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Invasion of murine respiratory tract epithelial cells by *Chryseobacterium meningosepticum* and identification of genes present specifically in an invasive strain

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SUMMARY

Chryseobacterium meningosepticum causes severe infections in infants or adults with underlying illness. The species is highly heterogeneous, genetically composed of subgroups with different pathogenicity. Eight strains of *C. meningosepticum*, representing four different genomic subgroups, were evaluated for their ability to penetrate Madin-Darby Canine Kidney (MDCK) epithelial cell monolayers and serum resistance. None of the strains showed cytotoxicity or penetration to the MDCK cells. All displayed resistance to the bactericidal activity of various normal human sera. A murine pulmonary infection model was used to compare the pathogenicity between a clinical isolate and an environmental isolate. *C. meningosepticum* were cleared from the lung of infected mice within 7 days following the intratracheal challenge. Electron microscopy demonstrated the large membrane protrusions, indicative of ruffles, and smaller, less organized membrane structures of the respiratory epithelial cells induced by the clinical isolate. Bacteria were observed to enter the cells as single entities in spacious vacuoles. Suppressive subtraction hybridization identified in the invasive isolate 35 distinct sequences associated with systems of energy production and conversion, transport, and secretion. In most cases, the identities between the references and the amino acid sequences deduced were low, suggesting that the functions of these sequences remain unknown.

KEY WORDS: *Chryseobacterium meningosepticum*, invasion, cytotoxicity, suppressive subtraction hybridization.

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INTRODUCTION

Chryseobacterium meningosepticum, formerly known as *Flavobacterium meningosepticum* or CDC II-a, is a gram-negative rod widely distributed in nature. The pathogen causes meningitis in premature and newborn infants (King, 1959; Maderazo *et al*., 1974; Thong *et al*., 1981) and pneumonia, endocarditis, postoperative bacteremia, and meningitis usually associated with severe underlying illness in adults (Olsen *et al*., 1965; Werthamer and Weiner, 1971; Bloch *et al*., 1997). Although *C. meningosepticum* infections are rare, accurate diagnosis is important because the bacterium is usually resistant to multiple antibiotics, including extended-spectrum β-lactam agents and aminoglycosides, generally prescribed for the treatment of aerobic, gram-negative bacterial infections. Moreover, epidemics may occur, and a death rate as high as 55% was reported in a nursery outbreak (von Graevenitz, 1985; Schreckenberger and von Graevenitz, 1999).

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Previous studies indicated that the species *C. meningosepticum* is highly heterogeneous and composed of many subgroups, which may be reclassified as separate species (Ursing and Bruun, 1987; Colding *et al.*, 1994; Chiu *et al.*, 2000; Lin *et al.*, 2004). Genetically defined subgroups within *C. meningosepticum* also differ in their pathogenicity (Colding *et al.*, 1994). On the basis of DNA-DNA hybridization, two main genomic relatedness groups (I and II) have been reported for a geographically varied collection of 52 strains of *C. meningosepticum* (Colding *et al.*, 1994). The genomic group II can be further divided into four subgroups (II:1 to II:4), and more importantly, clinical isolates that caused sepsis and meningitis in the neonatal intensive care units in Rigshospitalet, Copenhagen, Denmark invariably belonged to the same genomic subgroup, characteristic of genomic subgroup II:1 (Colding *et al.*, 1994). The present study was undertaken to evaluate the virulence mechanism of *C. meningosepticum* using cellular and molecular biological methods as well as an *in vivo* mouse respiratory challenge model. We also studied isolates from both genomic subgroup II:1 causing invasive infections in humans and those isolated from the environment belonging to other genomic subgroups.

MATERIALS AND METHODS

Bacterial isolates

Eight strains of *C. meningosepticum* kindly provided by Professor Brita Bruun, University of Copenhagen, Denmark, were used in this study. The designations of these strains, the sites of isolation, and genomic subgroups are as the following: 26774 and 27439 of the genomic subgroup II:1 isolated from cerebral spinal fluids and blood, respectively; CCUG7924 and CCUG12664 of the subgroup II:2 isolated from dog blood and water, respectively; AB1505 and AB1513 of the subgroup II:3 isolated from tracheal secretion and hospital environment, respectively; and HO1;J100 and AB1572 of the subgroup II:4 isolated from lung autopsy and sputum, respectively. The growth curves of these strains were determined by counting the viable bacteria in 5 ml Luria broth pre-inoculated with 100 µl of overnight bacterial cultures. All these strains grew into log phase within 6 h at 37 °C.

