INFECTION AND IMMUNITY, May 1994, p. 1623–1630
0019-9567/94/$04.00+0
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Vol. 62, No. 5

Salmonella typhimurium Loci Involved in Survival within Macrophages

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Received 10 October 1993/Returned for modification 27 January 1994/Accepted 9 February 1994

A set of Tn*10* mutants of *Salmonella typhimurium* which have a diminished capacity to survive in murine macrophages and decreased virulence in mice has been described previously. In this study, we characterized 30 of these mutants and determined map locations of Tn*10* insertions for 23 of these strains. In addition, short fragments of transposon-flanking DNA were cloned, and the nucleotide sequence was determined for 23 mutants. Seven mutants carried transposon insertions in known genes, representing six loci: *htrA*, *prc*, *purD*, *fliD*, *nagA*, and *smpB*. The possible roles of these genes in *Salmonella* virulence are discussed. One insertion was found to be in an unknown gene which shared homology with the open reading frames Bv′ and Bv located in the *pin* inversion system of *Shigella boydii*. In one mutant, Tn*10* was found to be inserted in a gene with significant homology to *adhE* of *Escherichia coli* and *Clostridium acetobutylicum*. The map location and degree of homology indicate that the *Salmonella* gene encodes a related, but different, dehydrogenase. In 14 of the mutants analyzed, Tn*10* was inserted into genes which had no significant homologies to entries in the DNA and protein data bases. In conclusion, 16 insertions define loci, termed *ims* for impaired macrophage survival, which have not yet been described in *S. typhimurium* but have been shown previously to be necessary for full virulence in mice. Although most *ims* loci are distributed randomly throughout the genome, a cluster was found between 75 and 78 min on the *Salmonella* chromosome.

Salmonella typhimurium evokes a systemic disease in mice called murine typhoid. In mice infected orally with *S. typhimurium*, the bacteria penetrate the intestinal mucosa and reach the Peyer's patches of the small intestine, the lymphatic drainage areas. From these foci, *S. typhimurium* disseminates via the lymphatics into the circulation, seeds the reticuloendothelial cell system, and replicates within splenic and hepatic tissue. A secondary bacteremia accompanied by rapid bacterial replication finally causes the death of the animal (11, 24).

Although the observation that *S. typhimurium* replicates within resident macrophages in splenic and hepatic tissue is relatively old, only limited data concerning the genetics involved in this interaction between host and pathogen are available. In the mouse, innate resistance or susceptibility to infection with *S. typhimurium* is under genetic control of the *ity* locus (for immunity to *S. typhimurium*) on chromosome 1 (34, 42, 43, 55). The bacterial genes involved in macrophage survival were identified by screening 9,516 independent Tn*10* mutants of *S. typhimurium* in an in vitro assay for survival within thioglycolate-elicited murine peritoneal macrophages (16). A total of 83 Tn*10* mutants were found to have a diminished capacity for intracellular survival (designated MS [for macrophage survival] mutants). All MS mutants were less virulent than the parent strain in vivo, demonstrating that survival within macrophages is essential for *Salmonella* pathogenesis (16). Twenty-two of 23 MS mutants tested multiplied normally within epithelial cells, indicating that different sets of genes are required for replication in phagocytic cells and epithelial cells (17). Although survival of *S. typhimurium* within macrophages varied with the source of cells, most MS mutants maintained their macrophage-sensitive phenotype within the murine macrophage cell line J774 (17) and within murine resident peritoneal, splenic, and bone-marrow-derived macrophages (6). The MS mutants were characterized with regard to several in vitro conditions thought to stimulate the macrophage intracellular environment. These included oxidative stress, nutrient-poor conditions, and the presence of defensins in the growth medium (15, 16). This analysis led to the identification of the *phoPQ* virulence genes as well as of several auxotrophs, which indicated that the original screening had selected Tn*10* insertions in bona fide virulence genes as well as in genes that encode housekeeping functions.

In this study, we describe the characterization of 28 attenuated nonauxotrophic MS mutants along with one nonmotile and one purine auxotroph MS mutant. The three *phoPQ* mutants, which were identified and mapped earlier, were used as controls so that a total of 33 MS mutants were analyzed (Table 1). Map locations from Tn*10* insertions in *phoPQ* mutants and in 23 additional MS mutants could be determined. Cloning and nucleotide sequence analysis of transposon-flanking DNA indicated that the majority of the identified loci were not sequenced previously in *S. typhimurium*. This analysis will facilitate the identification of new virulence factors, and further characterization of these loci will improve our understanding of *Salmonella* virulence.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. 14028r is a rough derivative of *S. typhimurium* ATCC 14028 (16). Macrophage-sensitive Tn*10* mutants of 14028r have been described earlier (16). *S. typhimurium* strains carrying MudP22 prophages are described elsewhere (5). *Escherichia coli* TA One Shot and DH5α were purchased from InVitroGen and GIBCO BRL, respectively. All bacteria were cultured aerobically at

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37°C. Antibiotics, when required, were included in the culture medium or plates at the following concentrations: carbenicillin, 100 mg/ml; kanamycin, 60 mg/ml; and tetracycline, 15 mg/ml. Analytical-grade chemicals were purchased from Sigma. Restriction endonuclease *Bln*I was purchased from TaKaRa Biochemical Inc., and all other enzymes were purchased from Boehringer Mannheim.

Minimal medium was prepared as described by Miller (38) with either 0.2% *N*-acetylglucosamine (Sigma) or 0.2% glucose (Sigma) as the sole carbon source.

Pulsed-field gel electrophoresis. Preparation of agarose-embedded chromosomal DNA for pulsed-field gel electrophoresis was based on a protocol from Liu and Sanderson (35). In brief, cells were harvested from 5 ml of an early-log-phase bacterial culture, resuspended in 0.5 ml of prewarmed (37°C) cell suspension buffer (10 mM Tris-HCl [pH 7.2], 20 mM NaCl, 100 mM EDTA), and mixed with 0.5 ml of 2% agarose (InCert) which was dissolved in phosphate-buffered saline (PBS) and precooled to 37°C. The mixture was poured into molds (Bio-Rad) and allowed to solidify. The molds were digested with lysozyme (1 mg/ml; New England Biolabs) in a volume of 7.5 ml for 1 h at room temperature in buffers provided by the manufacturer. The lysozyme solution was replaced by 7.5 ml of proteinase K (1 mg/ml; New England Biolabs) in buffer provided by the manufacturer and incubated at 55°C for 48 h with shaking. Agarose-embedded DNA prepared in this manner can be stored in 7.5 ml of TE buffer (10 mM Tris-HCl [pH 7.2], 5 mM EDTA) at 4°C for several months without degradation.

For restriction digests, an agarose block equivalent of about 0.05 to 0.1 ml was incubated for 15 min at room temperature in 0.2 ml of 1× KGB buffer (36) in which sodium acetate was replaced by potassium acetate. The buffer was replaced with 1× KGB buffer containing 20 U of a restriction endonuclease (*Xba*I or *Bln*I), and the samples were incubated for 4 h at 37°C. The agarose blocks were then loaded on an agarose gel for pulsed-field gel electrophoresis.

