Identification of a Locus Involved in Systemic Dissemination of *Yersinia enterocolitica*

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A putative LysR-type transcriptional activator, Hre20, was identified previously in an in vivo expression technology screen designed to identify factors which are expressed early during infection by *Yersinia enterocolitica* (G. M. Young and V. L. Miller, Mol. Microbiol. 25:319–328, 1997). An insertion in *hre20*, now designated *rscR*, resulted in increased splenic dissemination of bacteria during infection in a BALB/c mouse model. A nonpolar mutation was generated in *rscR*, and examination of this strain in the BALB/c mouse model demonstrated that the mutation in *rscR* was responsible for the increased dissemination to the spleen that was seen in the original experiments. RscR is homologous to the LysR family of transcriptional regulators; thus, a screen was undertaken to identify genes regulated by RscR. A strain containing an insertion in the chromosomal *rscR* gene and carrying *rscR* on a plasmid under the control of the inducible *araBAD* promoter was mutagenized with an mTn5Km-2 transposon containing a promoterless *lacZY*. Eighteen insertions were identified which appeared to respond to levels of RscR, and these were classified into four allelic groups based on Southern blot hybridization analysis. Representative members were sequenced from three allelic groups. Sequencing revealed insertions in an ORF with no known homologues, a homologue of OmpF of *Serratia marcescens*, and a locus (designated *rscBAC*) with similarity to the *hmwABC* locus of *Haemophilus influenzae*. The *hmwABC* locus promotes adherence of *H. influenzae* to host cells (S. J. Barenkamp and J. W. St. Geme III, Infect. Immun. 62:3320–3328, 1994; J. W. St. Geme III, S. Falkow, and S. J. Barenkamp, Proc. Natl. Acad. Sci. USA 90:2875–2879, 1993). A strain containing a deletion mutant of *rscA*, the *hmwA* homologue, exhibits increased splenic dissemination of bacteria during infection in a BALB/c mouse model, similar to the *rscR* mutant. This suggests that the phenotype of an *rscR* mutant is due to the loss of RscA.

Yersinia enterocolitica infects a wide range of animal hosts, including humans, and is transmitted through ingestion of contaminated food and water (4). In humans, the infection generally leads to an acute gastroenteritis which is self-limiting and manifests as fever, diarrhea, and abdominal pain. However, in patients who are iron overloaded or immunocompromised, a systemic infection, which frequently is fatal, may ensue. *Y. enterocolitica* is a lymphotropic organism. After ingestion, the bacteria are able to cross the intestinal epithelium by invasion of specialized M cells and colonize the underlying lymphoid follicles, called Peyer's patches (PP), that line the small intestine (1). From the PP, the bacteria can progress to the mesenteric lymph nodes (MLN) and eventually establish a systemic infection (5).

A large number of virulence factors reside on a well-characterized 70-kb virulence plasmid (7). Several of these genes encode a type III secretion system that exports factors, also encoded on the virulence plasmid, into host cells which disrupt cellular function. Other factors are chromosomally encoded, such as invasin, which has been shown to bind to β_1 integrins on the surface of M cells and in *Y. enterocolitica* is important in the initial steps of invasion (13, 21, 38). Previously, an in vivo

expression technology (IVET) screen was performed in order to identify additional chromosomally encoded factors which are expressed early during infection (36). In that study, random fragments of *Y. enterocolitica* DNA were transcriptionally fused to a promoterless *cat* gene and integrated onto the chromosome of wild-type *Y. enterocolitica*. Fusion of *cat* to an active promoter confers to the bacterium resistance to chloramphenicol (CHL). Strains containing *cat* fusions were used to orally infect BALB/c mice. *Y. enterocolitica* strains in which the promoter upstream of *cat* was transcriptionally active during infection (i.e., in vivo) were enriched for by administration of CHL to the mice. Fusions that were enriched in the animal were then screened for the absence of expression under standard laboratory conditions. One of the genes discovered by that screen was designated *hre20*. Hre20 was found by BLAST analysis to be 67% identical to YeiE from *Escherichia coli*, which is a hypothetical protein with homology to the LysR family of transcriptional regulators. An insertion in *hre20* resulted in increased splenic dissemination of bacteria during infection in a BALB/c mouse model, indicating a role for the *hre20* locus during infection. Based on the observed in vivo phenotype, *hre20* was designated *rscR* for reduced splenic colonization regulator.

Due to the apparent role in the progression of *Y. enterocolitica* infection, *rscR* was further characterized in the present study. Since RscR is proposed to be a transcriptional regulator, the genes which it regulates are likely to encode the effectors of the observed in vivo phenotype. A screen was undertaken to identify genes regulated by RscR in order to further understand the effect that these factors have on the course of infection.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Y. enterocolitica strains		
JB580v	r⁻m⁺ Nal^r	15
YVM557	*rscR::lacZYA* Cm^r	This study
YVM617	*rscR*::Ω(Sm/Sp)	This study
YVM690	Δ*rscR*	This study
YVM776	Δ*rscA*	This study
YVM777	*rscA*::Ω(Sm/Sp)	This study
YVM796	*rscA::lacZYA* Cm^r	This study
YVM797	*rscA::lacZYA* Δ*rscR* Cm^r	This study
YVM641	*rovA::erm*	8a
YVM885	*rscA::lacZYA rovA::erm* Cm^r	This study
Plasmids		
pBAD18	Inducible promoter vector; Kan^r	10
pBAD33	Inducible promoter vector; Cm^r	10
pEP185.2	Suicide vector	15
pFUSE	Suicide vector; *lacZYA*	3
pGY31	1.1-kb *Cla*I-*Eco*RI *rscR* in pWKS30; Amp^r	This study
pHG329	Cloning vector, medium copy number; Amp^r	29
pIVET8	Suicide vector; *lacZY*	18
pKN7	*rscR* in pFUSE; Cm^r	This study
pKN10.2	Δ*rscR* in pEP185.2; Cm^r	This study
pKN11	*rscR*::Ω(Sm/Sp) in pEP185.2; Cm^r Sm^r Sp^r	This study
pKN14	P$_{BAD}$−*rscR* Cm^r	This study
pKN15	P$_{BAD}$−*rscR* Kan^r	This study
pKN16	Δ*rscA* in pEP185.2 Cm^r	This study
pKN17	*rscA*::Ω(Sm/Sp) in pEP185.2; Cm^r Sm^r Sp^r	This study
pKN21	*rscA* in pFUSE; Cm^r	This study
pRev10	mTn5Km2-*lacZY*; Amp^r Kan^r	This study
pTn*Max2*	Tn*Max22* Erm^r	11
pUTmTn5Km2	mTn5Km2, Amp^r Kan^r	8
pWKS30	Cloning vector, low copy number; Amp^r	34

