MOLECULAR BIOLOGY OF YERSINIAE: A MODEL FOR GRAM NEGATIVE PATHOGENESIS

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Molecular Biology of Yersiniae: A Model for Gram Negative Pathogenesis

Plasmid DNA Relatedness Among Different Serogroups

CI 001 RESTRICTION ENDONUCLEASE ANALYSIS OF PLASMID DNA IN EPIDEMIOLOGICAL SURVEY OF *YERSINIA PSEUDOTUBERCULOSIS* INFECTION, Hiroshi Fukushima and Seiji Kaneko, Public Health Institute of Shimane Prefecture, Matsue, Shimane 690-01, Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo 160, Japan

We attempted to determine whether restriction endonuclease analysis of plasmid DNA could serve as an epidemiologic tool in *Yersinia pseudotuberculosis* infection. We compared the restriction endonuclease patterns of 42 Mdal virulence plasmids from isolates from patients, domestic animals, pets, wild animals, meat, water and soil in Shimane Prefecture. Moreover, we examined the isolates on each of four cases of *Y. pseudotuberculosis* infections. In intrafamilial outbreaks by serotype 4b, the isolates contained plasmid with identical restriction patterns, respectively. In a case of serotype 4b infection contracted through water contaminated by a wild animal, serotype 4b was isolated from a water sample derived from a mountain stream from which the patient had drank and from a rat trapped in the upper part of this stream. The restriction patterns of the plasmids in these isolates showed rat and patient isolates to be identical but distinct from the water isolate. In a case of serotypes 1b and 3 infection contacted through water contaminated by a cat, serotypes 1b and 3 were isolated from two patients, soil of the puddles from which the patients had drank and the sand and cat feces in the sandbox. The restriction endonuclease patterns of the plasmid in each strain of serotypes 1b and 3 were identical. These data suggest the transmission of *Y. pseudotuberculosis* through water contaminated by animals carrying this species and that this analysis is valid for use as an epidemiologic tool.

CI 002 COMPARISON OF PLASMID DNA AMONG DIFFERENT SEROGROUPS OF YERSINIA ENTEROCOLITICA AND YERSINIA PSEUDOTUBERCULOSIS, Seiji Kaneko, Tsutomu Maruyama and Hiroshi Fukushima, Department of Food Hygiene and Nutrition, The Tokyo Metropolitan Research Laboratory of Public Health, Hyakunin-cho 3-24-1, Shinjuku-ku, Tokyo 169, and Public Health Institute of Shimane Prefecture, Nishihamasada, Matsue, Shimane 690-01, Japan.

Virulence associated plasmids from various serogroups of Yersinia enterocolitica and Yersinia pseudotuberculosis which were isolated from human, swine, dog, cat, mouse and wild animals in Japan were analyzed by BamHI restriction endonuclease. Y. enterocolitica serogroups O3, O5:27, O8 and O9 were harboring 40 to 50 megadalton virulence plasmids and these plasmids were all correlated from virulence associated properties of autoagglutination(AGG) and calcium dependent growth(CAD). Restriction endonuclease analysis of these plasmids showed serogroup distinctively different patterns. Same analysis were carried out to Y. pseudotuberculosis serogroups 1b, 2a, 2b, 2c, 3, 4b, 5a and 5b. These strains were also harboring 40 to 50 megadalton plasmids and correlated from AGG and CAD. It was observed that restriction endonuclease digestion patterns of plasmids from Y. pseudotuberculosis serogroup 1b, 2a, 2b, 2c, 3, 4b, 5a and 5b were classified into 2, 1, 2, 1, 4, 9, 3 and 3 types, respectively. In comparison with virulence plasmids of Y. enterocolitica, Y. pseudotuberculosis strains were harboring various type of plasmids in spite of same serogroup.

Molecular Biology of Yersiniae: A Model for Gram Negative Pathogenesis

Molecular Basis of Invasiveness

CI 003 PURIFICATION OF THE YERSINIA ENTEROCOLITICA AIL PROTEIN AND CHARACTERIZATION WITH MONOCLONAL ANTIBODIES, JAMES B. BLISKA, DOROTHY E. PIERSON AND STANLEY FALKOW, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305 The pathogenic Y. enterocolitica 8081c strain encodes at least two distinct factors that promote attachment to, and invasion of, cultured epithelial cells. The inv (invasion) and ail (attachment-invasion locus) genes were cloned and characterized by their ability to convert E. coli into invasive bacteria. E. coli HB101 containing the ail gene on a multicopy plasmid was used as a source of the ail gene product for purification and antibody studies. A method for the rapid purification of an aggregating 17 Kd protein present in the membranes of HB101ail was devised. The N-terminal sequence of the purified protein confirmed its identity as the Ail protein, and revealed the site at which removal of a 23 amino acid signal sequence occurs. A battery of monoclonal antibodies directed against purified Ail were generated and used to screen for the expression of the protein in Y. enterocolitica 8081c and other Yersinia species. Ail is expressed on the surface of both Y. enterocolitica 8081c and HB101ail, and a protein antigenically related to Ail is present in Y. pseudotuberculosis. No expression of an Ail-like protein was detected in Y. pestis. Membranes purified from HB101ail exhibit cell binding activity, suggesting that a ligand-receptor interaction between Ail and the eukaryotic cell mediates cell binding and invasion. The interaction of Y. enterocolitica 8081c and HB101ail with a Human colonic cell line (Caco-2) and a variety of other cell types was explored. Y.enterocolitica 8081c and HB101-ail invade non-polarized Caco-2 cells, but are unable to efficiently penetrate the apical surface of the polarized cell sheet. This result implies that that the receptor(s) involved in in cellular invasion by Y. enterocolitica are restricted to the basolateral surface of polarized cells.

CI 004 DETECTION OF INVASIVE YERSINIA SPECIES USING OLIGONUCLEOTIDE PROBES. Peter Feng, Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204. Oligonucleotide probes targeted to plasmid and chromosomal virulence markers in yersiniae were used to detect pathogenic Yersinia species. The invasive phenotype is chromosomally encoded by the inv gene in Y. pseudotuberculosis, and the inv and ail genes in Y. enterocolitica. The probes INV-3 and INV-4 are 21 nucleotides in length and directed to the 5'- and 3'-termini of the Y. pseudotuberculosis inv gene, respectively. Colony hybridization analyses of 150 yersiniae and non-yersiniae isolates showed that the INV probes reacted only with Y. pseudotuberculosis isolates. Southern blots of total yersiniae DNA showed that the probes hybridized with a 4.5 kilobase (kb) BamHI fragment known to carry the inv gene in Y. pseudotuberculosis. Neither INV probe hybridized with Y. enterocolitica DNA. However, DNA fragments amplified from Y. pseudotuberculosis inv gene using polymerase chain reactions hybridized to a 9.4 kb or a 9.7 kb EcoRV fragment in various Y. enterocolitica serotypes. These results suggest that there are homologous sequences between the inv genes of these Yersinia species. The oligonucleotide probe PF-13 is 18 nucleotides in length and targeted to the 3'-terminus of the ail gene. Colony hybridization results showed PF-13 to be specific for selected Y. enterocolitica isolates. The ail marker is reported to be present only in this Yersinia species, and on a 1.3 kb AvaI - ClaI fragment. This was confirmed by Southern blotting analysis using the PF-13 oligo probe. The results of colony hybridization studies using invasion gene-specific oligonucleotides are compared using HeLa cell invasion assays. Preliminary results indicate that the ail marker-specific PF-13 probe can reliably detect invasive Y. enterocolitica. An oligonucleotide probe, P-12, directed to the 42 MDal virulence plasmid of Yersinia was also examined. The P-12 probe is 18 mer in length and is targeted to the plasmid region which encodes the gene(s) responsible for cytotoxicity and Sereny reactions. Colony hybridization studies followed by plasmid analyses confirmed that P-12 probe will only hybridize to isolates of Yersinia bearing the 42 MDal plasmid.

CI 005 ANALYSIS OF CELLULAR ENTRY MEDIATED BY THE *Yersinia pseudotuberculosis inv* GENE, John M. Leong, Robert S. Fournier, Susannah Rankin, and Ralph R. Isberg, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave, Boston, MA 02111.

The *inv* gene of *Y. pseudotuberculosis* promotes bacterial penetration into mammalian cells (1). The gene encodes a 986 amino acid protein, termed invasin, that is localized in the bacterial outer membrane and tightly binds mammalian cells (2,3). Several lines of evidence indicate that invasin binds multiple members of the integrin superfamily of cell adhesion receptors. Affinity chromatography of crude detergent extracts demonstrated that integrins containing the subunit structures $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha 6\beta 1$ bound to immobilized invasin. Furthermore, phospholipid vesicles containing isolated integrin proteins were able to attach to invasin. Antibodies directed against the $\alpha 5$ or $\beta 1$ integrin subunits blocked both invasin-mediated attachment and bacterial entry into mammalian cells.

