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Identification and Characterization of a *Salmonella typhimurium* Oxygen-Regulated Gene Required for Bacterial Internalization

BRADLEY D. JONES[1]* AND STANLEY FALKOW[1,2]

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305-5402,[1] and Microscopy Branch, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Montana 59840[2]

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Growth of *Salmonella typhimurium* in a low-oxygen environment induces the ability of these bacteria to enter mammalian cells. We have carried out a search for invasion genes that are expressed under low-oxygen conditions by using Tn*5lacZY* transcriptional fusions. Several noninvasive oxygen-regulated *lacZY* insertion strains have been identified. The invasion defect in one of these noninvasive *S. typhimurium* strains, BJ66, has been complemented by introduction of a cosmid (pBDJ125) from an *S. typhimurium* SL1344 gene bank. A 1.9-kb *Eco*RV DNA fragment subcloned from this cosmid, containing a single open reading frame (*orgA*), restores the ability of BJ66 to invade mammalian cells. Comparative searches of the GenBank and EMBL sequence data banks with the nucleotide sequence of the gene and deduced amino acid sequence of the protein reveal no significant similarities. Interestingly, hybridization of an *orgA* gene probe with a P22 chromosomal mapping library demonstrated that the *orgA* gene maps to a region on the chromosome between 57.5 and 60 min where other *Salmonella* invasion genes have been mapped. Other enteroinvasive bacteria (*Shigella flexneri*, *Escherichia coli*, *Yersinia* spp., and *Listeria monocytogenes*) lack sequences which cross hybridize to the probe. We have compared the virulence of *S. typhimurium* SL1344 and an isogenic *orgA* mutant in a mouse model of typhoid fever. The *orgA* mutant was as virulent as the wild-type strain was when inoculated intraperitoneally but is significantly reduced (>60-fold) in its ability to cause disease by an oral route of infection.

Salmonella species are enteric pathogens which must cross the mucosa of the small bowel to initiate disease (37). The passage of these bacteria through mammalian cell membranes occurs via a process which induces dramatic rearrangements of the host cell cytoskeleton (10–12, 14, 16, 20, 22, 46). Actin polymerization is known to be an integral component of this internalization process, since cytochalasins B and D, inhibitors of microfilament formation, block entry (9, 11, 15, 23, 24). Additionally, two groups have shown that bacterial uptake stimulates cellular calcium fluxes as part of the entry mechanism (16, 38) and a recent study has been published with data which preliminarily identifies components of the cellular signalling pathway (34).

Research efforts by several different laboratories have concentrated on identifying and characterizing *Salmonella* genes required for bacterial invasion. Elsinghorst et al. (7) have identified genes in *Salmonella typhi* located at 58 min on the chromosome which confer a low level of invasion on *Escherichia coli* HB101. Galán and Curtiss have found a separate group of four genes at 59 min on the chromosome that complement the invasion defect of a laboratory strain of *Salmonella typhimurium* (13). Subsequent work has expanded the number of *inv* genes known to be present in this region of the chromosome (1, 16). Recently, Groisman and Ochman (17) reported the presence of several more genes within this *inv* complex which share homology with the *mxi/spa* genes of *Shigella* species. The *Shigella mxi/spa* genes are believed to be involved in the presentation of invasion proteins. These investigators showed that the *spa24* gene from *Shigella flexneri* can restore the invasiveness of an *S. typhimurium spaP* mutant to wild-type levels.

We and others have shown that the ability of *Salmonella* organisms to enter mammalian cells is regulated by the concentration of oxygen in the growth medium such that high oxygen represses and low oxygen induces invasiveness (8, 27, 40). A third invasion locus, *hil*, has been identified by searching for mutants which are invasive even when grown in high-oxygen growth conditions (28). A *phoP*-repressed gene, *prgH*, which has been shown to be regulated by the *hil* locus maps within this same locus (2). We now report the identification of a gene which is activated by the same conditions which induce expression of the invasive phenotype and is essential for invasion and virulence of *S. typhimurium*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. An *S. typhimurium* strain with a deletion in the *oxrA* gene was constructed for Tn*5lacZY* transposon mutagenesis experiments. The *oxrA* gene encodes a transcription factor which activates 14 genes in low-oxygen conditions (42, 45). Therefore, to reduce the number of oxygen-regulated fusions obtained which are not part of the *Salmonella* invasion pathway, our experiments were carried out in an Δ*oxrA* strain background. It has been previously demonstrated that isogenic *oxrA*$^+$ and *oxrA* *S. typhimurium* strains are equally invasive for tissue culture cells (27). An *oxrA2* derivative of *S. typhimurium* EE251, designated BJ36, was made by transducing EE251 to tetracycline resistance with a P22HT Int$^-$ lysate prepared on *S. typhimurium* TN2336, which carries Tn*10* within the *oxrA* gene. Since imprecise excision of Tn*10* occurs at a frequency 10^2 to 10^5 higher than that of precise excision (3, 25), strain BJ36 was streaked on Bochner selection medium (3) to select for a tetracycline-sensitive *oxrA2* deletion of BJ36. The tetracycline-

* Corresponding author. Present address: Department of Microbiology, University of Iowa College of Medicine, Iowa City, IA 52242-1109.

