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19. Antibody RPA-199 was obtained as follows. HMG1 (300 μg) purified from rat liver (*10*) was separated by electrophoresis on a 15% SDS-polyacrylamide gel. The gel slice containing HMG1 was excised after staining with Coomassie blue, minced, and administered subcutaneously with Freund's adjuvant to a New Zealand White (NZW) rabbit over 3 weeks. The animals serum was incubated with 300 μg of HMG1 blotted onto a strip of Immobilon; the strip was washed extensively with TBSN (50 m*M* tris-HCl, *p*H 8.0, 150 m*M* NaCl, and 0.1% nonidet P-40); the specific antibodies were eluted with 0.1*M* glycine-HCl, *p*H 2.5, and immediately neutralized. Affinity-purified RPA-199 reacted with a single band with an apparent molecular size of 27 kD on blots of total extracts of rat, mouse, and human cells.
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A *Salmonella* Locus That Controls Resistance to Microbicidal Proteins from Phagocytic Cells

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Facultative intracellular pathogens pose an important health problem because they circumvent a primary defense mechanism of the host: killing and degradation by professional phagocytic cells. A gene of the intracellular pathogen *Salmonella typhimurium* that is required for virulence and intracellular survival was identified and shown to have a role in resistance to defensins and possibly to other microbicidal mechanisms of the phagocyte. This gene may prove to be a regulatory element in the expression of virulence functions.

PROFESSIONAL PHAGOCYTIC CELLS are important in the body's defense against pathogenic microorganisms; they engulf and kill invading microbes by both oxygen-dependent and oxygen-independent mechanisms. The phagocyte undergoes a burst of respiration upon initial contact with the invading organism and produces toxic metabolites of oxygen such as hydrogen peroxide and superoxide (*1*). Oxygen-independent mechanisms include acidification of the phagosome (the membrane-lined vacuole in which the ingested microorganisms are enclosed) and degradation of its contents by proteins or peptides with antimicrobial activity; these antimicrobial substances are released from cytoplasmic granules that fuse with the phagosome to form a phagolysosome (*2, 3*). Facultative intracellular pathogens avoid killing by phagocytic cells and can persist in the host, with debilitating diseases often the result (*4*). Survival mechanisms used by intracellular pathogens include inhibition of the respiratory burst, inhibition of phagolysosome fusion, and survival within or escape from the phagolysosome (*3*). The molecular basis of these survival strategies remains largely unknown. *Salmonella typhimurium* is a facultative intracellular pathogen of mice that has been extensively used as a model system for human typhoid, a worldwide health problem, with 12.5 million cases annually (*5*). In this report, we describe a *Salmonella* gene that is necessary for virulence in the mouse and survival in the macrophage in vitro, and we show that the gene is required for resistance to defensins, microbicidal peptides found in neutrophils and macrophages. These results may have clinical relevance since patients with "specific granule deficiency," who have frequent and severe infections, almost completely lack defensins (*6*).

A molecular genetic analysis of intracellular survival was started in our laboratory with the isolation of 83 Tn*10* insertion mutants of *S. typhimurium* that were unable to survive in mouse peritoneal macrophages in vitro. All of the mutant strains were subsequently shown to be attenuated in the mouse (*7*). A subset of these mutants, represented by MS4252s, MS5996s, and MS7953s (*8*), were of particular interest in that (i) they had the highest median lethal dose (LD_{50}) of all the mutants isolated (10^5 organisms compared to <10 for the parent strain by intraperitoneal injection of BALB/c mice) (*9*); (ii) they did not persist in the mouse (no bacteria were detectable 72 hours after injections), they did not stimulate a significant humoral or cell-mediated immune response, and they did not protect a mouse against challenge with the virulent parent strain. This is in contrast to other avirulent Tn*10* mutants isolated in the same study that persisted for up to 2 months and stimulated T cell and B cell responses as well as protective immunity against challenge with virulent strains (*10*). These results suggested that the mutants may be defective in an important virulence determinant.

