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Genetics

Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence

(transposon mutagenesis/defensin/magainin/cecropin/pathogenesis)

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ABSTRACT **The production of antibacterial peptides is a host defense strategy used by various species, including mammals, amphibians, and insects. Successful pathogens, such as the facultative intracellular bacterium *Salmonella typhimurium*, have evolved resistance mechanisms to this ubiquitous type of host defense. To identify the genes required for resistance to host peptides, we isolated a library of 20,000 MudJ transposon insertion mutants of a virulent peptide-resistant *S. typhimurium* strain and screened it for hypersensitivity to the antimicrobial peptide protamine. Eighteen mutants had heightened susceptibility to protamine and 12 of them were characterized in detail. Eleven mutants were attenuated for virulence *in vivo* when inoculated into BALB/c mice by the intragastric route, and 8 of them were also avirulent following intraperitoneal inoculation. The mutants fell into different phenotypic classes with respect to their susceptibility to rabbit defensin NP-1, frog magainin 2, pig cecropin P1, and the insect venom-derived peptides mastoparan and melittin. The resistance loci mapped to eight distinct locations in the genome. Characterization of the mutants showed that one had a defective lipopolysaccharide and another mutant harbored a mutation in *phoP*, a locus previously shown to control expression of *Salmonella* virulence genes. Our data indicate that the ability to resist the killing effect of host antimicrobial peptides is a virulence property and that several resistance mechanisms operate in *S. typhimurium*.**

Salmonellae are the etiologic agents of a variety of disease conditions collectively known as human salmonellosis. These include gastroenteritis, a nonsystemic infection of the intestinal tract and regional lymph nodes that gives rise to diarrhea, and enteric (typhoid) fever, a systemic disease in which the microorganism replicates within the cells of the reticuloendothelial system. Over the last 30 years there has been an increase in the number of cases of *Salmonella* gastroenteritis in the United States to an estimated rate of 1,000,000 cases per year; the number of typhoid fever cases has been calculated at over 12,000,000 per year worldwide, most of them in developing countries (1). *Salmonella typhimurium*, the most prevalent serotype causing gastroenteritis in humans, can also be considered an intracellular pathogen in its capacity to cause a typhoid-like disease in immunocompromised humans and mice. *Salmonella* infections generally result from the consumption of contaminated food or liquids, passage through the stomach, and entry of the bacteria into epithelial cells of the small intestine. *Salmonella* serotypes that cause systemic infection penetrate into deeper tissues and replicate specially within phagocytic cells of the liver and spleen (2). Facultative intracellular pathogens such as *S. typhimurium* have evolved distinct strategies to evade killing by the phagocyte oxygen-dependent and -independent mechanisms (3).

To cause disease, *Salmonella* must withstand the battery of short peptides with antibiotic activity present in phagocytic cells and other tissues. One such group of peptides is the defensins, which are abundant in the azurophilic granules of neutrophils and macrophages from rabbits, rats, guinea pigs, and humans and the crypt cells of mouse intestine (4). The importance of these peptides for host defense is underscored by the fact that patients with specialized granule deficiency—who lack defensins—have recurrent infections (5). *Salmonella* resistance to defensins appears to be regulated by PhoP (6), a transcription factor which controls gene expression in response to environmental signals detected by a second protein, PhoQ (7, 8). The integrity of the *phoPQ* operon is also required for virulence in mice (6), survival within macrophages (9), and resistance to low pH (10). None of the five known PhoP-regulated genes seems to play a direct role in defensin resistance (6, 7, 11).

Antimicrobial peptides have been isolated from other species and tissues. For example, magainins have been purified from skin (12) and gut (13, 14) of the African clawed frog *Xenopus laevis* and have been detected immunologically in human saliva (15); cecropins, the broad-spectrum antimicrobial peptides from the cecropia moth (16), have also been isolated from the pig intestine (17). Analogous antimicrobial peptides have been described in bovine neutrophils (18, 19), bovine tracheal mucosa (20), the hemocytes of the horseshoe crab (21), and the lymph fluid of honeybees (22, 23). We reason that successful pathogens, having been exposed to antimicrobial peptides for millions of years during their coexistence with mammalian and insect hosts, would have evolved mechanisms to resist or degrade the inhibitory and microbicidal activities presented by the host. For example, the fly pathogen *Serratia marscecens* is resistant to insect-derived cecropins, and *phoP* mutants of *S. typhimurium* are hypersensitive not only to defensins but also to magainins, cecropins, melittin, and mastoparan (24). To identify the bacterial products required for peptide resistance and to validate the correlation—suggested by the phenotype of *phoP* mutants—between peptide resistance and virulence, we have undertaken the isolation and characterization of mutants of *S. typhimurium* with defects in their response to host defense peptides. Our data suggest that several resistance mechanisms are operating in *Salmonella* and that resistance to this type of host defense strategy is a virulence property.

