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Genetic Approaches to Microbial Pathogenicity

Edited by W. Goebel

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Professor Dr. WERNER GOEBEL
Institut für Genetik und Mikrobiologie
Universität Würzburg
Röntgenring 11

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Preface

Important progress in the elucidation of the mechanisms influencing bacterial pathogenicity has recently been made through the introduction of modern genetic techniques. Molecular cloning allows the isolation of genes for phenotypes that epidemiological surveys have suggested play an important role in pathogenesis. The structural analysis of determinants for pathogenic traits can lead to the identification not only of the primary sequence but also of the possible secondary and tertiary structures for important virulence factors such as toxins and adhesins. From these data, the prediction of antigenic domains suitable for the development of new vaccines appears to be feasible. The regulation of virulence determinants by endogenous and exogenous factors can be more clearly understood through the functional analysis of the cloned virulence genes.

This volume surveys representative virulence properties of gram-positive and gram-negative bacteria to which the genetic approach has been successfully applied. The examples described here include important bacterial toxins (e.g., diphtheria toxin, cholera toxin, toxic shock syndrome toxin, hemolysins), adhesion structures from *E. coli* and *Neisseria gonorrhoeae*, and factors supporting iron uptake, serum resistance, and invasiveness in a variety of bacteria. Both the present state and the possible futural developments of these systems are described.

W. GOEBEL

List of Contributors

BÅGA, M., Department of Microbiology, University of Umeå, S-901 87 Umeå

BARON L.S., Department of Bacterial Immunology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20307-5100, USA

BILLYARD, E., Department of Molecular Biology, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

BINDEREIF, A., Department of Biochemistry, University of California, Berkeley, CA 94720, USA

BITTER-SUERMANN, D., Institute of Medical Microbiology, University of Mainz, D-6500 Mainz

BOULNOIS, G.J., Department of Medical Biochemistry, University of Geneva, CH-1211, Geneva, and Institute of Medical Microbiology, University of Leicester, United Kingdom

BUYSSE, J., Department of Bacterial Immunology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20307-5100, USA

CABELLO, F.C., Department of Microbiology, New York Medical College, Valhalla, NY, USA

CURTISS III, R., Department of Biology, Washington University, St. Louis, MO 63130, USA

DEAL, C., Department of Molecular Biology, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

DE GRAAF, F., Department of Microbiology, Biological Laboratory, Vrije Unviersiteit, De Boelelaan 1087, NL-1081 HV Amsterdam

FALKOW, S., Department of Medical Microbiology, Stanford University School of Medicine, Stanford, CA 94305, USA

GETZOFF, E., Department of Molecular Biology, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

GILMORE, M.S., Department of Microbiology and Immunology, University of Oklahoma, Health Sciences Center,

P.O. Box 26901, Oklahoma City, OK 73190, USA, and The Dental Research Institute, School of Dentistry, The University of Michigan, Ann Arbor, MI, USA

GÖRANSSON, M., Department of Microbiology, University of Umeå, S-901 87 Umeå

HACKER, J., Institut für Genetik und Mikrobiologie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg

HAGBLOM, P., Department of Molecular Biology, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

HUGHES, C., Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, United Kingdom

ISBERG, R.R., Department of Medical Microbiology, Stanford University, School of Medicine, Stanford, CA 94305, USA

KOPECKO, D.J., Department of Bacterial Immunology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20307-1500, USA

LINDBERG, F.P., Department of Microbiology, University of Umeå, S-901 87 Umeå

LORY, S., Department of Microbiology and Immunology, University of Washington, Seattle, WA 98195, USA

LUND, B., Department of Microbiology, University of Umeå, S-901 87 Umeå

MARTINEZ, R.J., Department of Microbiology, College of Letters and Science, University of California, Los Angeles, CA 90024, USA

MEKALANOS, J.J., Department of Microbiology and Molecular Genetics, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115, USA

MEYER, T. F., Department of Molecular Biology, Max-Planck-Institute, Jahnstraße 29, D-6900 Heidelberg

MONTGOMERIE, J.Z., Department of Medicine, Rancho Los Amigos Hospital, Downey, CA 90242, USA

MOOI, F.R., Department of Microbiology, Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, NL-1081 HV Amsterdam

