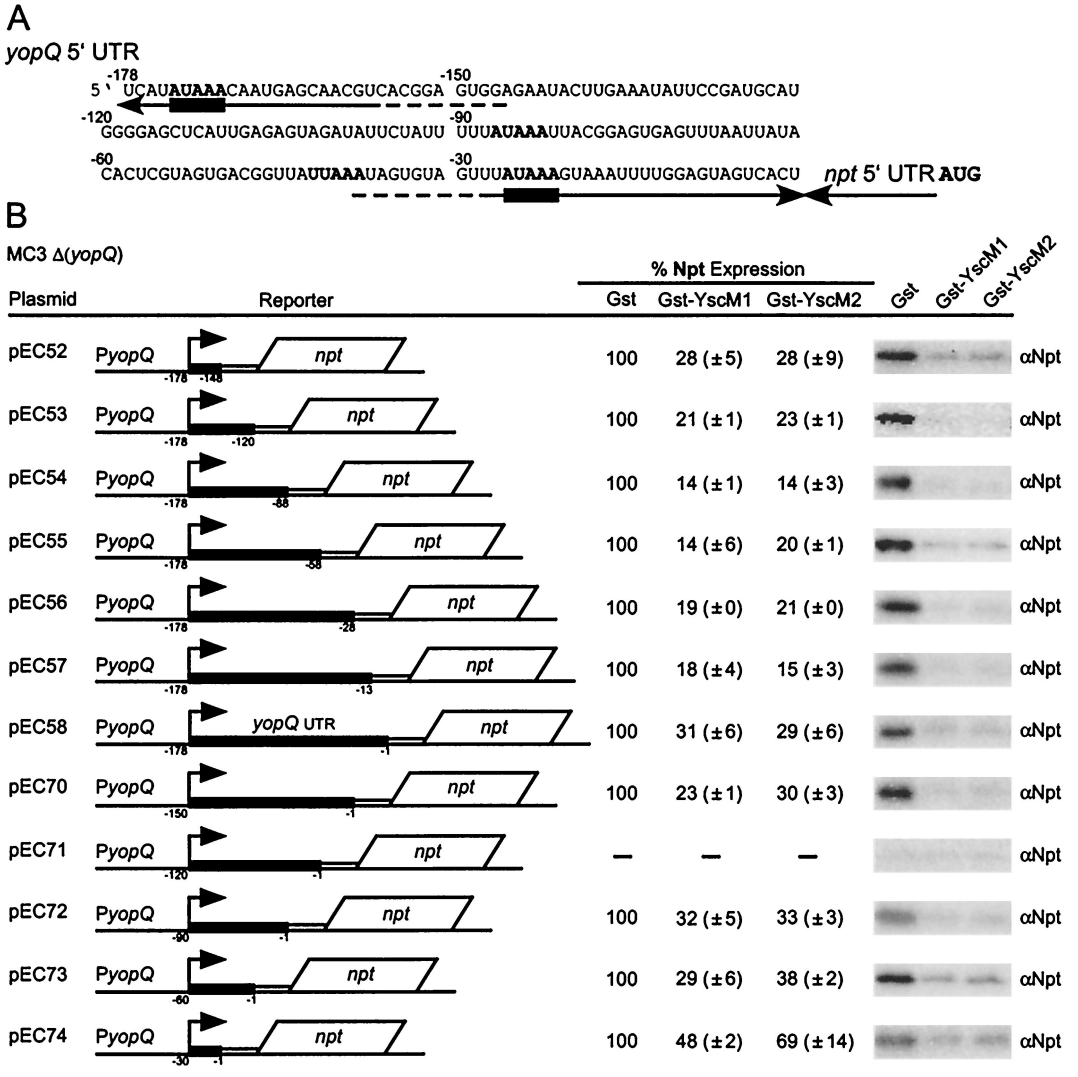

FIG. 10. The 5′-AUAAA-3′ elements of the 5′ UTR of *yopQ* are sufficient to impose Gst-YscM1- and Gst-YscM2-mediated repression when fused to the 5′ UTR of *npt*. (A) Illustration of the truncation strategy of the 5′ *yopQ* UTR. Sequences highlighted in boldface are predicted to be necessary for *yscM1*- and *yscM2*-mediated regulation. Arrows indicate the inclusion of conserved sequence (bold arrow) with truncations in both the 5′ and 3′ directions. Each truncation was appended to the 5′ end of the 5′ *npt* UTR. (B) *Y. enterocolitica* strain MC3 [Δ(*yopQ*)] harboring plasmids pEC52 to pEC58 and pEC70 to pEC74 was cultured in $-Ca^{2+}$ and analyzed for synthesis of Npt. *Y. enterocolitica* strains were transformed with pEC260 (encoding Gst), pEC347 (encoding Gst-YscM1), or pEC350 (encoding Gst-YscM2). The expression of pEC260-, pEC347-, or pEC350-carried genes was induced by the addition of 1 mM IPTG when shifting the temperature of bacterial cultures to induce the type III virulon. Quantification of immunoreactive signals is reported as the percent amount of the Npt signal compared to the signal for bacteria overexpressing Gst.

harbors a single 5′-AUAAA-3′ element in the UTR, the *yopE* gene is much less repressed than *yopQ*, containing at least four copies of 5′-AUAAA-3′.