Pulsed-field gel electrophoresis of a 1% agarose gel was performed in 0.5% Tris-borate-EDTA buffer at 200 V with pulse lengths of 6 to 150 s for 6 h, 6 to 12 s for 6 h, 26 to 36 s for 6 h, and 120 to 180 s for 6 h by using a CHEF-DRII apparatus from Bio-Rad.

Recombinant DNA techniques. Plasmid DNA was isolated by using ion-exchange columns from Qiagen. Standard methods were used for restriction endonuclease analyses and ligation and transformation of plasmid DNA (38). Sequencing was performed by the dideoxy chain termination method (48) by a protocol of Kraft et al. (32), using α-^{35}S-dATP (Amersham) for labeling.

Inverse PCR was performed by using agarose-embedded chromosomal DNA from MS mutants. An agarose block equivalent to approximately 0.05 to 0.1 ml was incubated for 15 min at room temperature in 0.2 ml of 1× KGB buffer (36) in which sodium acetate was replaced by potassium acetate. The buffer was replaced with 1× KGB buffer containing 50 U of a restriction endonuclease (*Alu*I, *Hin*dII, *Sph*I, or *Mae*I), and the samples were incubated for 4 h at 37°C. The samples were then incubated for 20 min at 72°C (to inactivate the restriction enzyme and to melt the agarose block). ATP (10 mM) and T4 DNA ligase (2 U) were added at 37°C, and ligation was performed overnight at 15°C. Inverse PCR was performed under standard conditions (39) in a total volume of 0.1 ml with 0.005 ml of the ligation mixture described above as a template and 0.1 ng of each of the following primers: 5′ CCAATTCT GCCCCGAATTAC 3′ and 5′ GTCGTGTAAAATATC GAGTTCG 3′ (Oligos Etc.). These primers are complementary to both IS*10* elements flanking Tn*10* (22) and could therefore be used to amplify DNA from either side of a Tn*10* insertion. PCR products were cloned into the vector pCRII and transformed into *E. coli* TA One Shot (TA-cloning kit; InVitroGen).

Prophage DNA isolation and hybridization. Preparation of DNA from phage particles of locked in MudP22 prophages has been described previously (60). In brief, a fresh 5-ml overnight culture of the desired lysogen was added to 25 ml of LB broth. Mitomycin (Sigma) was added to a final concentration of 0.002 mg/ml. After the mixture was shaken overnight at 37°C, 3 ml of $CHCl_3$ was added and the flask was shaken vigorously for 3 min. The LB broth was decanted from the $CHCl_3$, and the cell debris was removed by low-speed centrifugation (for 10 min at 8,000 rpm in a Sorvall SS34 rotor). Phage particles were pelleted from the supernatant by centrifugation at 37,000 × *g* for 70 min and resuspended in 1/10 volume of PBS. To extract DNA, 0.5 ml of this stock was mixed with an equal volume of $CHCl_3$. After the addition of 0.002 ml of diethylpyrocarbonate, 0.01 ml of 10% sodium dodecyl sulfate (SDS), and 0.05 ml of 2 M Tris-HCl (pH 8)–0.5 M EDTA, the mixtures were incubated at 65°C for 15 min. After 0.05 ml of 5 M KCl was added, the mixtures were placed on ice for 1 h and protein-SDS complexes were pelleted for 15 min in a microcentrifuge. The DNA in the supernatant was precipitated by the addition of 2 volumes of ethanol, wound onto the end of a sterile Pasteur pipette, dipped in a solution of 70% ethanol, blotted dry, and resuspended in 0.1 ml of TE buffer. Five microliters of this solution was loaded onto a nylon membrane by using the Minifold II slot blot system (Schleicher & Schuell).

Labeling of DNA probes, hybridization, and immunological detection were performed by using a DNA labeling and detection kit (nonradioactive) from Boehringer Mannheim. The DNA was labeled by randomly primed incorporation of digoxigenin-labeled dUTP. Hybridization to the slot blot was performed at 68°C in solutions without formamide. Hybrids were detected by an enzyme-linked immunoassay using an antibody conjugate (anti-digoxygenin alkaline phosphatase conjugate) and the substrate AMPPD [3-(2′-spiroademantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxethane; Boehringer Mannheim]. The light emitted by the dephosphorylated AMPPD was detected by X-ray film.

Computer analysis. The nucleotide sequences were compared with the SWISS-PROT, PIR(R), and GenPept data bases by using the program blastX and with the GenBank and EMBL data bases by using the program blastN (4).

Accession numbers. Nucleotide sequences were deposited at GenBank under the following accession numbers: MS1, U06133; MS1321, U06126; MS1413, U06127; MS1521, U06129; MS1523, U06128; MS1633, U06130; MS1668, U06131; MS2097, U06134; MS2101, U06135; MS3493, U06132; MS3747, U06137; MS4290, U06136; MS5076, U06138; MS6290, U06140; MS7481, U06139; MS8282, U06142; and MS9020, U06141.

RESULTS

Mapping the Tn*10* insertions of *S. typhimurium* MS mutants. Macrorestriction maps of the *S. typhimurium* genome with the enzymes *Bln*I and *Xba*I have been published recently (35, 59). These restriction endonucleases cut the *S. typhimurium* chromosome 11 and 23 times, respectively. Since Tn*10* contains restriction sites for both of these rare-cutting enzymes, a transposon insertion introduces new *Bln*I and *Xba*I restriction sites into the *Salmonella* genome. After a restriction digestion of chromosomal DNA from a Tn*10* mutant is per-

TABLE 1. Mapping data of *S. typhimurium* Tn*10* insertion mutants showing reduced macrophage survival

MS mutant	*Xba*I restriction map[a]		*Bln*I restriction map[b]		Hybridization with MudP22 prophages[c]	Sequence homology[d]	Map position (min)	Designation of gene or locus
	Fragment missing	Size (kb) of new fragment(s)	Fragment missing	Size (kb) of new fragment(s)				
1	I	155	B	190	ND[e]	*adhE*	50	*ims50*
3	D	320, 155	B	175, 730	ND	*fliD*	40	*fliD*
1321		28	E	55	TT15265	NH[f]	73	*ims73*
1413		60	E	260	TT15266	NH	78	*ims78*
1446	H	77	E	270	ND	ND	77	
1521			E	260	TT15266	NH	78	*ims78*
1523	H	125	E	275	TT15266	NH	75	*ims75*
1592	B	315, 400	A	750	ND	*htrA*	5	*htrA*
1601			E	160, 210	ND	ND	79	
1633	C	255, 420	D	270	ND	NH	61 or 66	*ims61-66*
1668	H	115, 155	E	340	TT15264	NH	76	*ims76*
2097	E	90, 300	A	270, ca. 1,200	ND	NH	98	*ims98*
2101	E	175, 200	A	210, ca. 1,300	ND	NH	96	*ims96*
3010	B	315, 400	A	750	ND	ND	5 or 6	
3493	D	240		65	TT15630	NH	37	*ims37*
3747	A	120, 690	C	235, 580	ND	NH	26	*ims26*
3792	A	130, 680	A	320, ca. 1,200	TT15237	*nagA*	15.5	*nagA*
4179	B	250, 460	A	650, 860	ND	ND	3 or 7.5	
4252	A	145, 670	C	210, 600	ND	*phoPQ*[g]	25	*phoPQ*
4290	D	240, 225		55	ND	*prc*	36	*prc*
4347		9.3			ND	*purD*	89	*purD*
4602	B	250, 460	A	650, 860	ND	ND	3 or 7.5	
4916	A	130, 680	C	225, 590	ND	ND	25.5	
5076	C	175, 500	D		ND	ORF Bv'	59.5 or 67	*ims60-67*
5996	A	145, 670	C	210, 600	ND	*phoPQ*	25	*phoPQ*
6290	A	120, 690	A	330, ca. 1,200	ND	NH	14.5	*ims15*
7342	B	140, 580	A	580, ca. 950	ND	ND	2 or 8	
7481		29	F1	35, 120	ND	*smpB*	55	*smpB*
7953	A	145, 670	C	210, 600	ND	*phoPQ*	25	*phoPQ*
8282	B	240, 460	A	670, 840	TT15231	NH	7	*ims7*
8467	C	255, 420	D	270	ND	NH	61 or 66	*ims61-66*
9020	A	120, 690	C	235, 580	ND	NH	26	*ims26*
9187		29	F1	35, 120	ND	*smpB*	55	*smpB*