MURPHY, J.R., Biomolecular Medicine Section, Evans Department of Clinical Research, and Department of Medicine, University Hospital, Boston University Medical Center, Boston, MA 02118, USA

NEILANDS, J.B., Department of Biochemistry, University of California, Berkeley, CA 94720, USA

NORGREN, M., Department of Microbiology, University of Umeå, S-901 87 Umeå

NORMARK, S., Department of Microbiology, University of Umeå, S-901 87 Umeå

PORTNOY, D.A., Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, NY 10021, USA

SEGAL, E., Department of Molecular Biology, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

SO, M., Department of Molecular Biology, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

TAI, P.C., Department of Metabolic Regulation, Boston Biochemical Research Institute, Boston, MA 02114, USA

TAINER, J., Department of Molecular Biology, Max-Planck-Institute, Jahnstraße 29, D-6900 Heidelberg

TIMMIS, K.N., Department of Medical Biochemistry, University of Geneva, CH-1211 Geneva

UHLIN, B.E., Department of Microbiology, University of Umeå, S-901 87 Umeå

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Genetic Analysis of Bacterial Virulence Determinants in *Bordetella pertussis* and the Pathogenic *Yersinia*

R.R. Isberg and S. Falkow

1 Introduction

With the advent of recombinant DNA technology, rapid advances have been made in understanding the molecular organization of bacterial pathogens (for review, see Macrina 1984). This has been especially true in the analysis of enterotoxin structure and function (So et al. 1976, 1978; Moseley and Falkow 1980; Pearson and Mekalanos 1982) as well as in the evaluation of bacterial pili and hemolysins in gastrointestinal or extraintestinal infections (Svanborg-Eden and Hansson 1978; Welch et al. 1981; Hartlein et al. 1983). Similarly, such studies have clarified the role of iron uptake in the infectious process (Crosa 1984) and have been helpful in understanding better the molecular epidemiology of disease agents (Hull et al. 1984). We suppose that in the near future most virulence factors defined by classic studies in medical microbiology will be analyzed at the genetic level in some manner or other.

The cloning of a bacterial virulence factor does not, in itself, determine how the factor contributes to the infection of an animal host. For example, while we could clone and subsequently introduce mutations into the IgA protease gene of *Neisseria gonorrhoeae* (Koomey et al. 1982), the absence of a convenient and inexpensive model infection system hampered our ability to demonstrate clearly that this interesting protein was essential for gonoccal virulence. Similar problems have been encountered in analyzing potential determinants of chlamydial virulence. In this latter case, not only are animal infection studies difficult, but the absence of a method of DNA exchange makes it currently impossible to perform meaningful mutational analyses that are necessary and desirable if specific gene products are to be evaluated for their role in virulence.

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, CA 94305, USA

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It seems to us that the analysis of microbial pathogenicity is most likely to proceed rapidly if one can construct mutants in the organism of interest and, in turn, if these mutants can be evaluated for virulence in either an animal host or in some kind of model infection system. This concept, though not at all original (for example, see BURROWS 1962), means that the true power of the recent advances in DNA technology lies as much in the construction and analysis of well-defined mutants as in the isolation and sequencing of genes. For instance, we believe one of the most useful avenues in our study of *Yersinia* virulence (PORTNOY and FALKOW 1981; PORTNOY et al. 1983; PORTNOY and MARTINEZ, this volume) was the ability to define the physical structure of avirulent mutants located on a plasmid in *Y. pestis*. Although such mutants had been originally isolated 20 years previously (HIGUCHI and SMITH 1961), their pleiotropic nature had remained a mystery. With the ability to isolate and map plasmid DNA, these mutations could be mapped physically, now rendering it a rather straightforward proposition to define the gene products that have been eliminated by these lesions.

Unquestionably, the increased application of genetic analysis to the study of bacterial pathogens will result in the discovery of new factors not previously known to play a role in virulence. This type of analysis permits us to go beyond the cloning of toxins and adherence factors into the realm of defining the roles various gene products play in allowing intracellular parasites to invade and survive within the tissues of their hosts. In order to define the factors that allow the intracellular pathway to take place, the investigator must rely on isolating mutants that eliminate or alter the phenotype of interest. In this sense, genetic analysis can achieve its full resolving power: it allows the investigator to discover new proteins whose mode of action may turn out to be quite novel. In our view, therefore, the goal of most analyses is to develop a method of screening or selecting for simple phenotypes that make possible the discovery of new gene products and the isolation of new classes of avirulent mutants.