DISCUSSION

LcrQ (YscM1 and YscM2) has previously been hypothesized to regulate the expression of *yop* genes in *Yersinia* species at the level of transcription (7, 43, 52). This model was based on the following observations. (i) Knockout mutations in *lcrQ* (*yscM1* and *yscM2*) increased the expression of *yop* genes compared to that in wild-type yersiniae (7, 43, 48, 52). (ii) Overexpression of *yscM1* and *yscM2* decreased the concentration of *yop* mRNA compared to that of wild-type yersiniae (48). (iii) Knockout mutations in *lcrQ* (*yscM1* and *yscM2*) increased the expression of reporter genes fused to *yop* genes compared to wild-type yersiniae (7, 43, 52). These experiments used insertions of the *luxAB* gene into *yopE* (18) or the fusion of the chloramphenicol acetyltransferase reporter gene to the 3′ end of *yopH*. Although the reported data were consistent with YscM1 and YscM2 (LcrQ) acting to prevent transcription, one could not discard the possibility of a posttranscriptional regulatory mechanism (43, 52).

Stainier et al. observed that the regulatory effect of *yscM1* and *yscM2* on *yop* expression did not involve type III secretion but required additional genes that were located on the *Yersinia* virulence plasmid (52). Rimpilainen et al. (48) as well as Williams and Straley (55) observed a requirement of *lcrH* and

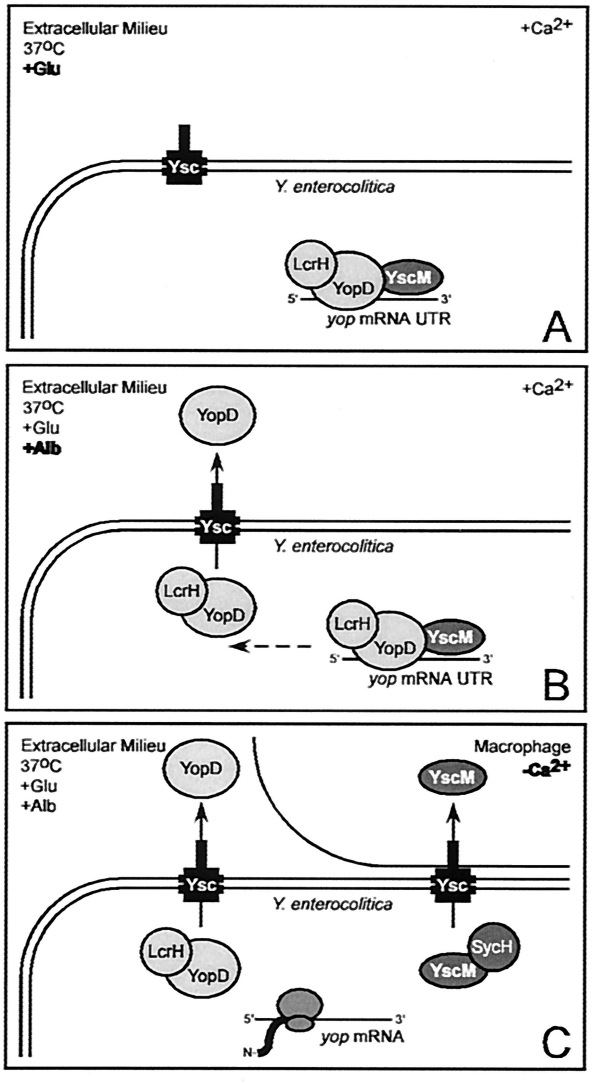

FIG. 11. Model for the regulation of *yop* genes by class II gene products. (A) Upon host entry and exposure to 37°C, glutamate (serum amino acid), and 1.2 mM calcium, YopD, LcrH, YscM1, and YscM2 prevent the translation of *yop* mRNAs by recognizing a feature in the 5′ UTR. (B) Albumin (serum protein) activates the type III secretion of YopD. (C) Insertion of type III needles into the plasma membrane of macrophages triggers a calcium signal that activates the type III targeting of YscM1 and YscM2 via binding to the SycH chaperone. Secretion of YopD and targeting of YscM1 and YscM2 relieve the inhibition of *yop* mRNA translation and activate *Yersinia* type III injection of effector Yops.