[a] Data based on article by Liu and Sanderson (35).
[b] Data based on article by Wong and McClelland (59).
[c] Data based on article by Benson and Goldman (5).
[d] For interpretation of sequence homologies, see text.
[e] ND, no data.
[f] NH, no significant homology with any entries of the data bank.
[g] The *phoPQ* insertion mutants have been mapped and characterized earlier (20) and served as a control of the methods used.

formed, the fragment in which the transposon is inserted will disappear, while two new, smaller fragments can be detected after separation by pulsed-field gel electrophoresis.

By analysis of *Bln*I and *Xba*I restriction digests, the map positions of 17 MS mutations were located on the *S. typhimurium* chromosome (Table 1; Fig. 1). As a control, the map positions of the *phoPQ* mutations in strains MS7953, MS5996, and MS4252 were determined by the same technique. Several genetic markers which had been physically mapped previously were used to align the physical and genetic maps of *S. typhimurium* (Fig. 1) (35, 59). As can be seen in Table 1 and Fig. 1, the determined map positions of MS7953, MS5996, and MS4252 corresponded well with the published position at 25 min. The map positions of 13 MS mutants could not be determined by this method because either (i) the Tn*10* insertions (of MS1321, MS1413, MS1521, and MS1523) were located in a restriction fragment which comigrates with other fragments of similar size (e.g., *Xba*I fragments H, I, and J) or (ii) in two places of the chromosome, *Bln*I and *Xba*I fragments are centered around the same midpoint (e.g., *Bln*I fragment A and *Xba*I fragment B; *Bln*I fragment D and *Xba*I fragment C; Fig. 1). Therefore, by physical mapping, a decision between two possible map positions could not be made for Tn*10* mutants mapping in these regions (MS1592, MS1633, MS3010, MS4179, MS4602, MS5076, MS7342, MS8282, and MS8467).

The Tn*10* insertions of those mutants which could not be located physically were located on the *Salmonella* genetic map by the method of Benson and Goldman (5). This mapping technique is based on a set of *S. typhimurium* strains carrying locked-in MudP22 prophages in different locations on their chromosome (60). After induction, these prophages package unidirectionally approximately three headfuls of chromosomal DNA on one side of the prophage insertion. DNA isolated from the phage heads is therefore about 100-fold enriched for DNA from this area of the chromosome. The phage DNA of 50 strains was isolated, and each was applied separately to a slot blot. Transposon-flanking DNA was cloned by inverse PCR and used as a probe for hybridization with the slot blot. A positive hybridization signal localized the corresponding Tn*10* insertion to a 3-min region on the *S. typhimurium* chromosome. By combining the results from physical and prophage mapping, map locations of six additional MS mu-

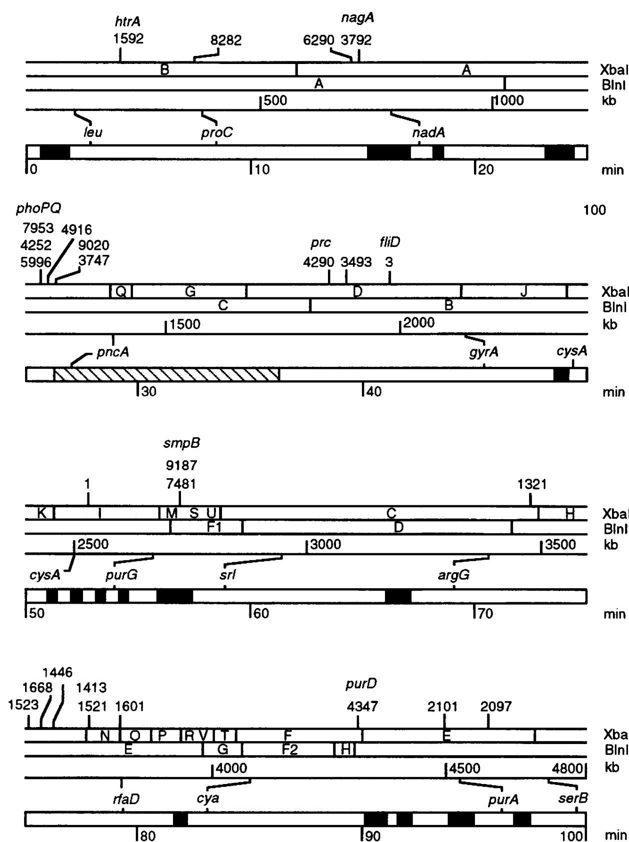

FIG. 1. Chromosomal location of Tn*10* insertions of MS mutants. *Xba*I and *Bln*I restriction sites are indicated above the physical map of the *S. typhimurium* chromosome in kilobases (horizontal lines). Bars below indicate the genetic map in minutes. Genetic markers drawn between the physical and genetic maps were used to align both maps (35, 59). The solid bars indicate large regions (loops) in the *Salmonella* genetic map which are not present in *E. coli*. The hatched bar marks the genetic region that is inverted relative to *E. coli* (46). The numbers above the chromosomal map indicate the locations of Tn*10* insertion sites in the corresponding MS mutants. If known, genes affected by the Tn*10* insertion are indicated above the insertion site.

tants were determined (Table 1; Fig. 1). The *Salmonella* chromosome contains approximately 4,000 genes. In a bank of 9,516 independent insertions, most genes should therefore be inactivated in more than one mutant. However, insertions of most MS mutants were found in distinct genes, indicating that the mutagenesis system was not saturated (Fig. 1).

Nucleotide sequence analysis of cloned transposon-flanking DNA. Transposon-flanking DNA from 23 MS mutants was cloned by inverse PCR, and the nucleotide sequence was determined. The transposon Tn*10* creates a 9-bp direct repeat at the insertion site in the target DNA. Comparison of the 9-bp repeat of various Tn*10* insertions revealed that the transposon integrates preferentially at the hot spot NGCTNAGCN or similar sequences (21). The 9-bp direct repeats created by Tn*10* insertions in the MS mutants analyzed were compared with the hot-spot consensus sequence, and four were found to be in perfect hot spots. The target sequence contained at least four of the six conserved residues for another 17 insertions (data not shown).