The region of invasin that is responsible for cell attachment was defined. Analysis of invasin deletion mutants and invasin fragments fused to *E. coli* maltose binding protein (MBP) revealed that the carboxyl terminal 192 amino acids of invasin are sufficient for cell attachment. Monoclonal antibodies directed against invasin were isolated, and all those that blocked cell binding recognized epitopes within this 192 amino acid region. One carboxyl terminal invasin fragment and seven MBP-invasin fusion proteins were purified, and all invasin derivatives that contained the carboxyl terminal 192 residues of invasin inhibited bacterial entry into cultured mammalian cells. Taken together, these results indicate that this region contains the integrin-binding domain of invasin, even though it does not contain the tripeptide sequence Arg-Gly-Asp. This 192 amino acid region of invasin is also sufficient for cellular entry, because bacteria coated with MBP-invasin fusion protein containing only this region of invasin efficiently entered mammalian cells.

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CI 006 THE INVASION GENES OF *YERSINIA ENTEROCOLITICA*. Virginia L. Miller. Department of Microbiology, University of California, Los Angeles. 405 Hilgard Ave., Los Angeles, CA, 90024.

The ability to enter, survive, and multiply within animal cells is a property common to many pathogenic bacteria. To study the first of these steps, invasion, we have been investigating the ability of *Yersinia enterocolitica* to invade human epithelial cells. Two genetic loci that confer an invasive phenotype on *E. coli*, *inv* and *ail*, have been cloned from *Y. enterocolitica*. The *inv* locus when present in *E. coli* promotes a high level of attachment and invasion of most tissue culture cells tested. Tn*phoA* translational fusions to *inv* were constructed and used to identify, characterize, and localize the *inv* protein product. Minicell analysis showed that *inv* encodes a protein of ~ 92,000 D; this protein is recognized by monoclonal antibodies directed against *Y. pseudotuberculosis* invasin. The *Y. enterocolitica* invasin is localized to the outer membrane and exposed on the surface of the bacterial cell.

The *ail* locus when present in *E. coli* promotes a high level of attachment to most cell types tested but promotes high levels of invasion for only a few of the cell types tested. The *ail* gene is expressed well in *E. coli* and encodes a protein of ~ 17,000 D. Ail has a typical prokaryotic signal sequence that is processed normally in *E. coli*. The *ail* promoter appears to be recognized by *E. coli*, because the same *ail* specific mRNA is observed in *E. coli ail* and *Y. enterocolitica* strain 8081c. In *Y. enterocolitica*, transcription of *ail* is greater when the bacteria are grown at 37°C as opposed to 23°C. The *ail* gene and the region surrounding it is found only in pathogenic strains of *Y. enterocolitica* and other pathogenic *Yersinia* species.

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Genetic Analysis of Virulence Plasmid Determinants

CI 007 GENETIC RELATIONSHIP BETWEEN THE SERENY REACTION AND HEp-2 CELL CYTOTOXICITY, Marianne D. Miliotis[1], J. Glenn Morris, Jr.[2] and Roy M. Robins-Browne[3], Division of Microbiology, U.S. FDA, Washington, DC 20204[1], Division of Geographic Medicine, University of Maryland School of Medicine, Baltimore MD 21201[2] and Division of Microbiology, University of Melbourne, Australia[3].

The presence of the virulence plasmid (pYV) of Yersinia enterocolitica is necessary for HEp-2 cell cytotoxicity and production of conjunctivitis in guinea pigs. In order to identify the genes associated with these phenomena BamHI and SalI restriction fragments of the virulence plasmid of Y. enterocolitica strain A2635 (serotype 0:8) (pYVA2635) were subcloned into derivatives of the broad host range vector, pRK290, and introduced into plasmid-negative Y. enterocolitica strains. Guinea pigs developed a mild, transient conjunctivitis twenty-four hours after intraconjunctival inoculation with strains containing either the 2.8-kb BamHI fragment of pYVA2635 or the 19.5-kb SalI fragment which includes the 2.8-kb BamHI fragment. These strains were also cytotoxic to HEp-2 cells. A mutant strain in which the 2.8-kb fragment had been deleted from pYVA2635 produced no effect in the guinea pig eye and was not cytotoxic to HEp-2 cells. These data demonstrate a 2.8-kb BamHI fragment of pYV is associated with development of conjuctivitis in guinea pigs and HEp-2 cell cytotoxicity.

CI 008 EFFECT OF INCREASED GENE DOSAGE ON THE LOW CALCIUM RESPONSE OF NALIDIXIC ACID-RESISTANT STRAINS OF YERSINIA PESTIS, Robert M. Zsigray, Department of Microbiology, University of New Hampshire, Durham, NH 03824. Conjugal transfer of the fertility factor (F) of Escherichia coli to Yersinia pestis strain EV76 results in the eventual integration of the approximately 47 megadalton, Vwa plasmid into the Y. pestis chromosome, along with the loss of the low calcium response (Lcr). On the other hand, transfer of F to isogenic strains of EV76 made resistant to nalidixic acid resulted in transconjugants that contained a cytoplasmic Vwa plasmid, all of which were predominantly Lcr^+. When preexisting strains of Y. pestis harboring an F'lac plasmid were plated on lactose indicator medium supplemented with nalidixic acid, several lac^+, nalidixic acid-resistant mutants were obtained and tested for plasmid contents and the expression of the lcr. With the exception of one isolate, termed EV76/3-nal, all of the lac^+, nalidixic acid-resistant clones remained Lcr^- and all contained an integrated Vwa plasmid. Although EV76/3-nal lacked a cytoplasmic Vwa plasmid, this strain was Lcr^+. Twelve lac segregants of EV76/3-nal were obtained and each tested for plasmid content and expression of the lcr. All were Lcr^+ (99.98%) and approximately 90% of the clones maintained the Vwa plasmid in the integrated state. These strains were then used for gene dosage studies to determine what effect an increase in copy number of the Vwa plasmid would have on existing Lcr^+ cells. Each isolate was transduced with $P1_{vir}$ lysates prepared with E. coli LE392 containing pYV019::Tn5 with the transposon being inserted either within (pYV019::Tn5 #1) or outside of (pYV 019::Tn5 #4) the lcr region of the Vwa plasmid. The Lcr status of at least one transductant per lac segregant was determined. Although the transductants harboring pYV019::Tn5 #4 were Lcr^+ relative to the total bacterial population, an increase in copy number of the Vwa plasmid reduced the number of Lcr^+ cells in the population by ten fold when compared to the controls (.26% vs .02%).

The transductants containing pYV019::Tn5 #1 were essentially Lcr^- (83.60%). This finding was unexpected since it was predicted that a functional lcr region would be dominant over a nonfunctional one. Experiments are currently being conducted to cure the cells of pYV019::Tn5 #1 to determine whether or not non-reciprocal, genetic recombination between the defective lcr region associated with the cytoplasmic Vwa plasmid and the functional lcr sequences located on the integrated plasmid had occurred. This possibility seems likely since isolates having a defective lcr region in the integrated state became Lcr^+ when the isolates harbored pYV019::Tn5 #4.

CI 028 MOLECULAR MIMIKRY BETWEEN A YERSINIA PROTEIN AND HLA-B27: NO REQUIREMENT FOR PRIMARY SEQUENCE HOMOLOGY, Uwe Groß*, Midori Hamachi Jin-Hai Chen, and David T. Y. Yu, Department of Medicine, University of California, Los Angeles, CA 90024, *Present address: Institute of Hygiene and Microbiology, University of Würzburg, Josef-Schneider-Str. 2, D-8700 Würzburg, Federal Republic of Germany.

The pathogenesis of reactive arthritis still has to be clarified. It was supposed that molecular mimikry between components of certain bacteria and HLA-B27 might be involved, because one pre-requisite of this disease is an episode of infection and most of the respecting patients are HLA-B27 positive.

Therefore, we were interested to further analyse the requirements for cross-reactivity between HLA-B27 and Yersinia. Using a polyclonal antibody reactive against a Yersinia protein as well as against HLA-B27 (1), a chromosomal library of Yersinia pseudotuberculosis was screened. One of the identified positive clones was reactive with several HLA-B27 specific monoclonal antibodies of the IgM and IgG class as was ascertained by three different immunological methods such as western blot, ELISA and immuno-precipitation. The reactive component was identified as being a 44,000 MW intracytoplasmic protein. DNA sequencing and the predicted amino acid sequence revealed only a very remote similarity between HLA-B27 and the bacterial clone. The reactive protein seemed to be highly antigenic, because it reacted with the vast majority of sera from patients with reactive arthritis as well as from healthy controls. Therefore, this Yersinia protein might not be involved directly in the pathogenesis of reactive arthritis.

Nevertheless, it was shown that for cross-reactivity between bacterial components and HLA-B27 an excessive homology of their DNA or amino acid sequences is not required. Instead, molecular mimicry might be determined by similarities in the secondary or tertiary structure of the two molecules.