TABLE 1. Bacterial strains

S. typhimurium strain	Genotype	Source
BJ36	Same as EE251 but with *oxrA2*::Tn*10*	This study
BJ37	Same as BJ36 but with Δ*oxrA2*	This study
BJ42	Same as BJ37 but with *orgA*::Tn*5lacZY*	This study
BJ62	Same as BJ37 but with Tn*5lacZY* (Lac$^-$)	This study
BJ63	Same as BJ37 but with Tn*5lacZY* (Lac$^+$)	This study
BJ66	Same as SL1344 but with *orgA*::Tn*5lacZY*	This study
EE251	*rpsL* isolate of SL4012	C. Lee (28)
EE421	Same as SL1344 but with *hil*::Tn*5*B50-380	C. Lee (28)
EE451	Same as SL1344 but with Δ*hil*	C. Lee (28)
SL1344	*hisG rpsL xyl*	B. Stocker (48)
TN1909	*pepT*::*lac zda-888*::Tn*10*	C. Miller (45)
TN2336	*oxrA2*::Tn*10* from *S. typhimurium* TN2063	C. Miller (45)

sensitive isolate, designated BJ37, was shown to be OxrA$^-$ by genetic backcrosses and confirmed to have normal levels of tissue culture invasiveness. The genotypes of other relevant strains are listed in Table 1.

S. typhimurium strains to be assayed for β-galactosidase activity or invasiveness were grown in conditions defined as high or low oxygen (21). Briefly, cultures were begun from a stationary-phase culture which had been grown overnight at 37°C on a roller drum (New Brunswick Scientific, New Brunswick, N.J.; 80 rpm). High-oxygen cultures were obtained by inoculating 3 ml of Luria-Bertani (LB) broth with 6 μl of a stationary-phase culture and growing at 37°C on the roller drum to early exponential phase (~10^8 CFU per ml). Low-oxygen cultures were started from 6 μl of a stationary-phase culture and grown statically at 37°C to a density of 4×10^8 to 5×10^8 CFU per ml.

Transposon mutagenesis of *S. typhimurium*. Transposon Tn*5*B21 is a tetracycline-resistant derivative of Tn*5* which was constructed to make *lacZ* fusions (41). A derivative of this transposon, which was used to make *lacZY* promoter fusions, was constructed in the following manner. Transposon Tn*5*B21 was moved by transposition from λ to the plasmid pRTP1 (44) in the nonsuppressing *E. coli* MC4100. Plasmid pRTP1 is an ampicillin-resistant ColE1 derivative which carries a streptomycin-sensitive allele for the ribosomal protein S12. The presence of the S12 gene on the vector can be used to positively select (in a strain carrying the *rpsL* allele) for loss of the plasmid. The resulting plasmid pRTP1::Tn*5*B21 (pBD J103) retained Amp^r and Str^s. Plasmid pBDJ103 was partially cut with *Eco*RI, ligated with an *Eco*RI fragment carrying the intact *lacY* gene, and transformed into *E. coli* DH5α. Plasmids were isolated from individual transformants and a plasmid, designated pBDJ108, which had *lacY* inserted behind the *lacZ* gene in the same orientation was selected for further use. Plasmid pBDJ108 was electroporated into *S. typhimurium* BJ37 in preparation for subsequent experiments.

The protocol followed to generate pools of *S. typhimurium* Tn*5lacZY* insertion mutants has been previously described (28). Briefly, a single colony of *S. typhimurium* BJ37 with pBDJ108 was inoculated into LB broth, and the culture was grown to the exponential phase of growth. Approximately 5,000 CFU were then plated on LB agar containing 20 μg of tetracycline per ml and grown overnight at 30°C. The following day, the colonies were replica plated to LB agar with 100 μg of streptomycin per ml and 20 μg of tetracycline per ml and grown overnight at 37°C to select for transposition and loss of plasmid pBDJ108. By this technique, each Tet^r Str^r colony represents at least one unique Tn*5lacZY* transposon insertion. All of the tetracycline- and streptomycin-resistant colonies from a single plate (~5,000) were pooled.

Characterization of Tn*5lacZY* insertional mutants. The *S. typhimurium* pools were screened for mutants containing oxygen-regulated *lacZY* fusions by plating ~1,000 CFU on MacConkey agar-lactose plates (Difco Laboratories, Detroit, Mich.). Putative oxygen-regulated *lacZY* fusion strains were identified as colonies with dark-red centers and white peripheries. The β-galactosidase activity of colonies with the "fish-eye" phenotype was quantitated by the method of Miller (32) following growth in high- and low-oxygen conditions.

Tissue culture conditions and invasion assay. Hep-2 tissue culture cells (33) were maintained in RPMI 1640 (Whittaker Bioproducts, Inc., Walkersville, Md.) and passaged every 2 to 3 days.

The invasiveness of *S. typhimurium* strains for Hep-2 cells was determined by a gentamicin resistance assay which has been previously described in detail (21).