MATERIALS AND METHODS

Growth conditions. All cultures were grown in Luria-Bertani (LB) broth unless otherwise noted. *E. coli* was grown at 37°C and *Y. enterocolitica* was grown at 26°C, with aeration on a roller drum. The following antibiotics were used at the indicated concentrations: ampicillin, 100 μg/ml; CHL, 12.5 μg/ml; kanamycin (KAN), 100 μg/ml; nalidixic acid (NAL), 20 μg/ml; streptomycin, 50 μg/ml; and spectinomycin, 50 μg/ml. As a chromogenic substrate for β-galactosidase, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 40 μg/ml. For regulation of the P$_{BAD}$ promoter, arabinose and glucose were used at 0.2% in LB.

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. A designation of "v" indicates the presence of the 70-kb virulence plasmid. Electroporations were performed as described previously (25). Restriction enzymes and DNA ligase were purchased from New England Biolabs. PCR was carried out using recombinant PFU (Stratagene). Sequencing was performed with BigDye Terminator (Amersham) and reactions were processed by the Protein and Nucleic Acid Chemistry Lab at Washington University.

Chromosomal *lacZYA* fusions to *rscR* and *rscA* were generated as follows. Cloned fragments or PCR products were subcloned into the vector pFUSE upstream of *lacZYA*. In the case of the *rscR* fusion, a 564-bp PCR fragment was amplified from the chromosome utilizing primers hre20-F1 (5′ GCTCTAGAG CGGTAACAGTCAGGATGTGAT 3′) and hre20-R1 (5′ CAATACGCTTTG CCGAAAACCACC 3′). hre20-F1 creates a cleavage site at the end for *Xba*I, and hre20-R1 leaves a blunt end. The product was digested with *Xba*I and cloned into the *Xba*I-*Sma*I site of pFUSE, and this construct was designated pKN7. For the *rscA* fusion, the 21B2 mutant clone (see "Cloning and sequencing" below)

was digested with *Xba*I/*Stu*I and cloned into the *Xba*I-*Sma*I site of pFUSE to generate plasmid pKN21. Replication of the pFUSE vector is *pir* dependent, and thus it cannot replicate in *Y. enterocolitica* which does not contain *pir*. The pFUSE suicide vector carrying these fragments was conjugated into the desired strain by mixing 500 μl of 16- to 18-h cultures of the donor and recipient strains and plating onto LB agar. After growth for 16 to 20 h at 26°C, the bacteria were collected from the plate and dilutions were plated onto LB containing NAL, to select for *Y. enterocolitica*, and CHL, to select for plasmid integration onto the chromosome. Integration was confirmed by Southern blot hybridization (data not shown).

The *rscR*::Ω(Sm/Sp) strain, YVM617, used in the screen was constructed as follows. A plasmid, pGY31 (pWKS30 plus *Cla*I-*Eco*RI *hre20*), was utilized which carried a partial sequence of *rscR*, from the upstream *Cla*I site to the *Eco*RI site within the gene. The Ω(Sm/Sp) cassette from pSmuC was cloned into the *Hin*dIII site of the *rscR* gene in pGY31. The pSmuC plasmid was provided by Hao Shen and was constructed by cloning the Ω(Sm/Sp) cassette from pRU875 into pMTL24 and then cloning the cassette into pUC1129 (32). The *rscR*-Ω(Sm/Sp) fragment was isolated by digestion with *Cla*I/*Sac*II and was ligated into these sites of the suicide vector pEP185.2 to generate the construct pKN11. This vector was conjugated as described above into strain JB580v. A double-crossover event to completely replace the wild-type copy of *rscR* was identified by selecting for the Ω(Sm/Sp) cassette and not CHL resistance, which would have selected for integration of the whole plasmid. Clones were then tested for sensitivity to CHL. The crossover was confirmed by Southern blot hybridization (data not shown). The plasmid pKN14 (see below) was electroporated into this strain.

The strains YVM690 (Δ*rscR*) and YVM776 (Δ*rscA*) were generated by the same strategy. Flanking regions to the deletion were amplified. The primers used to construct Δ*rscR* were as follows: IFD1 (5′ GGGAACAAAAGCTGGGTA CCG 3′) and IFD2 (5′ GGAAGATCTGCTGGCAGCAATGCGTAGTG 3′) to amplify the upstream region and IFD3 (5′ GGAAGATCTGAGCTTGGGAAT TCTGAGGC 3′) and IFD4 (5′ GCTCTAGAGGTCAACGGCGATAGTCACC 3′) to amplify the downstream region. The primers used to construct Δ*rscA* were as follows: del1 (5′ CCATCGATGGCTTACGGTATTGGCGAAG 3′) and del2 (5′ GGAAGATCTGCCATTCACCGGCAATGAAG 3′) to amplify the upstream region and del3 (5′ GGAAGATCTGTAGCGGGGTGGATATTGGC 3′) and del4 (5′ GCTCTAGACCTGAGGCTGCGTTATCTGC 3′) to amplify the downstream region. These fragments were cloned sequentially into pEP185.2, with a *Bgl*II linker joining the fragments, to create pKN10.2 and pKN16, and the deletions were confirmed by sequence analysis. These plasmids were conjugated into JB580v as described above. Each plasmid was integrated by a single-crossover event at the locus of interest, and this was confirmed by Southern blot hybridization (data not shown). Cycloserine enrichment was utilized to resolve the merodiploid (19). Southern blot hybridization then allowed identification of clones retaining the deleted copy of the gene. Additionally, the Ω(Sm/Sp) cassette was cloned into the *Bgl*II site of pKN16 (Δ*rscA*/pEP185.2), and this new construct, designated pKN17, was crossed onto the chromosome of JB580v by selection for the Ω(Sm/Sp) cassette. This strain was confirmed by Southern blot hybridization and was designated YVM777 [Δ*rscA*::Ω(Sm/Sp)] (data not shown).