MATERIALS AND METHODS

Strains, Phages, and Plasmids. Strain 14028s (9) was the virulent, peptide-resistant, wild-type strain used in this

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Abbreviation: LPS, lipopolysaccharide.

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study. Mutants were generated in this genetic background so that comparisons were always performed between isogenic strains. Strain MS7953s is a *phoP7953*::Tn*10* derivative of 14028s (6). Strain TT10288 is *hisD9953*::Mud*JhisA9944*:: MudI (25). Plasmid pEG5381 has been described (7). Gene designations are as summarized by Sanderson and Roth (26).

Bacterial Genetic Techniques. LB, MacConkey indicator, and M63 minimal media have been described (27). Kanamycin was used at 40 μg/ml, ampicillin at 50 μg/ml, chloramphenicol at 40 μg/ml, streptomycin at 100 μg/ml, and tetracycline at 10 μg/ml. δ-Aminolevulinic acid was added to LB medium at 20 μg/ml. Preparation and use of phage P22 lysates were as described (28). Isolation of MudJ insertion mutants was performed with a P22 lysate grown in strain TT10288 to infect 14028s cells (25). MudJ designates the MudI1734 defective transposon, which is deleted for the Mu transposition genes *A* and *B* and carries a gene (*kan*) encoding resistance to kanamycin and a segment of the *lac* operon to generate transcriptional gene fusions (29). To map the MudJ transposon insertions, genomic libraries were prepared in the pIBI25 vector (Pharmacia) by using either *Sal* I or *Bam*HI, which cut MudJ only once and to the right of the *kan* gene (Fig. 1), allowing the isolation of junction fragments as ampicillin- and kanamycin-resistant transformants (see Fig. 3). *Salmonella*-specific fragments were purified by gel electrophoresis, radiolabeled, and hybridized to a filter harboring segments of the *S. typhimurium* chromosome recovered after induction of a set of Mud–P22 prophages present at different positions in the genome (30). This collection of Mud–P22 lysogens was originally assembled by Benson and Goldman (30) and was obtained via K. Sanderson (Salmonella Genetic Stock Centre, Calgary, Canada). Further mapping was performed by classic P22 transduction experiments with markers located in the 3-min region identified by hybridization.

Peptide Susceptibility Assays. Assays were performed as described by Fields *et al.* (6) for defensin NP-1 and crude granulocyte extracts, and as reported by Groisman *et al.* (24) for magainin 2, cecropin P1, mastoparan, and melittin. Defensin NP-1 and crude granulocyte extracts were provided by R. I. Lehrer (UCLA) and J. Spitznagel (Emory University, Atlanta), respectively. Magainin 2, mastoparan, and melittin were purchased from Bachem, and cecropin P1 from Peninsula Laboratories.

Virulence Assays. Groups of four mice were inoculated either intraperitoneally or intragastrically with 100 μl of diluted bacteria in phosphate-buffered saline. Viability was recorded for at least 30 days.

DNA Biochemistry. Restriction endonucleases and phage T4 DNA ligase were purchased from Bethesda Research Laboratories, Boehringer Mannheim, or New England Biolabs, and used according to the suppliers' specifications. Other protocols were taken from Maniatis *et al.* (31).