yopD for *lcrQ*-mediated regulation. Further evidence for functional cooperation among *yscM1*, *yscM2*, *yopD*, and *lcrH* was provided by the observation that knockout mutations in all four genes could bypass the *Yersinia* requirement for an environmental amino acid signal (glutamate) to induce type III secretion (37). Recently, Anderson et al. reported that *yopD* and *lcrH* regulate the expression of *yopQ* at a posttranscriptional step that involves binding of YopD and LcrH to *yopQ* mRNA (2). It is reported here that *yscM1* and *yscM2* regulated the expression of *yopQ* and *yopE* at the same posttranscriptional step as did *yopD* and *lcrH*. The target of regulation was the 5′ UTR of *yop* mRNAs, which presumably contained a specific binding site for YopD and LcrH. The 5′-AUAAA-3′ sequence represents the target element, as mutations in the conserved sequence motif abolish class II gene-mediated regulation. Further, introduction of a single 5′-AUAAA-3′ into the 5′ *npt* UTR is sufficient to impose at least a twofold repression by the class II-mediated mechanism. Fusions of truncations of the 178-nucleotide 5′ *yopQ* UTR to the 5′ *npt* UTR and *npt* reporter gene, all containing at least one of the four aforementioned conserved sequence elements, are also sufficient for class II gene-mediated regulation. Future work will provide further characterization of this sequence element. Because expression of *yopQ* is more tightly regulated by the class II genes than by other type III substrates, it is likely that the presence of multiple conserved sequence elements in the relatively long 5′ *yopQ* UTR provides for the engagement of several repressor complexes compared to other substrates which are not as tightly regulated. It is also possible that the conserved sequence element found in other parts of the transcript, i.e., the coding region, could contribute to class II gene-mediated regulation. There is evidence of *yopD*-sensitive nucleotide substitution mutations in one of two conserved 5′-AUAAA-3′ sequences in the *yopQ* coding sequence (2, 46). This may explain why repression of the *npt* reporter by YscM1 or YscM2 overexpression is maximal only when *npt* is appended to the full-length coding sequence of *yopQ* (Fig. 5C).

It is yet unclear whether YscM1 and YscM2 are capable of binding *yop* mRNA or whether they contribute to the binding of YopD and LcrH. Alternatively, YscM1- and YscM2-mediated control of *yop* gene expression could be indirect. Wulff-Strobel et al. proposed a model whereby LcrQ may regulate the activity of the type III secretion machinery (56). The observations in Fig. 2 are consistent with this model. It is, however, also plausible that class II genes control the expression of a regulatory factor for the type III pathway in addition to regulating *yop* genes, as YscM1 or YscM2 overexpression lowers the expression of other factors in the *yop* virulon (7, 52). Our data are consistent with the notion that binding of YopD and LcrH to *yop* mRNA alone is not sufficient to prevent Yop synthesis but requires at least one additional factor, YscM1 or YscM2. Further, YscM1 and YscM2 can be viewed as negative regulators of *yop* expression while SycH functions as an activator.

We believe that the simplest model for the data presented here and elsewhere is the formation of a protein complex composed of YopD, LcrH, YscM1, and YscM2 on *yop* mRNA. The assembly of the complex likely prevents translation of the transcript and may even target mRNAs for degradation (2). Multiple events contribute to relieving the posttranscriptional repression of the *yop* virulon. Binding of SycH to YscM1 and YscM2 certainly suffices to induce *yop* expression. However, depletion of YopD, YscM1, and YscM2 from the bacterial cytoplasm via the type III transport is another possible mechanism of regulation. We reason that the assembly of a YopD, LcrH, YscM1, and YscM2 repressor complex may represent the setting of a switch. The switch may be set once *Yersinia* enters the host and the temperature is raised to 37°C. Flipping the switch, i.e., causing a large increase in *yop* expression, requires the environmental signals glutamate (amino acid), albumin (serum protein), and calcium. Three regulatory re-

sponse mechanisms seem coupled to these signals: (i) the secretion of YopD in the presence of calcium and albumin; (ii) the binding of SycH to YscM1 and YscM2 in the presence of the glutamate, albumin, and calcium signals; and (iii) the targeting of YscM1 and YscM2 into the cytoplasm of tissue culture cells. Available evidence suggests that all three mechanisms are reversible and perhaps not essential to the flipping of the switch. Other regulatory mechanisms could play a role. For example, temporal posttranslational modification of any one class II gene product, YopD, LcrH, or YscM1 and YscM2, could inactivate the entire complex, a possibility that will need to be addressed in future studies. The drawing in Fig. 11 summarizes our present knowledge and views of the events that regulate *yop* expression by the class II gene products YopD, LcrH, YscM1, and YscM2.

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