Penetration and cytotoxicity assay

MDCK cells were maintained in minimal essential medium with 10% fetal bovine serum, 100 U of penicillin G per ml, and 100 µg streptomycin per ml. Monolayers for penetration assays were prepared by seeding 1.5×10^5 cells into each well of a Transwell filter unit (Costar, Cambridge, MA, USA) containing 0.33-cm^2 porous filter membranes in 24-well tissue culture plates. The monolayer was grown in the antibiotic-free minimal essential medium with 10% fetal bovine serum and was ready for use after 4 days incubation at 37 °C in 5% CO_2.

The method described previously was used in the penetration assay (Hirakata *et al.*, 2000). Briefly, the monolayer was infected with bacteria by adding 5 µl of log-phase bacteria with a multiplicity of infection at approximately 100:1 (bacteria:cell). The plate was then incubated at 37 °C in 5% CO_2. The viable bacteria in the basolateral medium were counted at 3 h and 6 h after infection. *Salmonella enterica* serotype Typhimurium SL1344 and *Escherichia coli* RDEC-1, a non-invasive rabbit enterotoxigenic strain, were used as positive and negative controls, respectively. Each assay was performed in triplicate.

To quantify the cytotoxicity of bacterial isolates on the MDCK cells, the concentration of lactate dehydrogenase released from the MDCK cells into the medium, both apical and basolateral, in the filter units was measured using a dry chemistry method (Johnson & Johnson, Rochester, NY, USA) performed on an autoanalyzer, KODAK EKTACHEM 250. The assay was performed concurrently with the penetration assay. The viability of the remaining MDCK cells in the filter units was also checked by a trypan blue exclusion assay (Fleiszig *et al.*, 1996). *Serratia marcescens* NB36 (L.-H. Su, unpublished data) and *E. coli* RDEC-1 were used in the cytotoxicity assay as positive and negative controls, respectively.

Serum susceptibility

The serum bactericidal assay followed a method described previously (Criado *et al.*, 1990). Overnight broth cultures were diluted 1:10 to yield a suspension of approximately 10^8 viable bacteria per ml. A drop of 50 µl of each suspension was spread over half of a 90 mm blood agar plate (Difco Laboratory, Detroit, MI, USA).

The bacterial cultures were allowed to dry on the plates for about 10 min at room temperature. Two 50 µl drops of 50% normal human serum with or without heat-inactivation (56 °C, 30 min) were deposited on the plates separately. After another 10 min absorption at room temperature, the plates were incubated overnight at 37 °C with lid-side up. Serum susceptibility was indicated by a complete inhibition of bacterial growth or only a few discrete colonies observed in the area of the drop of normal human serum and normal growth in that of heat-inactivated serum.

Infection of mice with C. meningosepticum
Two strains of *C. meningosepticum*, 27439 and AB1513, were used in this study. Bacterial inocula were prepared by seeding 5 colonies of each strain to Luria broth and allowing them to grow for 18 h at 37 °C. Bacteria were collected by centrifugation and resuspended in 1 ml 1% gelatin-Hank's balanced salt solution (gel-HBSS) (Gibco BRL, Life Technologies, Rockville, MD, USA). The determination of the 50% infection dose was done by intratracheal injection of serial dilutions of bacterial suspension (Morissette *et al*., 1995). Balb/c mice were divided into groups of five. All mice were euthanized at day 3 and hematoxylin-and-eosin-stained lung sections were examined microscopically for any pathological changes. By intratracheal injection, the 50% infection dose of *C. meningosepticum* for Balb/c mice, determined by lung histology, was around 10^6 - 10^7. For the following pathological experiments, mice were challenged intratracheally with approximately 5 × 10^8 bacteria in 50 µl gel-HBSS (Morissette *et al*., 1995). At predetermined time points after infection, mice were sacrificed, and spleens, livers, and lungs were excised aseptically. Each organ sample was placed in 5 ml gel-HBSS, homogenized, diluted serially and inoculated onto Luria agar plates. Viable bacterial counts were determined after incubation at 37 °C for 48 h.

Histological examination
A small portion of the lung sample was fixed in 2.5% sodium phosphate-buffered glutaraldehyde and post-fixed in 1% osmium tetroxide for electron microscopic analysis. Another portion was fixed in a paraformaldehyde solution for pathological examination.

Electron microscopy
The fixed lung samples were dehydrated through a graded alcohol series. The tissues were then embedded in Epon, thin sectioned, and stained with oryanyl acetate and lead citrate. Specimens were examined with a Phillip 400 transmission electron microscope.

