al and recessive or partially recessive (*16*). In *Heliothis virescens* (Fabricius), resistance to a delta endotoxin of *Bt* subsp. *kurstaki* strain HD-1 was autosomal, incompletely dominant, but controlled by several genetic factors (*17*). The high level of resistance in *H. virescens* to CryIAc and to CryIAb was partially recessive and controlled by one or a few loci, but the low level of resistance to CryIIA was more dominant (*18*). In four colonies of *Plutella xylostella* (L.) (*6, 19*), inheritance of resistance to *Bt* subsp. *kurstaki* was recessive or incompletely recessive. Resistance was most likely autosomal and controlled by one or a few major loci. The genetic bases for resistance to *Bt* toxins appear to be different in different insects (*20*) and may even differ for different toxins. Our results agree with other studies in that Dipel ES resistance in *O. nubilalis* appears to be caused by a single autosomal gene (or by a few genes). However, our results differ in that resistance in this strain appears to be incompletely dominant rather than recessive.

These results could have important implications for resistance management of *O. nubilalis* in *Bt* maize. The currently proposed resistance management strategy for *Bt* maize, the high-dose/refuge strategy (*1, 2, 21*), requires (i) that plant tissue be very toxic so that heterozygotes for resistance are killed, (ii) that the resistance alleles be very rare, and (iii) that susceptible insects are within an effective mating distance of resistant insects. The high-dose/refuge strategy would not be useful for resistance management if the trait is dominant. However, the genetic dominance we observed is expressed over a specific range of Dipel ES doses; at higher dosages, all individuals are expected to be susceptible. The practical importance of this genetic dominance will depend on whether these insects can survive on *Bt* maize. The high-dose/refuge strategy is subject to a number of stringent prerequisites that may be difficult to meet in practice. More robust resistance management options are needed.

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7. These *O. nubilalis* strains were initiated with 48 egg masses collected in July 1995 from maize fields near St. John, in south central Kansas. The exposure of the *O. nubilalis* population to *Bt* pesticides in the field is unknown but is believed to be low (*3*). However, this *O. nubilalis* population was probably exposed to other non-*Bt* foliar pesticide treatments on an annual basis over the past 20 to 30 years. After one generation, the progeny were divided into two strains: KS-SC-S (the unselected control strain) and KS-SC-R (the resistant strain). The corn borers were reared on a meridic agar-based diet. The selected strain was reared on the same diet, but for each selected generation, neonates were exposed to the diet laced with Dipel ES at concentrations that caused 80 to 99% mortality. Larvae from both strains continued to develop normally on greenhouse-grown maize plants. Neonates of the Dipel ES–resistant *O. nubilalis* strain were able to cause more damage than susceptible insects when placed on certain greenhouse-grown *Bt* maize hybrids (F. Huang, R. A. Higgins, L. L. Buschman, unpublished data).
8. The reciprocal crosses of resistant and susceptible strains demonstrated that resistance to Dipel ES in *O. nubilalis* was incompletely dominant. Therefore, we backcrossed the F_1 with the susceptible strain. This choice increased our ability to distinguish the modes of inheritance (*6*).
9. Because the resistance was incompletely dominant, according to the data obtained from the reciprocal crosses (F_1), we crossed the hybrid and susceptible strains. A discriminating dose of 2.43 ml of Dipel ES per kilogram of diet was employed in the selection of offspring. At this concentration, the susceptible strain exhibited 99% mortality, whereas the RS hybrids in these tests experienced 91% survival.
10. Dipel ES was incorporated in the diet at 0.03, 0.09, 0.27, 0.81, 2.43, 7.29, and 21.87 ml of Dipel ES per kilogram of diet. There was also a no–Dipel ES control. The dilutions were prepared with 0.05% Triton X-100. The diets were poured into cells in a 128-cell tray, and a single neonate larva was placed in each cell. For each concentration, there were four replicates of 32 larvae (n = 128 larvae). The bioassay trays were maintained at 27°C under continuous light. Mortality was assessed on the fifth day.
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An Essential Role for DNA Adenine Methylation in Bacterial Virulence