In this article, we will describe how the development of genetic systems for the study of two organisms has made possible the description of new gene products involved in the infection process. Studies on these organisms relied on the ability to introduce mutations eliminating virulence in one of them (*Bordetella pertussis*) and the ability to select for a simple phenotype in the other (*Y. pseudotuberculosis*). We will conclude by suggesting some genetic techniques that have, in our judgment, been underused in the evaluation of virulence.

2 Insertion Mutagenesis To Define Virulence Factors

During the past decade the easiest factors to describe genetically have been those that could be cloned in *Escherichia coli* from another organism by selecting or screening for a particular phenotype. Unfortunately, this is not always possible particularly when dealing with fastidious pathogens; perhaps a simple phenotype does not exist or the desired gene does not express a product when cloned into *E. coli*. Under these circumstances, it is necessary to develop an alternative

method. One such approach is to isolate mutations in the pathogenic organism and analyze the lesions for their effects on virulence. The results will often suggest more fruitful approaches than cloning the desired genes (WEISS and FALKOW 1983).

We initiated our studies on the pathogenesis of *B. pertussis* infection by attempting to clone and characterize the genes encoding the pertussis toxin. Attempts to clone these genes using standard recombinant DNA technology failed (WEISS and FALKOW 1983), so it became apparent that more information had to be obtained concerning the genetic organization and expression of these genes. For this reason, mutants were isolated that lacked various virulence determinants, and the effects of these mutations on the pathogenesis of disease were evaluated. The availability of an animal model for *B. pertussis* infection made such a proposition feasible (PITTMAN et al. 1980).

Until recently, the isolation and mapping of mutations in an organism that had no established system of genetic exchange was a monumental task. Mutants could be isolated with hard work, but determining how many loci were affected by the lesions was often impractical. Fortunately, utilization of insertion mutagenesis now bypasses many of the classic problems of mutant isolation and mapping. The advantages of using transposable elements to isolate such mutants are manifold (KLECKNER et al. 1977). Insertion mutations usually result in totally eliminating the expression of the gene; their phenotype is rarely leaky. They are easy to map physically and genetically. The insertion of a drug-resistance transposon enables a mutation to be located either by changes in the restriction pattern of a DNA fragment or by genetically mapping the site of drug resistance. Finally, they are easy to isolate and are almost always the result of only a single insertional event, so having secondary mutations in the same strains is not a problem. For this reason, a protocol was devised for isolating insertions of the transposon Tn*5* into the chromosome of *B. pertussis* (WEISS et al. 1983).

Insertions of Tn*5* onto the chromosome were isolated by selecting for kanamycin resistance after introduction of a replication-defective plasmid that harbored the transposon (WEISS et al. 1983). Each kanamycin-resistant colony that grew was the result of a transposon-induced mutation, so relatively few colonies had to be screened in order to isolate lesions in the desired genes. Pools of insertions were then screened for their ability to produce pertussis toxin, hemolysin, an extracellular adenylate cyclase, a filamentous hemagglutinin (FHA), and dermonecrotic toxin. Mutants were isolated that reduced expression of hemolysin, pertussis toxin, and FHA individually, or reduced expression of the cyclase and hemolysin coordinately. Most importantly, several insertions were identified that eliminated expression of all of these factors simultaneously, implying that expression of these proteins is under the positive control of a single locus (WEISS and FALKOW 1983), as had been suspected from previous studies (KASUGA et al. 1953).

The mapping of mutations and the proof that they cause the phenotype being studied are the most important features of genetic analysis. There is no gross map of the *B. pertussis* chromosome, so mapping was performed by locating the site of insertion mutations on single restriction fragments, using Southern blot hybridization. It was found, in general, that each mutation that eliminated

the expression of a particular gene product mapped in the same restriction fragment, while mutations affecting different proteins were located on different DNA fragments. It appeared, therefore, that the affected genes were not closely linked physically. Proof that each mapped insertion was the cause of the negative phenotype was almost as straightforward. To accomplish this, one usually attempts to isolate and analyze revertants of the mutation. An alternative method had be pursued, however, since no selection existed that enabled spontaneous revertants to be isolated. To this end, it was possible to exploit a system of genetic exchange that involved mobilization of *B. pertussis* chromosomal genes by a conjugative plasmid integrated into the chromosome (WEISS et al. 1983). Crosses were performed between an avirulent Tn*5* insertion mutant and a fully virulent kanamycin-sensitive donor. Recombinants that regained virulence always simultaneously lost the Tn*5* insertion, showing that this insertion was the cause of the avirulent phenotype.

