Molecular Interaction of *Ornithobacterium rhinotracheale* with Eukaryotic Cells

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Moleculaire Interactie van *Ornithobacterium rhinotracheale* met Eukaryote Cellen

(met een samenvatting in het Nederlands)

ปฏิสัมพันธ์เชิงโมเลกุลของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* กับเซลล์ยูคาริโอต

(พร้อมบทสรุปภาษาไทย)

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ฝูงชนกำเนิดคล้าย คลึงกัน
ใหญ่ย่อมเพศผิวพรรณ แผกบ้าง
ความรู้อาจเรียนทัน กันหมด
เว้นแต่ชั่วดีกระด้าง ห่อนแก้ ฤาไหว

ร. ๕

Born men are we all and one
Brown, black by the sun culture
Knowledge can be won alike
Only the heart differs from man to man

M.R. Seni Pramoj

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

Infections of the respiratory tract of chickens and turkeys pose a major problem to poultry farms. High medication costs, mortality and condemnation rates, a drop in egg production, reduction of egg shell quality and decreased hatchability cause a considerable economic loss and urge the development of improved prevention strategies. Infectious agents that cause avian respiratory disease include fungi (Akan *et al.*, 2002), viruses (Alexander, 2000; Higgins, 1971; Ignjatovic *et al.*, 2002; McFerran and Adair, 1977; Swayne *et al.*, 2001) and bacteria (Blackall, 1999; da Rocha *et al.*, 2002; Noormohammadi *et al.*, 2002; Pruimboom *et al.*, 1996). One recently identified pathogen is the bacterium *Ornithobacterium rhinotracheale* (*ORT*). This pathogen was discovered as an etiologic agent of respiratory infections in broiler chickens in South Africa in 1991 by Jan DuPreez (van Beek *et al.*, 1994). Bacteriological examination of the diseased animals revealed a slow growing, pleomorphic, gram-negative rod that could not be classified as any of the known bacterial species (van Beek *et al.*, 1994). Further investigation indicated that a *Pasteurella*-like bacterium isolated from ducks suffering from respiratory disease in Hungary in 1987, and also *Riemerella anatipestifer*-like strains isolated from turkeys suffering respiratory disease in Germany in 1991 and 1992, were identical to the South African isolates both in appearance and biochemical characteristics. Similar Gram-negative rods could be isolated from turkey flocks and broilers in The Netherlands in 1993 (Szalay *et al.*, 2002). These animals showed signs of respiratory infection, growth retardation and sudden death (Hinz *et al.*, 1994; van Beek *et al.*, 1994). In the following years similar rods were isolated in association with avian respiratory disease in Belgium (Wyffels and Hommez, 1990), Canada (Joubert *et al.*, 1999), USA (Charlton *et al.*, 1993), France (Leroy-Sétrin *et al.*, 1998), Israel (Bock *et al.*, 1995), England (Amonsin *et al.*, 1997), Slovenia (Zorman-Rojs *et al.*, 2000), Mexico (Soriano *et al.*, 2003), Peru (Hung and Alvarado, 2001), Korea (Yoon *et al.*, 2000), Japan (Sakai *et al.*, 2000), Jordan (El-Sukhon *et al.*, 2002), Turkey (Erganis *et al.*, 2002; Turan and Ak, 2002) and Egypt (Devriese *et al.*, 2001). In 1994, Vandamme *et al.* proposed the name *Ornithobacterium* for this new genus and the species name *rhinotracheale* was assigned (Vandamme *et al.*, 1994). Interestingly, investigations of old culture collections in Germany revealed that *ORT* had already been isolated from the respiratory tracts of turkeys in 1981 and from rooks in 1983 (Vandamme *et al.*, 1994). It had also been isolated in Belgium (Wyffels and Hommez, 1990), the USA (Charlton *et al.*, 1993) and Israel (Bock *et al.*, 1995) before 1990, but so far no isolates from earlier than 1981 have been reported (Hinz and Hafez, 1997). Nowadays *ORT* is frequently isolated from cases of airsacculitis and purulent pneumonia in meat type turkeys and broiler chickens from all over the world (Odor *et al.*, 1997; Roepke *et al.*, 1998; Tahseen, 1997; Travers *et al.*, 1996; van Beek *et al.*, 1994; van Empel and Hafez , 1999) and also from wild birds including partridge, pheasant, pigeon, rook, quail, duck, chukar, ostrich, goose, guinea fowl, chicken and turkey (Buys, 1996; Charlton *et al.*, 1993; Devriese *et al.*, 1995; Vandamme *et al.*, 1994; van Empel and Hafez, 1999; van Empel *et al.*, 1997). The global occurrence of the bacterium is further confirmed by the presence of maternally derived antibodies against *ORT* in eggs and day-old birds from all over the world. At this time, *ORT* has not been found to be a threat to public health.