The inducible *rscR* construct, pKN14, was generated by amplifying the *rscR* open reading frame (ORF), including the ribosome binding site but excluding the putative promoter region. Primers hreR F2 (5′ CGGGATCCGATGATAG CGTCCTCCATTCT 3′) and hreR R2 (5′ GCTCTAGACACCAATTCAGGG AAGAAGG 3′) amplified a 934-bp product with an *Xba*I cleavage site at the 3′ end of the ORF. This was digested with *Xba*I and cloned into the *Sma*I-*Xba*I sites of pBAD33 to generate pKN14. The correct construct was confirmed by sequencing. The fragment was moved from pKN14 into the *Sac*I-*Pst*I sites of pBAD18 to generate pKN15. This was used for some experiments in order to have an inducible construct with Kan^r rather than Cm^r.

The transposon delivery plasmid, pRev10, was constructed as follows: the *Sph*I fragment of pIVET8, carrying *lacZY*, was cloned into the *Sph*I site of pUTmTn5-Km2. This was the delivery plasmid utilized in the mutagenesis to identify *rscR* regulated genes.

Animal experiments. Six- to seven-week-old BALB/c mice were used. Oral infections were performed using a 1-ml syringe with 2 in. of intramedic tubing encompassing a 21-guage needle. Bacteria were grown 16 to 18 h at 26°C and then resuspended in sterile phosphate-buffered saline to the desired concentration. Bacterial suspension (200 μl) was administered. On the indicated day postinfection, the tissues of interest were harvested and homogenized in sterile phosphate-buffered saline. The homogenates were diluted and plated to determine viable cell counts. *Y. enterocolitica* was selected for using NAL. Bacterial load was reported as CFU per gram of tissue. The significance of the results was assessed using the Mann-Whitney test.

Mutagenesis. The strain YVM618 was mutagenized by delivery of mTn5Km2-*lacZY* from pRev10. Conjugation was carried out by filter mating of YVM617 [*rscR*::Ω(Sm/Sp)] carrying pKN14 and *E. coli* S17-1λpir containing pRev10. An aliquot (100 μl) of a 16- to 18-h culture of each strain was added to 3 ml of sterile 10 mM $MgSO_4$ and was pushed through a 0.45-μm-pore-size filter. The filter was placed on minimal medium without glucose to prevent outgrowth and was incubated overnight at 26°C. Filters were vortexed in 1 ml of $MgSO_4$, and 100 μl of a 1:4 dilution was plated on LB containing NAL (to select against the *E. coli* donor strain), KAN (to select for transposition), and CHL (to select for pKN14). Plates were incubated at 26°C for 30 h, and then each was replica plated to two plates—one containing NAL, KAN, CHL, X-Gal, and 0.2% arabinose and one containing NAL, KAN, CHL, X-Gal, and 0.2% glucose. Colonies were compared visually, with inspection for differences in color after growth on arabinose compared to glucose. Colonies displaying potential differential β-galactosidase expression were purified, and enzyme assays were performed.

Enzyme assays. β-Galactosidase assays were performed as previously described (19). Cultures (1 ml) were grown for 16 to 18 h at 26°C in LB containing appropriate antibiotics and either 0.2% arabinose or 0.2% glucose as described above for the plate assays. In initial screening, single assays were performed with each mutant, and those displaying differential expression were then repeated and assays were performed in duplicate. Fusions selected for further characterization displayed fivefold or more induction in arabinose or glucose in the repeated assays.

Screen controls. To identify fusions to genes encoding products involved in arabinose utilization, bacteria containing fusions were patched onto minimal medium containing arabinose as the only carbon source. To identify fusions responding to glucose or arabinose rather than the presence or absence of RscR, the *rscR* expression plasmid, pKN14, was cured by culturing without selection for 3 days. These cultures were diluted and plated on LB agar containing no antibiotics. After 2 days of growth, colonies were patched to two plates, one containing KAN and one containing CHL. Clones which retained Kan[r] and lost Cm[r] were selected. If both resistances were lost, this indicated a transposon insertion on the plasmid. Selected plasmid-cured fusions were analyzed as above for differential β-galactosidase levels in arabinose and glucose. Fusions displaying induction after the loss of the plasmid were removed from further consideration as it was likely that the differential expression observed was due to the carbon source rather than the presence or absence of RscR.

Cloning and sequencing. Chromosome-transposon junctions were cloned as follows. Total genomic DNA preparations of the mutant strains were digested with restriction enzymes known to have a unique restriction site between the KAN resistance gene and the *lacZY* genes of the transposon. Using the KAN resistance gene as a probe, Southern hybridization analysis was used to determine which fragment contained the fusion. After identification of potential fragments for cloning, the genomic DNA was digested with the selected enzyme and separated on an agarose gel. The approximately sized fragments, as determined from the Southern blot, were excised from the gel and the DNA was purified by Gene-Clean. These fragments were then ligated into the cloning vector pHG329 which had been linearized with the same enzyme. After the clones were obtained, these were sequenced using a primer, P6, designed from the transposon end which allows sequencing through the junction and into the disrupted gene (12).

To obtain additional sequence for the *rsc* locus, a clone was obtained containing the wild-type locus from an existing cosmid library described previously (37), and the clone containing the junction of the 21B2 fusion contained 12 kb of the locus, beginning within the *rscB* gene, was utilized. Subcloning and Tn*Max* mutagenesis, as described previously (11), allowed compilation of the entire sequence of the region. The sequence of both strands of the DNA was determined.

Nucleotide sequence accession numbers. The sequences for *rscR* and *rscBAC* may be found under accession numbers AF394928 and AF394927, respectively.