Suppressive subtraction hybridization
Comparison of the genome sequences of non-pathogenic with pathogenic bacterial strains could yield valuable information. The comparison can be made by the recently developed PCR-based subtractive hybridization method (Duguid and Dinauer, 1990; Akopyants *et al*., 1998) in which the DNA sequences presented in the virulent but not in the non-virulent strain can be specifically amplified. The *C. meningosepticum* strains used in this subtraction study were 27439, a clinical isolate collected from blood specimens of a patient with sepsis (Colding *et al*., 1994), and AB1513, an environmental strain (Colding *et al*., 1994). The chromosomal DNA was prepared by incubating the bacteria grown overnight in a buffer containing 50 mM Tris-HCl (pH 8.0) and 0.6% sodium dodecyl sulfate followed by lysozyme digestion, phenol-chloroform extraction, and ethanol precipitation. The Bacterial PCR-Select DNA Subtraction Kit (Clontech, La Jolla, CA, USA) and a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA, USA) were used in the DNA subtraction, and the procedure was performed essentially according to the manufacturer's instructions. After the subtractive hybridization, DNA fragments unique to *C. meningosepticum* 27439 were amplified by PCR, subcloned into pUC18, and transformed into *E. coli* XL1-Blue, and the transformants were subjected to β-galactosidase activity selection. To verify whether the subcloned DNA fragments are indeed unique to *C. meningosepticum* 27439, a dot blot analysis was performed with the genomic DNA of *C. meningosepticum* 27439 and AB1513 as the probes. The nucleotide sequences of the *C. meningosepticum* 27439-specific DNA fragments were determined with an ABI-377 autosequencer. The sequence data were analyzed through the World Wide Web using the BLAST programs (Altschul *et al*., 1990), provided by the National Center for Biotechnology Information, USA.

RESULTS

Penetration, cytotoxicity and serum susceptibility

All strains displayed resistance to the bactericidal activity of normal human sera from newborns and adults. However, none of the *C. meningosepticum*, regardless of the sites of isolation and genomic subgroups, could penetrate the MDCK monolayer, in contrast to *S.* Typhimurium SL1344 that penetrated the monolayer readily 3 h after the incubation. *C. meningosepticum* was not cytotoxic to MDCK cells; the range of the lactate dehydrogenase levels in the media was 1 – 16 U/L at 6 h after incubation for all strains tested, compared to over 200 U/L generated by *S. marcescens* NB36. The MDCK cells appeared viable in trypan blue staining at 6 h after the addition of *C. meningosepticum*.

Murine model of pulmonary clearance

The virulence of *C. meningosepticum* was further examined *in vivo* using a mouse respiratory challenge model. Both strains of *C. meningosepticum* were cleared from the lungs of infected mice within 7 days following intratracheal challenge with a high dose of the bacteria. *C. meningosepticum* 27439, but not AB1513, persisted 3 days post infection in the lungs. There was no mortality of the mice. Neither 27439 nor AB1513 translocated from lungs to livers or spleens. The results of a representative experiment repeated in triplicate are displayed in Figure 1. The lung inflammatory response to *C. meningosepticum* infection was characterized by cellular infiltration of the bronchioles and adjacent lung tissues and edema within alveolar spaces (data not shown). The infiltrating inflammatory cells were predominantly polymorphonuclear neutrophils.