ORT may cause an acute contagious respiratory infection in turkeys and chickens (Roepke *et al.*, 1998; Tanyi *et al.*, 1995; van Empel and Hafez, 1999; van Veen *et al.*, 2000a; 2000b). The manifestations of infection such as its duration, severity, morbidity, and mortality rate, are extremely variable and appear aggravated by environmental factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene or concurrent infections. Nowadays *ORT* infections are recognized as a major burden to the poultry industry. However many *ORT* infections may still be missed, as conventional routine isolation methods for avian respiratory bacteria have to be changed for optimal isolation.

2. Bacteriology

Detailed bacteriological examination indicated that *O. rhinotracheale* is a Gram-negative, non-motile, pleomorphic, rod-shaped, non-spore forming bacterium of the rRNA superfamily V, in the taxonomic neighborhood of the genera *Cytophaga*, *Riemerella*, *Flavobacterium*, *Weeksella*, *Sporocytophaga* and *Capnocytophaga*. *ORT* was proposed as a separate bacterial species based on the phylogenetic position and various genotypic, chemotaxonomic and classical phenotypic characteristics of 21 Gram-negative avian isolates (Vandamme *et al.*, 1994). Most *ORT* strains grow under aerobic, micro-aerophilic, and anaerobic conditions. Optimal growth occurs on 5% sheep blood agar (van Empel and Hafez, 1999), chocolate agar, or Columbia agar (without blood or serum) under micro-aerophilic conditions (5 to 10% CO_2) at 37ºC. Under these conditions, *ORT* appears as pinpoint colonies (<1 mm in diameter) after 24 h of incubation. After 48 h, small, circular, butyrous, gray to gray-white colonies (1-2 mm in diameter) are formed, sometimes with a reddish glow and always with a distinct odor similar to that of butyric acid. Because the colonies are very small, especially after 24 h of incubation, they can be masked or overgrown by large numbers of other more robust bacterial species such as *E. coli* (Chin and Droual, 1997). Addition of gentamicin and polymixin (both 5 µg/ml) to the agar plates adds to the isolation of *ORT* although at this time only 90% of *ORT* strains are resistant to both of these antibiotics. Thus sheep blood agar plates without antibiotics should always be included in the isolation procedure (van Empel *et al.*, 1996). When grown in liquid media (Todd-Hewitt broth or Brain Heart Infusion broth), *ORT* will become more pleomorphic than when grown on agar and are of variable length (0.6-5 µm). The bacteria often form clumps that consist of up to several thousands of organisms (van Empel and Hafez, 1999). Some strains require a nutrient-rich medium such as Todd-Hewitt or Brain Heart Infusion broth supplemented with serum (van Empel and Hafez, 1999). Further growth characteristics of *ORT* are the lack of growth onto MacConkey agar, Gassner agar, Drigalski agar, or Simmon's citrate medium, and poor growth on TSI slants with no change in the butt or slant portions of the tube (Chin and Droual, 1997). Colonies are catalase-negative and oxidase-positive. *ORT* isolates are positive for enzymes such as alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-Bi-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and *N*-acetyl-β-glucosaminidase (Back *et al.*, 1996; Charlton *et al.*, 1993).

3. Antigenic properties of *O. rhinotracheale*

Immunological analysis of *ORT* strains using agar gel precipitation (AGP) and ELISA techniques (van Empel, 2002) with antisera raised against different *ORT* strains indicated the existence of up to 18 different serotypes. The majority of isolates are of serotype A, while 97% of strains belong to one of the four major serotypes A, B, D and E. Serotype A is found in 95% of the isolates from chickens, while turkey isolates showed more serotype diversity (van Empel *et al.*, 1997). van Empel *et al.*(1997) reported a relationship between the geographic origin and the serotype of *ORT*. The molecular basis of the serotyping is still unknown. In search for the serotype antigen, heat-stable or proteinase K antigen extractions showed to be suitable for serotyping of *ORT* isolates (Hafez and Sting, 1999), suggesting the carbohydrates (co)determine the serotype. Hyper-immune sera of different *ORT* serotypes has been used to differentiate distinct serotypes of *ORT* by Western blot analysis using isolated outer membrane proteins as antigens (Lopes *et al.*, 2000).

Immunoblots of *ORT* whole-cell proteins probed with sera from chickens naturally infected with *ORT* showed that the animals mounted an immune response during the natural infection. The antibodies reacted strongly with proteins with molecular masses of 33 and 42 kDa, and to a lesser extent with proteins of 52 and 66 kDa (Hung and Alvarado, 2001). Antibodies could be in 100% of infected birds until 8 wk post-infection.