RESULTS

The *rscR* mutation is responsible for increased dissemination to the spleen. The nucleotide sequence of *rscR*, formerly *hre20*, and the surrounding DNA was determined (Fig. 1). The predicted amino acid sequence of RscR is 68% identical to the hypothetical protein YeiE and 24% identical to LysR of *E. coli*. Upstream of *rscR* is a divergently transcribed ORF with 55% identity to YeiH, the hypothetical protein encoded upstream of *yeiE* in *E. coli*. YeiH has no homology to any other known
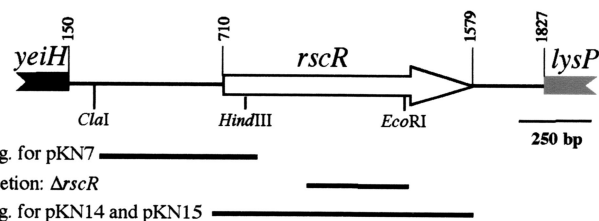

FIG. 1. Schematic of the *rscR* locus showing the gene arrangement. DNA fragments and restriction sites are indicated for relevant constructs. Only partial gene length is shown for the *yeiH* and *lysP* homologues. Numbering begins at left from position 0. The fragments (Frag.) subcloned to generate pKN7, pKN14, and pKN15 are shown. In addition the region deleted to generate the in-frame Δ*rscR* mutation is indicated.

proteins in the database. The potential translational start site of the *yeiH* homologue is 560 bp from the *rscR* initiation codon. Downstream of *rscR* is an ORF with 80% identity to LysP of *E. coli*, which is also located downstream of *yeiE* in *E. coli*. The translational start site of the LysP homologue is 244 bp from the stop codon of *rscR*.

The original *rscR* mutant contained an insertion which could have exerted polar effects on the *lysP* homologue. To test if an insertion in *rscR* affects transcription of the downstream *lysP* homologue, a Φ(*lysP-lacZYA*) fusion was constructed. This fusion was integrated into the parental strain (JB580v), a strain containing an in-frame deletion of *rscR* [Δ*rscR* (YVM690)] and an *rscR*::Ω(Sm/Sp) [YVM617] strain background (mutant construction described below). Comparisons of β-galactosidase activity from Φ(*lysP-lacZYA*) in the three strain backgrounds demonstrated a modest polar effect of an insertional mutation on the transcriptional levels of the *lysP* homologue. The *rscR*::Ω (Sm/Sp) strain had 50% less β-galactosidase activity than the wild type and the Δ*rscR* mutant (data not shown). These assays demonstrated that an insertion in *rscR*, *rscR*::Ω(Sm/Sp), did affect *lysP* transcription, while the in-frame deletion of *rscR*, Δ*rscR*, did not exert any polar effects.

To determine if the increased dissemination to the spleen of the originally reported *rscR* mutant was a result of the mutation in *rscR* rather than a result of polar effects on downstream genes, the animal infection studies were repeated using the *rscR* in-frame deletion strain, Δ*rscR*. To create Δ*rscR*, an *rscR* mutant containing a 360-bp deletion was exchanged for the wild-type *rscR* by homologous recombination (see Materials and Methods). Mice were inoculated orally with 5×10^7 CFU of either JB580v or the Δ*rscR* mutant. Mice were sacrificed on days 3, 5, and 7 postinfection. Viable cell counts of bacteria recovered from the PP, MLN, and spleen were determined. This experiment was performed twice and demonstrated increased dissemination to the spleen by the strain containing the Δ*rscR* mutation on day 5 postinfection. This result is similar to what was previously observed for the original insertional mutant (data not shown). Therefore, the *rscR* mutation, not the polar effect on downstream transcription, was responsible for the increased dissemination to the spleen.

Screening for *rscR*-regulated genes. *rscR* has been demonstrated to play a role in the course of infection by *Y. enterocolitica*. Since RscR has homology to the LysR family of transcriptional regulators, it was presumed that RscR does not
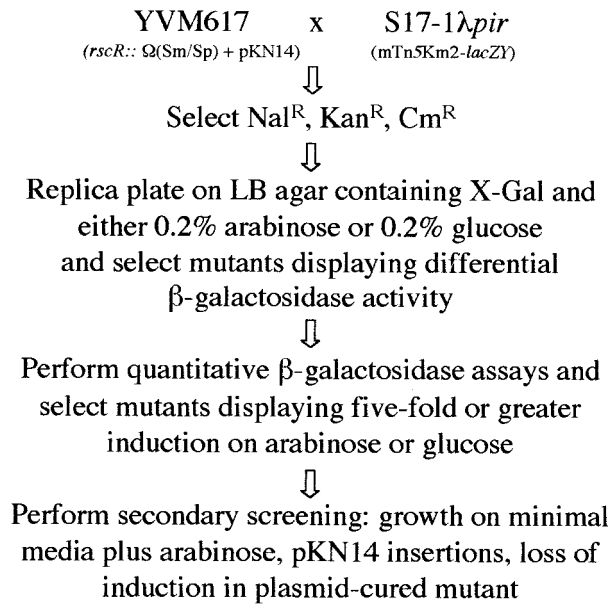
YVM617 x S17-1λ*pir*

(rscR:: Ω(Sm/Sp) + pKN14)   (mTn5Km2-*lacZY*)

⇩

Select Nal^R, Kan^R, Cm^R

⇩

Replica plate on LB agar containing X-Gal and
either 0.2% arabinose or 0.2% glucose
and select mutants displaying differential
β-galactosidase activity

⇩

Perform quantitative β-galactosidase assays and
select mutants displaying five-fold or greater
induction on arabinose or glucose

⇩

Perform secondary screening: growth on minimal
media plus arabinose, pKN14 insertions, loss of
induction in plasmid-cured mutant

FIG. 2. Flowchart depicting the screen for RscR-regulated genes.

have a direct effect on the course of infection but instead may regulate one or more genes involved in pathogenesis. Therefore, it was of interest to determine what genes are regulated by RscR, because these would be expected to be the effectors of the virulence phenotype. The screen for RscR-regulated genes utilized transposon mutagenesis (Fig. 2). The transposon used was an mTn*5* derivative containing a KAN resistance gene and a promoterless *lacZY* on the plasmid designated pRev10. Transposition into a given gene in the correct orientation will generate a transcriptional fusion to *lacZY*.

