can only speculate as to the causative influence of this defective Rb protein. The J82 point mutation we have documented leads to the synthesis of a protein that fails to bind the E1A and SV40 large T oncoproteins yet still retains an associated DNA-binding activity. If this latter activity is important in the normal physiology of p105-Rb, then we must assume that DNA binding alone may not be sufficient for the antineoplastic function of Rb.

Our analysis of the J82 Rb gene expands the catalog of genes in which point mutations create potentially tumorigenic proteins. To date, these have only been well documented in the *ras* and *neu* oncogenes (*23*). Use of the adenovirus E1A complex assay, PCR technology, and a detailed map of the Rb gene made this analysis possible in a relatively short period of time and indicates the possibility of rapid molecular analysis of mutant Rb alleles encountered in the future (*16, 18*).

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Epithelial Cell Surfaces Induce *Salmonella* Proteins Required for Bacterial Adherence and Invasion

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Salmonella **bacteria are capable of entering (invading) and multiplying within eukaryotic cells. Stable adherence to and invasion of epithelial cells by *S. choleraesuis* and *S. typhimurium* were found to require de novo synthesis of several new bacterial proteins. This inducible event appears to be a coordinately regulated system dependent on trypsin- and neuraminidase-sensitive structures present on the epithelial cell surface. Mutants of *S. choleraesuis* and *S. typhimurium* were unable to synthesize these proteins and did not stably adhere to nor invade eukaryotic cells. Two such *S. typhimurium* mutants were avirulent in mice, an indication that these proteins are required for *Salmonella* virulence.**

SALMONELLOSIS IS A MAJOR HEALTH problem throughout the world. In the United States this disease accounts for 40,000 reported cases, 500 deaths, and health care costs exceeding $50 billion annually (*1*). *Salmonella* species are intracellular parasites, and it is thought that these bacteria gain access to their host by penetrating through intestinal epithelial cells (*2*). However, the molecular mechanisms used by these organisms to adhere to, enter, and penetrate through epithelial cells remain poorly defined.

We previously demonstrated that *S. choleraesuis*, a highly invasive *Salmonella* species, could enter, multiply, and proceed through viable MDCK (Madin Darby canine kidney) epithelial cells grown on permeable filters (*3*). Inhibitors of bacterial RNA or protein synthesis blocked penetration in this model system. Other workers found that *Salmonella* adherence consists of two phases of attachment—reversible (bacteria can be removed by washing) and irreversible (resistant to washing)—and that addition of chloramphenicol inhibited stable (irreversible) adherence of *S. typhimurium* to small intestinal enterocytes from rats (*4*). These data were consistent with the idea that *Salmonella* penetrate through viable epithelial monolayers impermeable to other bacteria and that *Salmonella* species must synthesize proteins essential to this process. We now report that several bacterial proteins required for *Salmonella* internalization into host cells are induced by epithelial cells.

The kinetics of stable *S. choleraesuis* adherence and invasion of MDCK cells are presented in Fig. 1. Few bacteria adhered to MDCK monolayers within the first 2 hours, but binding then increased exponentially,

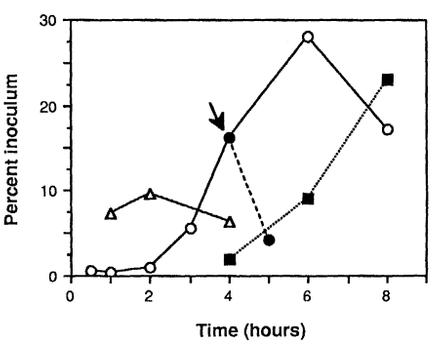

Fig. 1. Kinetics of *S. choleraesuis* adherence and invasion to MDCK epithelial cells. The percentage of bacteria adherent to glutaraldehyde-fixed monolayers (○) and the percentage of bacteria that invaded viable monolayers (■) were determined as described in Table 1. The effect of adding chloramphenicol or rifampin or cooling to 4°C for 1 hour after bacteria have bound to fixed monolayers for 4 hours is illustrated (●). The arrow indicates when these agents were applied. The percentage of bacteria bound to new fixed monolayers after incubation for 3 hours with a fixed monolayer followed by vigorous pipetting before incubation with the new fixed monolayer is shown (△). Values are an average of duplicate samples and are representative data from one of three experiments.