4. Diversity of *O. rhinotracheale*

Comparison of a large number of *ORT* strains by molecular DNA techniques such as 16S rRNA sequencing and rep-PCR typing, indicated considerable similarities at the genetic level and suggested that the species consists of a small group of closely related clones (Amonsin *et al.*, 1997; Lopes *et al.*, 2000). 16S rRNA sequencing of a large number of strains revealed more than 99% identity, while rep-PCR typing yielded only six different rep-PCR types that showed only minor differences (Amonsin *et al.*, 1997). DNA-DNA binding values and G+C content (37-39%) also showed high similarities between tested strains (Vandamme *et al.*, 1994). Ribotyping of 23 French strains showed a low discriminatory power. Random amplified polymorphic DNA (RAPD) with OPG11 (TGCCCGTCGT) as a primer was able to discriminate nine RAPD types with an similarity coefficient of 82% and five RAPD types with a similarity coefficient of 50% (Leroy-Sétrin *et al.*, 1998). In a study of 53 strains, the amplified fragment length polymorphism (AFLP) method was also found to be discriminative (van Empel and Hafez, 1999). At this time, no information about the *ORT* genome is available.

At the protein level, SDS-PAGE analysis revealed that the total protein (TP) and outer membrane protein (OMP) profiles of *ORT* strains were relatively conserved with similarity coefficients of more than 84% between strains of different origin and/or serotype (Amonsin *et al.*, 1997). The predominant OMP bands in SDS-PAGE are the 33 kDa and 42 kDa proteins that are highly immunogenic during the natural infection (Hung and Alvarado, 2001; Lopes *et al.*, 2000). Together, the DNA and protein analyses indicate the existence of only limited diversity among *ORT* strains.

5. Pathogenic potential of *O. rhinotracheale*

Initially, *ORT* was considered to be a secondary pathogen as it was unable to cause disease in the specific pathogen-free (SPF) chickens without priming with avian respiratory viruses, mainly Newcastle disease (ND) virus or turkey rhinotracheitis (TRT) virus (van Empel *et al.*, 1996; van Empel *et al.*, 1999). Later, in experiments with commercial birds, airsacculitis, pneumonia and increased mortality were observed after aerosol, intra-tracheal, intravenous and intra-thoracic *ORT* challenge without viral priming (Ryll *et al.*, 1997; Sprenger *et al.*, 1998). Furthermore, intravenous challenge of SPF birds with *ORT* did result in clinical disease such as meningitis and osteitis with up to 20% mortality (Goovaerts *et al.*, 1998). In 2000, van Veen *et al.* (2000b), finally did succeed to demonstrate that *ORT* is capable to induce respiratory lesions in SPF chickens after aerosol challenge without previous priming with virus. This classified *ORT* as a primary pathogen although it is evident that the establishment of disease is promoted by pre-existing viral infections. In field cases, bacteria such as *Bordetella avium* and *E. coli* have also been suspected to promote the establishment of *ORT* infections but viral infections appear more important (DeRosa *et al.*, 1996; Droual and Chin, 1997; Travers, 1996).

Aerosol challenge experiments using different combinations of *ORT* strains and viral priming revealed no major differences in pathogenicity among *ORT* strains. In an experimental study a series of *ORT* isolates infected chickens and turkeys equally well independent of their origin or serotype (van Empel *et al.*, 1996). Comparison of the pathogenicity of 18 different isolates (eight from chickens, seven from turkeys, two from partridges and one from a duck) also revealed comparable pathology in infected turkeys and broiler chickens after viral priming (van Empel and Hafez, 1999; van Empel *et al.*, 1996). In another study minor differences in pathogenicity between strains isolated from South Africa and Germany have been reported (Travers *et al.*, 1996; van Empel and Hafez, 1999). Similarly, van Veen *et al.* (2000a) and Travers *et al.* (1996) reported that Dutch isolates and a South African strain were more virulent than American strains of *ORT*.