Perhaps the most useful insight from this work was the results on the regulation of virulence factors and the relative contribution of distinct virulence determinants to pathogenesis. In both cases, progress depended on the development of a convenient system for the isolation and characterization of mutants. Inactivation of a single locus, *vir*, resulted in simultaneous elimination of expression of a whole battery of virulence-associated factors, including all known toxins and adhesins encoded by *B. pertussis*. This suggested that *vir* encoded a positive regulator of these factors, and that cloning of these factors in *E. coli* might be facilitated by the presence of *vir* in trans. Subsequently, this has proven to be the case (WEISS et al. 1985). Secondly, the use of an appropriate animal model, lethality in the infant mouse following intranasal inoculation, provided important insights into the mechanism of pathogenesis. For instance, mutants that were defective only in the production of pertussis toxin were avirulent, establishing conclusively the importance of this protein (WEISS et al. 1984). Similarly, for the first time, the adenylate cyclase protein could be demonstrated to play an essential role in disease.

We believe that the main lessons learned from studies on *B. pertussis* are applicable to the analysis of virulence factors encoded by most bacterial pathogens. The inability to clone a virulence factor does not imply that studies in molecular genetics are impossible for that organism. One should not overlook the fact that the use of insertion elements as mutagens has become almost as powerful a genetic technique as the use of recombinant DNA technology. In the case of *B. pertussis*, insertional analysis permitted the isolation of both specific and pleiotropic mutants that could be mapped physically and characterized genetically in a detailed manner. Previously, such mutants were either not isolatable or not as easily understood. In addition, this analysis suggested a new approach for the cloning of genes that were previously found to be refractory to straightforward recombinant DNA methods. The other lesson that is reemphasized from these results is that the genetic analysis of pathogenic traits is best done in conjunction with a reasonable animal infection model. When such applicable animal systems do not exist, or existing systems are not exploited, the relevance of even the most elegant genetic and molecular analysis may be unclear.

3 Genetics of Invasiveness

Our studies on the pathogenesis of *B. pertussis* illustrate how genetic analysis can be utilized to gain insight into well-recognized virulence factors such as toxins and adhesins. Can such an approach be used to investigate less familiar factors? In this section, we will describe how we have used a genetic approach to study the physiology and pathogenesis of a more poorly understood process. Using a simple infection system and insertion mutagenesis, we have investigated the genetic basis for the ability of a pathogenic bacterium to invade epithelial cells.

Many pathogenic species of bacteria are able to invade the tissues of their hosts. For some of these species, such as *Shigella dysenteriae*, *Mycobacterium tuberculosis*, and *Legionella pneumophila* (FORMAL et al. 1983; WONG et al. 1980), invasion appears to be central in the evolution of their host-parasite relationship. Invasiveness appears to be important to these bacteria for several reasons, not the least of which is that it provides the bacterium with a mechanism to avoid host defenses. Hence, the bacterium is sequestered from antibody, antibiotics, and phagocytes. In addition, the invading microorganism has the ability to evade intracellular killing mechanisms while multiplying intracellularly in a "safe" niche. Furthermore, the ability to invade enables the bacterium to cross epithelial barriers, enter the lymphatic system, and subsequently become systemically distributed. Although most workers agree that invasiveness is an important factor in the pathogenesis of disease, the factors encoded by the bacterium that allow it to be internalized by the animal cell had not been identified to date (SANSONETTI et al. 1983). Our interest in this problem stems from our belief that invasive bacteria represent a class of pathogens that is basically different from specifically adherent toxin-producers, such as *B. pertussis*, in its pathway of infection.