Molecular cloning and genetic techniques. Chromosomal DNA was isolated from bacteria by the procedure of Marmur (31). DNA flanking the Tn*5lacZY* insertion in *S. typhimurium* BJ42 was cloned by digesting genomic DNA and vector pACYC177 with *Hin*dIII, ligating, and selecting for *E. coli* DH5α transformants carrying the tetracycline resistance gene from Tn*5lacZY*. The *S. typhimurium* SL1344 gene bank was constructed by partial *Sau*3A digestion of chromosomal DNA, ligation to *Bam*HI-digested cosmid pHC79 (19), and packaging in vitro with the Gigapack II XL packaging extract (Stratagene Cloning Systems, San Diego, Calif.) This preparation was used to infect *E. coli* DH5α before selecting for transfectants on L agar with 100 μg of ampicillin per ml. Vectors used for subcloning *orgA* gene sequences were pBR329 (Amp^r Tet^r Cam^r), Bluescript II KS$^-$ (Amp^r) (Stratagene Cloning Systems), and the mini-F vector pMF3 (Amp^r) (30). DNA probes were labelled with [^{32}P]CTP by the nick translation procedure (29) and hybridized in 50% formamide (Sigma, St. Louis, Mo.) to DNA bound to nitrocellulose filters following published protocols (29).

The nucleotide sequences of both strands of the *orgA* gene were obtained with a combination of deletion subclones and walking primers. The standard dideoxy sequencing technique (39) was used in combination with Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio).

P22-mediated transductions were performed as previously described (5).

Animal experiments. Six- to eight-week-old female BALB/c mice were used for all animal infection experiments. Prior to oral inoculation, the pH of the mouse stomach was neutralized with 25 μl of 0.2 M sodium bicarbonate, pH 8.3. The bacterial inoculum was delivered orally (p.o.) by allowing the mouse to drink the bacterial suspension in a 25-μl volume directly from the end of a pipette tip. The doses for the p.o. route of infection were 10^5, 10^6, 10^7, 10^8, and 10^9 CFU and 10^1, 10^2, and 10^3 CFU for the intraperitoneal (i.p.) inoculations. The method of Reed and Muench (36) was used to determine the 50% lethal doses (LD_{50}s) for the p.o. and i.p. routes of infection with five mice per sample group.

Nucleotide sequence accession number. GenBank has assigned the *S. typhimurium orgA* gene sequence accession number L33855.

RESULTS

Identification of oxygen-regulated *lacZY* insertions within genes required for tissue culture cell entry. We have con-

TABLE 2. β-Galactosidase activities and quantitation of invasiveness of *S. typhimurium* Tn*5lacZY* mutants

S. typhimurium strain	β-Galactosidase activity (U)		β-Galactosidase induction ratio (low oxygen/aerobic)	% Invasion[a]	
	Aerobic growth	Low-oxygen growth		Aerobic growth	Low-oxygen growth
EE251 Δ*oxrA2* (BJ37)	ND[b]	ND	ND	0.0010	1.5000
EE251 Δ*oxrA2* (pool 1)	74	642	8.7	0.0003	0.0014
EE251 Δ*oxrA2* (pool 2)	91	634	7.0	0.0005	0.0012
EE251 Δ*oxrA2* (pool 3)	94	1,277	13.6	0.0003	0.0012
EE251 Δ*oxrA2* (pool 4)	72	1,060	14.7	0.0001	0.0012
EE251 Δ*oxrA2* (pool 5)	73	1,939	26.6	0.0003	0.0014
EE251 Δ*oxrA2* (pool 6)	86	1,050	12.2.	0.0002	0.0006
EE251 Δ*oxrA2* (pool 7)	119	442	3.7	0.0001	0.0030
EE251 Δ*oxrA2* (pool 8)	203	901	4.4	0.0002	0.0006
EE251 Δ*oxrA2* (pool 9)	125	718	5.7	0.0005	0.0006
EE251 Δ*oxrA2* (pool 10)	117	847	7.2	0.0001	0.0005
EE251 Δ*oxrA2* (pool 11)	86	394	4.6	0.0001	0.0010
EE251 Δ*oxrA2* (Lac^-)	38	47	1.2	ND	ND
EE251 Δ*oxrA2* (Lac^+)	593	944	1.6	ND	ND
TN1909 *pepT*::*lacZY*	50	592	11.8	ND	ND

[a] Percentage of 10^7 bacteria that entered 10^5 Hep-2 cells.
[b] ND, not determined.

ducted a search for invasion genes which are expressed under the same conditions that induce the invasive phenotype of *S. typhimurium* by using Tn*5lacZY* transcriptional fusions. We use the term oxygen regulated to describe these genes, although it is possible that the differential expression of these genes is controlled by an environmental condition that occurs simultaneously with oxygen deprivation in our growth experiments.