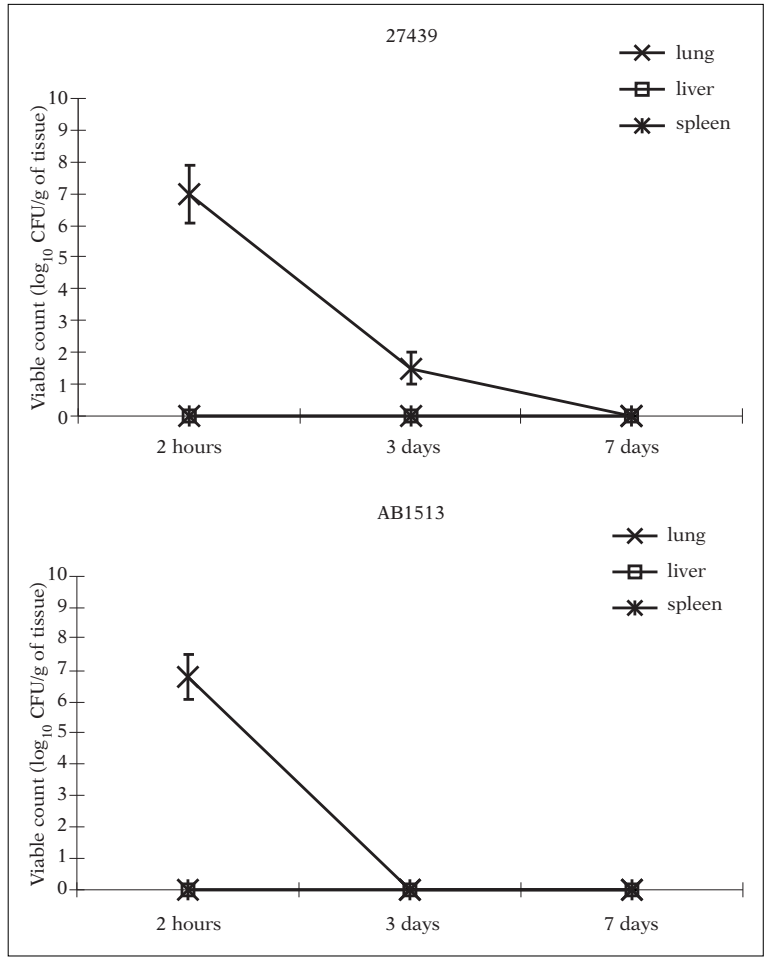

FIGURE 1 - *Intratracheal infection with C. meningosepticum of Balb/c mice. Balb/c mice were injected intratracheally with approximately 5×10^8 C. meningosepticum 27439 and AB1513 in 50 µl gel-HBSS and sacrificed by cervical dislocation at various times after infection (2 h, 3 days, and 7 days). Tissues were removed and homogenized, and the numbers of viable C. meningosepticum were assessed by the plate count detection method (three mice per sacrifice interval). Data are expressed as log CFU/g tissue (mean ± standard deviation).*

Histological examination by electron microscopy
Electron microscopy demonstrated the interaction between *C. meningosepticum* 27439 and respiratory epithelial cells at 2 h following the intratracheal infection. Figure 2 demonstrates large numbers of bacteria in close proximity to the ciliated respiratory tract epithelium (Fig. 2A); large membrane protrusions, indicative of ruffles, and smaller, less organized membrane structures were readily observed. *C. meningosepticum* were observed to enter the cells as single entities in spacious vacuoles (Fig. 2B). Intracellular invasion of the respiratory tract epithelial cells was not observed upon analysis of lung tissues infected by AB1513.

Molecular analysis
by suppressive subtraction hybridization
To determine whether any specific gene(s) may be associated with the virulence in *C. meningosepticum*, a PCR-based subtractive hybridization method was conducted to select the genes specially present in the virulent strain, 27439, a clinical strain isolated from a patient with sepsis, but not in the non-virulent strain, AB1513, an environmental strain. Through the method, approximately 120 white colonies were obtained and subjected for further analysis. The average size of the subtracted fragments was approximately 400 to 1,000 bp, as determined by restriction enzyme digestion and agarose gel electrophoresis. A total of 52 unique transformants were confirmed to contain DNA fragments specific to *C. meningosepticum* 27439, and 35 distinct sequences were identified (Table 1). The remaining sequences did not exhibit notable homologies with any data file in the GenBank. It appears that *C. meningosepticum* is genetically related to *Cytophaga hutchinsonii*, an organism common in soil that exhibits rapid gliding motility and is a member of the *Cytophaga-Flavobacterium-Bacteroides* group of bacteria. Among the 35 sequences, some were associated with energy production and conversion (fragments 41, 45, 63, and 82), some with transport systems (fragments 45, 58 and 63), and one with a secretion system (fragment 48). One sequence was found to match a previously identified gene encoding a transcriptional factor of the Crp family (fragment 115). A few sequences appeared to be metabolism-related (fragments 33, 38, 40, 43, and 80). In most cases, the identities between the references and the amino acid sequences deduced were low, suggesting that the function of these genes remains to be elucidated.

DISCUSSION

In this study we observed the inflammatory response following *C. meningosepticum* intratracheal infection in mice. We found, however, a rapid clearance of *C. meningosepticum* from tissues of mice within 3-7 days of the infection.