RESULTS

Isolation of Mutants Sensitive to Cationic Peptide Antibiotics. To identify genes required for resistance to small antimicrobial peptides, we constructed a bank of mutants of a mouse virulent, peptide-resistant *S. typhimurium* strain and screened it for susceptibility to protamine, a 32-amino acid cationic peptide from salmon sperm. Although we were particularly interested in defining genes involved in resistance to physiologically relevant compounds such as defensins, magainins, and cecropins, protamine was chosen as the prototype antimicrobial compound because we had previously found that it also preferentially killed *phoP Salmonella*, and—unlike the other compounds—it was inexpensive and easily available, to allow a large-scale screening. Twenty thousand kanamycin-resistant MudJ-insertion mutants were isolated and tested for protamine sensitivity by replica plating

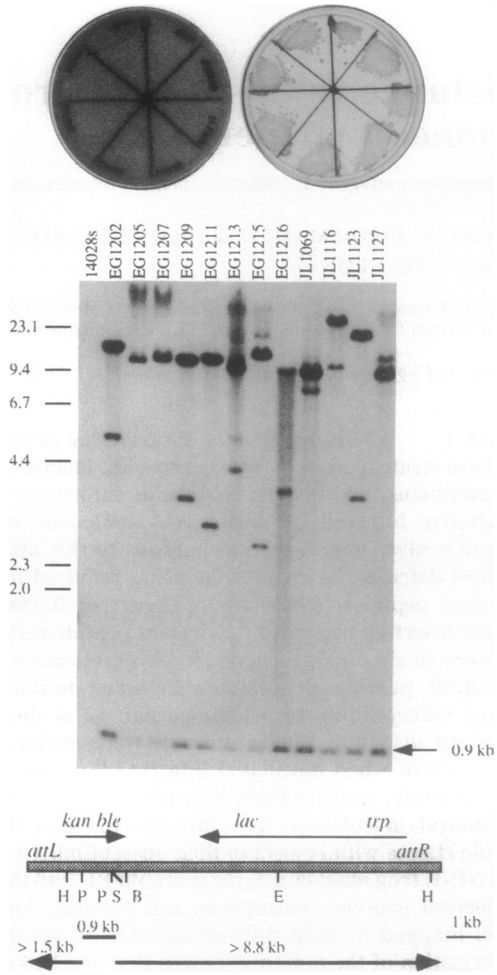

FIG. 1. (*Top*) Growth properties of wild-type and *sap* mutants in LB agar with (*Left*) or without (*Right*) protamine sulfate at 1 mg/ml. Strains are (counterclockwise from the arrow) 14028s (wild type), EG1202, EG1205, EG1207, EG1209, EG1211, EG1213, and MS7953s. (*Middle*) Southern hybridization analysis of DNA from wild-type and *sap* mutants. Chromosomal DNA (≈1 μg) was digested with *Pst* I, electrophoresed in a 0.8% agarose gel, and transferred to a Magna NT nylon membrane (Micron Separations, Westboro, MA). Two hours of prehybridization of the membrane at 65°C was followed by overnight hybridization with a ^{32}P-labeled probe corresponding to *Sal* I-linearized pEG7125 (pBC0::MudJ). The blotted and hybridized membranes were washed in 0.15 M NaCl/0.015 sodium citrate/0.1% sodium dodecyl sulfate once for 15 min at room temperature and twice for 30 min at 60°C and then were exposed to X-AR film (Kodak). There is a constant 0.9-kilobase (kb) band internal to the MudJ transposon and variable bands corresponding to MudJ–*Salmonella* junction fragments, which are predicted to be >1.5 kb and >8.8 kb for the left and right junctions, respectively. Each mutant contains a single transposon insertion per haploid genome as evidenced by the presence of only two junction fragments. As expected, 14028s (wild-type) DNA showed no hybridization to transposon sequences. (*Bottom*) Genetic and physical map of MudJ: *attL* and *attR* are the left and right ends of Mu, respectively; *trp–lac* is a DNA segment used to probe promoter activity in host sequences adjacent to *attL*; *kan* and *ble* are genes encoding resistance to kanamycin and bleomycin, respectively. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; S, *Sal* I.

onto LB agar plates containing protamine sulfate (0.5–1.0 mg/ml). Protamine-sensitive colonies were clonally purified and retested by streaking side by side with the wild-type and *phoP* controls onto media containing various (0.5, 0.625, 0.75, 1.0, and 2.0 mg/ml) concentrations of protamine (Fig. 1 *Top*). This step was necessary because protamine plates lost antimicrobial activity with time. While 71 mutants ap-

peared hypersensitive after the original replica plating, the streaking step showed that only 18 of them were more susceptible to protamine than the wild-type strain. The present study concerns the characterization of 12 of these *sap* (*s*ensitive to *a*ntimicrobial *p*eptides) mutants.