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reaching a maximum value at 6 hours. Virtually identical adherence kinetics were obtained with viable and glutaraldehyde-fixed monolayers (*5*). Use of fixed monolayers facilitated analysis of stable bacterial adherence without the accompanying invasion, as bacteria cannot enter fixed MDCK cells. The kinetics of invasion (entry into viable MDCK cells) paralleled the adherence kinetics, except that invasion required an additional 2 hours to achieve numbers of internalized bacteria comparable to numbers of adherent bacteria, presumably because of the lag time between bacterial adherence and internalization. If bacteria were incubated with a fixed monolayer for 3 hours, removed by vigorous agitation, and added to another fixed monolayer, stable binding occurred rapidly within 1 hour at levels similar to those that required 2 and 4 hours after addition to the new monolayer (Fig. 1). These results suggest that bacteria can be induced to bind much faster to monolayers without the characteristic lag period. If bacteria bound to the monolayers after 4 hours were treated with chloramphenicol or rifampin, or were cooled to 4°C for 1 hour, the number of stably bound bacteria decreased significantly (Fig. 1), indicating that, at this point, continuous bacterial protein synthesis is required for stable *Salmonella* adherence to monolayers. Hence, our results were consistent with the idea that an induction event requiring bacterial protein synthesis is required for stable *Salmonella* adherence and subsequent invasion into host cells.