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Role of Plasmid in Phagocytosis

CI 009 ANTI-PHAGOCYTIC ROLE OF PLASMID-MEDIATED CELL SURFACE COMPONENTS OF YERSINIA ENTEROCOLITICA, Chang-Joo Lian+ and Chik H. Pai*, Dept. of Surgery (Urology Division), University of Cincinnati Medical Center, OH 45267-0589+ and Dept. of Clinical Pathology, ASAN Foundation, Seoul Central Hospital, Seoul 134-040, Korea*

The role of the plasmid of Yersinia enterocolitica (YE) in phagocytosis was investigated using two isogenic strains that differed only in the presence (strain S) or absence (strain L) of a 42 Mdal plasmid. The plasmid, essential for the virulence of the organism, is expressed at 37°C in vitro with the appearance of plasmid-mediated cell surface components (pCSC) including novel outer membrane proteins (1,2). Our previous studies have shown a role for these pCSC in the interaction of YE with human neutrophils (PMNs)(3,4), as indicated by lower chemiluminescence (CL) response, phagocytosis-associated metabolic activities and phagocytosis by PMNs exposed to S strain grown at 37°C (S-37°C) as compared to the same strain grown at 25°C (S-25°C) or L strain grown at either temperature. Further findings suggested that the outer membrane fragments derived from S-37°C, but not S-25°C or L, inhibited the CL responses of PMNs to opsonized zymosan and pronase treatment of pCSC resulted in the elimination of the CL inhibition. Phagocytosis assay by direct microscopic examination showed that the ingestion and intracellular killing by PMNs were inhibited by pCSC. In vivo phagocytosis was examined by intradermal inoculation of S and L strains into the backs of rabbits. Histopathological examination of tissue sections showed total phagocytosis of L strain by PMNs and mononuclear cells, and almost complete absence of phagocytosis of S- strain. The mechanism of anti-phagocytic function of pCSC was examined by looking at their capacity for C3 binding. This binding was monitored by immunoelectron microscopy and fluorescent immunoassay following incubation with pooled normal human serum or purified C3. The amount of complement consumed by S-37°C (as measured by hemolytic complement activity) was 3-fold lower than avirulent L-37°C. The level of C3 deposited on S-37°C was several fold lower than S-25°C or L-37°C. Pretreatment with either pronase or specific heat-inactivated antiserum of S-37°C resulted in an increase in C3 binding to the same level as control strains. These data suggest that plasmid-mediated cell surface components, especially the outer membrane proteins, of YE interfere with the activation and binding of C3 and thereby confer resistance to phagocytosis.

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CI 010 EFFECTS OF *YERSINIA* ENCODED PRODUCTS ON EUCARYOTIC CELLS.

Roland Rosqvist[1], Åke Forsberg[2] and Hans Wolf-Watz[2],
1) Department of Cell and Microbiology, National Defence Research Establishment, S-901 82 Umeå, Sweden, 2) Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden

All three virulent *Yersinia* species can mediate a cytotoxic effect on cultured cells. The significance of the cytotoxic activity and the actual roles of the common gene products in this virulence process has not been revealed. We have here used three different model systems: mice, macrophages and HeLa cells, to investigate this event. A specific mutant of *Y. pseudotuberculosis* unable to express the plasmid-encoded protein YopE did not mediate a cytotoxic response on a confluent layer of HeLa cells. This ability was restored by the introduction of a hybrid-plasmid that carried the intact *yopE* gene. *Y. pestis* did not mediate cytotoxicity since it could not adhere to the surface of the HeLa cell showing that the YopE activity is contact dependent. Introduction of a plasmid that encoded either the invasin or the ModV (YopA) protein into *Y. pestis* generated cytotoxic strains that adhered to the HeLa cell surface. Only the extracellular cell-associated bacteria expressed significant amounts of the Yops and could mediate cytotoxicity. By the use of a *yopH/yopE* double mutant of *Y. pseudotuberculosis* it was shown that the YopE protein plays an important role in the ability of the pathogen to inhibit the phagocytic process of macrophages. Strains that showed reduced expression of YopE were avirulent after oral or intraperitoneal injection but were virulent following intravenous injection. Based on these analyses we propose a role of YopE in the virulence process of *yersinia*.

CI 011 IN VIVO IMPACT OF THE pYV PLASMID ON THE PATHOGENESIS OF *YERSINIA PSEUDOTUBERCULOSIS* INFECTION, Michel Simonet and Patrick Berche, Laboratoire de Microbiologie, Faculte de Medecine Necker-Enfants Malades, 156 rue de Vaugirard, F-75015, Paris FRANCE Virulence in the three pathogenic species of the genus *Yersinia, Yersinia pestis, Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, is controlled by a family of plasmids, approximately 70 kb, called pYV. They encode temperature-inducible, Ca^{2+} regulated proteins in the outer membrane (Yops) and in the cytoplasmic or periplasmic compartment (V-antigen). However little is known so far about the exact role of plasmid-mediated factors during the course of yersiniosis. The dramatic baterial growth observed in spleen, liver and lungs of mice inoculated by i.v. route with a pYV^+ strain of *Yersinia pseudotuberculosis* suggests that bacteria might escape the bactericidal mechanisms triggered by the host immune system during infection. In order to determine whether the virulent strain of *Y. pseudotuberculosis* can restrict in vivo immune mechanisms, we tested the capacity of mice to destroy an unrelated microorganism, *Listeria monocytogenes*, during the course of *Yersinia* infection. Mice were injected i.v. with a lethal challenge of strain pYV^+ (6. 10^2 bacteria). One day later, a sublethal dose of *L. monocytogenes* strain EGD (1. 10^4) bacteria was inoculated by the same route. Bacterial growth in the spleen was then followed over a 6-day period by differential counts of *Yersinia* and *Listeria* on selective culture media. With regard to *Listeria* survival in the spleen of these mice, it was observed at day 6 that *Listeria* counts in the spleen of *Y. pseudotuberculosis*-infected mice were *c* 1000 fold higher than those in the spleen of mice infected only with *L. monocytogenes*. This result suggests that immune mechanisms are strongly restricted during infection by *Y. pseudotuberculosis*. This immunosuppression is correlated with the presence of the pYV plasmid in bacteria since mice infected by the plasmidless derivative were able to eliminate *Listeria* from their tissues as efficaciously as control mice only infected with *Listeria*.
In the aim to better understand the in vivo role of the pYV plasmid on the pathogenesis of *Y. pseudotuberculosis* infection, we performed histological studies in mice infected by a pYV^+ strain or its plasmidless derivative. Bacteria harboring the pYV plasmid produce multiple necrotic abscesses poorly populated with inflammatory cells wheras pYV^- bacteria induced granulomas mostly constituted with polymorphonuclear and mononuclear cells. Strikingly, pYV^+ bacteria were only observed in extracellular sites by electron microscopy, suggesting that pYV plasmid might stimulate the extracellular bacterial multiplication in vivo through a severe restriction of the local immune response. The nature of the plasmid-encoded factors responsible for host immunosuppression remains to be determined.

Identification and Characterization of Plasmid-Mediated Expression of Outer Membrane Proteins

CI 012 CALCIUM DEPENDENCE AND EXPRESSION OF OUTER MEMBRANE PROTEINS BY VIRULENCE PLASMID IN YERSINIA ENTEROCOLITICA, Saumya Bhaduri, Eastern Regional Research Center, Agricultural Research Service, U. S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118

The relationship between calcium dependence and the expression of plasmid-encoded outer membrane proteins (POMPs) in virulent Yersinia enterocolitica is well characterized. To assess further this relationship, the expression of POMPs was investigated by growing virulent, plasmid-bearing Y. enterocolitica on low-calcium agarose-based media (238-311 μM: calcium-deficient) and high-calcium agar-based media (1400-1500 μM: calcium-adequate). POMPs expressed under these conditions were compared with polypeptides synthesized in vitro in a Y. enterocolitica virulence-plasmid-DNA dependent Escherichia coli cell-free coupled transcription-translation system. This permits identification of virulence plasmid-encoded gene products without interference from chromosomal gene products. Out of twenty-four polypeptides synthesized in vitro, ten were identified (based on identical molecular masses) as POMPs synthesized in vivo when virulent plasmid-bearing Y. enterocolitica cells were grown on these media. Two high-molecular weight POMPs synthesized in vivo by plasmid-bearing cells were not detected in vitro. Different plasmid-mediated POMPs were expressed in vivo in Y. enterocolitica grown on media containing different calcium concentrations. Y. enterocolitica grown on calcium-adequate media expressed twice the number of lower molecular weight POMPs as compared to the organism grown on calcium-deficient media. This is the first report in which a single serotype has been shown to synthesize all the reported virulence plasmid-encoded POMPs including three new proteins. The nutritional factors in the medium as well as the level of calcium appear to have a regulatory role in plasmid gene expression for lower molecular weight POMPs.