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Salmonella typhimurium lacking DNA adenine methylase (Dam) were fully proficient in colonization of mucosal sites but showed severe defects in colonization of deeper tissue sites. These Dam$^-$ mutants were totally avirulent and were effective as live vaccines against murine typhoid fever. Dam regulated the expression of at least 20 genes known to be induced during infection; a subset of these genes are among those activated by the PhoP global virulence regulator. PhoP, in turn, affected Dam methylation at specific genomic sites, as evidenced by alterations in DNA methylation patterns. Dam inhibitors are likely to have broad antimicrobial action, and Dam$^-$ derivatives of these pathogens may serve as live attenuated vaccines.

Methylation at adenine residues by Dam controls the timing and targeting of important biological processes such as DNA replication, methyl-directed mismatch repair, and transposition (*1*). In addition, Dam regulates the expression of operons such as pyelonephritis-associated pili (*pap*), which are an important virulence determinant in upper urinary tract infections (*2, 3*). The latter regulatory mechanism involves formation of heritable DNA methylation patterns, which con-

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trol gene expression by modulating the binding of regulatory proteins. Although Dam regulates pili gene expression, its role in microbial pathogenesis has never been tested.

To determine whether Dam plays a role in the pathogenesis of *Salmonella typhimurium*, we assessed the effect of an insertion in the *dam* gene (Mu*d*-Cm). The oral lethal dose required to kill 50% of the animals (LD_{50}) of this Dam$^-$ mutant was more than 10,000 times the LD_{50} for the wild type, and the intraperitoneal LD_{50} for the mutant was more than 1000 times that for the wild type (Table 1). Because the *dam* insertion could decrease the expression of downstream genes (polar effects), an in-frame, nonpolar *dam* deletion was constructed (*4*) and shown to have the same reduced virulence as the *dam* insertion. Thus, the attenuation was specifically attributable to the lack of Dam. Moreover, intraperitoneal inoculation of mice with a mixture of equal numbers of Dam$^+$ and Dam$^-$ *Salmonella* showed that Dam$^-$ mutants were completely eliminated during growth in the mouse (competitive index assay). Similar results were obtained with a strain that overproduces Dam from a recombinant plasmid, which suggested that precise amounts of the Dam methylase are required for full virulence. These results show that the Dam methylase is essential for bacterial pathogenesis.

Dam plays an essential role in methyl-directed mismatch repair (MDMR) because it allows discrimination between parental and daughter DNA strands (*1*). Thus, in the absence of Dam, bacteria show an increased mutation rate. To test the hypothesis that the reduction in virulence of Dam$^-$ *Salmonella* was due to a high mutation rate, we measured the virulence of *mutS Salmonella*, which lack MDMR and also have a high mutation rate. Table 1 shows that in both the oral LD_{50} and the competitive index virulence assays, *mutS Salmonella* were identical to the wild type, indicating that Dam does not affect pathogenesis via an increased mutation rate. Because more DNA exchange between species occurs in MutS$^-$ strains than in MutS$^+$ strains, they more readily acquire new virulence determinants (*1*). The fact that MutS$^-$ strains are fully virulent could explain the high frequency at which *mutS Escherichia coli* and *Salmonella* mutants are found among clinical isolates (*5*).

Dam controls the expression of Pap pili by modulating the binding of leucine-responsive regulatory protein (Lrp) to *pap* regulatory DNA sequences (*3*). Lrp is a global regulator of at least 35 genes in *E. coli* that include operons involved in metabolism, transport, and adhesion (*6*). To determine whether Dam affects *Salmonella* virulence through an Lrp-mediated pathway, we analyzed Lrp$^-$ *Salmonella* (Table 1). *Salmonella* lacking Lrp were fully virulent, as assessed by the LD_{50} and competitive index assays. These data show that Lrp is not required for virulence in a mouse model of typhoid fever.