We investigated the possibility that these mutants were more sensitive to specific antimicrobial mechanisms of the phagocyte. Crude extracts from human neutrophils and rabbit peritoneal macrophages had a strong microbicidal effect on these mutants (Fig. 1, A and B). The sensitivity to granule extracts was not due to a lipopolysaccharide (LPS) defect, since all the mutants showed the same phage sensitivity profile and had a complete LPS (smooth) identical to that of the parent strain when purified and visualized on polyacrylamide gel electrophoresis (PAGE) (*9*). In an attempt to identify a specific component of the extract to which the mutants were sensitive, we fractionated the rabbit extract on an anion exchange column and tested the mutants with individual fractions. Those fractions that were most active against the mutants contained low

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molecular weight proteins that might correspond to defensins (*11*).

Defensins are a family of small molecular weight cationic peptides of 29 to 34 amino acids that have antibacterial, antifungal, antiviral, and cytotoxic activities in vitro; they have been found in rabbits, humans, and guinea pigs (*12*). In mice, a defensin-like precursor mRNA has been identified as being among the most abundant transcripts in cells of the small intestine (*13*). Defensins are present in large amounts in granules of neutrophils and macrophages, and in human polymorphonuclear leukocytes they amount to more than 5% of total protein by weight. A homogeneously pure preparation of rabbit defensin NP-1 showed strong bactericidal effect on the mutants. The viability of the mutants was 100- to 1000-fold lower than that of the wild-type strain after incubation with purified NP-1 (Fig. 1C). The sensitivity of the mutants to defensins is fairly specific, since they showed a response to cathepsin G and lysozyme that was equivalent to that of the wild-type strain; these two compounds are also found in the host granules. The defect in these mutants is not a nonspecific alteration of membrane permeability, since they behaved like the wild-type strain in their sensitivity to the detergents sodium deoxycholate and sodium dodecyl sulfate, to the antibiotics ampicillin, erythromycin, and novobiocin, to the small molecular weight bacteriocins colicin V and microcin B17, and to a number of oxidizing and DNA-damaging agents (*11*). The mutants but not the wild-type strain were extremely sensitive to magainins (*14*). Magainins are microbicidal peptides 23 amino acids in length found in frog skin (*15*).

Molecular and genetic analyses of the mutants were performed to determine which gene or genes were altered. Southern hybridization analysis of DNA from the three mutants indicated that each of the mutant strains contained only one Tn*10* transposon, that the insertions were present in the same 6-kb Pst I fragment, and that they were clustered within 500 bp of each other (Fig. 2). These results were later confirmed by analysis of restriction enzyme digests of plasmid clones of each of the Tn*10* insertions and of the wild-type clone (*16*). Conventional phage P22 transduction experiments showed that the Tn*10* insertions in the three mutants were linked to *purB* at 25 min on the *S. typhimurium* chromosome (*17*). Three genes have been mapped to this region of the *S. typhimurium* genome: *purB*, involved in purine biosynthesis; *phoP*, necessary for the production of nonspecific acid phosphatase; and *pepT*, encoding a tripeptidase (*18*). A series of experiments showed that the defensin-sensitive phenotype of the three mutants was due to mutation of the *phoP* gene. (i) The Tn*10* mutants did not have nonspecific acid phosphatase activity and were prototrophs showing no requirement for purines. The Tn*10* insertions of