The invasive phenotype can be easily studied in vitro by assaying for the endocytosis of the pathogen in cultured epitheloid cells (DEVENISH and SCHIEMANN 1981; SANSONETTI et al. 1983). The most convenient assay simply involves the addition of bacterial cells to a monolayer of animal cells for a few hours. The monolayer is then washed exhaustively, and fresh medium containing the antibiotic gentamicin is added. This antibiotic kills extracellular bacteria; bacteria survive if they have invaded the epithelial cell, since gentamicin does not efficiently cross the animal cell membrane. The degree of invasion can then be quantitated by gently lysing the animal cells in nonionic detergent, releasing the intracellularly located bacteria so they can be titered on a suitable bacteriological growth medium. We will show that this simple assay allows a rather detailed analysis of a factor that promotes invasion of a pathogen.

We have chosen *Yersinia pseudotuberculosis* as a model system for studying the invasive phenotype. This microorganism usually causes a zoonotic illness (MERCHANT and PARKER 1962), although it can cause an enteric disease in humans that leads to mesenteric lymphadenitis. A fatal disease that parallels this systemic illness can be induced in the mouse or guinea pig with this organism. We believe there are three advantages to studying *Y. pseudotuberculosis*. First it efficiently invades cultured epithelial cells (BOVALLIUS and NILSSON 1975).

Second, it is a member of the *Enterobacteriacae*, (as is *E. coli*) and is sensitive to coliphage P1, allowing DNA to be easily introduced into the bacterium via transduction (BOLIN and WOLF-WATZ 1984). Third, animal models exist that allow the pathogenicity of mutants to be evaluated (BOLIN et al. 1982).

It would be desirable to define all the determinants encoded by this bacterium that promote the invasion of animals cells. As a first step, we decided to take a simple approach and attempt to clone the genes responsible for this process into *E. coli* K12, an organism that is normally noninvasive. To this end, we introduced into *E. coli* a cosmid clone bank of the *Y. pseudotuberculosis* chromosomal DNA, and enriched for strains that were able to invade cultured HeLa cells. To enrich for the desired *E. coli* strain, a mixed culture containing representatives from the entire clone bank was pooled and allowed to infect a monolayer of HeLa cells. After allowing time for binding and invasion of the desired clone into the monolayer, the cells were washed exhaustively to remove noninvasive organisms. Bacteria that had bound or invaded the animal cells were then released from the monolayer by gentle detergent treatment.

Using the above protocol, about one-half of all the bacteria that survive the enrichment contain recombinant plasmids encoding *Y. pseudotuberculosis* gene products that convert *E. coli* K12 into a HeLa cell-invasive organism. Each of the clones surviving the enrichment invade cultured animal cells about as efficiently as *Y. pseudotuberculosis*, implying that the relevant factors encoded on the plasmids in these strains are expressed efficiently and localized properly in *E. coli*. Furthermore, the kinetics of invasion appear identical for *Y. pseudotuberculosis* and the *E. coli* clone.

In order to localize the region of DNA that encodes the factors conferring invasiveness, Tn*5* insertion mutations were isolated in the plasmid in a manner similar to that described above for *B. pertussis*. All of these mutations mapped in a 3.2-kilobase region of the cosmid clone. Deletion and more insertion mutations, this time with the nonpolar Tn*1000* (IIDA et al. 1984), were isolated in this region and used in functional complementation tests with previously isolated Tn*5* mutations. All mutations were found to fall into only one complementation group, indicating that only one protein may be necessary to confer the invasive phenotype.

We have begun physiological and electron microscopic studies of *E. coli* cells containing either the intact invasion gene or various insertion mutations in that gene (Fig. 1). Thin-section electron microscopy shows that the bacteria first bind HEp-2 cells at 0 °C without invading (Fig. 1b). This is identical to what is seen with *Y. pseudotuberculosis*. The bacteria are soon surrounded by microvilli after being bound by the animal cell. When the temperature is raised to 37 °C, vacuoles containing large numbers of bacteria are observed within the HEp-2 cells, although some vacuoles containing only one bacterium are also present. There may be an occasional bacterium in vacuole-free cytoplasmic environments, but these are distinctly in the minority. Thin sectioning of an infection by an insertion mutant, in contrast, shows no bacteria present in the environment of the animal cell. When radiolabeled bacteria were used to study binding to the epithelial cells, we found that each insertion mutation that had lost the invasive phenotype was also unable to bind the animal cells