The results discussed above show that adenine methylation is critical for *Salmonella* pathogenesis. DNA methylation of cytosine residues appears to be important for the regulation of biological processes in both plants

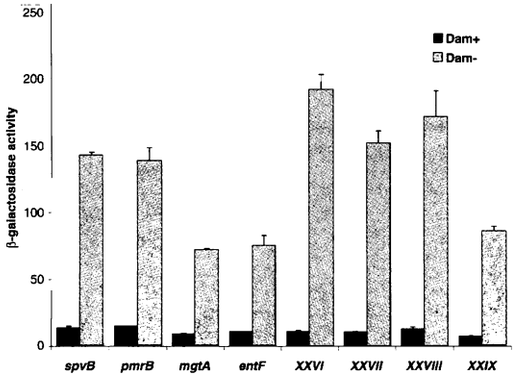

Fig. 1. Dam regulates in vivo induced genes. β-galactosidase expression from *S. typhimurium ivi* fusions in Dam$^+$ and Dam$^-$ strains, grown to saturation in LB medium as described (*27*), was measured. The vertical axis shows β-galactosidase activities [micromoles of *o*-nitrophenol (ONP) formed per minute per A_{600} unit per milliliter of cell suspension $\times 10^3$]. The β-galactosidase activities were assayed as described (*28*).

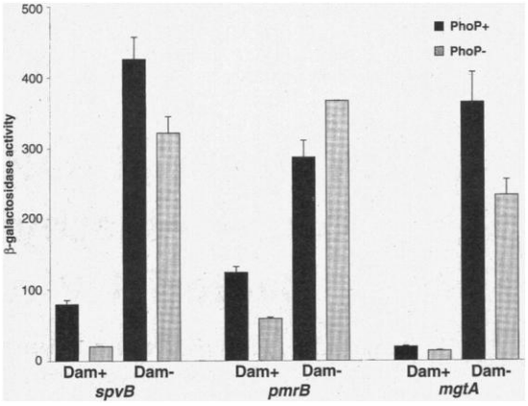

Fig. 2. Dam represses PhoP-activated genes. β-galactosidase expression from *S. typhimurium ivi* fusion strains, grown in minimal medium (pH 5.5, 50 μM Mg^{2+}) as described (*27*), was measured. The vertical axis shows β-galactosidase activities (calculated as in Fig. 1). The β-galactosidase activities were assayed as described (*28*).

Table 1. Dam is required for *Salmonella* virulence. ND, not determined.

Relevant genotype*	Oral LD_{50}†	Intraperitoneal LD_{50}†	Competitive index‡
Wild type	10^5	<10	–
dam102::Mu*d*-Cm	>10^9	>10^4	<10^{-4}
damΔ232 (nonpolar deletion)	>10^9	>10^4	<10^{-4}
Wild type (pTP166) (Dam overproducer)	10^8	>10^4	<10^{-4}
mutS121::Tn*10*	10^5	ND	0.9
lrp31::Km	10^5	ND	9.4
dcm1::Km	10^5	<10	0.2

*All bacterial strains used in this study are derivatives of *S. typhimurium* 14028. Mutant strains are isogenic to the wild type and were obtained or constructed as described (*4*). Strains used in infection studies were grown overnight in LB with shaking (*9*). The Dam-overproducing strain contains *E. coli dam* on a recombinant plasmid (pTP166) in a wild-type background (*30*). †The LD_{50} assay for each of these strains was compared to that for the wild type. The peroral LD_{50} via gastrointubation for all derivatives was determined by infecting at least 12 BALB/c mice; the intraperitoneal LD_{50} was determined by infecting at least six mice. ‡At least five BALB/c mice were intraperitoneally infected with a 1:1 ratio of mutant to wild type, as described (*9*). Five days after infection, the bacterial cells were recovered from the spleen; the competitive index is the ratio of mutant to wild-type bacteria recovered.