In the field, *ORT* is acquired via horizontal bird-to-bird transmission through aerosols (van Empel *et al.*, 1996) or via vertical transmission. Horizontal transmission appears to be influenced by a number of environmental factors such as stress, inadequate ventilation, poor hygiene, and high ammonia levels (van Empel, 2002). Consistent with vertical transmission *ORT* has been reported to survive in the ovary and

oviduct without clinical signs (Back *et al.*, 1996; 1998; Nagaraja *et al.*, 1998). Furthermore, hatched chicks from serologically positive breeder flocks with a known history of *ORT* infection that were kept in total isolation during the investigation and that were given sterile food and water, showed positive for *ORT* after aerosol exposure to ND or TRT virus. In another study, *ORT* had been found on eggshells and yolk sacs of 1-day-old birds albeit at a low frequency (van Empel *et al.*, 1997). These data strongly suggest that the egg can transmit *ORT*, either trans-ovarially or by cloacal contamination (van Empel *et al.*, 1997). As *ORT* specific antibodies can be detected in eggs and day-old chicks from all over the world, it is likely that the establishment of *ORT* infections in young birds is limited due to the presence of maternally derived antibodies (van Empel and Hafez, 1999).

6. Diagnosis of *ORT* infection

ORT infections are diagnosed on the basis of the clinical signs associated with the disease, ELISA results and/or post-mortem lesions in combination with isolation of the bacterium (Naeem *et al.*, 2003). Clinical signs of *ORT* infection are not sufficient for diagnosis as they are rather variable and resemble to those of other respiratory pathogens such as *E. coli* or *Riemerella anatipestifer,* or other agents causing joint infections such as *E. coli, Staphylococcus aureus* or *Streptococcus feacalis*. Brain infections caused by *ORT* are most of the time not even noticed as possible cause of death and therefore easily missed.

ORT can often successfully be isolated from diseased animals at an early stage of the infection. Recovery of *ORT* at a late stage of infection often fails (van Empel, 2002) due to the existence of secondary infections caused by microorganisms that grow faster and are less fastidious than *ORT*. In one study it has been reported that *ORT* caused 70% of the cases with respiratory symptoms in broiler chickens but that with bacteriology and/or serology only 30% of the cases could be connected to *ORT*. More recently, immuno-histological examination has indicated that joint-infections in turkeys can regularly be attributed to *ORT* (van Empel, 2002).

The most frequently used method to isolate *ORT* in the laboratory is with use of Columbia agar plates enriched with blood that are placed at 37^{o}C in a 5-10% CO_2 atmosphere. *ORT* colonies are catalase-negative and oxidase-positive. Gram staining will reveal characteristic pleomorphic, Gram-negative bacteria. Additional characterization can be done with biochemical tests, polymerase chain reaction and serology (Charlton *et al.*, 1993; Vandamme *et al.*, 1994; van Empel and Hafez, 1999). Serological tests that have been developed include a rapid agglutination test (RAT) (Back *et al.*, 1998) a serum agglutination test (SAT), a dot immunobinding assay (DIA), and more sensitive ELISA tests (Hafez, 1996; Lopes *et al.*, 2002). In the ELISA, boiled-extract of *ORT* is often used as antigen. This method has been successfully applied for screening purposes and to detect maternal antibodies in day-old chickens and turkeys (van Empel *et al.*, 1999). The ELISA is particularly attractive for screening as with this method antibodies can be detected from shortly after the start of a field infection up to 8 weeks post infection (Lopes *et al*., 2000). This is important as titers will usually peak between 1 to 4 weeks post infection and then rapidly decline. The serotype specificity of the ELISA is a disadvantage but commercial ELISA's are available with which most serotypes can be detected. In the field, *ORT* infections suspected on the basis of serology can be confirmed with a sensitive immuno-histochemical staining of the infected tissues.

7. Pathology associated with *O. rhinotracheale* infection

Broilers are most susceptible to *ORT* infection at 3 to 4 weeks of age. Broiler breeders are most prone to infection between 24 to 52 weeks of age. The initial clinical signs of young chickens infected with *ORT* include relatively mild respiratory stress. In turkeys, infections in 2 week-old birds manifest as nasal discharge, followed by facial edema and swelling of infraorbital sinuses (Hafez, 1996). Later, birds appear depressed with ruffled feathers and have decreased feed and water consumption. *ORT* also can cause sudden deaths in young birds through infections of the brains and the skull, featuring totally weakened

skull-bones. Sometimes, a subcutaneous edema over the cranium with a severe bacterial osteitis is found without respiratory tract infection (Goovaerts *et al.*, 1998).

Older birds (>14 weeks) generally show more severe lesions (Hafez, 1996) and may develop acute pneumonia with mortality rates from 1 to 15%, and sometimes even up to 50% (DeRosa *et al.*, 1996; 1997; Tahseen, 1997). Macroscopic post-mortem examinations of these animals show a foamy, white, "yogurt"-like exudate in the air sacs, commonly accompanied by unilateral pneumonia. When the animals survive, the symptoms can disappear within one week but the situation may also deteriorate due to the occurrence of secondary infections.