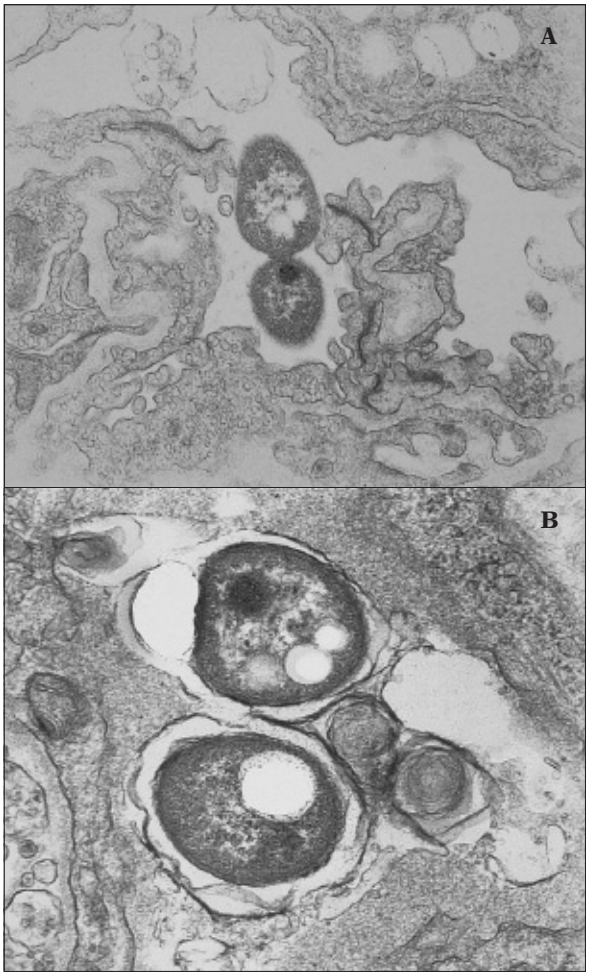

FIGURE 2 - *C. meningosepticum 27439 in close proximity to and invasion of the respiratory tract epithelium. (A) Large membrane protrusions, indicative of ruffles, and smaller, less organized membrane structures are readily observed (magnification = 12,000 ×). (B) C. meningosepticum are observed to enter the cells as single entities in spacious vacuoles. (magnification = 15,000 ×).*

TABLE 1 - *Characteristics of* C. meningosepticum *27439-specific DNA fragments*

Fragment	*Insert size (bp)*	*Sequence with similarity and the source*	*Identities (%)*
33	704	glycine cleavage system protein P (pyridoxal-binding), N-terminal domain, *Cytophaga hutchinsonii*	63
38	857	oxidoreductase, *Bacteroides thetaiotaomicron* VPI-5482	42
39	988	1,4-alpha-glucan branching enzyme, *Nostoc* spp. PCC 7120	55
40	1024	N-acetylglucosaminyltransferase, *Bradyyrhizobium* sp. ISLU256	100
41	275	DNA segregation ATPase FtsK/SpoIIIE and related proteins, *Cytophaga hutchinsonii*	86
43	1110	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase, *Chlamydophila pneumoniae*	53
45	1191	cation transport ATPase, *Cytophaga hutchinsonii*	70
46	350	Uncharacterized protein conserved in bacteria, *Novosphingobium aromaticivorans*	52
47	446	inositol 1,4,5-trisphosphate receptor type 1, *Rattus norvegicus* (Norway rat)	30
48	974	signal peptidase I, *Cytophaga hutchinsonii*	74
49	921	putative sulfatase, *Bacteroides thetaiotaomicron*	45
52	1065	CapadS protein, *Porphyromonas gingivalis*	42
54	921	nicotinate phosphoribosyltransferase, *Pseudomonas putida*	50
58	508	ABC transporter, ATP-binding protein, *Porphyromonas gingivalis* W83	48
60	614	putative outer membrane protein, probably involved in nutrient binding, *Bacteroides thetaiotaomicron* VPI-5482	34
61	820	hypothetical protein, *Vibrio parahaemolyticus*	46
63	794	cation transport ATPase, *Cytophaga hutchinsonii*	63
66	439	envelope glycoprotein, human immunodeficiency virus 1 (HIV-1)	47
67	657	hypothetical protein, *Chlamydophila pneumoniae* AR39	46
68	344	UDP-N-acetylmuramate-alanine ligase, *Cytophaga hutchinsonii*	49
69	678	ATPase involved in DNA repair, *Cytophaga hutchinsonii*	31
72	1150	immunoreactive 84 kDa antigen PG93, *Porphyromonas gingivalis* W83	42
73	867	Leucyl-tRNA synthetase, *Cytophaga hutchinsonii*	57
74	217	HDC09939, *Drosophila melanogaster* (fruit fly)	40
77	1486	hypothetical protein, *Chloroflexus aurantiacus*	44
78	762	conserved hypothetical protein, *Corynebacterium efficiens* YS-314	73
80	949	Uronate isomerase, *Bacteroides thetaiotaomicron*	59
82	977	Ca++ ATPase, *Kluyveromyces lactis*	33
83	636	hypothetical protein, *Plasmodium falciparum* 3D7	33
87	535	hypothetical protein lin 2603, *Listeria innocua*	33
88	1053	hypothetical protein, *Bdellovibrio bacteriovorus*	31
106	416	hypothetical protein, *Bdellovibrio bacteriovorus*	57
110	477	conserved hypothetical protein, *Caulobacter crescentus* CB15	58
115	527	transcriptional regulator, Crp family, *Porphyromonas gingivalis* W83	70
117	348	*yqiW*, Bacillus subtilis subsp. *subtilis* strain 168	51