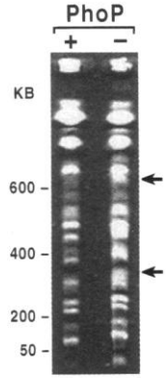

Fig. 3. PhoP affects the formation of *Salmonella* DNA methylation patterns. DNA methylation patterns formed in PhoP$^+$ and from PhoP$^-$ strains in minimal medium. Genomic DNA prepared from PhoP$^+$ and from PhoP$^-$ strains embedded in agarose was cleaved with Mbo I (which cleaves nonmethylated Dam-target sites) and subjected to pulsed-field gel electrophoresis (*29*). The arrows indicate two DNA fragments that were present in PhoP$^-$ *Salmonella* but were absent in PhoP$^+$ *Salmonella*.

and animals. Although *Salmonella* contain a DNA cytosine methylase (Dcm), the role of cytosine methylation in this organism is unclear. The *dcm*$^-$ mutant was virulent in the LD_{50} and competitive index assays (Table 1). These results demonstrate that methylation of adenine but not cytosine residues is required for *Salmonella* pathogenesis.

DNA adenine methylation has been shown to directly control virulence gene expression (*7*). Therefore, we determined whether Dam regulates *Salmonella* genes that are preferentially expressed in the mouse [designated as in vivo–induced (*ivi*) genes (*8–11*)]. Dam significantly repressed the expression of more than 20 *ivi* genes (by a factor of 2 to 18) when grown in rich medium (Fig. 1). Four of the eight fusions in Fig. 1 are in known genes, all of which have been shown to be involved or have been implicated in virulence: *spvB* resides on the *Salmonella* virulence plasmid and functions to facilitate growth at systemic sites of infection (*12*); *pmrB* is involved in resistance to antibacterial peptides termed defensins (*13*); and *mgtA* and *entF* are involved in the transport of magnesium and iron, respectively (*14, 15*). Additional *ivi* genes of unknown function were also Dam-regulated. These results indicate that Dam is a global regulator of *Salmonella* gene expression and that the *dam*-regulated *ivi* genes constitute a *dam* regulon (*1*).

Salmonella pathogenesis is known to be controlled by PhoP, a DNA binding protein that acts as both an inducer and repressor of specific virulence genes [reviewed in (*16*)]. To determine whether the Dam and PhoP regulatory pathways share common genes, we tested the effect of Dam on seven PhoP-activated *ivi* genes, including *spvB*, *pmrB*, and *mgtA*. Dam repressed the expression of these three genes by a factor of 2 to 19 (Fig. 2), and this repression was not dependent on the PhoP protein. Dam did not significantly affect the expression of the remaining four PhoP-activated genes (*17*). These results indicate that Dam and PhoP constitute an overlapping global regulatory network controlling *Salmonella* virulence.

Table 2. Dam$^-$ *Salmonella* serve as effective live attenuated vaccines.

Immunization with Dam$^-$ *S. typhimurium**	Challenge with 10^9 wild-type *S. typhimurium*
None	12/12 dead
dam102::Mud-Cm	17/17 alive
damΔ232 (nonpolar deletion)	8/8 alive

*BALB/c mice were perorally immunized via gastrointubation with a dose of 10^9 Dam$^-$ *S. typhimurium*. Five weeks later, the immunized mice were challenged perorally with 10^9 wild-type *S. typhimurium* as described. No visible effects of typhoid fever were observed after immunization with Dam$^-$ *Salmonella*, nor were there visible effects after the wild-type challenge.

Binding of regulatory proteins to DNA can form DNA methylation patterns by blocking the methylation of specific Dam target sites (GATC sequences) (*18*). Therefore, we further investigated the interactions between Dam and PhoP by determining whether the binding of PhoP (or a PhoP-regulated protein) to specific DNA sites blocks methylation of these sites by Dam, resulting in an alteration in the DNA methylation pattern. Analysis of PhoP$^+$ and PhoP$^-$ *Salmonella* showed distinct differences in DNA methylation patterns. Digestion of genomic DNA from PhoP$^-$ bacteria with Mbo I (which cleaves only at nonmethylated GATC sites) resulted in the appearance of DNA fragments that were not present in DNA from PhoP$^+$ bacteria, indicating that the PhoP protein (or a PhoP-regulated gene product) blocks Dam methylation at specific GATC-containing sites in the *Salmonella* genome (Fig. 3, arrows). Recent data have shown that although catabolite gene activator protein binds to a DNA sequence containing GATC, it does not protect this site from methylation (*18*). Thus, not every protein that binds to a Dam target site protects the GATC sequence from methylation. It is also possible that PhoP$^+$ and PhoP$^-$ strains have different amounts of Dam activity, which in turn could affect DNA methylation patterns. However, this regulation does not occur at the transcriptional level because Dam does not alter PhoP expression, nor does PhoP alter Dam expression (*17*). Further analysis will determine whether these PhoP-protected sites are within regulatory regions of virulence genes, and whether DNA methylation directly affects the PhoP regulon by altering DNA-PhoP interactions.