Screens of mutant pools identified colonies (~5% of the total) with red centers and white peripheries ("fish-eye") on MacConkey agar with lactose. Two types of *lacZY* insertions which could give a "fish-eye" phenotype were found: β-galactosidase expressed from a weak promoter or oxygen-regulated expression of *lacZY*. Colonies of the second type have a white periphery because they do not express β-galactosidase in the aerobic portion of the colony, and they have a red center because the middle of the colony has oxygen-limiting conditions. To distinguish between the two possibilities, each of the "fish-eye" colonies was examined under a dissecting microscope. Approximately one-third of the putative "fish-eye" colonies from each pool had a sharp boundary or line between the red and white portions of the colony, suggesting that β-galactosidase activity was being controlled by an environmental condition. The β-galactosidase activity of isolates which fit this criteria was quantitated following growth in high- and low-oxygen conditions. About half of the colonies screened had a β-galactosidase induction ratio of ≥4 (low oxygen/high oxygen) and were subsequently screened for tissue culture invasiveness. Prior to assaying the ability of the *S. typhimurium* BJ37 Tn*5lacZY* mutants to enter tissue culture cells, the transposon from each mutant was moved into *S. typhimurium* SL1344 by transducing the strain to tetracycline resistance. These genetic crosses were carried out to reduce the possibility that a mutant carried more than one transposon insertion and to ensure that each transposon insertion was responsible for any mutant phenotype that might be observed. Transductants were shown to carry an oxygen-regulated *lacZY* insertion by growing on MacConkey agar with lactose and confirming that the isolate had a "fish-eye" phenotype. The ability of the mutants to enter Hep-2 cells was measured following growth in high- and low-oxygen conditions. About one in three isolates carrying an oxygen-regulated *lac* fusion was found to be noninvasive for tissue culture cells. A single mutant was saved from each pool to avoid the possibility of studying siblings. Listed in Table 2 is the relevant data obtained for each of the oxygen-regulated, noninvasive mutants. The *S. typhimurium* Lac^- and Lac^+ strains, BJ62 and BJ63, respectively, are described in Table 1. These strains carry *lacZY* insertions at unknown locations in the chromosome delivered by the Tn*5lacZY* transposon described in Materials and Methods.

Complementation of the invasive defect of *S. typhimurium* BJ66. Following transposon mutagenesis and preliminary characterization of the mutants from each pool, the oxygen-regulated noninvasive mutant from pool 3 was selected for more extensive characterization. To identify the gene responsible for the invasive defect in strain BJ66, the tetracycline resistance of the transposon and flanking DNA was cloned from the chromosome. A 1.4-kb *Bam*HI DNA fragment, consisting primarily of *S. typhimurium* DNA along with 53 bp of the transposon, was identified. This was used as a probe to identify cosmids with homologous sequences from an *S. typhimurium* SL1344 gene bank. Seven cosmids which hybridized to the probe were transformed into strain BJ66 and tested for the ability to restore invasion. One cosmid, designated pBDJ125, fully restored the ability of BJ66 to enter tissue culture cells when grown in low-oxygen conditions (Fig. 1) and high-oxygen conditions (data not shown). The *orgA* gene was localized to a region on cosmid pBDJ125 by the following strategy. Southern blot experiments had previously shown that the *orgA* gene probe hybridized to a 6.0-kb *Bam*HI-*Eco*RI fragment (data not shown). Plasmid pBDJ125 was cut with *Bam*HI and *Eco*RI and found to have a 6.0-kb fragment. This fragment was isolated and subcloned into pBR329 by directed cloning. The resulting plasmid, pBDJ127, was transformed into *S. typhimurium* BJ66 and was found to complement the invasion defect of the *orgA* mutant (Fig. 1). Plasmid pBDJ127 was cut to completion with *Hin*dIII and religated to delete a 3.1-kb fragment. The resulting plasmid, pBDJ134, retained the ability to restore the invasive phenotype to BJ66 (Fig. 1). Finally, restriction mapping and sequence data were used to show that a 1.9-kb *Eco*RV DNA fragment cloned into Bluescript II KS^- (pBDJ143) carried a functional *orgA* gene (Fig. 1). Complementation of the invasion defect was also dependent upon the level of oxygen in the growth medium, indicating that the 1.9-kb fragment carried the necessary sequences for regula-