CI 013 **GENETIC EVIDENCE THAT YERSINIA OUTER MEMBRANE PROTEIN YOP1 INHIBITS THE ANTI-INVASIVE EFFECT OF INTERFERON**, Geir Bukholm, Georg Kapperud and Mikael Skurnik, Rikshospitalet, University of Oslo, National Institute of Public Health, Oslo, Norway, and Department of Medical Microbiology, University of Turku, Turku, Finland

All three Yersinia species that are pathogenic for man, harbor a 40-50 Mdal plasmid which is essential for virulence. Among the properties encoded by this virulence plasmid are calcium dependent growth restriction and production of a series of ancillary outer membrane proteins (Yops), the biological role of which is incompletely understood. In this work, HEp-2 cell monolayers were challenged with genetic variants of Y. pseudotuberculosis strain YPIII and Y. enterocolitica strain W22708. Both strains were represented by plasmid-bearing and plasmid-cured isogenic derivatives and by two transposon mutants constructed by insertional inactivation of the plasmid genes coding for calcium dependent growth and outer membrane protein Yop1, respectively. All variants localized intracellularily in HEp-2 cells within 3 h. However, when the cells were pretreated with recombinant alpha-2 interferon, only bacteria expressing Yop1 invaded the cells, whereas plasmid-cured derivatives and Yop1-negative transposon mutants remained extracellular. The ability to invade HEp-2 cells was restored, however, when a sterile filtered sonicate of Yop1 producing bacteria was added to the cell cultures. Likewise, the protective effect of interferon in cell cultures challenged with Salmonella typhimurium was eliminated when the filtrate was added. To ensure that Yop1 was responsible for the interferon inhibition observed, the Yop1 gene (yopA) was cloned into plasmid pBR322 which was transferred to a Yop1-negative insertion mutant of Y. pseudotuberculosis and to its plasmid-cured counterpart. Both transformants regained the ability to invade interferon treated HEp-2 cells. The results provide direct genetic evidence indicating that Yop1 is capable of inhibiting the anti-invasive effect of interferon in the HEp-2 cell model.

CI 014 The secretion of Yop proteins by Yersiniae

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Upon incubation at 37°C in the absence of Ca^{++} ions, virulent strains of *Yersinia* cease growing and produce large amounts of plasmid-encoded proteins involved in virulence [1]. These proteins are called Yops (for "Yersinia Outer-membrane Proteins")[2,3,4]. They are detected in both the outer-membrane fraction and the culture supernatant [5].

We investigated the exportation mechanism of Yops from *Yersinia enterocolitica O:9*. The study of the kinetics of Yops production as well as solvent extraction experiments indicate that Yops are not membrane-anchored but are true released proteins. The detection of Yops in the outer-membrane fraction likely results from the co-purification of aggregated Yops with membranes or from the adsorption of released proteins to the cell surface. The secretion of Yops by *Yersiniae* occurs through a new and specific exportation mechanism that involves neither the removal of a N-terminal signal sequence nor the recognition of a C-terminal secretion domain. The secretion step is inhibited by Ca^{++} ions. The lack of homology between the sequences of different Yops suggests that the secretion signal is conformational rather than sequential. The exportation machinery of *Yersiniae* allows efficient secretion of hybrid proteins.

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Mapping of Plasmid-Encoded Outer Membrane Proteins

CI 015 IDENTIFICATION, MAPPING AND SITE-SPECIFIC MUTAGENESIS OF VIRULENCE PLASMID ENCODED GENES OF Yersinia pseudotuberculosis,
Åke Forsberg, Hans Wolf-Watz, Ingrid Bölin, Lena Norlander and Thomas Bergman, Unit for Applied Cell- and Molecular Biology, University of Umeå, S-901 87 Umeå, Sweden.

The pathogenic species of the genus Yersinia, Y.enterocolitica, Y.pestis and Y.pseudotuberculosis all carry related virulence plasmids. All three plasmids encode a number of Ca^{2+} and temperature regulated genes (encoding Yops and V-antigen). Six of these genes were mapped on the different plasmids. All the structural genes but one mapped outside the Ca^{2+} region. The structural genes were scattered around the plasmids and contained within both monocistronic (yopE and yopH) and polycistronic operons (lcrV yopB yopD and lcrE/yopN). A number of specific insertion mutants were constructed in Y.pseudotuberculosis by insertion of a kanamycin resistance gene into the respective structural genes. All the insertions within the polycistronic operons also affected the common regulation of the yop genes. These mutants were all temperature-sensitive for growth at 37°C and therefore not possible to evaluate in the mouse virulence model. The yopE and yopH mutants, however showed wild-type phenotype with respect to in vitro growth and were avirulent. An insertion immediately downstream of the yopE gene was fully virulent, showing that the yopE insertion did not affect downstream genes. DNA sequencing revealed a divergently transcribed gene upstream of the yopE gene. An insertion in this gene, denoted yerA, resulted in a lowered YopE expression in vitro, and also loss of virulence. Both the yopE and the yerA genes could be transcomplemented to wild type expression levels in vitro, but could not be transcomplemented to virulence. This is likely to be due to the fact that the virulence plasmid and the transcomplementing plasmid are not stably maintained at 37°C. The yopH mutant on the other hand could be transcomplemented back to virulence by the cloned yopH gene. Thus, so far the YopE and YopH proteins have been clearly shown to be virulence determinants in Y.pseudotuberculosis, while the role of the other Yops and V-antigen has to be adressed by constructing nonpolar mutations in these genes.

CI 016 MOLECULAR BIOLOGY AND ROLE OF Yop1 ENCODED BY THE yopA GENE OF Yersiniae, Mikael Skurnik, Department of Medical Microbiology, University of Turku, SF-20520 Turku, Finland

The virulence plasmid of Yersinia encodes a number of temperature-inducible outer membrane proteins called Yops. One of these, Yop1, is involved in binding of bacteria to various substrates. Yop1 expression has been observed in the oral pathogens Y. enterocolitica and Y. pseudotuberculosis but not in Y. pestis. The yopA-gene has been sequenced from four virulence plasmids. The inability of Y. pestis to express the Yop1 protein is caused by only one base pair deletion in the coding region of the yopA gene of Y. pestis. We have recently shown that introduction of a functional yopA gene into Y. pestis leads to decreased virulence of this species. Insertion mutation in the yopA gene decreases the virulence of Y. enterocolitica; however, similar mutation in Y. pseudotuberculosis does not. Thus, the role of Yop1 in determining virulence is variable in these three three Yersinia species.

In attempts to elucidate the biological role of Yop1, many experimental models have been used. Initially, Yop1 was associated with autoagglutination, and with adherence onto epithelial cells in tissue culture. Later the fibril structure of Yop1 was established in electron microscope. Also, some role of Yop1 in the serum resistance of Y. enterocolitica was noticed. Recently, data has been obtained of the interactions of Yop1 with host factors such as collagen, fibronectin and interferon, and also of direct binding of Yersinia to intestinal tissue disks or setions.

CI 017 REGULATION OF PLASMID ENCODED VIRULENCE DETERMINANTS (Yops) OF YERSINIA PSEUDOTUBERCULOSIS, Hans Wolf-Watz, Åke Forsberg, Ingrid Bölin, Kerstin Ericksson, Thomas Bergman, Sebastian Håkansson, Marja Rimpiläinen and Mikael Skurnik, Unit for Applied Cell- and Molecular Biology, University of Umeå, S- 901 87 Umeå, Sweden.

Y. pestis, Y.pseudotuberculosis and Y.enterocolitica harbour related virulence plasmids that encode a number of inducible virulence determinants (Yops). These proteins are not expressed at 26°C but are induced at 37°C. When grown at 37°C, at Ca^{2+} concentrations exceeding 2,5 mM Ca^{2+}, the Yop expression is low and the proteins can only be detected in small amounts in the outer membrane of the pathogen. In contrast when Ca^{2+} is removed, the expression is very high and the Yops can be recovered in large amounts in the outer membrane fraction as well as in the culture supernatant. Thus, the expression of the Yops are regulated by the environmental stimuli Ca^{2+} and temperature. The structural genes of the Yops are scattered around the plasmid. The yop-genes are regulated by transacting negative and positive elements encoded by the 25 kb Ca^{2+} region. The positive loop is solely regulated by temperature and involves an activator LcrF that shows homology to AraC (1). LcrF is regulated by at least one chromosomal and one plasmid gene (lcrI). The negative control loop is involved in the cellular response to Ca^{2+}. The extracellular concentration of Ca^{2+} determines the intracellular level of repressor that inhibits the transcription from pyop. The regulation of the repressor is complex and involves at least 5 different plasmid encoded loci. One of these encodes LcrE and a lcrE mutant shows low repressor concentration at 37°C even the presence of 2,5 mol Ca^{2+} resulting in derepressed Yop expression at 37°C. The DNA-sequence of lcrE was determined and it could be shown that LcrE is identical to Yop4b/YopN. Thus, LcrE is an outer membrane/secreted protein involved in the low calcium response, suggesting that this protein is sensing the extracellular Ca^{2+} concentration and responds accordingly.

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Molecular Biology of Yersiniae: A Model for Gram Negative Pathogenesis

Role of Plasmid-Encoded Outer Membrane Proteins in Virulence

CI 018 FURTHER STUDIES OF THE 24,000-DALTON PLASMID-INDEPENDENT SURFACE PROTEIN OF Yersinia enterocolitica. M. Iriarte, M. A. Díaz-Laviada, J. R. Toyos, M. C. Frades and R. Díaz, Department of Microbiology, University of Navarra, Aptdo. 273, 31080 Pamplona, Spain.

When grown at 37°C on a solid medium supplemented with glucose and sucrose, the pathogenic serogroups of Y. enterocolitica synthesize a plasmid-independent protein (24,000 dalton) which behaves as a common surface antigen (CSAg) (1,2). The relationship of the CSAg to the V antigen and the Yersinia outer membrane proteins (YOPs) was studied with antisera from rabbits infected with either Ca^{2+}-dependent or independent cells from representative strains of pathogenic serogroups, and with avirulent serogroups. Only the rabbits infected with pathogenic serogroups showed antibodies to CSAg, no matter whether the infecting cells were Ca^{2+}-dependent or independent. The results obtained by gel precipitation, coagglutination, and Western blot analysis with non-absorbed and sera absorbed with the corresponding antigenic preparations, showed that the CSAg is different from the V antigen and the YOPs.