Fusions to genes which are regulated by RscR would be expected to display differential β-galactosidase activity in response to different levels of RscR. Conditions permitting control of the levels of RscR needed to be identified. Additionally, it was of interest to characterize the expression patterns of *rscR* since under standard in vitro growth conditions (LB, 26°C) *rscR* was not expressed. Various in vitro conditions were examined using β-galactosidase assays in order to identify those which induced expression of Φ(*rscR::lacZYA*) and thus could be utilized in the screen for RscR-regulated genes. The conditions tested were the following: temperature (26 and 37°C), O_2 levels (high aeration in a flask, low aeration in a screwcap tube), pH {20 mM morpholineethanesulfonic acid (MES), pH 5.5; 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), pH 6.5; 20 mM *N*-tris[hydroxymethyl]methyl-3-amino-propanesulfonic acid (TAPS), pH 8.0}, RPMI, minimal medium, iron-depleted LB (100 μM dipyridyl), calcium-depleted LB (20 mM $MgCl_2$, 20 mM Na oxalate), and growth phase (2-h time points for 10 h, beginning at an optical density at 600 nm of 0.05). None of the conditions tested affected activity from the *rscR* reporter more than twofold, and therefore none were suitable to screen for RscR-regulated genes (data not shown). To control levels of RscR, plasmid pKN14, which carried the *rscR* gene under control of the *araBAD* promoter (P_{BAD}), was constructed (see Materials and Meth-

ods). This construct allowed for the identification of genes whose transcription was affected by RscR.

The strain that was mutagenized was *rscR*::Ω(Sm/Sp) (YVM617), which contains an Ω(Sm/Sp) cassette disruption in the *Hin*dIII site of the *rscR* gene. *rscR*::Ω(Sm/Sp) was created by allelic exchange utilizing selection for the resistance marker (see Materials and Methods). pKN14 was electroporated into *rscR*::Ω(Sm/Sp) and allows differential expression of *rscR*; growth on arabinose induces expression, and growth on glucose represses expression. pRev10 was mated into the strain *rscR*::Ω(Sm/Sp) containing the plasmid pKN14, and transposon insertions were selected using KAN. Kan^r colonies were then replica plated to plates containing X-Gal and either arabinose or glucose. Approximately 40,000 colonies from 38 independent matings were examined for a difference in color on arabinose or glucose, to identify fusions to genes whose expression responds to the presence or absence of RscR. Approximately 250 mutants displaying differential expression of *lacZY* on plates containing arabinose or glucose were then analyzed by β-galactosidase assays. Thirty-two mutants demonstrated an induction of fivefold or more on either arabinose or glucose and were examined further.

Several control assays were performed on these 32 mutants to identify fusions that were differentially expressed due to factors other than RscR levels. Mutants were tested for ability to grow on minimal medium supplemented with arabinose in order to identify genes whose products are involved in arabinose utilization. Two mutants were unable to grow on minimal medium supplemented with arabinose. Additionally, mutants were cultured without selection in order to cure the pKN14 plasmid and then tested for induction by arabinose or glucose. This allowed identification of genes responding to the presence of arabinose or glucose and not to RscR; three mutants fell into this group. Curing the plasmid also allowed identification of transposition events in which the transposon inserted into the P_{BAD} region of pKN14, which resulted in *lacZY* expression that was controlled by the P_{BAD} promoter. Nine mutants were identified which contained plasmid insertions. After these control assays, 18 mutants remained which appeared to respond to levels of RscR.

Identification and sequencing of RscR-regulated genes. Southern blot analysis indicated that the eighteen mutants belong to four allelic groups (Fig. 3). Southern blotting was performed using the KAN resistance gene as a probe. Allelic groups were determined on the basis of the same size fragments containing the KAN resistance gene for at least two different restriction enzyme digests (data not shown). To identify the potential RscR-regulated genes, transposon-chromosome junctions were cloned. Sequence data were obtained by using a primer that annealed to sequences at the transposon end. This allowed the DNA sequence of the cloned junction to be determined. The sequence was then analyzed for potential ORFs and used to search available databases for sequence homology. Other than the mutant 39A5, representative sequence data were obtained for each allelic group.

The chromosome-transposon junction of mutant 24A2 was cloned and sequenced. An ORF was present but displayed no significant homology to any proteins in the database. No apparent motifs were present to indicate function. Sequence collected from the 3I6, 23A3, and 23A4 allelic group indicates
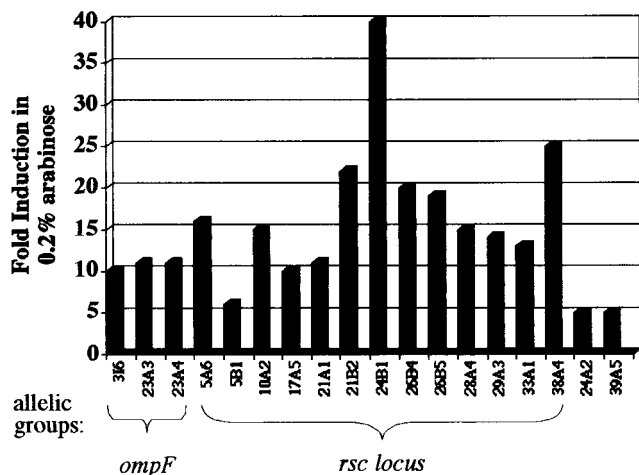
FIG. 3. Chart displaying levels of induction of putative RscR-regulated fusions. Fold induction represents β-galactosidase activity in 0.2% arabinose divided by β-galactosidase activity in 0.2% glucose. The allelic groups are represented by braces, and identified loci are indicated. Representative assays are shown. Assays were performed three times on at least two different days.