Another type of *ORT* infection particularly in older turkeys and chickens involves the dissemination of the bacteria to other body sites. Frequently arthritis, osteitis and osteomyelitis develop with the formation of a purulent, slimy exudate in the joints of the lame birds.

A more mild manifestation of *ORT* infection has been observed in flocks of turkey breeder birds. Here the infection manifests as a slightly increased overall mortality, a drop in egg production of 2-5% (Chin and Droual, 1997) and a decrease in the egg quality. Because of its relatively mild nature, this type of infection is probably seldom recognized as being caused by *ORT* (van Empel *et al.*, 1996).

Immunohistochemistry on experimentally infected SPF chickens one week after viral priming with ND and after subsequent aerosol challenge with *ORT*, showed that the lesions in the air sacs, lungs and, to a lesser extent, in the trachea are caused by *ORT* and not by the ND virus. At the first 2 days after *ORT* challenge, microorganisms have been found attached to the epithelium on the respiratory side of the air sacs. Later, the air sacs thickened, and edema and an acute granulomatous airsacculitis developed. At this stage, *ORT* and bacterial fragments have been found in abundance within single and aggregated macrophages. Occasionally, distinct parts of the bronchial associated lymphoid tissue (BALT) in the lungs became infiltrated and necrotic, while other regions of the BALT in the same lung remained unaffected. In general, the air-sacculitis and pneumonia induced after *ORT* challenge of ND primed birds were fully established at 5 to 7 days post-*ORT* challenge (van Empel *et al.*, 1999).

8. Treatment and control of *ORT* infections

In general, bacterial infections in poultry can be successfully treated and/or controlled with anti-microbial drugs in combination with hygienic measures. Unfortunately, most *ORT* strains are now resistant to most types of antibiotics. This resistance has likely developed as a result of intensive and inadequate use of antibiotics in the field as sensitive strains have been isolated from wild birds (Devriese *et al.*, 2001). The anti-microbial drug sensitivity of *ORT* seems to vary dependent on the type of bird and geographical region. When *ORT* strains isolated from gallinaceous birds were compared with isolates from rooks using the agar dilution method, the minimal inhibitory concentrations for penicillin-cephalosporin antibiotics differed 5- to 20-fold (Devriese *et al.*, 1995). Similarly, Nagaraja *et al.* (1998) reported that the sensitivity of *ORT* isolates from the USA to a panel of antibiotics differed significantly from isolates from Germany (Lopes *et al.*, 2000). Isolates from Europe have been found to be often resistant to trimethoprim / sulphonamide, gentamicin, spectinomycin, neomycin, enrofloxacin, and colistin (Hafez, 1996; van Beek, 1994; van Empel, 2002) but sensitive to ampicillin, ceftiofur, doxycyline, lincomycin, tetracycline, chloramphenicol, amoxycillin, penicillin and tylosin (Chin and Droual, 1997; Devriese *et al.*, 1995; Hafez, 1996). As in most parts of the world *ORT* is sensitive to doxycycline and tetracycline (Devriese *et al.*, 1995; Hafez, 1996; Nagaraja *et al.*, 1998; van Beek, 1994), administration of chlortetracycline via the drinking water for 4 to 5 days is often used to treat *ORT* infections. Administration of amoxicillin via the drinking water for 3 to 7 days have also been reported to give satisfactory results under field conditions in some regions (Hafez, 1997).

Treatments of infected turkeys generally shows poor results with the use of conventional oral therapeutics, especially when pneumonia was prevalent. On some cases, injections with tetracycline and synthetic penicillin is an effective but often medication fails resulting in a loss of up to 25% of the animals within a few weeks (van Beek, 1994).

Adequate control of *ORT* infections is pursued with the use of chemical disinfectants to which most *ORT* strains are still highly sensitive (van Empel and Hafez, 1999). As *ORT* appears to have become endemic on many farms, thorough cleaning and disinfecting of houses is essential before introducing new stocks, especially in multiple age farms and in areas with an intensive poultry production. The most commonly used disinfectants are based on the antibacterial effects of different organic acids such as formic and glyoxyl acids, and on aldehydes that readily inactivate *ORT in vitro* (van Empel, 2002).

9. Vaccination against *ORT* infection

Probably the best way to control *ORT* infections is by vaccination. In the past several types of vaccines (live attenuated bacteria, formalin-killed bacteria) have been developed and have shown promise for protecting at-risk chickens. Lopes *et al.* (2002a; 2002b) developed a temperature-sensitive (Ts) mutant of *ORT* for use as a live vaccine. This vaccine is based on the ability of the mutant to colonize the upper respiratory tract but not the lower respiratory tract. In turkeys, the Ts vaccine has been reported to elicit a secretory immune response (IgA) and to evoke a protective response against experimental *ORT* challenge. Three weeks after administration of the vaccine via the drinking water or oculo-nasal instillation, antibodies could be demonstrated. Vaccination with live *ORT* also appears to induce protection against *ORT* challenge of broilers when the maternal antibody levels were low (van Empel and van den Bosch, 1998).