To show genetic linkage between the MudJ insertion and the protamine-sensitive phenotype, we used phage P22-mediated transduction to transfer the MudJ element into a wild-type genetic background. For 9 of the mutants, we established that 6 out of 6 kanamycin-resistant transductants tested were sensitive to protamine. Two other mutants, JL1116 and JL1123, could not have their MudJ transduced into a wild-type genetic strain because they were either resistant to phage P22 (JL1123) or unable to sustain phage growth (JL1116). For the 12th mutant, only half of the kanamycin-resistant transductants were protamine-sensitive, suggesting that it harbored two MudJ insertions. A protamine-sensitive transductant was purified and named EG1202; protamine-resistant transductants were not studied further. Southern hybridization analysis of the 12 mutants indicated that each of them harbored a single transposon insertion per haploid genome and that the MudJ transposon had inserted in distinct locations of the *Salmonella* chromosome (see Fig. 1 *Middle* for results with *Pst* I chromosomal digests; similar results were obtained with *Hin*dIII and *Bgl* II).

Peptide-Susceptibility Profile of Protamine-Sensitive Mutants. Initially, we performed liquid assays to determine the minimal inhibitory concentration for protamine; we found that the wild-type strain 14028s grew in the presence of protamine at 200 μg/ml but showed no growth at 400 μg/ml. This strain had shown resistance to protamine at 1 mg/ml in solid medium, which suggested that agar contained compounds that counteracted the killing effects of protamine; an alternative explanation is that growth in solid medium induced expression of products conferring resistance to this peptide. In this assay, only mutants EG1202, EG1205, EG1207, EG1209, EG1213, EG1216, JL1069, and JL1123 appeared more susceptible than the wild-type strain. We then investigated the mutants for their pattern of susceptibility to defensin NP-1, magainin 2, cecropin P1, mastoparan, and melittin and against crude granulocyte extracts from human neutrophils (Fig. 2). One mutant, EG1202, was extremely sensitive to all peptides tested, and strain JL1123, which also exhibited extreme sensitivity to melittin, mastoparan, and cecropin P1, showed relatively higher resistance to defensin NP-1 and magainin 2 than EG1202. The remaining mutants were resistant to mastoparan and cecropin P1, but some were susceptible to melittin, magainin 2, and defensin NP-1.

Virulence Properties of Protamine-Sensitive Mutants. To test the correlation between virulence and resistance to small peptide antibiotics—implied in the finding that avirulent *S. typhimurium phoP* mutants were hypersusceptible to the small antimicrobial peptides described in Fig. 2—we investigated the mutants for their ability to cause a lethal infection in mice. Eleven out of the 12 *sap* mutants were avirulent when inoculated by the intragastric route with doses corresponding to 1000 × median lethal dose (LD_{50}) for the virulent parent strain (Table 1). That 8 of the mutants were also avirulent when inoculated intraperitoneally at doses > 1000 × LD_{50} (Table 1) implied that the remaining 3 mutants might be susceptible to products present in the stomach or the gut. These data show that the ability to resist host defense peptides is a virulence property of *Salmonella* and suggest the presence of diverse sets of antibacterial peptides in phagocytic cells and the gastrointestinal tract (see *Discussion*).

Genetic and Phenotypic Characterization of Protamine-Sensitive Mutants. To establish whether loci involved in resistance to small antimicrobial peptides were clustered in one region of the *S. typhimurium* chromosome or distributed around the genome, we determined the location of the MudJ