FIG. 4. Comparison of the *H. influenzae hmwABC* locus and the *Y. enterocolitica rscBAC* locus. Nucleotides are numbered starting from the putative translational start sites of *hmwA* and *rscB*. The fragment utilized for creation of Φ(*rscA::lacZYA*) and the fragment deleted in Δ*rscA* are indicated.

insertions in an *ompF* homologue most closely related to *ompF* from *Serratia marcescens*. The partial DNA sequence obtained from the clones was 71% identical and 84% similar to the *S. marcescens* OmpF. This allelic group retained threefold induction after curing of the plasmid but was still included because the original induction in the presence of RscR was 10-fold or greater. β-Galactosidase assays were performed comparing activity without NaCl and with 0.3 M NaCl. Expression was 10-fold higher without NaCl, as would be expected from a fusion to an *ompF* homologue (data not shown) (23). These insertions have not been studied further.

The remaining 13 mutants contained insertions in a homologue of *hxuB* from *Haemophilus influenzae*. The chromosome-transposon junctions were cloned from mutants 5A6, 21B2, and 38A4. Utilizing an internal fragment of the *hxuB* homologue as a probe, the remaining insertions were confirmed by Southern blot analysis to be disrupted at the same locus. Additionally, localization of each fusion to an approximately 3-kb *Xba*I fragment indicated that all insertions were in the *hxuB* homologue within this locus. Sequence data were compiled which encompassed 1.5 kb upstream and 8.5 kb downstream of the 1.8-kb *hxuB* homologue. This revealed two additional ORFs downstream of the *hxuB* homologue which appeared to be in an operon with this gene. The products of these ORFs were homologous to HmwA and HmwC of *H. influenzae*. These have been designated as follows: the HxuB homologue, RscB; the HmwA homologue, RscA; and the HmwC homologue, RscC (Fig. 4). In *H. influenzae*, the locus contains *hmwABC* in that order. The first ORF, *rscB*, in the *Y. enterocolitica* locus is 31% identical to *hxuB*, which is a homologue of *hmwB* of *H. influenzae*. HmwB and HxuB of *H. influenzae* are 23% identical and belong to a family of transporters which function to secrete an effector across the outer membrane (30). ShlB of *S. marcescens* is a well-characterized member of the family that includes HmwB and HxuB and functions to activate and secrete the ShlA hemolysin across the outer membrane
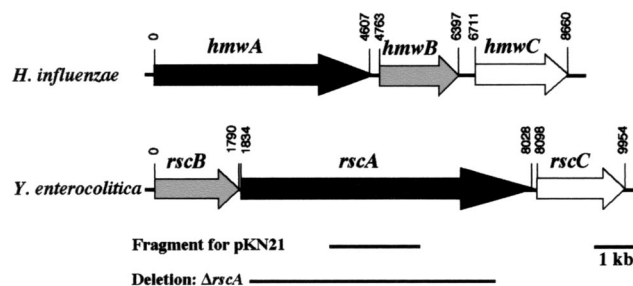
(27). HxuB is proposed to secrete the heme-hemopexin binding protein HxuA (6), and HmwB has been demonstrated to secrete the adhesin HmwA across the outer membrane of nontypeable *H. influenzae* (30). RscA is 24% identical to HmwA, and although *rscA* is 1,587 bp greater in length than *hmwA*, the homology extends across the entire *rscA* ORF. The role of HmwC is not clear, but it is essential for proper processing and secretion of HmwA (30). RscC is 41% identical to HmwC and is the first described homologue of HmwC.

Expression of the *rsc* locus. The 13 insertions in the *rscBAC* locus all map to the *hxuB* homologue, *rscB*, although the gene arrangement suggests an operon structure in which insertions could have been isolated from each of the three ORFs. To begin to address the question of why transposon insertions were not isolated from *rscA* and *rscC*, a chromosomal *lacZYA* fusion to *rscA*, the putative effector, was generated by integration of a suicide plasmid, pFUSE, carrying a fragment of *rscA*. Φ(*rscA::lacZYA*) was generated in JB580v, Δ*rscR*, and *rovA::erm* (YVM641), to create strains YVM796, YVM797, and YVM885, respectively. RovA regulates the expression of *inv* and is expected to regulate other genes in *Y. enterocolitica* (24). Levels of expression from the *rscA* promoter as determined by monitoring β-galactosidase activity from Φ(*rscA::lacZYA*) from these three strains were equivalent (141 ± 2, 140 ± 3, and 141 ± 2 Miller units, respectively [results are given as means $\pm$ standard deviations]). To recreate the conditions of the screen, plasmid pKN15 containing an arabinose inducible *rscR* was moved into the Δ*rscR* mutant containing Φ(*rscA::lacZYA*). When *rscR* was induced with 0.2% arabinose the β-galactosidase activity from Φ(*rscA::lacZYA*) was 318 ± 31 Miller units. When *rscR* expression was repressed by 0.2% glucose, the β-galactosidase activity from Φ(*rscA::lacZYA*) was 84 ± 2 Miller units. In the presence of RscR, *rscA* was induced fourfold, which is an induction level that would have been excluded in the original screen. As a control, plasmid pBAD18 was also moved into the Δ*rscR* mutant containing Φ(*rscA::lacZYA*). No induction was observed with the control plasmid; β-galactosidase activity from Φ(*rscA::lacZYA*) was 84 ± 3 Miller units in arabinose and 100 ± 3 Miller units in glucose. This suggests that transposon insertions in *rscA* or *rscC* may not have displayed sufficient differential expression to be selected in the screen, and this could explain why none were identified.
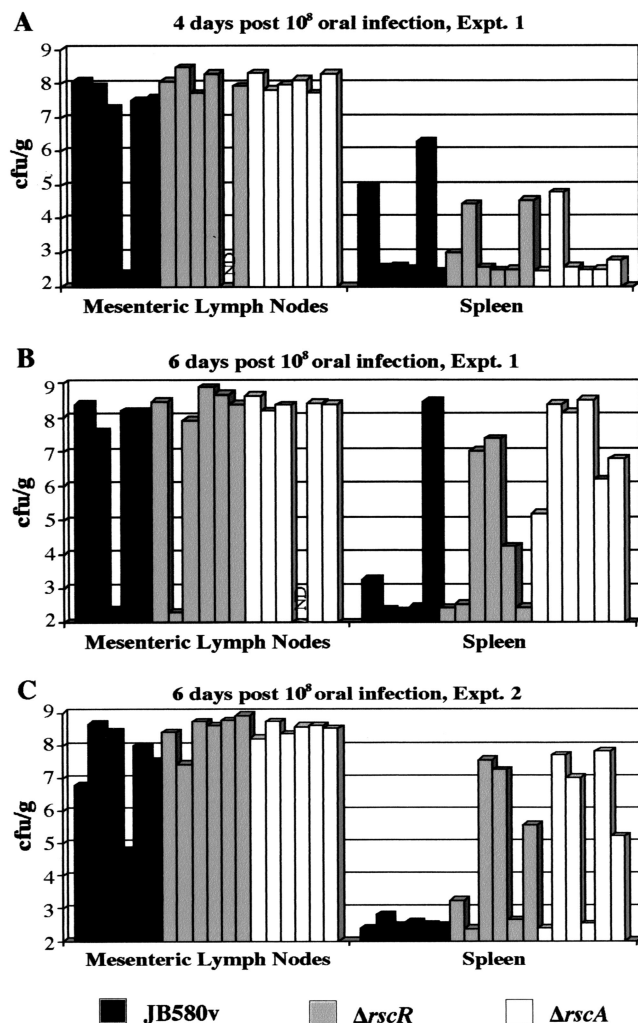
A   4 days post 10^8 oral infection, Expt. 1