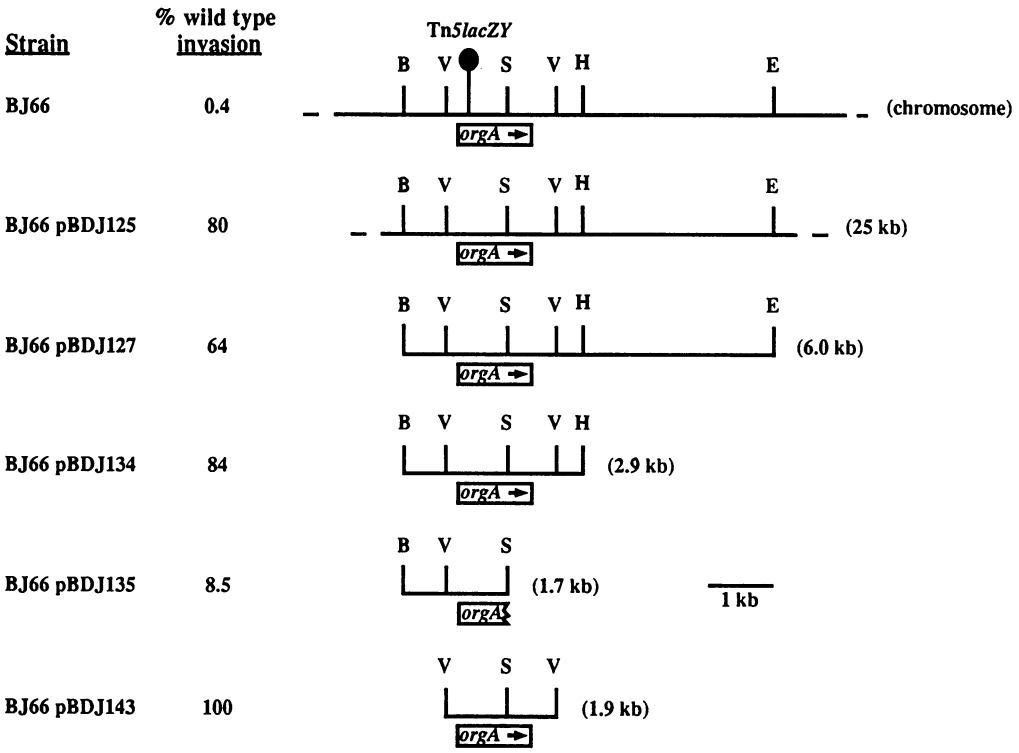

FIG. 1. Localization of the *S. typhimurium orgA* gene by restriction mapping and subcloning. Cosmid pBDJ125, which restored the invasive phenotype of *S. typhimurium* BJ66, was digested with *Bam*HI and *Eco*RI, and the 6-kb band was subcloned (pBDJ127) and shown to retain complementing activity. The intact *orgA* gene was found to reside on a 1.9-kb *Eco*RV fragment (pBDJ143). The site of the original Tn*5lacZY* insertion in BJ66 is indicated by the symbol. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; V, *Eco*RV.

tion. The same 1.9-kb *Eco*RV fragment cloned into the mini-F vector pMF3 could also completely complement the invasion defect of *S. typhimurium* BJ66 (data not shown).

Chromosomal mapping of the complementing invasion gene. The chromosomal location of the *orgA* gene was determined with the aid of strains carrying Mud-P22 insertions at defined positions of the *Salmonella* chromosome (49). Hybridization of the *orgA* probe to phage particle DNA from 70 *S. typhimurium* Mud-P22 strains revealed that the Tn*5lacZY* insertion in *orgA* mapped between *proU* at 57.5 min and *cysHIJ* at 60 min of the chromosome (Fig. 2A). To more precisely define the position of the *orgA* gene, genomic DNA from *S. typhimurium* EE451, which contains a 10-kb deletion at 59 min, was hybridized to the *orgA* probe. The probe hybridized to a 10-kb *Eco*RI fragment from *S. typhimurium* SL1344 but failed to hybridize to a fragment of any size from strain EE451 (data not shown). This result indicates that the *orgA* gene has been deleted from *S. typhimurium* EE451 and is located in the region of the 10-kb deletion. Comparison of restriction enzyme maps of this chromosomal region with restriction enzyme maps of the cloned complementing activity reveals that the *orgA* gene maps between the Tn*5*B50-380 insertion (*hil*) (28) and the *prgH* gene (2), as shown in Fig. 2B.

Prevalence of the *orgA* gene among invasive bacterial species. Hybridization studies were conducted, using the *orgA* gene probe, to determine whether other *Salmonella* strains as well as other invasive bacterial strains carried the *orgA* gene. Genomic DNA from *Salmonella cholerae-suis* (two strains), *S. dublin* (one strain), S. *enteriditis* (two strains), S. *gallinarum* (one strain), S. *pullorum* (one strain), S. *typhi* (two strains), and S. *typhimurium* (four strains) was isolated and probed for the presence of the *orgA* gene. All 13 of the *Salmonella* strains tested hybridized to the *orgA* gene probe (data not shown). In addition, chromosomal DNA from several enteroinvasive bacterial strains (*Shigella flexneri*, *E. coli* RDEC-1, enterohemorrhagic *E. coli*, enteroinvasive *E. coli*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Listeria monocytogenes*) was hybridized to the *orgA* probe. None of these bacterial species had sequences which were recognized by the probe under high-stringency hybridization conditions (data not shown), but it is possible that the use of less-stringent conditions might have detected some homology.

Regulation of the *orgA* gene. We have demonstrated that the *orgA* gene maps between the Tn*5*B50-380 (*hil*) transposon insertion and the *prgH* gene. In a recent report describing the *prgH* gene, Behlau and Miller found that a *prgH*::Tn*phoA* fusion was constitutively expressed in a Tn*5*B50-380 background (2). Because of the physical proximity of the *orgA* gene to the *hil* locus and the *prgH* gene, we examined the effect of the Tn5B50-380 transposon insertion on the expression of the *orgA*::Tn*5lacZY* fusion. The *orgA*::Tn*5lacZY* fusion was cloned onto the single-copy mini-F vector pMF3, and the resulting plasmid, pBDJ142, was electroporated into *S. typhimurium* SL1344 and EE421. The production of β-galactosidase was assayed for each strain after growth in high- and low-oxygen conditions. As shown in Fig. 3, the expression of β-galactosidase from the strain carrying the *orgA*::*lacZY* fusion following growth in high-oxygen conditions was 520 U and was induced ~4.3-fold in low-oxygen conditions to give 2,221 U. However, the β-galactosidase units of EE421(pBDJ142) after growth in high-oxygen conditions were 1,272 and after growth in low-oxygen conditions were 2,181, giving a ratio of 1.7, which was