The nucleotide sequences of the cloned 83-bp DNA fragments from two mutants, MS7481 and MS9187, were found to be identical. The nucleotide sequence and the deduced amino acid sequence had 91 and 97% identity to *smpB* (for small protein) of *E. coli* (13), respectively. A computer search with the amino acid sequence of *smpB* from *E. coli* identified a homologous (41% identity; Fig. 2A) open reading frame (ORF) from *Enterococcus hirae* (49). The map position determined for the *S. typhimurium* homolog (Fig. 1; Table 1) corresponds to about 56.5 min on the *E. coli* genetic map, i.e., the location of *smpB* (40, 47).

The nucleotide sequence of 124 bp flanking the transposon insertion in MS3792 was determined and found to share 88% homology with *nagA*, the gene for *N*-acetylglucosamine-6-phosphate deacetylase of *E. coli* (44). The Tn*10* insertion site of this mutant corresponded to nucleotides 842 to 850 of the coding sequence of the *E. coli* homolog. The *nag* locus of *S. typhimurium* and the *nagA* gene of *E. coli* have both been mapped previously at 15 min (25, 47).

The nucleotide sequence of the cloned transposon-flanking DNA of MS1592 (490 bp) was identical to base pairs 28 to 518 of the nucleotide sequence of *htrA*, encoding a heat shock protein of *S. typhimurium* (29). The 9-bp repeat created by the Tn*10* insertion extended from base pair 114 to base pair 122 of the ORF of *htrA*. In *E. coli*, *htrA* is located at 3.7 min (33), which corresponds to 5 min on the *Salmonella* genetic map (46).

The nucleotide sequence of 119 bp flanking the transposon insertion of MS4290 was determined and displayed 81% homology to *prc*, a gene encoding a periplasmic protease of *E. coli*. The 9-bp repeat of the Tn*10* insertion site corresponded to nucleotides 1140 to 1148 of the coding sequence of the *E. coli* homolog. The *prc* locus maps to 40.4 min in *E. coli* (23).

Transposon-flanking DNA from a purine auxotroph mutant (MS4347) and a nonmotile mutant (MS3) was sequenced. Strain MS4347 was initially reported to have no phenotype (16) but was later found to be defective in purine biosynthesis (5a). The nucleotide sequence of the DNA flanking the transposon insertion in MS4347 was identical to *purD*, the gene encoding the 5′-phosphoribosyl glycinamide synthetase of *S. typhimurium* (3). The 9-bp repeat created by the transposon insertion covered base pairs 898 to 906 of the ORF of *purD*. The nucleotide sequence of transposon-flanking DNA from MS3 was identical to *fliD*, a flagellar hook protein of *S. typhimurium* (26). The 9-bp repeat created by the transposon insertion extended from base pair 86 to base pair 94 of the ORF of *fliD*. Mutants in *purD* and *fliD* are defective in purine biosynthesis and assembly of flagella, respectively. Therefore, the observed phenotypes of MS4347 and MS3 can be explained by Tn*10* insertions in these genes. Furthermore, the chromosomal locations determined by physical mapping (Table 1; Fig. 1) corresponded well with the published positions for *purD* (89 min) and *fliD* (40 min).

The nucleotide sequence of a 443-bp fragment flanking the Tn*10* insertion in strain MS5076 was found to overlap two ORFs. One of these had significant homology to ORF Bv′ and ORF Bv (23 and 31% identity, respectively); the second had significant homology to ORF B177 and ORF B175 (27 and 25% identity, respectively) of *Shigella boydii* (52). Tn*10* was inserted in the Bv′ and Bv homologs (Fig. 2B).

The nucleotide sequence of a 1,040-bp fragment flanking the Tn*10* insertion in strain MS1 contained a continuous ORF. The deduced amino acid sequence displayed homology to *adhE* of *E. coli* (37% identity; Fig. 2C) (30) and *adhE* of *Clostridium acetobutylicum* (26% identity; Fig. 2C; accession number x72831). These genes encode the alcohol dehydrogenases of *E. coli* and *C. acetobutylicum*, respectively.