B   6 days post 10^8 oral infection, Expt. 1

C   6 days post 10^8 oral infection, Expt. 2

■ JB580v   ▨ ΔrscR   □ ΔrscA

FIG. 5. Kinetics of infection of wild type, Δ*rscR*, and Δ*rscA*. Viable cell counts recovered from the MLN and spleen are indicated (log scale). Each bar represents data from one animal. ND, no data. (A) Viable cell counts recovered from mice at day 4 postinfection. (B) Viable cell counts recovered from mice at day 6 postinfection. Results are from the same experiment as shown in panel A. (C) Viable cell counts recovered from mice at day 6 postinfection. These results are from a separate experiment from that shown in panels A and B.

Analysis of an *rscA* mutant. In order to determine if the *rscBAC* locus encodes the factor responsible for the increased splenic colonization observed for an *rscR* mutant, a deletion was made in the *rscA* gene, designated Δ*rscA* (YVM776). This gene was chosen because by analogy to *H. influenzae* RscA would be the predicted effector and RscB and RscC would be accessory proteins. The ORF of *rscA* is 6,195 bp, and the deletion removes 5,131 bp to create the Δ*rscA* mutant (see Materials and Methods). BALB/c mice were infected orally with 10^8 CFU of the Δ*rscA* mutant, the Δ*rscR* mutant, or JB580v; the Δ*rscR* strain and JB580v were tested in parallel for comparison. For each strain, 12 mice were infected. On days 4 and 6 postinfection, six mice per strain were sacrificed, and the MLN and spleen were harvested and viable cell counts were assessed for each tissue. On day 4, mice infected with the

parental strain looked similar to mice infected with the Δ*rscA* mutant or the Δ*rscR* mutant (Fig. 5A). By day 6, more mice infected with either the Δ*rscA* mutant or the Δ*rscR* mutant had a high bacterial load in the spleen compared to mice infected with the parental strain (Fig. 5B). A second experiment again demonstrated increased bacterial dissemination to the spleen of mice infected with the Δ*rscA* mutant or the Δ*rscR* mutant compared to JB580v at day 6 postinfection (Fig. 5C). These results were significantly different compared to those from wild-type-infected mice, with P values of 0.008 and 0.03 for Δ*rscA* and Δ*rscR*, respectively. In both experiments, it appeared that the *rscA* mutation caused a more severe phenotype than the *rscR* mutation ($P = 0.06$). These results demonstrate that a mutation in *rscA* mimicked the phenotype observed when mice were infected with the *rscR* mutant and potentially a mutation in *rscA* causes an even more severe phenotype than a mutation in *rscR*.

DISCUSSION

A previous study by Young and Miller examined the effect on infection of an insertion in *rscR* (formerly called *hre20*) and found that the mutant spread more rapidly to the liver and spleen of infected mice than did the parental strain (36). Sequencing of the region surrounding *rscR* showed a gene arrangement that is similar to the organization of *yeiE* and *lysP* in *E. coli*, with a homologue of *lysP* downstream of *rscR*. In this study, we demonstrated that an in-frame deletion in the *rscR* gene also results in increased dissemination of bacteria to the spleen after oral infection of BALB/c mice. This suggests that the phenotype of the previously described *rscR* mutant was not a result of polar effects on the downstream gene. This was confirmed by comparing the kinetics of infection for mice infected with wild-type bacteria to mice infected with a mutant containing the nonpolar Δ*rscR* allele.

After establishing that RscR plays a role in the course of infection, we wanted to identify genes that were regulated by RscR, because RscR is predicted to be a LysR-type transcriptional regulator. Members of the LysR family of transcriptional regulators typically utilize a coinducer for optimal function (26). Hence, it was of interest to determine in vitro conditions which could produce optimal levels of RscR and would more likely be permissive for a coinducer to be present if one is required. In vitro conditions which allowed expression of *rscR* from its own promoter were not identified, and the presence or absence of a necessary coinducer can only be speculated. This made it necessary to artificially induce expression of *rscR* from a P_{BAD} promoter for the purpose of screening for genes regulated by RscR. A strain containing an inducible *rscR* was mutagenized with the mTn5Km2-*lacZY* transposon. However, because RscR levels may not be physiological or a necessary coinducer may not be present, some genes regulated by RscR may have been missed in the screen and the level of regulation observed is not likely to be representative of true in vivo values.