Native CSAg was resistant to pronase and proteinase K digestion, did not enter in the resolving gel (12%) in non-denaturing polyacrylamide gel electrophoresis (PAGE), and was sedimented by ultracentrifugation (85,000 x g, 6 h). Upon heating at 100°C for 10 min., the denatured CSAg, did not sediment under the same conditions, and was digested by pronase and proteinase K. Moreover, the heat-denatured CSAg gave a ladder pattern in non-denaturing PAGE. These results suggets that the CSAg forms a polymer in the native state.

The CSAg is a virulence marker of pathogenic serogroups of Y. enterocolitica (2) but its role in the mechanism of pathogenicity is unknown. Preliminary experiments of immunization suggest that CSAg could play some role in the invasion of the intestinal epithelium of mice by pathogenic strains of Y. enterocolitica.

1. Díaz, R. et al. 1985. J. Clin. Microbiol. 22: 1035-1039.
2. Toyos, J. et al. 1986. J. Clin. Microbiol. 23: 804-805.

CI 019 THE ROLE OF Yersinia enterocolitica PORINS IN PERMEABILITY OF OUTER MEMBRANE FOR BETA-LACTAM ANTIBIOTICS, Jerzy Hrebenda, Katarzyna Brzostek, Institute of Microbiology, Warsaw University, 00-046 Warsaw, Poland, Roland Benz, University of Wurzburg, FGR, Winfried Boos, University of Konstanz, FRG.

Most beta-lactam antibiotics penetrate through the outer membrane at well defined sites involving diffusion channels formed by proteins known as porins. A single diffusion channel is usually formed by three protein subunits (1). Y. enterocolitica produces two general porins YOMP-C and YOMP-F. Mutants YOMP-C^-, YOMP-F^- and YOMP-C^-, YOMP-F^+ are characterized by reduced uptake of cephaloridine and (^{3}H) glucose and increased resistance to beta-lactam antibiotics (especially cephaloridines) and tetracycline. The synthesis of YOMP-C and YOMP-F is subject to osmoregulation. Inhibition of YOMP-C synthesis in YOMP-C^-, YOMP-F^+ mutants is compensated for overproduction of YOMP-F. Both proteins are peptidoglycan-associated (2).

Protein YOMP-F (corresponding to OmpC of E. coli) was isolated and purified to homogeneity. At room temperature it occurs in the form of oligomers but at 95°C dissociates into monomers with mol. wt 36 kDa. In reconstitution experiments with black lipid bilayer YOMP-F was found to form water filled diffusion channels with estimated diameter of 1 nm. The channels are not voltage gated and channels formed by YOMP-F allow the selective passage of cations. Polyclonal antibodies against YOMP-F react only with nondenaturated YOMP-F but cross react with OmpC and OmpF of E. coli (3).

It can be concluded that the outer membrane of Y. enterocolitica constitutes a permeability barrier for beta-lactam antibiotics and that porins YOMP-C and YOMP-F play an important role in the maintenance of this structure.

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2. Brzostek K., Hrebenda J., 1988 J. Gen. Microbiol. 134, 1535-1540
3. Brzostek K., Hrebenda J., Benz R., Boos W., 1989 Res. in Microbiol. (in press)

Molecular Biology of Yersiniae: A Model for Gram Negative Pathogenesis

Genetic Analysis of the Low Calcium Response

CI 020 SIGNIFICANCE OF POST-TRANSLATIONAL MODIFICATION, Brubaker, R. R. and Une, T. Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824. It is established that the Lcr plasmid of medically significant yersiniae mediates restriction of cell division in vitro at 37°C in Ca^{2+}-deficient medium while promoting synthesis of virulence factors including V antigen and certain Yops (Lcr^{+}). The latter, which undergo net accumulation in the enteropathogenic species (Yersinia pseudotuberculosis and Yersinia enterocolitica), are known to exhibit rapid post-translational degradation in Yersinia pestis, the causative agent of bubonic plague. The presence of Yops as major surface structures of the enteropathogenic yersiniae suggested that antibody directed specifically against these structures might provide opsonic protection against disease. To prove this point, outer membranes possessing accumulated Yops were purified and used to prepare antiserum in rabbits. After absorption with Lcr^{-} yersiniae, all remaining antibodies were directed against Yops as judged by immunoblotting. This antiserum exhibited absolute passive protection against Y. pseudotuberculosis but failed to promote resistance to challenge by Y. pestis. This observation indicates that steady-state renewal of the outer membrane surface of Y. pestis via concomitant synthesis and degradation of Yops functions as a significant mechanism of immunosuppression.

CI 021 STUDY OF THE GENE CODING FOR HMWP2: AN IRON-REGULATED PROTEIN SPECIFIC TO THE HIGHLY PATHOGENIC *YERSINIA*, Elisabeth Carniel, Odile Mercereau-Puijalon, Annie Guiyoule and Henri H. Mollaret, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15. France.

The pathogenic *Yersiniae* can be divided into highly pathogenic species (*Y. pestis* , *Y. pseudotuberculosis*, *Y. enterocolitica* serovar 0:8 (Ye 0:8), and poorly pathogenic strains (Ye 0:3 and 0:9). This difference in the degree of pathogenicity is not mediated by the 70 kb virulence plasmid and we suggested that it could be related to iron. We investigated this point and demonstrated that under iron starvation, all the *Yersiniae* express novel polypeptides. Two proteins of high molecular weight: HMWP1 and HMWP2 are synthesized only by the highly pathogenic species and not by the poorly and non pathogenic strains.

The HMWPs were purified and specific antibodies were obtained. We thus demonstrated that 1) the proteins are located on the outer membrane but are not exposed on the cell surface, 2) disulfide bonds are involved in their conformation, 3) the two HMWPs share common epitopes, 4) these polypeptides are antigenically related among the highly pathogenic species, 5) the poorly pathogenic strains do not synthesize even an altered form of the HMWPs.

The HMWPs are chromosomally encoded. A genomic library of Ye 0:8 was constructed in lambda gt11 and screened with HMWPs specific antibodies. A 1 kb fragment of the gene coding for the HMWP2 (= *irp2* gene) was obtained and used as a probe. We therefore demonstrated the following points: 1) the *irp2* gene is conserved among the highly pathogenic strains, 2) this gene is absent from the poorly and non virulent species, 3) iron regulates the transcription of the *irp2* gene, 4) the size of the DNA specific to the highly pathogenic *Yersinia* is greater than 14 kb, 5) the 14 kb region is at least partly conserved among these species.

The *irp2* gene and its promoter were cloned on a pUC18 plasmid and introduced into *E. coli*. The HMWP2 protein was expressed, exported to the outer membrane and regulated by iron in *E. coli*. These results demonstrate that the machinery able to recognize the signals of exportation and regulation by iron of the HMWP2 is at least partly similar in *Yersinia* and in *E. coli*. The cloned *irp2* gene was introduced by electroporation into HMWPs defective mutants of *Y. pestis* and *Y. pseudotuberculosis* both strains expressed the HMWP2.

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CI 022 ROLE OF THE *pla* GENE IN THE VIRULENCE OF *YERSINIA PESTIS*. Jon D. Goguen[1], Ola Sodiende[1], Thomas Quan[2], and Bao Youdi[1]. [1]Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester,MA 01655. [2]Center for Infectious Disease, Fort Collins,CO 80522-2087. It has been previously shown that the *pla* gene of *Yersinia pestis* encodes a protein which promotes both fibrinolysis and coagulation in vitro. This gene is carried by a 9.5 kb plasmid found in virulent *Y. pestis* strains. Loss of this plasmid, which also encodes a bacteriocin (pesticin) and pesticin immunity, has been shown to dramatically reduce the LD_{50} of strains injected into mice subcutaneously. To determine if the *pla* gene is the important virulence determinant on the plasmid we constructed a series of mutants, including an in-frame deletion mutant, in which the *pla* gene was inactivated. These mutants were otherwise isogenic to a fully virulent control. Each of the mutants had an LD_{50} about 100,000 times higher than that of the virulent control. A strain containing the cloned *pla* gene but no other portion of the native 9.5 kb plasmid had an LD_{50} similar to that of the control. Although *pla* mutants clearly established an infection and caused a lesion at the inoculation site, they were found infrequently and in low numbers within liver and spleen. We have also established that the Pla protein is a plasminogen activator both in reactions with purified plasminogen and in plasma samples, that it fails to coagulate human or rat plasma, and that it coagulates rabbit plasma only at high concentrations.

CI 023 REGULATION OF VIRULENCE GENE EXPRESSION IN YERSINIA PESTIS BY CALCIUM AND NUCLEOTIDES, Susan C. Straley, Department of Microbiology and Immunology, University of Kentucky, Lexington, KY 40536.