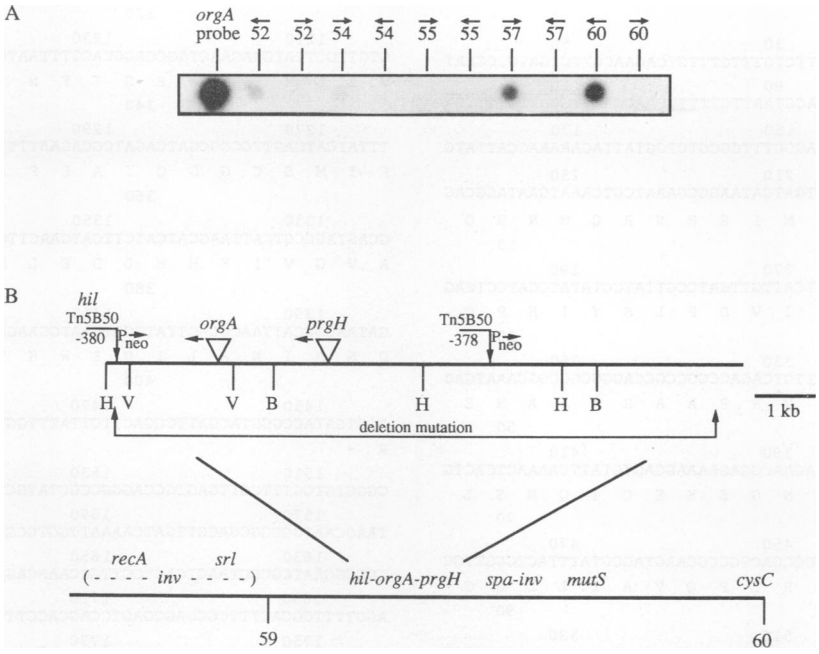

FIG. 2. Chromosomal mapping of the *orgA* gene. (A) An *orgA* gene probe hybridized to phage DNA from two *S. typhimurium* strains carrying Mud-P22 insertions at 57.5 and 60 min. Probe DNA was used as a positive control for hybridization. The arrows indicate the direction of DNA packaging by the "locked-in" P22 phage. (B) The exact chromosomal position of the *orgA* gene was determined by Southern hybridization and restriction mapping.

similar to that observed for strains carrying unregulated *lacZY* fusions. These results indicate that the *hil* locus partially regulates the expression of the *orgA* gene in an oxygen-dependent manner.

Sequencing of the *orgA* gene. The nucleotide sequences of both strands of the 1.9-kb EcoRV fragment carried on plasmid pBDJ143 were determined and the coding strand is shown in Fig. 4. The open reading frame (ORF) corresponding to the *orgA* gene was identified from the insertion site and orientation of the Tn*5lacZY* transposon insertion. The ORF is capable of encoding a protein of 412 amino acids and 47,806 Da. A potential ribosome binding site (GAAAGG) begins at bp 191 of the nucleotide sequence and is 11 bp upstream of the first methionine residue within the ORF. No signal sequence appears to be present at the N terminus of the deduced amino acid sequence. Hydropathy analysis of the amino acid sequence indicated that the protein is hydrophilic with no striking hydrophobic regions.

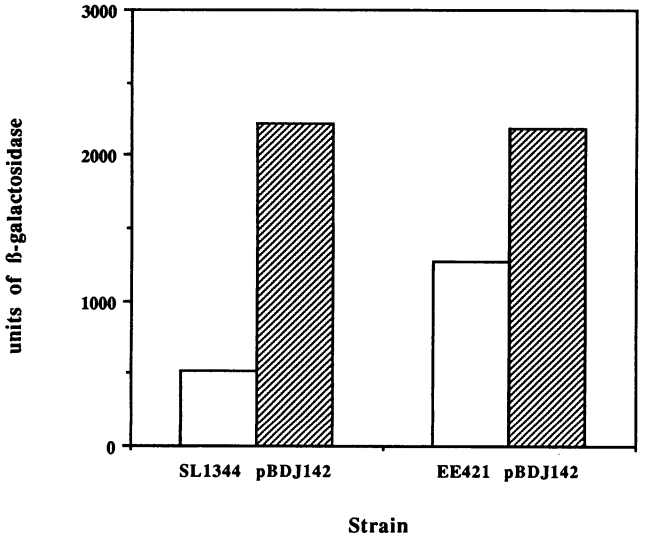

FIG. 3. Effect of *hil*::Tn*5*B50 insertion on the expression of *orgA*::*lacZY* from plasmid pBDJ142. Bacteria were grown under high-oxygen (open bars) or low-oxygen (hatched bars) conditions before measuring β-galactosidase activity by the method of Miller (32). SL1344 carries *hil*$^+$; EE421 carries *hil*::Tn*5*B50-380.

Since plasmid pBDJ143, which carries the 1.9-kb *Eco*RV fragment, complements the invasion defect of *S. typhimurium* BJ66 in an oxygen-dependent manner, it is likely that the first 208 bp of sequence contain the signals for oxygen regulation of the *orgA* gene. Two direct repeats (TTGGATTnnnTTGATT) have been found in this region and are located in the middle of a putative promoter with a −35 sequence (TTGCAA) and −10 sequence (TATTTT). The significance of these repeats is currently being investigated.