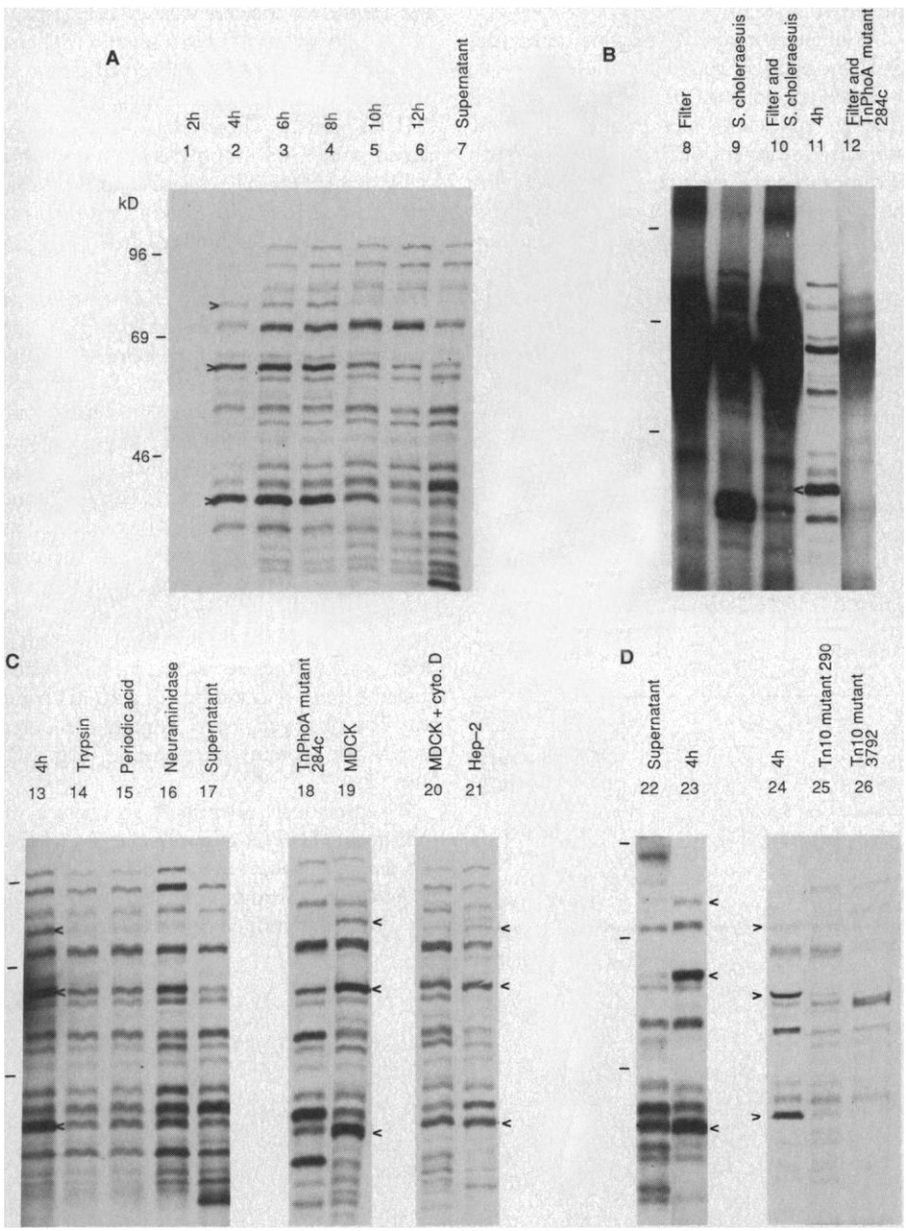

Fig. 2. Autoradiographs of 10% polyacrylamide gels containing [^{35}S]methionine-labeled bacteria, illustrating the proteins synthesized in the presence of epithelial cells. Induced proteins are marked by arrows. (**A**) *Salmonella choleraesuis* was incubated for indicated times with fixed MDCK monolayers before being labeled for 1 hour (lanes 1 to 6); unbound bacteria taken from the supernatant of the 4-hour sample (lane 7). (**B**) Fixed monolayers (lane 8), *S. choleraesuis* supernatant (lane 9), *S. choleraesuis* (lane 10), and Tn*phoA* mutant [no. 284c (*9*)] (lane 12) bound to fixed monolayers were surface-iodinated as described (*12*). Lanes 11 and 13 are the same as lane 2. (**C**) Lanes 14, 15, and 16 are monolayers pretreated with trypsin, periodic acid, and neuraminidase before fixing, incubating with *S. choleraesuis* for 4 hours, and labeling. Lane 18 contains *S. choleraesuis* Tn*phoA* mutant no. 284c (*9*) incubated and labeled in a similar manner with a fixed monolayer. Viable MDCK (lanes 19 and 20) and Hep-2 (lane 21) monolayers were treated with cycloheximide [and cytochalasin D (MDCK cells, lane 20)] before addition of *S. choleraesuis* and labeling. (**D**) The proteins produced by *S. typhimurium* bound to fixed MDCK monolayers (lanes 23 and 24) or in the supernatant (lane 22) are shown, as are two Tn*10* mutants [SL290, lane 25; SL3792, lane 26 (*9*)].

Salmonella choleraesuis treated with chloramphenicol (protein synthesis inhibitor), rifampin (RNA synthesis inhibitor), gentamicin (aminoglycoside), or low temperature did not adhere to fixed monolayers, nor did they invade viable MDCK cells (Table 1). However, treatment with naladixic acid (DNA synthesis inhibitor) had no effect on these processes. Similar results were found with *S. typhimurium* (*5*), indicating that *Salmonella* species must synthesize RNA and proteins, but not DNA, to adhere to and invade MDCK epithelial cells.

We attempted to identify induced bacterial proteins by pulse-labeling bacteria with [^{35}S]methionine in the presence and absence of fixed epithelial monolayers (Fig. 2). When the protiens synthesized by *S. choleraesuis* bound to fixed monolayers are compared to those synthesized by organisms in the supernatant of the same sample, numerous differences in these protein profiles are present (Fig. 2A, lane 2 versus lane 7). Several proteins synthesized by the bound bacteria are not produced by bacteria in the supernatant or in control medium. In addition, synthesis of some proteins was apparently shut off in the bound bacteria. These differences were also observed with *S. typhimurium* (Fig. 2D, lane 22 versus lane 23). We used two-dimensional electrophoresis to determine more accurately the number of proteins being regulated by this event (Fig. 3). At least six new proteins were synthesized by bound *S. choleraesuis* (Fig. 3A), whereas a minimum of seven proteins were no longer produced when compared to bacteria in the supernatant (Fig. 3B). We found the same results for *S. typhimurium* (*5*).