In *E. coli*, almost all GATC sites protected from methylation are in 5′ noncoding DNA regions presumably involved in the control of gene expression (*19, 20*). Thus, it is likely that the DNA methylation patterns identified in *Salmonella* (Fig. 3) are also within gene regulatory regions. Methylation of specific GATC sites in the regulatory regions of virulence genes could affect the binding of regulatory proteins to DNA. Such altered protein-DNA interactions can affect gene expression, as has been shown for the *pap* virulence operon in *E. coli* (*7, 18*). Similarly, Dam methylation could directly or indirectly affect the expression of PhoPQ-regulated genes in *S. typhimurium*.

Because Dam$^-$ mutants were highly attenuated, we determined whether Dam$^-$ *Salmonella* could serve as a live attenuated vaccine. Table 2 shows that all (17/17) mice immunized with a *S. typhimurium* Dam$^-$ insertion strain survived a wild-type challenge of 10^4 above the LD_{50}, whereas all nonimmunized mice (12/12) died after challenge. Moreover, because all (8/8) mice immunized with *Salmonella* containing the *dam* deletion survived challenge, these data indicate that protection was specifically due to the absence of Dam methylase. Preliminary experiments indicate that mice immunized with Dam$^-$ *S. typhimurium* showed cross-protection against another pathogenic strain of *Salmonella* (*17*). The virulence attenuation and effectiveness of Dam$^-$ mutants as a vaccine (Tables 1 and 2) could be due to the ectopic expression of virulence determinants (Figs. 1 and 2), which would likely be deleterious to the growth (or survival) of *Salmonella* during infection.

Dam$^-$ *Salmonella* could have been avirulent as a result of multiple defects in basic cellular processes that reduced viability. This hypothesis was tested by comparing the survival of Dam$^+$ and Dam$^-$ *Salmonella* in mouse tissues. As shown in Fig. 4, Dam$^-$ bacteria were fully proficient in colonization of a mucosal site (Peyer's patches) but showed severe defects in colonization of deeper tissue sites. Five days after infection, we observed a reduction of three orders of magnitude in numbers of Dam$^-$ *Salmonella* in the mesenteric lymph nodes (relative to numbers of Dam$^+$ bacteria) and a reduction of eight orders of magnitude in numbers of Dam$^-$ *Salmonella* in the liver and spleen.

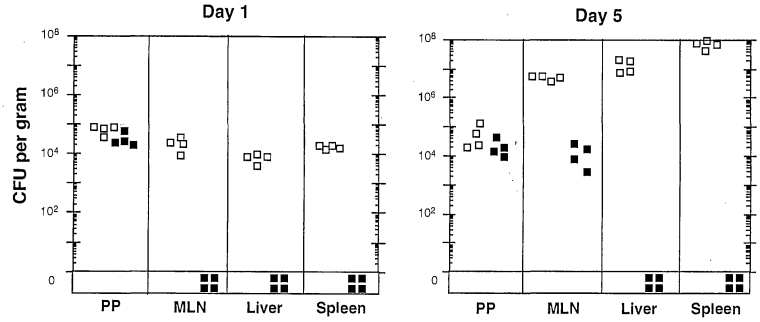

Fig. 4. Colonization of mouse tissue sites by Dam$^-$ *Salmonella*. BALB/c mice were infected via gastrointubation at a dose of 10^9 Dam$^+$ (open boxes) or Dam$^-$ (closed boxes) *S. typhimurium*. After 1 day or 5 days after infection, mice were killed and bacteria were recovered from the host tissues indicated. PP, Peyer's patches (the four patches proximal to the ileal-cecal junction); MLN, mesenteric lymph nodes; CFU, colony-forming units.