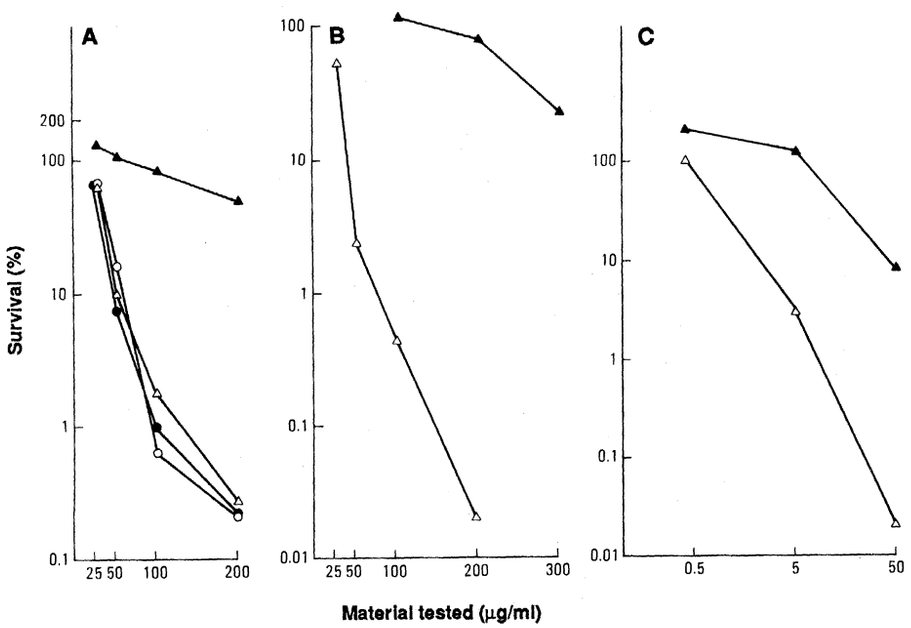

Fig. 1. Sensitivity of mutants to products of the phagocyte. (**A**) Sensitivity to crude granule extracts from human neutrophils (*26*). (**B**) Sensitivity to crude granule extracts from rabbit peritoneal macrophages (*27*). (**C**) Sensitivity to purified rabbit defensin NP-1. For (A) and (B), log phase cells were pelleted, washed once with phosphate-buffered saline (PBS), and diluted to 2×10^4 cfu/ml in 0.5% tryptone and 0.5% NaCl. Diluted material (50 μl) was placed in 96-well microtiter dishes, and granule extract was added in 50 μl of tryptone-NaCl to the final concentration shown. Plates were incubated at 37°C for 1 hour with shaking. A portion was plated on an LB plate to determine the number of viable bacteria. Data are presented as percent survival relative to cells incubated without extract. Survival of more than 100% shows the growth of the organisms during the incubation period. For (C) the experimental protocol was similar except that the cells were diluted to 5×10^4 cfu/ml in 1% tryptone, the rabbit defensin NP-1 was resuspended in 0.01% acetic acid to the indicated concentration, and the incubation was carried out for 2 hours at 37°C with shaking. A homogeneous pure preparation of defensin NP-1 purified by reversed-phase high performance liquid chromatography, and confirmed by PAGE and amino acid sequence analysis, was used for these experiments (*28*). (▲) 14028s, (○) MS4252s, (●) MS5996s, and (△) MS7953s. Mutants MS4252s and MS5996s behaved like mutant MS7953s in their sensitivity to neutrophil extracts, to defensin NP-1, virulence in vivo in mice, and lack of production of nonspecific acic phosphatase. Mutant MS7953s is presented as the prototype of this family of *phoP* mutants. The results are for a typical experiment from three independent trials.