Comparison of the nucleotide sequences of transposon-

A

```
MS7481                          SATIALNKR-RHEYFIEEEFEAGLALQGWEVKSLRAGK
                                ::::::::: ::::::::::::::::::::::::::::
E. coli   SmpB    M----TKKKAHKPGSATIALNKRARHEYFIEEEFEAGLALQGWEVKSLRAGKANIS
                  :      .  :    :: ::.:::.: : .  :::. ::: :.::.:   .  :.
E. hirae   19k    MNTSLEECHMPKGDGKLIAQNKKARHDYSIIDTMEAGMVLQGTEIKSIRNSRINLK

E. coli   SmpB    DSYVLLRDGEAFLFGANITPMAVASTHVVGDPTRTRKLLLNQRELDSLYGRVNREG
                  : .: .:.:::::     .:.:           :: :::::::.....   :      :
E. hirae   19k    DGFVRIRNGEAFLHNVHISPYEQG-NIFNHDPLRTRKLLLHKKQISRLETETKNTG

E. coli   SmpB    YTVVALSLYWKNAWCKVKIGVAKGKKQHDKRSDIKEREWQVDKARIMKNAHR 160
                   :.: : .: .. . :: ::.::::: .::: :.: ..        : .:: :
E. hirae   19k    VTIVPLKEYIRDGYAKVLIGLAKGKKSYDKREDLKRKDVDRQIDRTLKNFSR 163
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B

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S. boydii Bv'     RNGGIFYRSARDGYGFEADWSEFYTTTRKP-SAGDVGAYTQAECNSRFITGIRLGG
                        . ::                :          :           :  ..  :: :
MS5076            LSAFVASKPARQYITMVGVYQNDKTKPFMLHDDGSGVFLATTDMLSGYVQSIRFGA
                    :                  :   ::.   .   :. : :.:::::  ::.  .: :
S. boydii Bv      NKQFGIYMINNSRTANGTDGQAYMDNNGNWLCGSQVIPGNYGNFDSRYVKDVRLGS

S. boydii Bv'     -LLSV-QTWNGPGWSDRSGYVVTGSVNSNRDE-LIDTTQA--RPGIQYCINGTWYN
                   .     .  ::..:. :::.::   :  .  ..    :    :     :    .:   .:: ::
MS5076            -VEHG-NLYRSPGFADQLGYVITGVENGDSND-TPDRIQ---RRLLQLKVNGQWYT
                     : :  ...   :     :::.::.     :.     .  :  :     :  .:   .:: ::
S. boydii Bv      QQYYGVNNWQTWNFQCPSGYVLTGINVQDTGKNSADNIAGVHYRPVQKYINGTWYN

S. boydii Bv'* B177   AGSI  *  MMHLKNISAGNPETKEQYQLTKQFNIKWLYTEDGKN
                       :.        : : :: .      :  : .:.. .:... : : .
MS5076                VGA   *  MRHFKNFTKTTELTLVQQKLSENCSIQFIQDESGVD
                       :        : : ::       :. : .:.. .  ..  : : .
S. boydii Bv * B175   VASI  *  MMHLKNIKAGNAKTVEQYELTKKHGVIWLYSEDGKN
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C

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C.a. AdhE   EDKVIKNHFAGEYIYNKYKDEKTCGIIERNEPYGITKIAEPIGVVAAIIPVTNPT
            .::  ::  :         .  .  ..               :  :::::..  :  ::::.
MS1         DDKFAKNV-AQAARGTPGVECLSPQVLTGDN-GLTLIENAPWGVVASVTPSTNPA
            .::  ::  :         .   .  ::. :    :.      :  :..   .  :.::::.
E.c. AdhE   EDKVIKNHFASEYIYNAYKDEKTCGVLSEDDTFGTITIAEPIGIICGIVPTTNPT

C.a. AdhE   STTIFKSLISLKTRNGIFFSPHPRAKKSTILAAKTILDAAVKSGAPENIIGWIDE
            .: :   ..  .    :  .  :.:::  :::  .   :   .  .:  :       .      .
MS1         ATVINNAISLIAAGNSVVFAPHPAAKKVSQRAITLLNQAVVAAVVRKICWSPWRT
            .: :   ..  .    :...:.:::  :::.  .  .:   ::  ::  .::       :
E.c. AdhE   STAIFKSLISLKTRNAIIFSPHPRAKDATNKAADIVLQAAIAAGAPKDLIGWIDQ

C.a. AdhE   PSIELTQYLM--QKADITL--ATGGPSLVKSAYS-SGKPAIGVGPGNTPVIIDES
                  ..    .    : :    ::: . :. :          :   :   :  ::  :::..::.
MS1         ---RISNAQRLFKYPGIGLLVVTGGEAVVEPARKHTNKRLIAPGAGNPPVVVDET
                  .:::    . : : :.   :::   .:. :        . :   :   ::::  :::.:::
E.c. AdhE   PSVELSNALM--HHPDINLILATGGPGMVKAAYS-SGKPAIGVGAGNTPVVIDET

C.a. AdhE   AHIKMAVSSIILSKTYDNGVICASEQSVIVLKSIYNKVKDEFQERGAYIIKKNEL
            :  .   :   ::.    ..:: .::: :. .::   :.    ..       .  .  :   .    .
MS1         ADLPPAAQSIVKGASFDNNIICADEKVLIVVDSVAEQLMRLMEGQHAVKLTAAQR
            ::.   :   :..    .::: .::: :. ..::::: .  .                 :    .
E.c. AdhE   ADIKRAVASVLMSKTFDNGVICASEQSVVVVDSVYDAVRERFATHGGYLLQGKEL
                                .

C.a. AdhE   DKVREVIFKD------GSVNPKIVGQSAYTIAAMAGIKVPKTTRILIGEVTSLGE
            .... :. :.         :.:.    ::. :   :::   :. ::   ::.:   : :
MS1         EQLQPVLLKNIDERGKGTVSRDWVGRDAGKIAAAIGLNVPDQTRLLFVE-TPA--
            . .: :.:::         : ..    ::. : :::     : .::. :..:   : :
E.c. AdhE   KAVQDVILKN------GALNAAIVGQPAYKIAELAGFSVPENTKILIGEVTVVDE

C.a. AdhE   EEPFA-HEKLSPVLAMYEADNFDDALKKAVTLINLGGLGHTSGI-YAD-EIKARD
            . :::   :     ::: . :   :  ..:.        .:         .:          :  .  :
MS1         NHPFAVTEMMMPVLPVVRVANVEEAIAWRFSLKAVAPIGGDALAQYRQHEPDGER
              :::   :     :  :  .  :    .  :.:.        :  :.   ::          :  :  .  .  :
E.c. AdhE   SEPFA-HEKLSPTLAMYRAKDFEDAVEKAEKLVAMGGIGHTSCL-YTDQDNQPAR

C.a. AdhE   KIDRGCGSWGGNSISENVGPKHLL   438
            .  .               :  . .:
MS1         HRHQHFRQKRAVHCRAWIGRRRLD
                 :  ::
E.c. AdhE   --VSYFGQKMKTARILINTPASQG   404
```

FIG. 2. Alignment of the deduced amino acid sequences of transposon-flanking DNA from three MS mutants (indicated by their number) with published amino acid sequences from a data bank. Amino acids conserved in the *Salmonella* homolog are indicated by a colon; those with similar biochemical properties are indicated by a period. Dashes indicate gaps introduced to improve the alignment. (A) Alignment of the complete amino acid sequences of SmpB (*E. coli* SmpB) and 19k (*E. hirae* 19k) with the deduced amino acid sequence of transposon-flanking DNA from MS7481; (B) alignment of the amino acid sequence of the first ORF deduced from transposon-flanking DNA of MS5076 with the C-terminal part (the C terminus is indicated by an asterisk) of ORF Bv (*S. boydii* Bv) and ORF Bv′ (*S. boydii* Bv′) and alignment of the amino acid sequence of the second ORF deduced from transposon-flanking DNA of MS5076 with the N terminus of ORF B177 (*S. boydii* B177) and ORF B175 (*S. boydii* B175); C) alignment of the amino acid sequence deduced from transposon-flanking DNA of MS1 with the corresponding regions of AdhE of *E. coli* (E.c. AdhE) and AdhE of *C. acetobutylicum* (C.a. AdhE). The Tn*10* insertion site is at the C-terminal end of the sequence shown. Numbers at the ends of the sequence indicate the positions of amino acids in AdhE.

flanking DNA from 16 MS mutants with entries in a current data base indicated that the corresponding Tn*10* insertions defined loci which have not yet been sequenced in *S. typhimurium*. Each of these loci was designated by *ims* (for impaired macrophage survival), followed by a number indicating its approximate map location on the *S. typhimurium* chromosome in minutes (Table 1). The designation of loci rather than genes seems to be more appropriate since a polar effect of Tn*10* on the expression of downstream genes cannot be excluded. The nucleotide sequences of transposon-flanking DNA from 14 of these MS mutants displayed no significant homologies to any entries in the data base (Table 1). In some cases, this might have been due to the small size of the cloned transposon-flanking DNA (usually around 200 bp), which allowed detection of significant homologies only in highly conserved sequences, i.e., genes from *S. typhimurium* or closely related genera like *Escherichia*. The insertion sites of MS1633 and MS8467 were identical.

Phenotypic characterization of MS mutants. The map position and the nucleotide sequence from flanking DNA of the Tn*10* insertion in MS3792 indicated that the transposon was inserted in *nagA*, the gene encoding the *N*-acetylglucosamine deacetylase. This gene is necessary for the metabolism of *N*-acetylglucosamine. Therefore, *nagA* mutants are unable to degrade this amino sugar (58). We tested MS3792 and its parent (14028r) for growth on minimal medium with *N*-acetylglucosamine as the sole carbon source. In contrast to 14028r, MS3792 was not able to grow in defined medium when this amino sugar was provided as the sole carbon source (data not shown). No differences in growth were observed when glucose was the sole carbon source (data not shown). The observed phenotype confirmed the results from the genetic and molecular analyses, which had indicated that MS3792 carries an insertional mutation in the *nagA* gene.