Vaccination with formalin-killed *ORT* (bacterin) of broiler breeders induced long-standing immunity and protected their progeny against experimental challenge with *ORT* for 30 days (van Empel and van den Bosch, 1998). With double subcutaneous vaccination of 12 and 18 weeks old broilers high mean *ORT* antibody titers were obtained that remained high for the entire production period (Cauwerts *et al.*, 2002). The vaccinations also caused high levels of maternal antibodies in the progeny, resulting in good protection against experimental challenge of the hatched broilers up to 30 days of age (van Empel and van den Bosch, 1998). In this study it has been observed that antibody titers in unvaccinated flocks increased during the study period suggesting that there was circulation of the bacterium among broiler breeders. As maternal immunity may protect chicks against lesions and clinical disease resulting from *ORT* infection up to the age of 4 weeks under experimental condition (van Empel and van den Bosch, 1998), vaccination should be started at 2-4 weeks to protect birds at older ages.

In turkeys, both live and killed *ORT* vaccines have been demonstrated to protect susceptible animals against development of pathologic lesions. In addition, serum samples from turkeys vaccinated with live or killed *ORT* vaccines contained antibodies to the organism within 1 week of vaccination. These antibodies remained present for 8 weeks (bacterin) or 14 weeks (live vaccine) after vaccination (Sprenger *et al.*, 2000). The most practical approach in programming a vaccine for preventing the *ORT* infection in broilers may be to vaccinate breeders with an inactivated bacterin combined with a live vaccination of broilers at 2-3 weeks of age.

A major problem with the current vaccines is that they do not provide broad cross-protection against the various serotypes of *ORT* (Bock *et al.*, 1995; 1997). Vaccination of turkey broilers with autogenous bacterins successfully reduced the number of outbreaks of *ORT* infections in the field (Bock *et al.*, 1997). However, repeated infections caused by other serotypes regularly occur during the long rearing period (van Empel, 2002). Development of a vaccine that provide long lasting strong cross-protection against all *ORT* serotypes (van Empel and Hafez, 1999) likely awaits knowledge of the bacterial and host factors that are essential for the establishment of *ORT* infections.

10. Outline of this thesis

Despite the emergence of *ORT* as a bacterial pathogen, its wide-spread resistance towards most current anti-microbial drugs, its economic importance, and the limited window of protection provided by the available vaccines, virtually no studies have been designed to better understand the molecular basis of

ORT infections. For other pathogens, bacterial colonization of the mucosa and the breaching of the mucosal barrier either via bacterial invasion and/or the production of toxins, are crucial steps in the establishment of an infection. Well-known bacterial adhesins found on many bacterial pathogens are the hair-like structures that protrude from the bacterial cell surface (fimbriae) and cell surface adhesins (outer membrane proteins, lipopolysaccharide, or even capsule) that specifically interact with distinct host cell surface receptors. These interactions may elicit a strong inflammatory response that contributes to the pathology. Although *ORT* has been seen in close association with the respiratory epithelium and within macrophages in infected tissues, the mechanisms underlying these interactions as well as the nature of the local host cell response are still completely unknown. Up to now, no special structures such as fimbriae or bacterial surface adhesins have been reported for *ORT* (Leroy-Sétrin *et al.*, 1998) and the basis of the host and tissue specificity of *ORT* infection, remain to be defined. Similarly, the bacterial factors that contribute to the potent inflammatory response and the clinical pathology observed during experimental and natural *ORT* infections (Chin and Droual, 1997; van Empel *et al.*, 1997), await investigation.

The work described in this thesis was designed to learn more about the molecular interaction between *ORT* and host cells in the belief that development of adequate novel infection intervention and prevention strategies is served best by fundamental scientific knowledge rather than by the thus far followed trial-and-error approach. Specific objectives were:

1. Development of an *in vitro* infection model to better understand the interaction between *ORT* and mucosal epithelial cells and the kinetics of infection at the cellular level (Chapter 2).
2. Characterization of the interaction of *ORT* with macrophages of different species and the induction of an inflammatory response using nitric oxide production as a marker of activation of the innate host defense (Chapter 3).
3. Identification of host cell receptors exploited by *ORT* and of natural inhibitors of *ORT* infection (Chapter 4).
4. Construction of an *Escherichia coli* - *ORT* shuttle vector to allow genetic manipulation of *ORT* (Chapter 5).