Comparison of the kinetics of the synthesis of these induced proteins indicated that they were analogous to the adherence kinetics (Fig. 2A, lanes 1 to 6); these new proteins were first detected after a minimum

of 4 hours of incubation with monolayers (Fig. 2A, lane 2), and were synthesized from 4 to 8 hours after infection (Fig. 2A, lanes 2 to 4) but were not being synthesized at either 10 or 12 hours after bacterial addition to the monolayer (Fig. 2A, lanes 5 and 6). However, if bound bacteria were labeled at 4 hours and incubated for an additional 6 or 8 hours, the induced proteins were still present, indicating that, although their synthesis was stopped after 10 to 12 hours, these proteins are still present in the bound bacterium (*5*).

Salmonella species contain a large plasmid that is essential for virulence but not for penetration of the intestinal epithelium (*6*). We tested two pairs of plasmid and plasmid-less isogenic *S. typhimurium* strains for the production of these proteins and found that this plasmid was not required for regulation and synthesis of the proteins (*5*). We also incubated *S. choleraesuis* at 42°C for 10 min before labeling to determine whether the bacterial proteins regulated by the presence of epithelial cells were similar to those induced by heat shock (*7*). No similarities were observed between proteins induced by these two systems.

As an alternative to using glutaraldehyde-fixed monolayers to induce these proteins, we used monolayers of MDCK or Hep-2 (human epithelial) cells grown on plastic surfaces. Treatment of the monolayers with cycloheximide or emitine inhibited labeling of eukaryotic proteins but did not affect the levels of *S. typhimurium* or *S. choleraesuis* adherence or invasion (*5*). We found that the same proteins were regulated in the presence of viable epithelial monolayers with these bacteria [Fig. 2C, lanes 19 and 20 (MDCK cells) and lane 21 (Hep-2 cells)]. These proteins were not induced by Chinese hamster ovary cells (*5*), indicating that specific epithelial surface receptors are required for this induction. When we treated MDCK and Hep-2 monolayers with cytochalasin D [to inhibit bacterial internalization (*8*)] and cycloheximide, we again observed the same proteins being regulated [Fig. 2c, lane 20 (MDCK cells)]. These data, like those obtained with fixed monolayers, indicate that stable adherence of *Salmonella* to epithelial cells is associated with production of these proteins, and bacterial internalization is not required.

We used iodine-125 to label bacteria bound to fixed monolayers to determine whether these induced proteins were exposed on the bacterial surface. Several proteins were masked by iodinated eukaryotic proteins (Fig. 2B, lane 8), but one of the induced proteins (40 kD) was exposed on the surface of bound bacteria (Fig. 2b, lane 10), with an intensity of labeling slightly less than that of the outer membrane proteins (36 kD and 38 kD) normally present in *S. choleraesuis* (Fig. 2B, lanes 9 and 10). When the surface of a mutant of *S. choleraesuis* that does not synthesize these proteins (discussed below) was iodinated, a 40-kD band was not observed, even though the outer membrane proteins were visible (Fig. 2B, lane 12).

We previously identified six classes of transposon Tn*phoA* mutations in *S. choleraesuis* that are unable to stably adhere, invade, or penetrate through epithelial monolayers (*9*). We also identified two transposon Tn*10*

Table 1. Effect of various inhibitors on *S. choleraesuis* adherence and invasion of MDCK epithelial cells. Bacteria and monolayers were treated separately with antibiotics or at 4°C for 15 min before infection, and these drugs were present for the duration of the experiment. Viable monolayers were washed with phosphate-buffered saline (PBS) and then treated with trypsin or periodic acid in PBS for 10 min at 37°C and again washed before medium and bacteria were added. Neuraminidase treatment was done in 100 m*M* sodium acetate, *p*H 5.0, for 30 min at 37°C. Polarized MDCK monolayers grown on filters (*3*) were washed in PBS and then fixed in 2% glutaraldehyde at 4°C for 2 hours. Monolayers digested with trypsin, neuraminidase, or periodic acid were treated before fixation. [^{35}S]Methionine-labeled bacteria (*3*) were added to fixed monolayers and incubated for 4 hours. Monolayers were washed and the remaining radioactivity was counted to determine stable adherence. Adherence values are expressed as the percentage of total counts added that remain associated with the fixed monolayer ± SD and represent the average of three samples. Invasion assays are described elsewhere (*8*). Invasion values are expressed as the percentage of the initial inoculum of bacteria that are gentamicin resistant ± SD and represent the average of three samples. ND, not determined.