DISCUSSION

When *S. typhimurium* enters the macrophage, it goes through various environmental changes, such as exposure to reactive oxygen species and to changes in pH, nutrient supply, and osmolarity. Thus, survival in this hostile environment requires differential expression not only of genes involved in defense against macrophage-killing mechanisms but also of other genes necessary for adaptation and maintenance of the microbe. These include genes necessary for repair of macrophage-induced damage and for adaptation to stresses induced by changes in pH, osmolarity, and differences in nutrient status. Mutations in all of these genes should be present in a library of mutants with a decreased ability to survive within macrophages, and several of these genes have already been identified. The *recA* and *recBC* genes, which are involved in repair of DNA damage, have recently been shown to be necessary for *Salmonella* survival within phagocytes and for full virulence in mice (8). *S. typhimurium* has been shown to express several stress-induced genes upon contact with macrophages, including *dnaK* and *groEL* (1, 7). Some of the macrophage-induced proteins were shown to be regulated by *phoPQ*, a two-component regulatory system similar to those that control environmental stresses in other bacteria (20, 37).

phoPQ mutants are attenuated and do not survive in professional phagocytes (15). Genes involved in de novo biosynthesis of nutrients like purine and aromatic compounds were also shown to be essential for intracellular survival (16). To identify other genes involved in macrophage survival, we characterized 30 MS mutants of *S. typhimurium* with regard to map location and nucleotide sequence homology. Most Tn*10* insertions analyzed during this study were dispersed randomly throughout the *Salmonella* genetic map (Fig. 1). However, the insertions of six MS mutants were located within a 3-min region (75 to 78 min) on the *Salmonella* genome. This is a silent region on the *S. typhimurium* chromosome in which only a few markers have been described to date (47). The localization of several *ims* loci here raises the possibility that this region of the *Salmonella* genome contains a virulence cluster.

Short fragments of chromosomal DNA adjacent to the Tn*10* insertion of 23 different MS mutants were cloned, and the nucleotide sequences were determined. The reliability of the cloning technique was confirmed by using a nonmotile mutant (MS3) and a purine auxotroph mutant (MS4347). The nucleotide sequences of DNA flanking the Tn*10* insertions of these mutants were found to be identical to *S. typhimurium fliD* and *purD*, respectively. Thus, molecular characterization provided a plausible explanation for the observed phenotypes as well as confirmation of the map locations determined for these two Tn*10* insertions.

S. typhimurium induces the synthesis of more than 30 stress proteins during infection of macrophages. It was reported that MS4347 fails to synthesize at least six macrophage-induced proteins detected in wild-type *S. typhimurium* (7). This mutant was later found to have a defect in purine biosynthesis (5a), which was confirmed by our finding that MS4347 carries Tn*10* inserted in *purD*. A purine auxotroph may be able to synthesize normal protein levels early during infection of macrophages. However, when available purines have been utilized for mRNA synthesis, bacterial protein synthesis will cease. Therefore, the stress proteins which are not expressed in MS4347 might be encoded by genes which are induced in later stages of macrophage infection. Alternatively, these proteins may have a short half-life and may therefore escape detection soon after cessation of protein biosynthesis.

MS3 is attenuated after intraperitoneal injection in *ity*^{s} mice, having a 10^3-fold-higher 50% lethal dose (LD_{50}) than the parent when tested in a smooth-strain background (17). The decrease in virulence cannot be explained by the lack of flagella since nonflagellated *Salmonella* mutants have been shown to be as virulent as their flagellated parents (9). Recently, a different class of nonflagellated *S. typhimurium* mutants, carrying a deletion termed Δ*flgABCDE25*, has been reported to be attenuated (10). Like *fliD* mutants, *S. typhimurium* Δ*flgABCDE25* mutants were more sensitive to intracellular killing mechanisms of murine macrophages (57). Interestingly, mutations in *fliD* and Δ*flgA-J* deletions have in common that they prevent flagellum assembly but not flagellin production (18, 19). In contrast, strains carrying monocistronic mutations in the flagellar middle genes *flgA*, *flgB*, *flgC*, *flgD*, and *flgE* produce no flagellin and have been shown to be fully virulent (9, 18). One possible explanation for the attenuation of *fliD* mutants might therefore be that the expression of flagellin (which is produced in several thousand copies per cell) in the absence of flagellum assembly is detrimental during growth of the bacteria within macrophages of the host.

S. typhimurium strains carrying a mutation in *htrA* (high temperature requirement) have been shown by others to be attenuated (29). The impaired ability to survive or replicate in host tissues led those authors to the suggestion that *htrA* mutants might be impaired in macrophage survival. The finding that one MS mutant (MS1592) carries a Tn*10* lesion in *htrA* now confirms this hypothesis. However, the possibility that the observed phenotype is due to polar effects on as-yet-unidentified genes located downstream of *htrA* cannot be excluded. The heat shock gene *htrA* of *E. coli* encodes a protease which has been shown to degrade abnormally folded proteins located in the periplasm (51). Mutants in *htrA* exhibit a pleiotrophic phenotype which includes temperature sensitivity and leakage of periplasmic proteins into the culture medium (33). The latter phenotype can be suppressed by a secondary mutation in *pldA* which encodes the outer membrane phospholipase A of *E. coli* (12). A mutation in a distinct locus, termed *prc*, causes similar phenotypic changes in *E. coli*. The *prc* gene encodes a proteolytic enzyme that also functions in the periplasm. Like *htrA*, loss of *prc* function results in temperature sensitivity of bacterial growth and leakage of periplasmic proteins. The latter phenotype of *prc* mutants can be suppressed by a *pldA* mutation (23). Mutant MS4290 had a mutation in *prc*. Thus, mutations in two distinct genes, both with similar phenotypes, were selected in the screening for macrophage sensitivity. The periplasmic proteases encoded by *htrA* and *prc* may serve as stress response proteins by degrading abnormally folded peptides generated as a result of macrophage-induced environmental stress, thus preventing their accumulation to toxic levels in the periplasm.

MS3792 is attenuated after intraperitoneal injection in *ity*^{s} mice, having a 10^4-fold-higher LD_{50} than the parent when tested in a smooth-strain background (17). We found that in this mutant, Tn*10* is inserted in *nagA*, the gene encoding the *N*-acetylglucosamine-6-phosphate deacetylase of *S. typhimurium*. The *nag* locus has been studied in detail in *E. coli* and consists of the operon *nagBACD* and the divergently transcribed gene *nagE* which encodes enzyme II of the *N*-acetylglucosamine phosphotransferase system (44). *N*-Acetylglucosamine-6-phosphate, which is bound by the repressor *nagC* as the inducer, accumulates in the absence of *N*-acetylglucosamine-6-phosphate deacetylase. Therefore, mutations in *nagA* cause a constitutive expression of all *nag* genes (45, 56). The repressor *nagC* also controls the mannose phosphotransferase system, the second import system involved in amino sugar uptake. To prevent a futile cycle, the degradation and biosynthesis pathways for amino sugars cannot be expressed simultaneously. However, the mechanism responsible for this regulation has not been elucidated. Thus, the attenuation of *Salmonella nagA* mutants might be the direct result of the defect in utilization of *N*-acetylglucosamine as a carbon source. More likely, however, is the possibility that it results from an indirect effect of reduction in biosynthesis of amino sugars, which are essential components of the cell wall and outer membrane.