Yersinia pestis, the causative agent of plague, has a multicomponent virulence property called the low-Ca^{2+} response. This is encoded by a 75 kilobase virulence plasmid called pCD1 and consists of at least 8 lcr loci that are thought to constitute a regulatory cascade and at least 13 virulence genes. The lcr loci cause the virulence genes to be regulated in expression in response to the environmental cues of temperature, Ca^{2+}, and nucleotides such as ATP. The products of the lcr loci are thought to cause the unusual Ca^{2+}- and nucleotide-dependence that Y. pestis has for growth at 37°C. Our model for regulation in the low-Ca^{2+} response is that virulence operons such as the V antigen-encoding lcrGVH are induced to strong expression through a cascade containing lcrB, C, D, and F gene products. lcrGVH is downregulated by ATP through the action of lcrH and by Ca^{2+} through the action of a cascade in which lcrH, lcrR, and lcrE participate.

Very recent findings concern the functions of lcrR, V antigen, and of the low-Ca^{2+} response-encoded outer membrane protein YopM. LcrR has a dual regulatory role: in the absence of Ca^{2+}, it functions posttranscriptionally in the expression of LcrG; when Ca^{2+} is present, it functions in the downregulation of lcrGVH at the transcriptional level.

Several lines of evidence indicate that it is expression of V antigen that causes the growth requirement for Ca^{2+} or nucleotides at 37°C. For example, a specifically V^- mutant is Ca^{2+}- and nucleotide-independent in its growth.

YopM has sequence similarity to the thrombin- and von Willebrand factor-binding domain of platelet surface protein GPIb. The yopM operon is monocistronic and regulated at the transcriptional level by Ca^{2+}. A specifically $YopM^-$ mutant is strongly decreased in virulence, showing that YopM is a virulence determinant of Y. pestis.

Insertion Mutagenesis to Define Virulence Factors

CI 024 Genetics of Yops production in *Yersinia enterocolitica*

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Upon incubation at 37°C in the absence of Ca^{++} ions, virulent strains of *Y.enterocolitica* cease growing and release large amounts of 10 plasmid-encoded proteins called Yops. At 37°C, they also produce a 200-240 kDa outer membrane protein called P1 and a 30 kDa outer membrane lipoprotein that we called YlpA for "*Yersinia* lipoprotein A". YlpA has a typical lipoprotein signal sequence. It is very similar to the protein TraT, encoded by the conjugative plasmid pED208.

The *yop* genes were mapped by transposon mutagenesis using Tn*3*, Tn*813*, mini-Mu d*lac* and Tn*2507*. The latter elements generate operon fusions, and allowed to monitor transcription in the mutated genes. The *yop* genes are scattered around pYV. *Yop44, yop41* and *yop37* form an operon presumed to be involved in the regulation of growth by Ca^{++} because some mutations in this operon are lethal in the presence of Ca^{++}. Genes *yop30* and *yop84* form a second operon while the other *yop* genes are isolated. Transcription of the *yop* genes is strongly induced by temperature and partially repressed by Ca^{++}.

Any mutation in a continuous 17kb region of pYV abolishes the production of Yops but not of P1. This region includes several genes called *virA,B,C,F,G,* and *H.* Gene *virF* encodes a 31 kDa transcription activator related to AraC. Protein VirF binds to DNA sequences in the promoter region of *yop* genes. VirF also controls *virC.* It does not control *virA, virB* nor *yopA.*
Transcription of *virF* is thermoregulated which explains the thermoregulation of *yop* genes. The role of Ca^{++} ions remains mysterious.

CI 025 CHROMOSOMAL GENES OF YERSINIA ENTEROCOLITICA MEDIATING MOUSE VIRULENCE: THE IRON UPTAKE DETERMINANT fyu, Jürgen Heesemann, Tilman Vocke, Sören Schubert, Klaus Hantke*, and Uwe Groß, Institute of Hygiene and Microbiology, University of Würzburg, Josef-Schneider-Str. 2, D-8700 Würzburg, * Institut of Microbiology, University of Tübingen, Federal Republic of Germany.

Human enteropathogenic Yersinia spp. (Y. enterocolitica of serotypes O:3, O:9, O:5,27, O:8, O:13, O:20 and Y. pseudotuberculosis of serotypes I-VI) can be divided into two groups of different virulence potential: Mouse-lethal serotypes and non-mouse-lethal serotypes. The recently described chromosomal genes inv and ail do not correlate with the mouse lethal trait of Yersinia. However, we have identified a chromosomal siderophore determinant, fyu, in Y. enterocolitica, serotype O:8 for the first time, which controlls mouse virulence (1). Fyu encodes for siderophore production and an outer membrane protein (Omp) of 65 kilodalton, FyuA, which is iron-inducible. By Tn5-transposon insertion mutants have been isolated which are deficient in expression of siderophore and FyuA. These mutants have also lost the mouse lethal trait. Specific antiserum against the purified FyuA recognized an iron-inducible Omp of 65 kdal with all mouse-lethal enteropathogenic Yersinia spp., but not with non-mouse-lethal strains. In contrast the non-mouse-lethal Y. enterocolitica strains of European serotypes O:3, O:9 and O:5,27 express 80 kdal Omp, cross-reacting with anti-FyuA serum, but do not produce siderophores. Moreover, derepressed mutants (fur) and siderophore transport mutants (ton B, fhu B, C, D,) have been constructed and tested for virulence: These mutations did not change significantly the virulence potential of Y. enterocolitica serotype O:8. Results of gene cloning experiments concerning the pathogenic role of fyu will be discussed.

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CI 026 THE USE OF Tn*phoA* MUTAGENESIS TO IDENTIFY AND STUDY THE REGULATION OF *Yersinia enterocolitica* CHROMOSOMALLY ENCODED OUTER-MEMBRANE PROTEINS. R. Claire Campbell, Gordon L. Wilson, and Scott A. Minnich*, Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho, 83843.

A common factor in the expression of virulence properties among pathogenic yersiniae is the well documented transition of the outer membrane protein (omps) profile induced by conditions of elevated temperature (37°C) and limiting calcium concentrations. *Yersinia enterocolitica* normally expresses two major omps at 25°C which are repressed when shifted to 37°C under limiting calcium concentrations. This latter effect does not occur in cultures cured of the virulence plasmid. One of the questions we are addressing is the mechanism by which the virulence plasmid is able to repress chromosomal genes. Using Tn*phoA* mutagenesis of *Y. enterocolitica* 0:8, we have isolated several hundred *phoA* fusions to exported proteins. These mutants are being analyzed with respect to temperature and osmotic responses . Additionally we have determined that the two major porin protein genes of *E. coli*, *ompF* and *ompC*, as well as their regulatory genes, *ompR* and *envZ* hybridize to *Y. enterocolitica* DNA. Using these genes as probes we have screened all the Tn*phoA* mutants by southern blotting and identified several potential *phoA* fusions to these *E. coli* homologs. Further analysis of these specific mutants will determine if these genes identify the two major omps in *Y. enterocolitica* grown at 25°C and provide the basis for conducting experiments to determine their mode of regulation.

CI 027 **ENHANCEMENT OF MOUSE VIRULENCE AFTER MUTATIONS IN SERENY-ASSOCIATED PLASMID GENE SEQUENCES IN YERSINIA ENTEROCOLITICA**
J. Glenn Morris, Jr., Roy M. Robins-Browne, Sam Cianciosi, and Marianne D. Miliotis. Division of Geographic Medicine, Department of Medicine, University of Maryland School of Medicine, Baltimore, Md., 21201, and Department of Microbiology, University of Melbourne, Parkville, Victoria, 3052, Australia

We have previously shown that gene sequences on a 2.6kb BamHI fragment (the #7 BamHI fragment) of pYV from Y. enterocolitica strain A2635 are associated with the Sereny reaction and HEp-2 cytotoxicity. A strain containing a virulence plasmid from which the #7 BamHI fragment had been deleted, with insertion of a kanamycin resistance gene, had a lower 50% lethal dose after intraperitoneal inoculation in iron-loaded mice than did the parent strain. When inoculated orogastrically into mice (not iron treated), the strain containing the deletion/insertion was isolated significantly more frequently at all extraintestinal sites sampled (spleen, liver, retroperitoneal lymph nodes, and blood); isolation rates and counts from gut contents for the mutated and parent strains were comparable. Complementation of the deletion with the cloned #7 BamHI fragment resulted in a corresponding decrease in virulence in mice. Expression of plasmid-associated outer membrane proteins was not affected by the mutation or subsequent complementation; the deleted gene sequences did not hybridize with yopA. TnphoA was used to mutagenize the #7 BamHI fragment in E. coli. Based on data on direction of transcription from our TnphoA mutants, the #7 BamHI fragment appears to contain multiple operons, at least one of which is transcribed only at 37°C in E. coli.

Our data indicate that genes on the #7 BamHI fragment of pYV affect virulence and dissemination of Y. enterocolitica in mice, with virulence enhanced by mutations that result in loss of a Sereny reaction and HEp-2 cytotoxicity. The exact structure of gene sequences on the #7 BamHI fragment and the function of their products is under investigation.