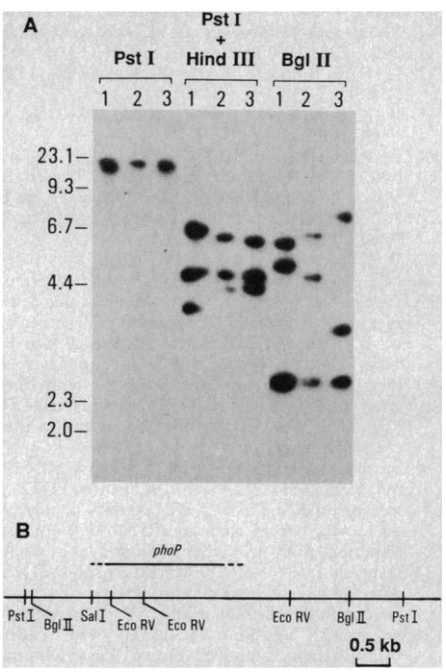

Fig. 2. Analysis of transposon insertions in the *S. typhimuirum phoP* region. (**A**) Southern hybridization of DNA from (lanes 1) MS4252s, (lanes 2) MS5996s, and (lanes 3) MS7953s, digested with the enzymes indicated and probed with plasmid pNK370, which contains a Tn*10* transposon deleted between positions 4827 and 8964. This deletion removes two of the three Hind III sites of Tn*10* so that hybridization to the *Salmonella*-Tn*10* junction fragments and to the 4.7-kb internal fragment, but not to the 500-bp Hind III internal fragment, is observed. There are no Pst I sites in Tn*10*, and the same single band is seen for the three mutants when digested with Pst I. There are two Bgl II sites in Tn*10*, so hybridization is observed for the 2.9-kb internal fragment and to the *Salmonella*-Tn*10* junction fragments. A complete restriction map of transposon Tn*10* can be found in (*29*). (**B**) Restriction map of the wild-type *phoP* region. The transposon insertions were mapped to the small 500-bp Eco RV fragment for MS4252s and MS7953s, and immediately to the right of this fragment, in the large 2100-bp Eco RV fragment for MS5996s.

these mutants showed 95% linkage to *purB*, the same as previously described for other *phoP* alleles (*19*). Furthermore, plasmid clones of the original Tn*10* insertions complemented a *purB* deficiency (*16*). (ii) A *pepT* mutant remained fully virulent in vivo, but a strain with a transposon insertion originally described as linked to *pepT* (TnΔ10Cm^r zce::1620) lacked nonspecific acid phosphatase activity, and was avirulent in vivo with the same LD_{50} as the Tn*10*-generated mutants (*20*). (iii) Strains with the chemically induced *phoP12* (ICR-372–induced) or *phoP22* (diethyl sulfate–induced) alleles (*19*) lacked nonspecific acid phosphatase and were as sensitive to the defensin NP-1 as the Tn*10*-generated mutants (Fig. 3). (iv) Plasmid DNA clones corresponding to the wild-type gene, when introduced into the mutant strain MS7953, complemented the defensin-sensitive phenotype of the Tn*10* mutant, made it virulent in vivo with the same LD_{50} as that of the wild-type strain, and also rendered it *phoP*$^+$ (*16*).

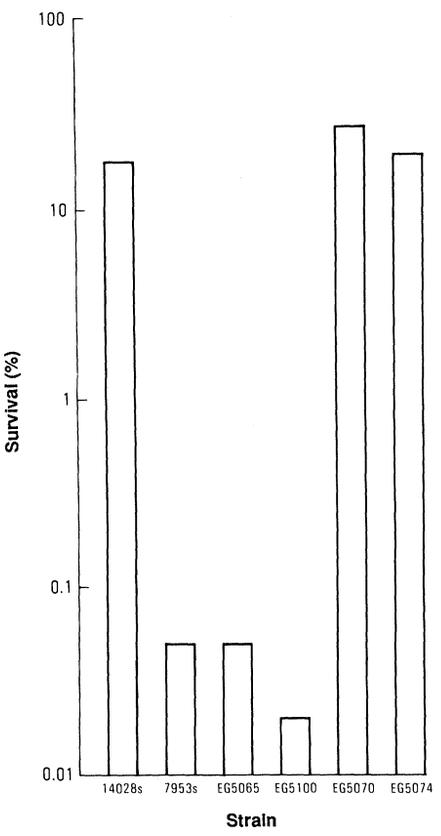

Fig. 3. Sensitivity of wild-type *phoP* and *phoN* strains to purified defensin NP-1. Relevant genotype of the strains tested (*20*): EG5065, *phoP12*; EG5100, *phoP22*; EG5070, *phoN2*; and EG5074, *phoN3*. Experiments were performed as described in the legend to Fig. 1, except that a single concentration of defensins (50 μg/ml) was used. Isogenic strains harboring the wild-type alleles for *phoP* and *phoN* linked to the same transposon insertion used to construct the mutant strains were also tested and behaved like the wild-type strains (*14*). The results are for a typical experiment from three independent trials.