Treatment	Adherence (%)	Invasion (%)
Untreated	16.2 ± 1.6	1.9 ± 0.2
4°C	0.89 ± 0.23	ND
Chloramphenicol (30 μg/ml)	0.56 ± 0.09	0.004 ± 0.003
Rifampin (32 μg/ml)	0.49 ± 0.01	0.002 ± 0.001
Gentamicin (100 μg/ml)	0.53 ± 0.04	0 ± 0
Naladixic acid (20 μg/ml)	15.8 ± 2.1	1.8 ± 0.3
Periodic acid (10 m*M*)	5.3 ± 0.2	0 ± 0
Trypsin (0.025%)	4.5 ± 1.2	ND
Neuraminidase (5 U/ml)	9.6 ± 1.3	0.12 ± 0.01

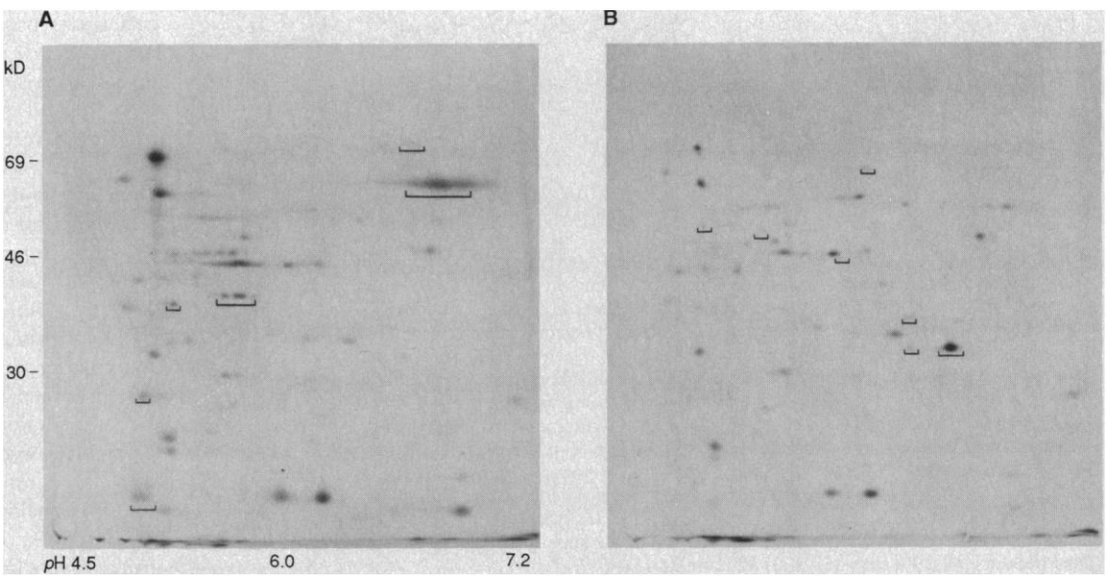

Fig. 3. Autoradiograph of two-dimensional gels containing (**A**) extracts of [^{35}S]methionine-labeled *S. choleraesuis* bound to the apical surface of MDCK monolayers for 4 hours or (**B**) unbound bacteria removed from the supernatant of sample in (A). Gels were prepared and run as described (*13*).

mutations in *S. typhimurium* that show decreased levels of invasion in epithelial cells and macrophages (*9*). We examined these mutant strains to determine whether any of the regulated proteins were affected by these mutations. We found that bacteria belonging to one class (class 6) of the Tn*phoA* mutations of *S. choleraesuis* no longer made the regulated proteins in the presence of fixed monolayers (Fig. 2C, lane 18), and only small numbers of bacteria adhered. The other five classes, including rough mutations in core and O–side chain lipopolysaccharides, all synthesized the induced proteins (*5*). The two *S. typhimurium* Tn*10* mutants did not synthesize the induced proteins in the presence of epithelial cells (Fig. 2D, lanes 25 and 26), nor did they adhere well. These two mutants had a median lethal dose (LD_{50}) five orders of magnitude greater than that of the parent strain when injected intraperitoneally into mice (*9*), an indication that these proteins are required for virulence of *S. typhimurium*. The results obtained with these mutants confirm that *Salmonella* must synthesize these proteins to stably adhere to and invade epithelial cells.