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Molecular Mechanisms of Pathogenesis

CI 100 *CHLAMYDIA TRACHOMATIS:* A MOLECULAR DISSECTION OF ITS LIFE CYCLE, Joanne Engel, Eve Perrara, Jon Pollack, and Don Ganem. Department of Microbiology and Immunology, UCSF, San Francisco, CA., 94143. *Chlamydia trachomatis*, a gram negative bacteria of major medical significance and intrinsic biologic interest, is an obligate intracellular parasite of eukaryotic cells. To better understand its pathogenesis, we have begun an orderly dissection of this parasite's unique intracellular life cycle. Signals regulating gene transcription (i.e. promoters) were defined and appear to differ from those of other bacteria. The genes encoding the subunits of RNA polymerase were cloned and purification of chlamydial polymerase is underway. Strategies developed to define the pattern of regulated gene expression during the chlamydial intracellular life cycle have resulted in the isolation of several developmentally regulated genes including two genes expressed during heat shock and a gene whose product is expressed late in the developmental cycle. Surprisingly, DNA sequencing has revealed that this latter gene encodes a protein with significant homology to eukaryotic histone H1 and may represent the first example of horizontal gene transfer from a host to its parasite. We are currently investigating whether other histone-like proteins exist in chlamydia and whether this protein functions as a DNA binding protein. In addition, the response of chlamdyia to heat shock has been characterized. We have shown that the induction of gene transcription by heat shock proceeds by at least two distinguishable pathways. Lastly, we are attempting to develop methods of gene transfer into chlamydia, as this organism has no naturally occurring genetics.

CI 101 DNA HYBRIDIZATION AND HOMOLOGY BETWEEN *YERSINIA PESTIS* STRAIN KIM SEQUENCES AND RAT HEME OXYGENASE STRUCTURAL GENE, Michael L. Pendrak and Robert D. Perry, Dept. of Microbiology and Immunology, Louisiana State University Medical Center, Shreveport, LA 71130

An essential component of bacterial infections is the ability to acquire iron from the mammalian host. While *Yersinia pestis* is unable to obtain inorganic iron from transferrin and lactoferrin, it does utilize most mammalian heme compounds as sole iron sources. However, liberation of inorganic iron from the porphyrin ring is necessary for heme alone to satisfy all bacterial iron requirements. In mammalian systems, heme oxygenase catalyzes the cleavage of the heme ring to yield α-biliverdin and inorganic iron. A similar bacterial activity would serve to liberate inorganic iron from the porphyrin ring. We have used the cDNA clone of rat heme oxygenase isoform 1 (pRHO1, obtained from S. Shibahara) to probe the *Y. pestis* genome for related DNA sequences. Under stringent hybridization conditions, an ~2.5 Kb region of the *Y. pestis* plasmid pPCP1 hybridizes to a probe containing only rat heme oxygenase isoform 1 (RHO1) coding sequences. Preliminary sequence analysis of a 300 bp region from pPCP1 shows forty percent homology to a region of RHO1 proposed to encode a heme binding site. Finally, preliminary results suggest that a heme oxygenase-like activity is present in cell extracts of *Y. pestis* KIM. These results indicate that a heme oxygenase-like enzyme may be encoded on plasmid pPCP1. This enzyme activity may be important in iron acquisition from the iron-deficient environment of mammals. This is the first report of a heme oxygenase-like gene in procaryotic cells.

Invasion of Eukaryotic Cells by Yersiniae and Other Pathogenic Bacteria

CI 200 DNA FROM THE rfa REGION OF SALMONELLA TYPHI TY2 RESTORES THE SMOOTH PHENOTYPE AND THE ABILITY OF TY2 ROUGH STRAINS TO ADHERE TO HELA CELL MONOLAYERS. M. J. Mroczenski-Wildey*, J. Di Fabio, and F. C. Cabello. New York Medical College, Valhalla, N.Y. 10595; *current address: Lederle Laboratories, Pearl River, N.Y. 10965. Previous experiments have shown that rough mutants of Salmonella typhi Ty2 fail to adhere to and invade HeLa cell monolayers. Plasmids carrying the **pyrE-rfa-cysE** region from S. typhi Ty2 were obtained using the vector pULB113 (RP4::mini-Mu). One R-prime plasmid, pSTA103, restored the smooth phenotype to the rough mutant MMW011 as determined by phage sensitivity, agglutination by specific antisera, and the presence of O-antigen ladders in qualitative analysis of the LPS in SDS-PAGE. Further quantitative analysis of the complemented rough mutant MMW011(pSTA103) revealed core sugars and O-polysaccharide side chains containing sugars identical to those found in Ty2. Although MMW011(pSTA103) contained the sugars glucose, mannose, rhamnose, galactose, and tyvelose in its O-side chain, the ratio of O-side chain to core was 0.83 as compared to 2.41 for Ty2. Adherence and invasive abililties were also restored in the complemented mutant MMW011(pSTA103) but not to the same degree as Ty2. Curing of MMW011(pSTA103) was accompanied by loss of the smooth phenotype and the ability to adhere to and invade the HeLa monolayer. An EcoRI digest of pSTA103 revealed a DNA fragment that hybridized with a 4.0 kb EcoRI fragment isolated from S. typhimurium LT2 DNA in the **rfa**G and **rfa**B region. Since pSTA103 has been shown to complement known S. typhimurium **rfa** rough mutants, these data suggest that the defect of MMW011 is in the **rfa** region. These experiments indicate that S. typhi LPS is involved in the ability of S. typhi Ty2 to adhere to and invade eukaryotic HeLa cell monolayers.

Molecular Biology of Yersiniae: A Model for Gram Negative Pathogenesis

CI 201 THE NON-INVASIVE PHENOTYPE OF NON-DISEASE ASSOCIATED ISOLATES OF YERSINIA ENTEROCOLITICA IS DUE TO A DEFECT IN inv-HOMOLOGOUS SEQUENCES, Dorothy E. Pierson and Stanley Falkow, Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305-5402

The inv and ail genes were cloned from a disease associated strain of Y. enterocolitica using their ability to convert a normally non-invasive E. coli laboratory strain into one that can invade mammalian tissue culture cells in vitro. All disease associated Yersinia isolates examined have sequences homologous to both of these genes. Non-disease associated isolates lack any ail-homology but they do have inv-homologous sequences. inv-homologous sequences were cloned from four different nonpathogenic isolates and shown to be unable to confer the invasive phenotype to E. coli. No inv-homologous RNA was detected in the four nonpathogenic strains. When the inv clone from the disease associated isolate was transferred into three of these strains on a plasmid, the resulting recombinants expressed inv-homologous RNA and invaded tissue culture cells in vitro. When the ail clone from the same disease associated isolate was transferred into these three strains, these recombinants did not become invasive. However, they did produce ail-homologous RNA. At least one of these recombinants produces Ail protein as detected with an anti-Ail monoclonal antibody. These results demonstrate that expression of inv but not ail is essential for invasion of tissue culture cells by Y. enterocolitica.

CI 202 **ENTEROPATHOGENIC YERSINIA ENTEROCOLITICA (YE) AND AUTOIMMUNE THYROID DISEASES (AITD), Wenzel B.E., Heufelder A., Franke T., *Heesemann J., and Scriba P.C.,** Cell- and Immunbiol. Lab., Dept. Internal Medicine, Med. University Lübeck and *Institute Hygiene and Mikrobiology, University Würzburg, FRG

Our recent studies have shown that 1) in AITD a high prevalence of antibodies (Ab) to YE-plasmid encoded released proteins (RP) is found; 2) YE-RPs have binding sides for thyrotrophin. We now investigated: 1) antigenic homologies between YE and thyroid epithelial cells (TEC); 2) immuno-sensitisation and -modulation of lymphocytes by YE-RPs; 2) the induction of lymphocytic thyroiditis (in BB/Wistar rats) by immunization with YE-RPs. Results: In the indirect immunofluorescence RP-Ab display a bright intracellular stain with TECs from patients with AITD. This is reflected by specific, TSH inducable bands of RP-Ab in WESTERNs with TEC-homogenates, thyroid peroxidase (TPO), thyroglobulin, thyroid microsomes and -mitochondria. With YE-antigens intrathyroidal lymphocytes react with a proliferative cellular immune response. YE-RPs can suppress macrophage function in a non-thyroid related immune response to tetanus toxoid antigen, while with lymphocytes from patients with AITD this it not observed. BB/W-rats develope a LT in higher frequency and at an earlier stage, when immunized chronically with YE-RP. Occurrance of thyroglobulin-Ab, however, is independent from immunization of rats with YE-RP. Our findings suggest a role of YE-plasmid encoded antigens at the onset of AITD via modulation of macrophage function in genetically predisposed individuals and via antigenic homologies of YE and TECs.

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Diagnostic DNA Probes for Virulent Strains; Recent Developments in the Immunodiagnosis and Other Methods in the Detection of Virulent Strains

CI 300 EVALUATION OF VIRULENCE TEST PROCEDURES FOR YERSINIA ENTEROCOLITICA, Saumya Bhaduri, U. S. Department of Agriculture, ARS, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, PA 19118

Human pathogenic strains of Yersinia enterocolitica harbor a particular species of 40 to 48 megadalton plasmid directly involved in virulence. In the development of a rapid, simple and efficient method to identify virulent plasmid-bearing clones of Y. enterocolitica, two assays were evaluated using a calcium-deficient agarose medium. The two tests thus developed: calcium dependency (CAD) and congo red pigmentation (CR), were compared with crystal violet binding assay (CV) for detection and identification of virulent strains. CV was the best predictor of virulence followed by CR. CAD test was less clearcut. CR, however, has advantages in that it allows a rapid isolation of viable plasmid-bearing virulent strains for subsequent investigation. CR also permits simultaneous detection of CAD and CR absorption in Y. enterocolitica clones.