A search of the GenBank and EMBL data banks using the FASTA program (35) failed to identify any significant similarity with previously reported gene sequences. In an effort to identify some possible function for the OrgA protein, the Motifs program from the GCG sequence analysis software (University of Wisconsin, Madison) and the Blocks program (47) were used to search the OrgA deduced amino acid sequence for functional protein domains. Neither of these two programs identified any motif which suggest a function for OrgA.

Virulence studies. Since the invasiveness of *S. typhimurium* strains correlates with their ability to cause murine typhoid fever, we compared the virulence of *S. typhimurium* BJ66 (*orgA*) with that of wild-type *S. typhimurium* SL1344. We have recently demonstrated that *S. typhimurium* BJ66 is unable to

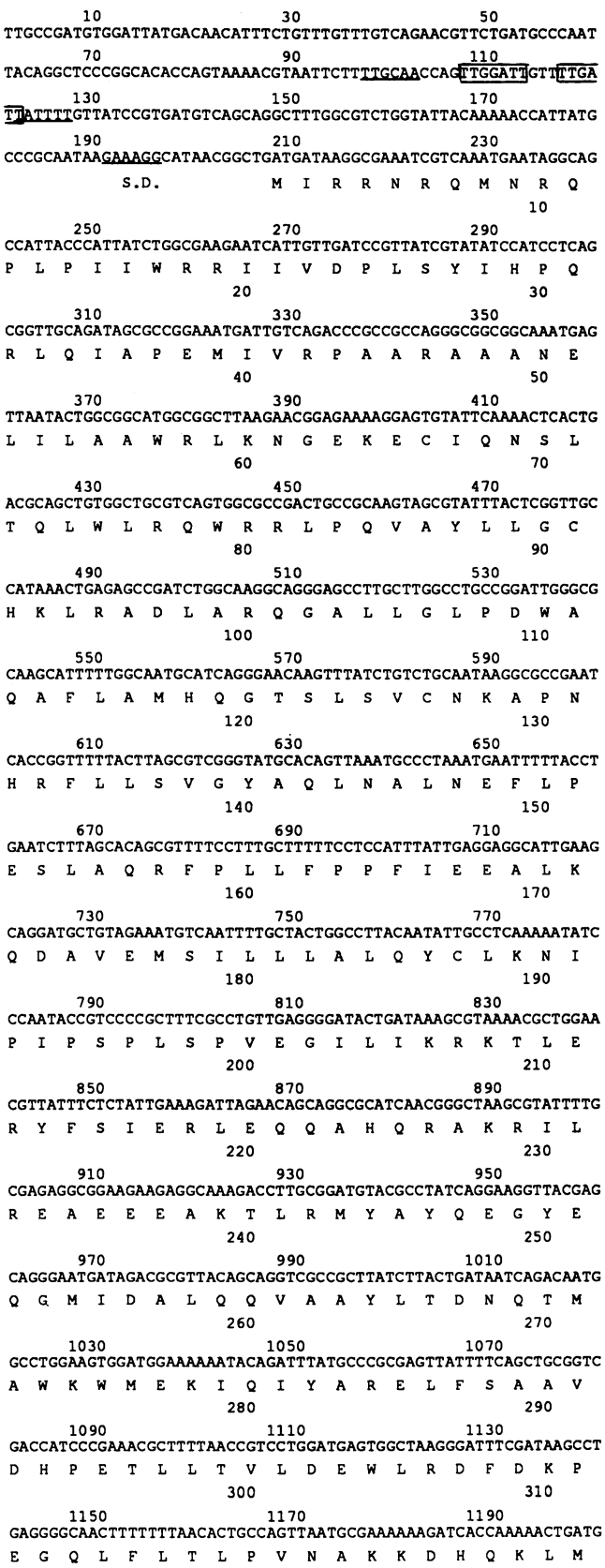

FIG. 4. Nucleotide sequence of the 1.9-kb *Eco*RV fragment containing the *orgA* ORF. A potential promoter site for the *orgA* gene at nucleotides 97 to 102 (−35) and 122 to 127 (−10) is underlined. The direct repeats at nucleotides 107 to 113 and 117 to 122 are indicated with boxes. A potential ribosome binding site beginning at position 191 is labelled S.D. The Tn*5lacZY* transposon inserted between nucleotides 578 and 579.

invade and destroy the M cells of murine Peyer's patches up to 3 h postinoculation (20). In contrast, virulent *S. typhimurium* SL1344 enters and destroys these specialized epithelial cells 60 min following inoculation into ligated loops (20). We have now measured the LD_{50}s for *S. typhimurium* SL1344 and BJ66 following p.o. or i.p. inoculation of BALB/c mice. The measured LD_{50}s of SL1344 (wild-type strain) was 8×10^5 and 3×10^1 CFU for the p.o. and i.p. routes, respectively. The i.p. LD_{50} for strain BJ66 (*orgA*) was roughly the same as that for the parent strain (4.5×10^1 CFU), while the p.o. LD_{50} was 5×10^7 CFU, greater than 60 times higher than for strain SL1344. We were interested in determining whether plasmid pBDJ143 could restore full virulence to *S. typhimurium* BJ66. However, testing the virulence contribution of a gene on a multicopy vector appears to have created experimental problems. Three separate animal experiments failed to establish the ability of the *orgA* gene to restore virulence to BJ66. Plasmid instability or a reduced growth rate due to the presence of the plasmid could explain our results. Experiments are now under way to construct a derivative of BJ66 which possesses a single functional *orgA* gene in the chromosome. The virulence of this strain will then be compared to the wild-type strain and BJ66 in the mouse typhoid model.