The potential significance of the work presented in this thesis as a basis for future intervention and prevention of *ORT* infections is discussed in Chapter 6.

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CHAPTER 2

LPS-Sensitive Adhesion of the Avian Pathogen *Ornithobacterium rhinotracheale* to Mucosal Epithelial Cells

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ABSTRACT

The recently identified Gram-negative bacterium *Ornithobacterium rhinotracheale* (*ORT*) is an emerging bacterial pathogen that appears to exclusively colonize birds. *ORT* infections cause major losses to the poultry industry. In search for factors contributing to the pathogenesis of *ORT* infections, the interaction of *ORT* with epithelial cells was investigated. *In vitro* infection experiments demonstrated that *ORT* efficiently adheres to various types of cultured epithelial cells with levels up to 80 adherent bacteria per cell at 3 h of infection. Comparison of different *ORT* isolates indicated that all tested strains exhibited adherence properties but with various characteristics. One strain switched between an epithelial cell and chicken red blood cell adherent phenotype, dependent on the growth conditions. Analysis of outer membrane profiles of adherent strains by SDS-PAGE revealed strain variation in both protein and lipopolysaccharide (LPS) electrophoretic mobility. Infection assays in the presence of LPS purified from *ORT* showed a LPS dose-dependent inhibition of *ORT* adhesion. This effect was strain-specific i.e. no inhibition was observed with LPS from other strains. These data suggest that the adhesion of *ORT* to epithelial cells is conferred via a unique strain-specific and LPS-sensitive mechanism.

INTRODUCTION

In 1994, the Gram-negative bacterium *Ornithobacterium rhinotracheale* (*ORT*) was proposed as a new bacterial species (Vandamme *et al.*, 1994). The bacterium had been demonstrated to reside in a wide variety of domestic and wild birds, and appeared associated with bacterial respiratory disease in poultry. Later studies suggested that it first emerged as a pathogen at least 20 years earlier (van Empel and Hafez, 1999). Nowadays, *ORT* is a major problem to the poultry industry causing major economic losses due to increased mortality and condemnation rates, growth retardation, decreased egg production and egg shell quality, and decreased hatchability (Charlton *et al.*, 1993; Hinz *et al.*, 1994; Vandamme *et al.*, 1994; van Empel and Hafez, 1999).

The primary natural niche of *ORT* appears to be the avian respiratory tract (Hinz *et al.*, 1994). From this site, the bacteria spread to the environment or to other body sites such as the brain and joints. Infections with *ORT* have been most often associated with chronic respiratory syndromes with severe bilateral bronchopneumonia and airsacculitis and, in some cases, pericarditis, hepatomegaly and arthritis. The cause of this syndrome has long been considered to be multifactorial as concurrent viral and bacterial infections seemed to enhance *ORT* pathogenicity. Challenge experiments however, have demonstrated that *ORT* can act as a primary pathogen in chicken (van Veen *et al.*, 2000).

The mechanisms via which *ORT* causes disease are largely unknown. Pathology on infected animals suggests that the bacteria colonize the respiratory tract of the host by adhering to the host mucosa. Prolonged *ORT* exposure is followed by influx of polymononuclear granulocytes and macrophages at the site of infection, a thickening of the air sacs, and the formation of granulomatous tissue (van Empel *et al*., 1999). Treatment of the disease is complicated as most isolates of *ORT* are resistant to most commonly used antibiotics. Vaccination is an effective form of infection prevention. Available vaccines composed of killed *ORT* however, are assumed to give only partial protection against a limited number of strains.

A major problem in developing novel strategies to combat *ORT* infections is the virtually complete lack of knowledge about the pathogenesis of the disease. As a first step to better understand the initial step in the colonization, we investigated the adhesive properties of *ORT* towards epithelial cells. *In vitro* infection assays with cultured epithelial cells demonstrated that *ORT* strains efficiently adhere to epithelial cells. Further analysis indicated that adhesion is conferred via a strain-specific and LPS-sensitive mechanism.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

ORT strains designated as 7, 29, 30 and 41 (generally provided by Dr. P. van Empel, Intervet, Boxmeer, The Netherlands) were isolated from infected birds. Strain 7 (serotype A) was isolated from guinea fowl. Strains 29 and 30 (serotype D) and strain 41 (serotype E) was isolated from turkeys. Bacteria were stored at -80°C in Todd-Hewitt (TH) broth (Oxoid, Hampshire, England) containing 15% glycerol. Bacteria were grown (37°C, 48 h) on Columbia (CO) agar (Oxoid) or TH broth supplemented with 2% Noble agar (Difco, Detroit, USA) in a humidified atmosphere of 5% CO_2 in air, or in 45 ml of TH broth in 50 ml polypropylene tubes on an orbital shaker (150 rpm).