Several RscR-regulated fusions were identified in the screen and placed into four allelic groups based on Southern blot analysis. The 39A5 mutant was not cloned or sequenced. The second group also contained only one fusion, 24A2, and although this junction was cloned and sequenced, the sequence did not display significant similarity to any known proteins in

the databases. The third group contained three fusions to a gene exhibiting homology to *ompF*. These fusions demonstrated differential expression in response to RscR levels (≥10-fold induction in arabinose) but also displayed differential expression to arabinose and glucose in the absence of RscR (threefold induction in arabinose). Studies in other systems have shown *ompF* to be regulated by many factors, including the carbon source (23). The fourth group of 13 insertions was located in a 12-kb region of the chromosome. This region was sequenced, and three ORFs were identified which were designated *rscBAC* (for reduced splenic colonization).

Although indirect, the observed in vivo phenotype of the Δ*rscA* mutant indicates that RscR regulates expression of *rscBAC* in the host environment. If the presence of RscR is necessary for proper expression of the *rscBAC* locus, then one would predict that a mutation in the regulator would have an effect on infection similar to that of a deletion of one of the genes in the locus. A comparison of the kinetics of infection of the parental strain, the Δ*rscR* mutant, and the Δ*rscA* mutant demonstrated that for both mutants there were increased numbers of mice with bacterial colonization of the spleen, with the Δ*rscA* mutant colonizing the largest number of spleens by day 6 postinfection. The phenotype of a Δ*rscA* mutant appears slightly more severe than that of a Δ*rscR* mutant, which could be explained if there is a basal level of transcription of the *rscBAC* locus in the absence of RscR. If this were the case, then a Δ*rscR* mutant would be producing a low level of RscA during infection and this could prevent the phenotype from being as severe as a Δ*rscA* mutant which expresses no functional RscA. These data along with the reporter fusion studies suggest that RscR, directly or indirectly, activates *rscA* expression.

The *rscBAC* locus is most similar to the *hmwABC* locus of *H. influenzae*. HmwA is a surface-expressed protein which promotes adherence to various epithelial cell lines in vitro and has been demonstrated to be expressed in clinical isolates from patients with acute otitis media (17). HmwA is expressed as a 150-kDa protein which contains a nontraditional signal sequence of 68 amino acids (9). These 68 amino acids are cleaved after secretion across the inner membrane by the general secretory pathway. A second cleavage event takes place after amino acid 441, concurrent with secretion across the outer membrane, to produce the mature 125-kDa protein, though this processing has been shown not to be essential for adherence. HmwB is essential for surface expression and the second cleavage of HmwA (30). Mutations in *hmwB* lead to the presence of unprocessed HmwA and probable degradation in the periplasm. Mutations in *hmwC* result in reduced levels of HmwA, and the protein is present in the unprocessed form. Null mutations in both *hmwB* or *hmwC* result in loss of adherence.

RscA has a putative nontraditional signal sequence which contains a number of charged residues in the first 47 amino acids followed by a typical signal sequence with a predicted cleavage site after residue 69. This is the same distribution of residues present in the HmwA 68-amino-acid signal sequence (9). Additionally, the second cleavage event of HmwA at amino acid 441 contains WLLDP, and this motif is present in RscA at residues 460 to 464, with predicted cleavage after residue 463. Another motif, NPNGI, is present in the amino terminus of HmwA (residues 150 to 154) as well as three related proteins: ShlA of *S. marcescens*, HpmA of *Proteus mirabilis*, and HhdA of *Haemophilus ducreyi* (20, 27, 30, 33). This family of proteins is grouped together based upon the mechanism of processing and secretion. Each protein has an associated outer membrane protein that functions in processing the protein to its mature form and in transport across the outer membrane. The NPNGI sequence is suggested to be a point of contact with the outer membrane transporter, which is identified as HmwB for HmwA (14, 28). The NPNGI motif is also present in RscA at residues 154 to 158, suggesting that RscA is a new member of this family of proteins. The *hmwABC* locus is unique in that it encodes a third functional protein, HmwC, which does not have homologues in the other related loci (2). RscC is the first identified homologue of HmwC, and this could suggest that HmwABC and RscBAC are functionally similar. RscA has the highest similarity to the adhesin HmwA, and the similarity spans the entire protein. However, RscA does have significant homology to other outer membrane and some secreted proteins with diverse functions and activities. For example, RscA is similar to a filamentous hemagglutinin-like protein of *H. ducreyi* which is suspected to be involved in forming lesions in the rabbit model of infection (35). RscA is also similar to the ShdA protein of *Salmonella enterica*, which is involved in fecal shedding of the bacteria and believed to be important in the spread of the bacteria among livestock and domestic fowl populations (16).

Adhesins have been shown to be important in the pathogenesis of numerous bacteria. Initial colonization of a pathogen at a particular site often requires a surface molecule which promotes adherence to the host cells. From these experiments it does not appear that RscA would function in initial colonization. Kinetic analysis of the Δ*rscR* and Δ*rscA* did not demonstrate a defect in the colonization of the PP, the initial site of a *Y. enterocolitica* infection (data not shown). In contrast, a mutation in *inv*, the gene encoding the surface protein invasin, results in greatly decreased colonization of the PP at early time points postinfection (21). Adhesins can function in later steps of infection as well. The YadA adhesin of *Y. enterocolitica* has been demonstrated to be important in the persistence of bacterial colonization in the PP (22). It is possible that RscA could function as an adhesin for a particular cell type during infection, and loss of adherence in an *rscA* mutant results in the altered kinetics seen in this study. Future experiments will examine the ability of the *rscBAC* locus to promote adherence to different cell types in vitro.

This study demonstrated that the RscR protein affects the normal progression of disease in the BALB/c mouse model of *Y. enterocolitica* infection. The *rscBAC* locus was identified in a transposon mutagenesis screen to be regulated by RscR, and a deletion in the *rscA* gene results in an alteration of the normal infection kinetics of *Y. enterocolitica* similar to that of an *rscR* mutant. The rapid dissemination to the spleen of the *rscR* and *rscA* mutants is an unusual phenotype. This alteration of infection kinetics could impact the virulence of *Y. enterocolitica*. Further study should elucidate more specifically the role that the *rscBAC* locus and related loci in other pathogenic bacteria play during infection.

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