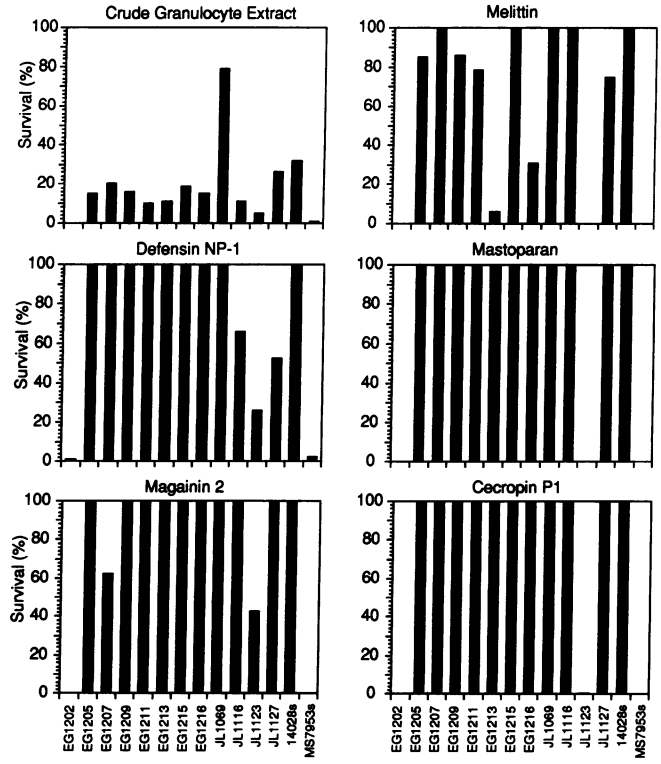

FIG. 2. Susceptibility of *sap* mutants to crude granulocyte extract (150 μg/ml), defensin NP-1 (50 μg/ml), magainin 2 (50 μg/ml), melittin (20 μg/ml), mastoparan (10 μg/ml), and cecropin P1 (4.5 μg/ml). The results are for a typical experiment from three independent trials conducted in duplicate. The primary sequences of the peptides tested are as follows: cecropin P1, STLSKTAKKLENSAKKRISEGIAIAIQGGPR; defensin NP-1, VVCACRRALCLPRERRAGFCRIRGRIHPLCCRR; magainin 2, GIGKFLHSAKKFGKAFVGEIMNS; mastoparan, INLKALAALAKKIL-amide; and melittin, GIGAVLKVLTTGLPALISWIKRKRQQ-amide.

insertion for 11 avirulent mutants. We used a mapping strategy which consisted of hybridizing the DNA present adjacent to the MudJ to a filter harboring an ordered array of segments of the *Salmonella* genome (Fig. 3). Our approach extends the mapping strategy described by Benson and Goldman (30) by allowing mapping of DNA sequences for which mutant alleles are not available. Since this technique allows mapping with a resolution of ≈150 kb, further localization was conducted using classical P22 transduction experiments with genetic markers present in the identified

Table 1. Virulence properties of protamine-sensitive mutants

Strain	Affected gene	LD_{50} oral	LD_{50} i.p.
EG1202	*phoQ*	$>4 \times 10^8$	$>1 \times 10^5$
EG1205	*sapG*	$>4 \times 10^8$	$>1 \times 10^3$
EG1207	*sapJ*	$>4 \times 10^8$	$<5 \times 10^2$
EG1209	*sapD1*	$>4 \times 10^8$	$>1 \times 10^3$
EG1211	*sapH*	$<4 \times 10^8$	$<5 \times 10^2$
EG1213	*sapC*	$>4 \times 10^8$	$>2 \times 10^5$
EG1215	*sapI*	$>4 \times 10^8$	$<5 \times 10^2$
EG1216	*sapD2*	$>4 \times 10^8$	$>1 \times 10^3$
JL1069	*sapE*	$>4 \times 10^8$	$>1 \times 10^5$
JL1116	*sapL*	$>4 \times 10^8$	$>5 \times 10^3$
JL1123	*sapM*	$>4 \times 10^8$	$>1 \times 10^5$
JL1127	*sapK*	$>4 \times 10^8$	$<5 \times 10^2$
MS7953s	*phoP*	$>4 \times 10^8$	$>1 \times 10^5$
14028s	—	6.4×10^5	$<1 \times 10^1$

Virulence assays were performed with oral or intraperitoneal (i.p.) doses of bacteria as described in *Materials and Methods*. Median lethal dose (LD_{50}) was calculated as described (32).