We used various agents to alter the surface of epithelial cells to determine the nature of the compounds of the eukaryotic cell that were required for induction of these proteins. Treatment of polarized MDCK monolayers with periodic acid, trypsin, and neuraminidase before fixation all caused lowered levels of stable bacterial adherence (Table 1). Treatment of viable monolayers with periodic acid and neuraminidase also significantly decreased invasion levels (Table 1). If *S. choleraesuis* or *S. typhimurium* was added to fixed monolayers that had been treated with any of these three agents, the induction of these proteins was no longer observed (Fig. 2C, lanes 14 to 16). Thus if the epithelial cell surface is modified by proteolytic or neuraminidase treatment, these bacteria bind poorly and do not synthesize proteins essential for stable adherence and invasion, indicating that glycoprotein-like structures are necessary for these processes. These experiments do not distinguish whether the receptor responsible for initial adherence or that required for stable adherence is disrupted, because altering either receptor would produce the observed results.

Bacteria contain many operons that are regulated by different environmental signals (*10*). These systems usually allow the bacterium to synthesize the proteins required to cope with the new environments and are often regulated by conserved two-component systems, one of which interacts with the stimulus while the other regulates the necessary genes (*10*). The interaction of *Salmonella* species with epithelial cell surfaces, and the resultant changes in bacterial proteins, may be similar to these other regulated systems. We believe these bacteria adapt to intracellular life by synthesizing proteins required for entry into and possibly intracellular growth within this niche. Although the concept of pathogenic organisms adapting to new environments inside hosts is an attractive one, there are few reported examples (*11*). We think that the system that we have reported represents one such system, and, as the tools become available, more systems analogous to this one will be identified in other intracellular pathogens.

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A Family of Putative Potassium Channel Genes in *Drosophila*

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Mutant flies in which the gene coding for the *Shaker* potassium channel is deleted still have potassium currents similar to those coded by the *Shaker* gene. This suggests the presence of a family of *Shaker*-like genes in *Drosophila*. By using a *Shaker* complementary DNA probe and low-stringency hybridization, three additional family members have now been isolated, *Shab*, *Shaw*, and *Shal*. The *Shaker* family genes are not clustered in the genome. The deduced proteins of *Shab*, *Shaw*, and *Shal* have high homology to the *Shaker* protein; the sequence identity of the integral membrane portions is greater than 50 percent. These genes are organized similarly to *Shaker* in that only a single homology domain containing six presumed membrane-spanning segments common to all voltage-gated ion channels is coded by each messenger RNA. Thus, potassium channel diversity could result from an extended gene family, as well as from alternate splicing of the *Shaker* primary transcript.

THE *SHAKER* GENE OF *DROSOPHILA melanogaster* codes for a potassium channel of the transient type (*1*) that is present in both nerves and muscles (*2*). In addition to the primary sequence of the peptide, the cloning of the K^+ channel coded by the *Shaker* gene revealed a possible mechanism for generating the diverse K^+ currents in different cells and tissues: the *Shaker* gene primary transcript is alternately spliced so as to produce a host of transcripts that encode proteins differing at their amino and carboxyl termini (*3*). Expression of these transcripts in *Xenopus* oocytes has shown that the alternative peptides produced by this gene form channels with different biophysical properties (*4*).

In addition, *Shaker* may be but one member of a K^+ channel gene family. Other ion currents in *Drosophila* with similar biophysical properties to the current coded by the *Shaker* gene are coded by at least one additional gene (*5, 6*). Solc *et al.* (*6*) reported that at least one *Shaker*-like current was present in larval neurons of some *Shaker* mutants. We have extended these studies and have found that a variety of *Shaker*-like currents are present in pupal neurons after the *Shaker* gene is removed genetically (*7*).

We isolated several *Shaker*-like cDNAs by screening a cDNA library at low stringency; a *Shaker* cDNA probe containing all pre-

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