DISCUSSION

Pathogenic *Salmonella* species initiate infection of a host by penetrating the intestinal mucosa, primarily at the lymphoid

follicles of the Peyer's patches (4, 18). Recent data suggest that these bacteria pass through the epithelium of a host by preferentially invading and destroying the M cells of Peyer's patches (20, 26). Previous work by our group and others has shown that low-oxygen environments, such as that present in the small intestine, induce the ability of *Salmonella* species to enter mammalian cells (8, 27, 40). We have now identified invasion genes which are induced by low-oxygen conditions, using β-galactosidase fusions. Preliminary characterization of these mutants has confirmed that many of these transposon insertions disrupt the ability of *S. typhimurium* to enter tissue culture cells. The defect from one of these mutants, BJ66, has been complemented, and the corresponding gene (*orgA*) has been cloned and sequenced. Searches of the GenBank data bank with the nucleotide and protein sequences failed to identify any significant similarities to other sequences present in the bank. Work is currently under way to identify a function for the OrgA protein and to characterize the other noninvasive oxygen-regulated *lacZY* fusion mutants.

We have analyzed a 200-bp region upstream of the *orgA* ORF for transcription signals. Two direct repeats (TTGGAT TnnnTTGATT) have been found which are similar to those identified for *fnr* (*oxrA*)-dependent promoters (6, 43). These repeats overlap a possible promoter for the *orgA* gene, suggesting that they may play a role in the oxygen regulation of the *orgA* gene. While we have no evidence to suggest that these repeats play a regulatory role in the expression of the *orgA* gene, it is tempting to speculate that these repeats are part of an unidentified oxygen regulatory system which controls the expression of this gene and invasion. In addition, we have found that repression of an *orgA*::*lacZY* fusion in high-oxygen conditions is partially relieved in *S. typhimurium* EE421 (*hil*::Tn*5*B50-380). Behlau and Miller (2) also found that the *prgH* gene, which maps immediately upstream of *orgA*, is constitutively expressed in the same strain background. These results indicate that the *hil* locus acts to regulate at least two genes involved in bacterial entry.

Hybridization of an *orgA* probe with a *Salmonella* chromosome mapping library has localized this gene to 58 to 60-min region of the chromosome. Several other genes necessary for bacterial invasion have been localized to the same region of the *Salmonella* chromosome. Elsinghorst et al. (7) have described four invasion genes (*invA* to *-D*) that lay near *recA* and *srlC*. Another large group of genes required for bacterial entry lies near the *mutS* gene and includes *inv* genes (1, 13, 16) and *spa* genes (17). Southern blot analysis and restriction mapping has shown that the *orgA* gene is part of yet another group of invasion genes which includes the *hil* locus (28) and the *prgH* gene (2) (Fig. 2). The clustering of so many genes within such a small area suggests that the entire 58- to 60-kb region of the *Salmonella* chromosome encodes genes involved in mammalian cell entry. Perhaps, as has been suggested previously (14, 28), *Salmonella* entry occurs via an invasion complex which has regulatory, transport, assembly, and structural elements.

The invasiveness of *Salmonella* strains is a major contributing factor to the development of disease (37). We have therefore tested the contribution of the *orgA* gene to the virulence of *S. typhimurium* SL1344 by using a murine model of typhoid fever. Our results showed clearly that BJ66, the *orgA* isogenic mutant of strain SL1344 has an LD_{50} 60-fold higher than that of the wild type when administered p.o. However, the noninvasive *orgA* mutant and SL1344 were equally virulent for mice challenged by an i.p. route. Galán and Curtiss obtained similar data when testing the effect of an *invA* mutation on the virulence of invasive *Salmonella* strains (13). They found that noninvasive *invA* derivatives of strains SL1344 and SR11 had LD_{50}s 50- to 60-fold higher than those of the parent strains. However, these noninvasive strains were as virulent as wild-type strains when introduced i.p. The loss of infectivity of noninvasive mutants by the p.o. route correlates with recently published work which demonstrated that BJ66 has lost the ability to invade and destroy M cells of the Peyer's patches (20). It appears that the ability of a strain to invade tissue culture cells in vitro correlates with its abilities to invade and destroy M cells in vivo and to cause systemic disease following oral infection. However, it seems that the function of the *orgA* gene (and the *invA* gene) are required for passage through the intestinal epithelium and the Peyer's patches, not for later stages of the infection process.

We have identified and partially characterized an oxygen-regulated gene involved in the *Salmonella* entry process. The specific function of this gene, *orgA*, is currently under investigation. In addition, we have identified and are currently studying several other loci which affect the ability of *S. typhimurium* to enter tissue culture cells. Our results demonstrate that the oxygen regulation of bacterial entry is a useful key for the identification of individual components of the *Salmonella* invasion machinery.

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