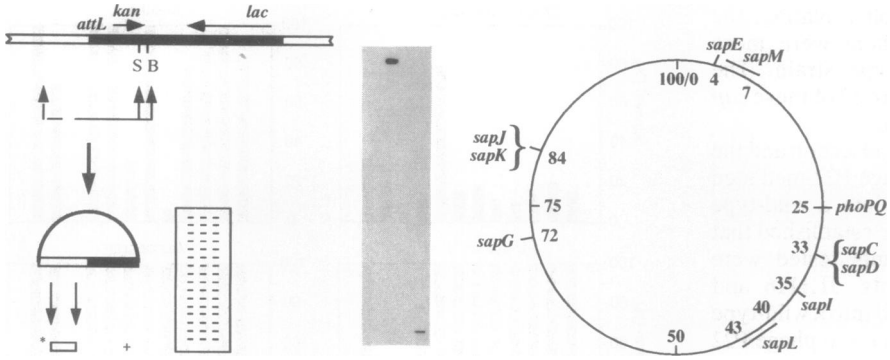

FIG. 3. Mapping of MudJ insertions. (*Left*) *Salmonella* DNA sequences adjacent to the *attL* side of MudJ were used to probe filters harboring an ordered array of segments of the *Salmonella* genome. (*Center*) Results of one such experiment. The strong band corresponds to a segment adjacent to MudJ in mutant JL1116 hybridizing to DNA prepared from induced TT15250 MudP22 lysogen (30), and the weak band at bottom right corresponds to total *S. typhimurium* DNA included as control. (*Right*) Location (in minutes) of the MudJ insertions in the *S. typhimurium* chromosome (see *Results*).

region. The MudJ insertions were mapped to eight different locations in the *Salmonella* chromosome (Fig. 3).

Three MudJ insertions, corresponding to *sapC*, *sapD1*, and *sapD2*, mapped to the 33-min region; they were 16%, 22%, and 24% linked to *pyrF*, respectively, but showed no linkage to *trp* at 34 min. Although these data revealed that the MudJ insertions must be present within the same operon or in tightly linked loci, the molecular and phenotypic data suggest that they are affecting at least two different genes. First, Southern hybridization analysis showed that these mutants harbored insertions in different locations (Fig. 1), and second, the *sapC* mutant appeared to be less virulent and more susceptible to some of the peptides than either *sapD1* or *sapD2*. Mutant EG1205 (*sapG1*) harbored a MudJ insertion that showed 39% linkage to *rpsL* at 72 min, 1% cotransduction with *aroE*, but none with *argR* (0/204) or *ompR* (0/212). Since EG1205 formed normal-size colonies and showed no defects in utilization of sugars under catabolite repression, the MudJ could not be in *crp*, a locus previously implicated in virulence (33). Both *sapJ1* and *sapK1* MudJ insertions were linked to *ntrC* (18% and 16%, respectively) and *metE* (0.5% and 1%, respectively) at 84 min, but they did not cotransduce with the virulence gene *cya* (0/204). However, their Southern hybridization profile and pattern of susceptibility to defensin NP-1 and magainin 2 indicated that the MudJ elements for these strains were present at distinct locations. The MudJ in *sapI* showed 96% linkage to *hemA* in the 35-min region, 0.5% cotransduction with *tre*, but none with *oppB* (0/216). The affected locus was not *hemA*, a gene recently shown to be involved in virulence (34), since EG1215 was able to grow in the absence of δ-aminolevulinic acid.

Mutant EG1202 was extremely sensitive to all the antimicrobial peptides tested, had one of the highest LD_{50} values of all the mutants isolated in this study, and mapped to the 25-min region, where the *phoP* locus is located. This mutant harbored a MudJ insertion in the *phoPQ* operon since (*i*) it could not produce nonspecific acid phosphatase, (*ii*) its protamine-sensitive defect could be complemented by the $phoPQ^+$ plasmid pEG5381 (7), and (*iii*) DNA sequence analysis of the MudJ–*Salmonella* joint revealed that the transposon had inserted within the *phoQ* coding region (after nucleotide 1882 of the sequence in figure 2 of ref. 8). Two insertions, corresponding to mutants JL1069 and JL1123, mapped to the 3′-6′ region; however, their Southern hybridization pattern and their phenotypic properties clearly indicated that the MudJ transposon insertions had disrupted different genes. Mutant JL1123 was resistant to phage P22 and unable to grow in MacConkey agar plates, probably due to hypersusceptibility to bile salts. Although these properties, as well as the lipopolysaccharide (LPS) profile of strain JL1123, were characteristic of deep rough mutants (data not shown), the MudJ insertion mapped to a region harboring no known genes involved in LPS biosynthesis.