There was no translocation of the organisms to livers or spleens following pulmonary challenge with a large number of bacteria. The same phenomenon has been reported in mice following *Pseudomonas aeruginosa* intratracheal infection (Morissette *et al*., 1995). This clearance was associated with an early inflammatory response as expressed by the recruitment of polymorphonuclear neutrophils in the lungs. Polymorphonuclear neutrophils appear to play a critical role in the defense of the lung infections caused by glucose-non-fermenting bacteria, including *C. meningosepticum*, in mice.

Previous clinical studies indicated that *C. meningosepticum* is an opportunistic pathogen, usually causing infections in newborn infants and immunocompromised hosts (King, 1959; Olson *et al*., 1965; Werthamer and Weiner, 1971; Maderazo *et al*., 1974; Thong *et al*., 1981; Bloch *et al*., 1997; Chiu *et al*., 2000; Lin *et al*., 2004). Results of the current *in vitro* study concur with these clinical observations, as *C. meningosepticum*, irrespective of the sites of isolation and genomic subgroups, were not capable of penetrating MDCK cell monolayers and not cytotoxic to the cells. However, this study also suggested that *C. meningosepticum* is more pathogenic to the susceptible hosts compared to the usual commensal bacteria. *C. meningosepticum* were found to express some characteristics generally considered to be properties of true pathogens, such as serum resistance and intracellular invasion. We observed in the mouse infection model that *C. meningosepticum* 27439, an invasive isolate, was capable of infecting and invading murine respiratory tract epithelial cells. More interestingly, membrane ruffles appeared to be induced in response to such infection. Ruffling is the result of a complex interaction between a bacterium and a host cell and is associated with a triggering mechanism that leads to macropinocytosis (Silverstein *et al*., 1977; Francis *et al*., 1993; Goosney *et al*., 1999). *Salmonella* and *Shigella* have been shown to induce membrane ruffling in a contact-dependent manner in which a type III secretion system allows for the secretion of numerous effector proteins that initiate the cellular response required for the observed cytoskeletal rearrangements (Francis *et al*., 1993; Goosney *et al*., 1999). A type III secretion system has not been described for *C. meningosepticum*. Suppressive subtraction hybridization was performed aiming to look for the possible existence of homologs of *Salmonella* and *Shigella* type III secretion systems and effector proteins. An invasive isolate of *C. meningosepticum* yielded, however, no significant matches to ruffling-associated proteins from the database. Previous studies showed that genes of the type III secretion system of *Salmonella* and *Shigella* are highly conserved and clustered in pathogenicity islands of the chromosomes of these pathogens (Goosney *et al*., 1999). We believe that the complete genome sequence of *C. meningosepticum* available in the near future would shed insights into the genetic mechanism of ruffle induction and intracellular invasion by this organism.

Suppressive subtraction hybridization showed that genetically *C. meningosepticum* is closely related to *C. hutchinsonii*, which is an industrially important organism (McBride and Baker, 1996). This method, on the other hand, identified several sequences in the genome of an invasive isolate (genomic subgroup II:1) that may be involved in the virulence of *C. meningosepticum*. Three of the fragments with known functions had homology to genes involved in the prokaryotic transport systems and one to a gene of a secretion system (Poquet *et al*., 1993; Rahman *et al*., 2003), suggesting that the genes may be involved in the pathogenesis of *C. meningosepticum* infection. Further experiments investigating the physiological and pathogenic roles of these DNA sequences identified are underway.

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