The *phoP* gene product has been hypothesized to be a regulator of *phoN*, the structural gene for nonspecific acid phosphatase (*19*). Mutants that lack nonspecific acid phosphatase because they have the *phoN2* or *phoN3* alleles (*19, 20*) were fully virulent in the mouse (*14*) and behaved like the wild-type strain in their sensitivity to defensin NP-1 (Fig. 3). This clearly shows that the lack of this phosphatase is not responsible for avirulence and sensitivity to defensins. The production of nonspecific acid phosphatase is induced by carbon, sulfur, phosphorus, or nitrogen starvation (*21*), and *phoP* mutants showed poor growth when succinate was present as the sole carbon source (*14*). Thus, *phoP* may code for a regulator that controls expression of *phoN* as well as other genes in *S. typhimurium*, some of them virulence loci, in response to environmental stimuli. The deduced amino acid sequence of the *phoP* gene product is similar to sequences of regulatory proteins such as PhoB and OmpR (*22*) of the family of two-component regulators of gene expression that have been described for several operons in prokaryotes (*23*).

We believe that *phoP* is the first *Salmonella* gene to be described that is responsible for resistance to a known antimicrobial mechanism of the host—that is, the production of defensins. The *phoP* mutants should be an important tool for the molecular elucidation of the mechanism of action of defensins and magainins. Both peptides, though structurally unrelated, are believed to act by inserting into the membrane and causing an increase in the permeability to ions (*24*). It is interesting that the *phoP* mutants, when present in a complete core rough background, are hypersensitive to serum complement (*7*). As the membrane attack complex of complement is also believed to act by forming transmembrane channels (*25*), the *phoP* mutants may lack the defense mechanism against several unrelated pore-forming molecules. The *phoP* mutants will provide a useful assay for the isolation and purification of products of the mouse macrophage and neutrophil that have an antimicrobial activity functionally equivalent to that of the defensins.

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Reciprocal Effects of Hyper- and Hypoactivity Mutations in the *Drosophila* Pattern Gene *torso*

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In *Drosophila*, five "terminal" polarity genes must be active in females in order for them to produce embryos with normal anterior and posterior ends. Hypoactivity mutations in one such gene, *torso*, result in the loss of the most posterior domain of *fushi tarazu* expression and the terminal cuticular structures. In contrast, a *torso* hyperactivity mutation causes the loss of central *fushi tarazu* expression and central cuticular structures. Cytoplasmic leakage, transplantation, and temperature-shift experiments suggest that the latter effect is caused by abnormal persistence of the *torso* product in the central region of the embryo during early development. Thus, the amount and timing of *torso* activity is key to distinguishing the central and terminal regions of the embryo. Mutations in the *tailless* terminal gene act as dominant maternal suppressors of the hyperactive *torso* allele, indicating that the *torso* product acts through, or in concert with, the *tailless* product.

THREE CLASSES OF MATERNAL EFFECT pattern genes specify the anterior-posterior axis of the *Drosophila* embryo (*1, 2*). When females carry "anterior" mutations, their progeny lack acronal, gnathal, and thoracic structures. Loss of the abdominal region (and the germ cells) occurs in offspring of the "posterior" or grandchildless-*knirps* class mutants. The "terminal" or *torso*-like genes comprise six loci that are involved in specifying the asegmental anterior (acron) and posterior (telson) structures of the embryo (Fig. 1, A and B) (*1–4*). Five of these are maternal effect genes [*torso* (*1*), *trunk* (*1*), *torsolike* (*5*), *fs* (*1*) *Nasrat* (*3*), and *fs* (*1*) *polehole* (*3*)] and one is zygotic in action [*tailless* (*4*)]. Here we focus on the mechanism of *torso* (*tor*) gene action. We show that hyper- and hypoactivity mutations in *tor* lead to reciprocal pattern defects. We investigate the basis for these effects and discuss their implications for the role of *tor* in establishing embryonic pattern.