Two MS mutants, MS7481 and MS9187, carried transposon insertions in a small ORF termed *smpB* in *E. coli* and the 19k gene in *E. hirae* (13, 49). The most striking feature of the predicted proteins is their high, positive charges which, in the case of SmpB, results in a calculated pI of 9.9. Properties of mutants in *smpB* or the 19k gene have not yet been described nor have functions for the corresponding gene products been proposed. However, the conservation among gram-positive and gram-negative bacteria suggests a housekeeping function of *smpB*.

A significant homology to *adhE* of *E. coli* and *C. acetobutylicum* indicated that strain MS1 carried an insertion in a gene encoding a dehydrogenase-like protein (30). However, the map location (27 min; the map location of *adhE* in *E. coli* corresponds to about 35 min on the *Salmonella* genetic map;

the Tn*10* insertion in MS1 is located at 50 min) and the relatively low sequence conservation between *E. coli adhE* and this gene suggested that it is different from *adhE*. Additional experiments are needed to elucidate whether this gene fulfills one of the dehydrogenase-related functions believed to play a role during infection. For example, some dehydrogenases have been shown to help in maintaining a reducing environment in the cell, thus preventing oxidative damage (reviewed in reference 14). Furthermore, a dehydrogenase has recently been found to be associated with the communication between host and parasite during infection (41).

The transposon insertion in MS5076 is located in a region which shares homology to the B segment-*pin* gene (B-*pin*) inversion system of *S. boydii* (52). In *Salmonella* spp., the phase variation of flagella is controlled by the H-*hin* system (50). However, the B-*pin* system is different in organization and more closely related to inversional switching systems in bacteriophages Mu (G-*gin*) and P1 (C-*cin*) (28, 31). The invertible segments (G or C) of both phages encode two different sets of tail fiber genes. Upon inversion, the amino-terminal part of one tail fiber gene (gene S of phage Mu or gene 19 of phage P1), which is located outside the invertible region, is fused to a different carboxy-terminal part inside the region. This mechanism controls the host specificity of the phage (27, 53). Interestingly, the amino-terminal part of this tail fiber protein, which in phage Mu is responsible for the attachment of the tail fiber to the base plate, is not conserved in the *S. boydii* system (52). This led to the speculation that the *Shigella* B-*pin* element might be part of a defective prophage which converted to a system more useful to the bacterial host (54). Experiments are under way in our laboratory to determine whether Tn*10* has inserted in a functional inversion system in strain MS5076. An inversion system would provide an attractive explanation for the observation that *S. typhimurium* consists of two different populations inside macrophages (2).

The fact that the majority of transposon insertions affecting macrophage survival were found to be in *Salmonella* genes that have not been sequenced explains why our understanding of the virulence mechanisms of this otherwise-well-studied organism is still incomplete. Since many *Salmonella* genes are organized in operons, Tn*10* insertion may in some cases be polar and therefore define loci rather than genes involved in virulence. Our laboratory is currently engaged in further characterization of *ims* loci to evaluate their role in *Salmonella* pathogenesis.

ACKNOWLEDGMENTS

We thank R. Tsolis and S. Lindgren for critically reading the manuscript and K. Sanderson for kindly providing *Salmonella* strains carrying MudP22 prophages.

A.J.B. was supported by fellowship Ba1337/1-2 from the Deutsche Forschungsgemeinschaft. J.G.K. was supported by a fellowship of the Royal Netherlands Academy of Arts and Sciences, The Netherlands.

REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. J. Bacteriol. **175:**3734–3743.
2. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Growth rate paradox of *Salmonella typhimurium* within host macrophages. J. Bacteriol. **175:**3744–3748.
3. **Aiba, A., and K. Mizobuchi.** 1989. Nucleotide sequence analysis of genes *purH* and *purD* involved in the *de novo* purine nucleotide biosynthesis of *Escherichia coli*. J. Biol. Chem. **264:**21239–21246.
4. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
5. **Benson, N. R., and B. S. Goldman.** 1992. Rapid mapping in *Salmonella typhimurium* with Mu*d*-P22 prophages. J. Bacteriol. **174:**1673–1681.

5a. **Buchmeier, N.** Unpublished data.

6. **Buchmeier, N. A., and F. Heffron.** 1988. Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. Infect. Immun. **57:**1–7.
7. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248:**730–732.
8. **Buchmeier, N. A., C. J. Lipps, M. Y. H. So, and F. Heffron.** 1993. Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. Mol. Microbiol. **7:**933–936.
9. **Carsiotis, M., B. A. D. Stocker, D. L. Weinstein, and A. D. O'Brien.** 1989. A *Salmonella typhimurium* virulence gene linked to *flg*. Infect. Immun. **57:**3276–3280.
10. **Carsiotis, M., D. L. Weinstein, H. Karch, I. A. Holder, and A. D. O'Brien.** 1984. Flagella of *Salmonella typhimurium* are a virulence factor in infected C57BL/6J mice. Infect. Immun. **46:**814–818.
11. **Carter, P. B., and F. M. Collins.** 1974. The route of enteric infection in normal mice. J. Exp. Med. **139:**1189–1203.
12. **Cavard, D., C. Lazdunski, and P. Howard.** 1989. The acylated precursor form of the colicin A lysis protein is a natural substrate of the DegP protease. J. Bacteriol. **171:**6316–6322.
13. **Chauhan, A. K., and D. Apirion.** 1989. The gene for a small stable RNA (10Sa RNA) of *Escherichia coli*. Mol. Microbiol. **3:**1481–1485.
14. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress response in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
15. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1062.
16. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
17. **Gahring, L. C., F. Heffron, B. B. Finlay, and S. Falkow.** 1990. Invasion and replication of *Salmonella typhimurium* in animal cells. Infect. Immun. **58:**443–448.
18. **Gillen, K. L., and K. T. Hughes.** 1991. Negative regulatory loci coupling flagellin synthesis to flagellar assembly in *Salmonella typhimurium*. J. Bacteriol. **173:**2301–2310.
19. **Gillen, K. L., and K. T. Hughes.** 1991. Molecular characterization of *flgM*, a gene encoding a negative regulator of flagellin synthesis in *Salmonella typhimurium*. J. Bacteriol. **173:**6453–6459.
20. **Groisman, E. A., E. Chiao, C. L. Lipps, and F. Heffron.** 1989. *Salmonella typhimurium phoP* is a transcriptional regulator. Proc. Natl. Acad. Sci. USA **86:**7077–7081.
21. **Halling, S. M., and N. Kleckner.** 1982. A symmetrical six base pair target site sequence determines Tn*10* insertion specificity. Cell **28:**155–163.
22. **Halling, S. M., R. W. Simons, J. C. Way, R. B. Walsh, and N. Kleckner.** 1982. DNA sequence organization of IS*10*-right of Tn*10* and comparison with IS*10*-left. Proc. Natl. Acad. Sci. USA **79:**2608–2612.
23. **Hara, H., Y. Yamamoto, A. Higashitani, H. Suzuki, and Y. Nishimura.** 1991. Cloning, mapping, and characterization of the *Escherichia coli prc* gene, which is involved in C-terminal processing of penicillin-binding protein 3. J. Bacteriol. **173:**4799–4813.
24. **Hohman, A. W.** 1978. Intestinal colonization and virulence of *Salmonella* in mice. Infect. Immun. **22:**763–770.
25. **Holmes, R. P., and R. R. B. Russell.** 1972. Mutations affecting amino sugar metabolism in *Escherichia coli* K-12. J. Bacteriol. **111:**290–291.
26. **Homma, M., D. J. DeRosier, and R. M. Macnab.** 1990. Flagellar hook and hook-associated proteins of *Salmonella typhimurium* and their relationship to other axial components of the flagellum. J. Mol. Biol. **213:**819–832.
27. **Iida, S.** 1984. Bacteriophage P1 carries two related sets of genes