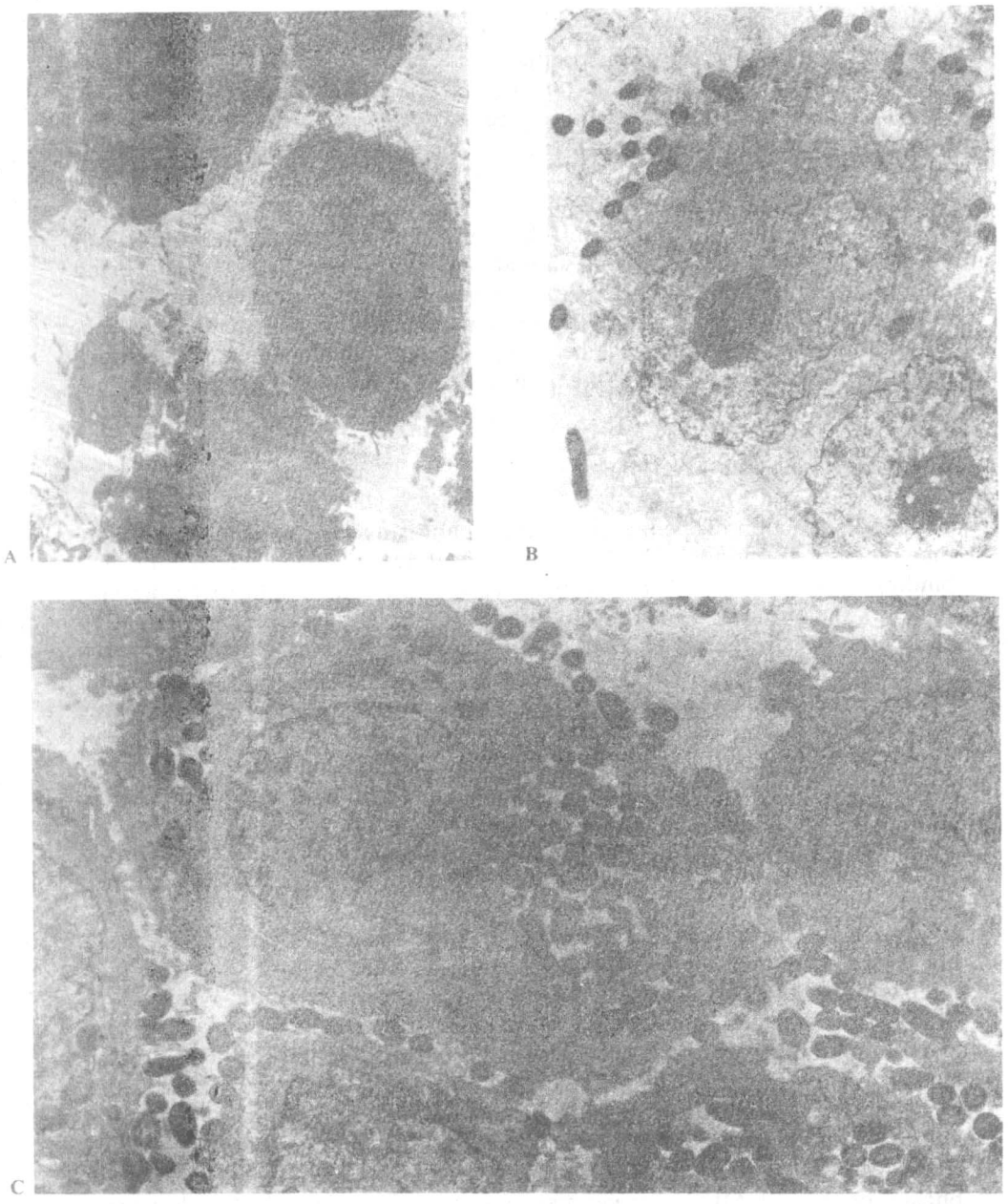

Fig. 1A–C. Thin section electron microscopy of the invasion of HEp-2 cells by *E. coli* strains. HEp-2 cells were infected with *E. coli* strains that harbored either a plasmid encoding the factor required for tissue invasion or an insertion mutation in this plasmid that eliminated invasion. Infection by an insertion mutant (**A**); infection at 0 °C by strain harboring intact invasiveness plasmid (**B**); infection at 37 °C by strain harboring intact invasiveness plasmid