Mothers homozygous for the $spliced^{RL3}$ mutation (*6*) produce embryos that lack thoracic and abdominal structures and have only an acron and a telson (Fig. 1E and Table 1B). The *spliced* mutation is semidominant: at 25°C heterozygous females produce offspring lacking up to three abdominal segments, with the second and third abdominal segments showing the greatest sensitivity (Table 1D). The *spliced* phenotype is complementary to that produced in progeny of females homozygous for hypoactivity mutations in the terminal class genes (compare in Fig. 1, B with C through E) (*1–4*). We have used chromosomal deficiencies and a duplication for the *tor* region to demonstrate that the *spliced* mutation maps to the same cytological region as *tor* (43C3-43E7) (*1*), and that it is a hypermorphic (7) (hyperactivity) *tor* allele (Table 1). Specifically, when a *spliced* homozygous or heterozygous mother carries an extra dose of the wild-type tor^+ gene, the embryos exhibit a more extreme phenotype, with a consistent loss of an additional one to two abdominal segments (compare in Table 1, A with B and C with D). In contrast, reduction of the number of copies of the *spliced* gene in *spliced* hemizygous mothers rescues the central region defects, resulting in the presence of the wild-type number of abdominal segments (compare in Table 1, B and G). Further, hypoactivity mutations in the *tor* gene also rescue the abdominal defects of *spliced* individuals (compare in Table 1, D with E and F). These genetic tests are consistent with the conclusion that *spliced* is a hyperactivity allele of *tor*, which we, therefore, designate as tor^{splc} (*8*). Although hypoactivity of *tor* causes a loss of the acron and telson and respecification of these regions to form central structures (*1*), hyperactivity of *tor* has no effect on the termini and causes loss of the thorax and abdomen (*9*).

The tor^{splc} allele is heat-sensitive. This fact enabled us to observe a progressive deletion of central embryonic regions due to increased *tor* expression in embryos from tor^{splc} homozygous mothers (Fig. 1, C through E). The cuticular defects correlated well with alterations in the expression of the zygotic pair-rule segmentation gene, *fushi tarazu* (*ftz*), an earlier, molecular marker of embryonic pattern (Fig. 2, A through D) (*10–13*). In wild-type embryos *ftz* is expressed early in embryogenesis in a pattern of seven stripes (Fig. 2A) (*10–12*). In embryos (from tor^{splc} females) raised at low temperatures (18° to 19°C), zero to three of the abdominal segments are missing, with A2 and A3 exhibiting the greatest sensitivity (Fig. 1C and Table 1B). This correlates with a reduction, or complete absence, of *ftz* stripe 4 (Fig. 2B). At intermediate temperatures (21° to 23°C) most of the eight abdominal segments are absent, whereas acronal, gnathal, and thoracic structures develop normally anteriorly, and A7, A8, and the telson develop normally posteriorly (Fig. 1D and Table 1B). At these temperatures, there is a compression and fusion of *ftz* stripes 2, 3, and 5 with a concomitant broadening of stripe 7 (Fig. 2C). At high temperatures (25°C) the progeny of homozygous mothers develop as bags of cuticle with acronal and gnathal structures, such as mouth hooks and pharynx anteriorly and a telson with filzkörper posteriorly (Fig. 1E and Table 1B). Only *ftz* stripe 1 and the broad posterior stripe remain in these embryos (Fig. 2D). About 25% of the embryos that are allowed to develop at 25°C show no activation of *ftz* expression. The embryonic fate map of embryos from tor^{splc} females, reflected by the pattern of *ftz* expression, thus suggests that the absence of stripes 2 to 5 likely corresponds to loss of segments T1 through A5. The presence of stripe 1 and the most posterior stripe (the expanded and fused stripes 6 and 7) are consistent with the presence of acronal, gnathal, and telson structures. The alterations in *ftz* expression and the loss of central structures caused by hyperactivity of *tor* contrasts with the loss of *ftz* stripe 7 and terminal structures caused by hypoactivity alleles of *tor* (Figs. 1B and 2G) (*11, 14*).

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