determining its host range in the invertible C segment of its genome. Virology **134**:421–434.
28. **Iida, S., J. Meyer, K. E. Kennedy, and W. Arber.** 1982. A site-specific, conservative recombination system carried by bacteriophage P1. Mapping the recombinase gene *cin* and the cross over sites *cix* for the inversion of the C segment. EMBO J. **1**:1445–1453.
29. **Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5**:401–407.
30. **Kessler, D., I. Leibrecht, and J. Knappe.** 1991. Pyruvate-formate-lyase-deactivase and acetyl-CoA reductase activities of *Escherichia coli* reside on a polymeric protein particle encoded by *adhE*. FEBS Lett. **281**:59–63.
31. **Koch, C., G. Mertens, F. Rudt, R. Kahmann, R. Kanaar, R. Plastere, P. van de Putte, R. Sandulache, and D. Kamp.** 1987. The invertible G segment, p. 75–91. *In* N. Symonds, A. Tousaint, P. van de Putte, and M. M. Howe (ed.), Phage mu. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand.** 1988. Using mini-prep plasmid DNA for sequencing double stranded templates with sequenase. BioTechniques **6**:544–546.
33. **Lipinska, B., O. Fayet, L. Baird, and C. Georgopoulos.** 1989. Identification, characterization, and mapping of the *Escherichia coli htrA* gene, whose product is essential for bacterial growth only at elevated temperatures. J. Bacteriol. **171**:1574–1584.
34. **Lissner, C. R., R. N. Swanson, and A. D. O'Brien.** 1983. Genetic control of the genetic resistance of mice to *Salmonella typhimurium*: expression of the *ity* gene in peritoneal and splenic macrophages in vitro. J. Immunol. **131**:3006–3013.
35. **Liu, S.-L., and K. E. Sanderson.** 1992. A physical map of the *Salmonella typhimurium* LT2 genome made by using *Xba*I analysis. J. Bacteriol. **174**:1662–1672.
36. **McClelland, M., J. Hanish, M. Nelson, and Y. Patel.** 1988. KGB: a single buffer for all restriction endonucleases. Nucleic Acids Res. **16**:364.
37. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243**:916–922.
38. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Ochman, H., M. M. Medhora, D. Garza, and D. L. Hartl.** 1990. Amplification of flanking DNA by inverse PCR, p. 219–227. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. Academic Press, Inc., San Diego, Calif.
40. **Oh, B. K., A. K. Chauhan, K. Isono, and D. Apirion.** 1990. Location of a gene (*ssrA*) for a small stable RNA (10Sa RNA) in the *Escherichia coli* chromosome. J. Bacteriol. **172**:4708–4709.
41. **Pancholi, V., and V. A. Fischetti.** 1993. Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. Proc. Natl. Acad. Sci. USA **90**:8154–8158.
42. **Plant, J. E., and A. Glynn.** 1976. Genetics of resistance to infection with *Salmonella typhimurium* in mice. J. Infect. Dis. **133**:72–78.
43. **Plant, J. E., and A. Glynn.** 1979. Locating *Salmonella* resistance gene on mouse chromosome 1. Clin. Exp. Immunol. **37**:1–6.
44. **Plumbridge, J. A.** 1989. Sequence of the *nagBACD* operon in *Escherichia coli* K12 and pattern of transcription within the *nag* regulon. Mol. Microbiol. **3**:505–515.
45. **Plumbridge, J. A.** 1991. Repression and induction of the *nag* regulon of *Escherichia coli* K-12: the roles of *nagC* and *nagA* in maintenance of the uninduced state. Mol. Microbiol. **5**:2053–2062.
46. **Riley, M., and S. Krawiec.** 1987. Genome organization, p. 967–981. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
47. **Sanderson, K. E., and J. A. Hurley.** 1987. Linkage map of *Salmonella typhimurium*, p. 877–918. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
49. **Shibata, C., T. Ehara, K. Tomura, K. Igarashi, and H. Kobayashi.** 1992. Gene structure of *Enterococcus hirae* (*Streptococcus faecalis*) F_1F_0-ATPase, which functions as a regulator of cytoplasmic pH. J. Bacteriol. **174**:6117–6124.
50. **Silverman, M., J. Zieg, M. Hilmen, and M. Simon.** 1979. Phase variation in *Salmonella*: genetic analysis of a recombinational switch. Proc. Natl. Acad. Sci. USA **76**:391–395.
51. **Strauch, K. L., and J. Beckwith.** 1988. An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. Proc. Natl. Acad. Sci. USA **85**:1576–1580.
52. **Tominaga, A., S. Ikemizu, and M. Enomoto.** 1991. Site-specific genes in three *Shigella* subgroups and nucleotide sequences of a *pinB* gene and an invertible B segment from *Shigella boydii*. J. Bacteriol. **173**:4079–4087.
53. **Van de Putte, P., S. Cramer, and M. Giphart-Gassler.** 1980. Invertible DNA determines host specificity of bacteriophage Mu DNA. Nature (London) **286**:218–222.
54. **Van de Putte, P., and N. Goosen.** 1992. DNA inversions in phages and bacteria. Trends Genet. **8**:457–462.
55. **Vidal, M. V., D. Malo, K. Vogan, E. Skamene, and P. Gros.** 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for *Bcg*. Cell **73**:469–485.
56. **Vogler, A. P., and J. W. Lengeler.** 1989. Analysis of the *nag* regulon from *Escherichia coli* K12 and *Klebsiella pneumoniae* and its regulation. Mol. Gen. Genet. **219**:97–105.
57. **Weinstein, D. L., M. Carsiotis, C. R. Lessner, and A. D. O'Brien.** 1984. Flagella help *Salmonella typhimurium* survive within murine macrophages. Infect. Immun. **46**:819–825.
58. **White, R. J.** 1968. Control of amino sugar metabolism in *E. coli* and isolation of mutants unable to degrade amino sugars. Biochem. J. **106**:847–858.
59. **Wong, K. K., and M. McClelland.** 1992. A *Bln*I restriction map of the *Salmonella typhimurium* LT2 genome. J. Bacteriol. **174**:1656–1661.
60. **Youderian, P., P. Sugiono, K. L. Brewer, N. P. Higgins, and T. Elliott.** 1988. Packaging specific segments of the *Salmonella* chromosome with locked in Mu*d*-P22 prophages. Genetics **118**:581–592.