CI 301 THE EFFECT OF SELECTIVE MEDIA ON CONGO RED BINDING, A VIRULENCE MARKER IN SHIGELLA, James L. Smith, Eastern Regional Research Center, Agricultural Research Service, U. S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118

Loss of congo red binding (CRB), a plasmid mediated trait which correlates with virulence in wild-type Shigella, results in avirulence and appearance of white colonies on congo red (CR) agar. The effect of selective media on retention of CRB was determined utilizing S. flexneri 5348 (SF). Plating SF onto selective agars and incubating at 37 C for 24-48 h followed by replica plating onto CR agar resulted in the appearance of red colonies but no white colonies. Prior growth in liquid selective media (agar was removed by centrifugation) led to appearance of white colonies. Ratios of white colonies/red colonies ranging from 0.001 to 1.0 were found after growth in the liquid test media. Testing inorganic and organic salts, sugars, anionic and non-ionic detergents, dyes, and EDTA in liquid medium indicated that only tergitol 7, sodium lauryl sulfate, bile salts #3, crystal violet, eosin Y, and methylene blue (at levels much higher than present in selective media) induced loss of CRB with W/R ratios ranging from 0.15 to 200.0. CRB appears to be relatively stable and the use of appropriate selective media should permit isolation of virulent (CRB+) shigellae from food or clinical samples.

CI 302 NUCLEIC ACID PROBES FOR *CAMPYLOBACTER FETUS* AND *CAMPYLOBACTER HYOINTESTINALIS* BASED ON 16S RIBOSOMAL RNA (16S rRNA) ANALYSIS. Irene V. Wesley. USDA, ARS, National Animal Disease Center, Ames, IA

The genus *Campylobacter* is composed of spirally curved gram negative pathogens which are metabolically inert and therefore are misidentified using conventional methods. The goal of this study was to develop DNA probes based on 16S rRNA sequence analysis for *Campylobacter fetus*, which induces abortion in livestock, and *Campylobacter hyointestinalis*, an agent of proliferative ileitis of swine.

Specificity of oligonucleotide probes constructed from 16S rRNA sequences was tested in a slot blot format using genomic DNA extracted from *C. fetus* (n=54) and *C. hyointestinalis* (n=52) isolates. The resultant *C. fetus* probe (17-mer) hybridized with 50 of 54 isolates of *C.fetus* but not with either *C. jejuni* or *C. hyointestinalis*. Isolates (n=4) which did not react with the probe were reidentified as *C. sputorum bubulus* (n=2), *C. hyointestinalis* (n=1) and *C. jejuni* (n=1). The *C. hyointestinalis* probe (29-mer) reacted with 44 of 52 isolates of *C. hyointestinalis*. Isolates (n=8) which did not hybridize were reidentified as *C. coli* (n=4) and *C. fetus* (n=3) and not identified further (n=1).

In Southern blot hybridization of Bgl II digests of genomic DNA, the respective probes reacted with 3 restriction fragments of either *C. hyointestinalis* (10.1, 8.2, 7.2 kb) or *C. fetus* (9.0, 7.7, 7.0 kb).

Regulation and Expression of Virulence Factors

CI 400 **MOLECULAR CLONING OF rfb REGION OF Yersinia enterocolitica O:3, Ayman Al-Hendy, Paavo Toivanen and Mikael Skurnik, Department of Medical Microbiology, Turku University, SF-20520 Turku, Finland.**

Lipopolysaccharide O-side chain is reported to be a virulence factor in many enteric bacteria. We have cloned the chromosomal gene cluster rfb, encoding for enzymes involved in biosynthesis of Lipopolysaccharide O-side chain, of Yersinia enterocolitica O:3 (Yeo3) in Escherichia coli C600.

An Yeo3 O-Ag specific monoclonal antibody was used to screen the genomic library formed of Yeo3 in pBR322. Restriction mapping and deletion analysis showed that about 12.7 kb is essential for the synthesis of O-Ag. This 12.7 kb fragment did not encode for LPS-core material as evidenced by negative immunoblotting using a Yeo3 core-specific monoclonal antibody. However, we could still show by immunofluorescence microscopy, slide agglutination and phage sensitivity experiments that YeO3 O-Ag is assembled in a normal fashion on E.coli core.

Interestingly, strong signal, in colony hybridization using the cloned insert as a probe, was detected mainly in pathogenic Y.enterocolitica serotypes .

CI 401 ANALYSIS OF THE *lcrF* GENE OF *Y. pestis* : Nancy P. Hoe, F. Chris Minion and Jon D. Goguen, Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01655 Previously, we identified the *lcrF* locus of *Y. pestis* as being responsible for the thermal induction of *yopE*. In *E. coli*, a *yopE-lacZ* fusion was found to be regulated in a temperature-dependent manner only in the presence of the BamHI G fragment of *Y. pestis* plasmid pCD1 which contains the *lcrF* locus. We used this activity in *E. coli* to locate the *lcrF* gene. Sequencing revealed an open reading frame capable of encoding a protein of 30.8 KDa and this was confirmed using a T7 expression system. As expected, LcrF exhibited 96.7% homology to VirF of *Y. enterocolitica*, and significant homology to the carboxy terminus of AraC, as had been reported for VirF. We identified several other proteins exhibiting homology to the carboxy terminus of LcrF. These could be divided into two classes according to function; those regulating operons involved in catabolism of carbon and energy sources, and those involved in regulating virulence genes. A majority of LcrF was found associated with the membrane fraction in *E. coli*. To investigate the mode of LcrF regulation by temperature, several *lcrF-lacZ* transcriptional fusions were constructed and analyzed in *Y. pestis* and *E. coli*. The activity of the fusions was the same in pCD1$^+$ and pCD1$^-$ *Y. pestis* strains. Although *virF* exhibits thermal induction in *Y. enterocolitica*, it appears that in *E. coli.*, induction of *lcrF* transcription is not required for temperature-dependent activation of *yopE* transcription.

CI 402 EFFECT OF LOW CONCENTRATIONS OF ENOXACIN AND OTHER QUINOLONE ANTIMICROBIALS ON THE PRODUCTION OF BACTERIAL VIRULENCE FACTORS, Stephen A. Sonstein, Jeffrey C. Burnham and Clifford Renk, Eastern Michigan University, Ypsilanti, MI 48197 and Medical College of Ohio, Toledo, OH.
This study determined whether low concentrations of enoxacin, lomefloxacin, and ciprofloxacin effected the production of virulence factors in several genera of bacteria. Factors studied were nuclease and alpha toxin production in S. aureus; adherence of S. typhimurium, E. coli and Ps. aeruginosa to urinary epithelial cells, and the major virulence factor in Y. pseudotuberculosis. The effect of low levels of enoxacin on phagocytosis of S. aureus by human polymorphonuclear leukocytes was also studied. Following exposure to subinhibitory levels of quinolones, significant reduction in activity or elimination was seen for chromosomally located virulence factors and enhancement of phagocytic activity by human PMNs, little was seen for the plasmid located virulence factor. These data suggest that exposure to enoxacin and other quinolones at concentrations below the MIC disrupts the regulatory mechanisms which control bacterial plasmid maintenance, enzyme production and surface structure. This may mean that certain virulent organisms which survive exposure to quinolone antibiotics may be less likely to produce or maintain the disease state in susceptible hosts.

CI 403 THE REGULATION OF THE *AIL* LOCUS OF *YERSINIAE ENTEROCOLITICA*, Marian R. Wachtel, Karen B. Beer, and Virginia L. Miller, Department of Microbiology, University of California, Los Angeles, 405 Hilgard Avenue, Los Angeles, California 90024.

Yersinia enterocolitica is a facultative intracellular pathogen which causes mild to severe gastroenteritis. Two separate loci have previously been identified, each of which confers an invasive phenotype to the normally non-invasive *E. coli* strain HB101. The *ail* locus confers a high degree of adhesion to many cell lines, as well as promoting different invasion phenotypes for different cell lines. Transcription of the *ail* gene expressed in *E. coli* HB101 cells increased when the growth temperature was raised from room temperature to 28°C, and finally to 37°C. The *ail*-encoded protein showed similar expression patterns. Transcription from the *ail* gene in *Yersinia enterocolitica* or *E. coli* appears to initiate from the same site(s). In primer extension analysis two products were observed which were separated by 18 base pairs. Current evidence suggests the shorter "transcript" is a pause site for reverse transcriptase. Fusions of *ail* to *lacZ* are being used to study regulation of *ail* expression further. When the expression of the *ail* gene was studied in a collection of American and Non-American *Yersinia enterocolitica* serotypes it was found that both types synthesized an *ail* specific transcript. However, monoclonal antibodies raised to purified AIL (from an American serotype) do not detect any *ail* product from Non-American serotypes.