DISCUSSION

Resistance to Antimicrobial Peptides and Virulence. To proliferate in host tissues, a successful parasite must be able to manufacture nutrients which are not available and to resist the inhibitory and microbicidal activities mounted by the host. This paper describes the isolation and characterization of *S. typhimurium* mutants defective in their ability to resist the killing effects of host defense peptides. Our data establish that the ability to resist antimicrobial peptides is a virulence property of *Salmonella*, as originally suggested by the finding that *phoP* mutants were hypersusceptible to defensins (6). Eight out of 11 mutants were avirulent by both the oral and the intraperitoneal routes. The 3 remaining mutants may be susceptible to antimicrobial agents that are present in the gut but absent from professional phagocytic cells. There is evidence for the presence of several antimicrobial compounds in the gastrointestinal tract of mammals: defensin (35) and defensin-like molecules (36) have been detected in the crypt cells of the mouse, magainins (15) and the histidine-rich histatins (37) in human saliva, and a cecropin has been purified from pig intestine (17). Finally, the possibility that mutant EG1211 had an LD_{50} only 10–100 times higher than that of the wild-type parent remains to be examined because Inv^- *S. typhimurium* mutants, which are unable to invade host epithelial cells, have LD_{50} values only 50–60 times higher than wild type (38). That several *sap* mutants were susceptible to crude granulocyte extracts but resistant to the particular peptides tested implies that there are probably additional—yet undescribed—antimicrobial compounds present in neutrophil granules whose activity was mimicked by protamine; an alternative possibility is that known host defense products act in a synergistic fashion.

Evolution of Bacterial Resistance to Host Defense Peptides. Salmonellae have been isolated from >100 different species, including amphibians, insects, birds, and a variety of mammals. Therefore, it is conceivable that they might have evolved resistance mechanisms against cationic peptides with antimicrobial activity present in these species. In the past, the production of such compounds had been associated with species lacking the specific type of immunological response mediated by antibodies or T cells which is characteristic of mammals. However, recent data indicate that this defense strategy has been conserved in evolution: defensin homologs have been isolated from insects (39–41) and cecropins have been purified from both the cecropia moth and pigs (16, 17).

The peptides utilized in this study do not share primary sequence similarity but have the same postulated mechanism of action: the capacity to adopt amphiphilic α-helical structures in hydrophobic environments and to form voltage-gated, anion-specific channels in artificial membranes (42–44). The pattern of susceptibility to different antimicrobial peptides, the virulence properties, and the distribution of resistance loci in the *S. typhimurium* genome argue for the presence of several resistance mechanisms against the various peptides. One of the mutants recovered in our screening

harbored a mutation in the *phoPQ* operon, suggesting an involvement of PhoP in the control of gene(s) necessary for resistance to defensin NP-1 and also to magainin 2, cecropin P1, mastoparan, and melittin. Another mutant (JL1123) harbored a defective LPS and had phenotypes characteristic of deep rough mutants, but interestingly, its mutation mapped to a region where no loci involved in LPS biosynthesis had been described. A role of LPS in resistance to magainins has been suggested (45). Complementation analysis will be required to establish whether strain JL1123 harbors a second mutation affecting the LPS biosynthesis apart from the single MudJ insertion in the 4- to 7-min region. Finally, the three mutations which mapped to the 33-min region probably affected two genes: the *sapC* mutant had a higher LD_{50} and was more susceptible to melittin and protamine than either the *sapD1* or the *sapD2* mutant. These loci are unlikely to be alleles of *mviA*, a virulence-related locus originally mapped to the 33-min region but recently shown to be not linked to *pyrF* (46). Moreover, the wild-type strain used in our study harbored the avirulent *mviA*$^+$ allele, and mutations in this locus would result in an increase rather than a decrease in LD_{50} for BALB/c mice (46). Recent molecular analysis of *sapD* revealed that it encodes a product homologous to the "ATP-binding cassette" family of transporters such as that encoded by the *mdr* locus (47), suggesting peptide transport as a mechanism of resistance (C.P.-L., M. Baer, and E.A.G., unpublished data).

It has been suggested that one of the bacterial resistance strategies against magainins is mediated by proteases present in the surface of the microorganism (48) but such proteases have not been purified, nor has their presence been demonstrated by genetic means. Interestingly, mutant EG1207 exhibited increased susceptibility to magainin 2 and was mapped to a region that includes the *pepQ* locus, which encodes an X-Pro peptidase (49). Further genetic and molecular analysis of the *sap* mutants will be required to elucidate the molecular mechanisms governing resistance to this ubiquitous form of host defense.

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