These data show that Dam⁻ *Salmonella* survive in Peyer's patches of the mouse small intestine for at least 5 days, providing an opportunity for elicitation of a host immune response. Dam⁻ *Salmonella*, however, were unable to cause disease; they either were unable to invade systemic tissues or were able to invade but could not survive.

DNA adenine methylases are potentially excellent targets for both vaccines and antimicrobials. They are highly conserved in many pathogenic bacteria that cause significant morbidity and mortality, such as *Vibrio cholerae* (*21*), *Salmonella typhi* (*22*), pathogenic *E. coli* (*23*), *Yersinia pestis* (*22*), *Haemophilus influenzae* (*24*), and *Treponema pallidum* (*25*). In addition, because Dam is a global regulator of genes expressed during infection (Fig. 1), Dam⁻ mutants may ectopically express multiple immunogens that are processed and presented to the immune system. Such ectopic expression could elicit a cross-protective immune response between related bacterial strains that share common epitopes. Finally, because the Dam methylase is essential for bacterial virulence, Dam inhibitors are likely to have broad antimicrobial action, hence Dam is a promising target for antimicrobial drug development.

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31. We thank J. Roth for the *dam102*::Mud-Cm allele, T. Cebula for the *mutS121*::Tn*10* allele, R. Ballester for critically reading the manuscript, and D. Hillyard for constructing the *lrp31* mutant. Supported by NIH grant AI36373 and a Beckman Young Investigator Award (M.J.M.) and NIH grant AI23348 (D.A.L.).

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Sources of Mathematical Thinking: Behavioral and Brain-Imaging Evidence

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Does the human capacity for mathematical intuition depend on linguistic competence or on visuo-spatial representations? A series of behavioral and brain-imaging experiments provides evidence for both sources. Exact arithmetic is acquired in a language-specific format, transfers poorly to a different language or to novel facts, and recruits networks involved in word-association processes. In contrast, approximate arithmetic shows language independence, relies on a sense of numerical magnitudes, and recruits bilateral areas of the parietal lobes involved in visuo-spatial processing. Mathematical intuition may emerge from the interplay of these brain systems.

> Will it ever happen that mathematicians will know enough about the physiology of the brain, and neurophysiologists enough of mathematical discovery, for efficient cooperation to be possible? [Jacques Hadamard (*1*)]

Until recently, the only source of information about the mental representations used in mathematics was the introspection of mathematicians. Eloquent support for the view that mathematics relies on visuo-spatial rather than linguistic processes came from Albert Einstein, who stated: "Words and language, whether written or spoken, do not seem to play any part in my thought processes. The psychological entities that serve as building blocks for my thought are certain signs or images, more or less clear, that I can reproduce and recombine at will" (*2*). Many mathematicians report similar experiences (*1, 3*), but some have stressed the crucial role played by language and other formal symbol systems in mathematics (*4*). Still others have maintained that the critical processes giving rise to new mathematical insights are opaque to consciousness and differ from explicit thought processes (*1, 3, 5*).

We address the role of language and visuo-spatial representation in mathematical thinking using empirical methods in cognitive neuroscience. Within the domain of elementary arithmetic, current cognitive models postulate at least two representational formats for number: a language-based format is used to store tables of exact arithmetic knowledge, and a language-independent representation of number magnitude, akin to a mental "number line," is used for quantity manipulation and approximation (*6, 7*). In agreement with these models, we now demonstrate that exact calculation is language-dependent, whereas approximation relies on nonverbal visuo-spatial cerebral networks.

We first used behavioral experiments in bilinguals to examine the role of language-based representations in learning exact and approximate arithmetic. In one experiment, Russian-English bilinguals were taught a set of exact or approximate sums of two two-digit numbers in one of their two languages (*8*). In the exact addition condition, subjects selected the correct sum from two numerically close numbers. In the approximate addition condition, they were asked to estimate the result and select the closest number. After training, subjects' response times for solving trained problems and novel problems were tested in their two languages. Performance in both tasks improved considerably with training (response times dropped, in

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