Cell culture

Chang conjunctiva epithelial cells (ATCC CCL-20.2) and INT 407 intestinal epithelial cells (ATCC CCL-6) were routinely maintained in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 5% fetal bovine serum (FBS) (Bodinco B.V., Alkmaar, The Netherlands) at 37°C in a humidified atmosphere of 5% CO_2 in air. For use in infection experiments, cells were seeded onto circular 12 mm glass coverslips in 24-well tissue culture plates (Costar, New York, USA) 48 h prior to infection and grown to near confluence (approximately 10^5 cells per well), unless indicated otherwise.

Infection assay

For use in infection assays, broth cultures (48 h) or solid phase grown (48 h) *ORT* were (re)suspended into Dulbecco's phosphate-buffered saline (DPBS) and added to the monolayers of epithelial cells maintained in 1 ml of RPMI 1640 (without serum) at a m.o.i of 100, unless indicated otherwise. At various times of incubation (37°C, 5% CO_2), the culture medium containing non-adherent bacteria was removed and the cells were rinsed three times with PBS. Then, the cells were fixed in 2% formaldehyde in DPBS for 1 h and either stained with 0.005% crystal violet in distilled water for at least 3 h, or subjected to the immunogold-silverstaining procedure that allows microscopic discrimination of adherent and intracellular microorganisms (see below). All specimens were viewed in an Olympus BH-2 microscope. All experiments were repeated at least three times and data were statistically analyzed with the independent Student's *t*-test using SPSS 9.0 software.

Immunogold-Silver Staining (IGSS) procedure and fluorescence microscopy.

The IGSS procedure is based on the inability of antibodies to diffuse into paraformaldehyde-fixed eukaryotic cells enabling selective immuno-staining of adherent microorganisms (van Putten, 1993). For use with *ORT*, the coverslips with the infected cells were inverted onto 100 µl drops of *ORT*-serotype specific hyper-immune rabbit sera (1/1000 dilution in DPBS) placed on Parafilm for 30 min at 20°C. Then, the coverslips were sequentially incubated (3 x 5 min) onto 3 drops of 100 µl of DPBS to remove unbound antibody, and placed onto 50 µl of 10 nm gold-conjugated protein A solution. After an additional 30 min of incubation, the coverslips were incubated three times for 5 min onto 100 µl drops of DPBS and two times for 5 min onto 100 ml drops of distilled water. Silver enhancement of the bound gold-particles was achieved by adding developing solution (composition: 70 ml of citrate buffer (2.55 g citric acid monohydrate + 2.35 g tri-sodium citrate dihydrate in distilled water), 15 ml of reducing agent (0.85 g hydroquinone) and 15 ml of silver acetate (0.11 g)) for 2-10 min. The coverslips were counterstained with 0.005% crystal violet and kept in this solution at 4°C until viewed in the microscope. For visualization of adherent bacteria in the fluorescence microscope, essentially the same procedure was followed except that the coverslips were incubated with goat anti-rabbit Alexa green (Molecular probes, Leiden, The Netherlands) instead of gold-conjugated protein A prior to mounting in DPBS + 5% glycerol.

Haemagglutination assay

Haemagglutination was performed with freshly isolated chicken red blood cells (RBC) (Hsiung, 1982) as well as glutaraldehyde inactivated cells (Eaves *et al.*, 1989) in PBS. RBCs were collected from heparinized blood derived from male White Leghorn, washed 3 times with DPBS and kept as 10% stock suspension at 4°C until use. Glutaraldehyde fixed RBC (GA-fixed RBC) were prepared as a 1% RBC suspension containing 1% GA solution (Polysciences, Warrington, PA) in DPBS for 30 min (4°C), followed by centrifugation (400 x g, 10 min, 4°C) to remove the fixative. After five additional washes with 0.15 M NaCl, the cells were suspended in DPBS and stored at 4°C until further use. Haemagglutination (HA) was tested by mixing fifty microliters of RBC suspension (1% final concentration) with an equal volume of 2-fold serial dilutions (1:2 to 1:16) of *ORT* suspensions ($5x10^7$ cfu/ml, 48 h plate-grown or broth cultures) in DPBS in 96 well-U-shaped disposable microtiters plates (Costar, New York, USA). After 2 h of incubation at 20°C, HA was scored. The HA titer was defined as the highest dilution of bacterial suspension still capable of causing partial or complete hemagglutination (HA).

Extraction of bacterial outer membranes

For isolation of bacterial outer membranes, *ORT* was grown (48 h, 37°C, 5% CO_2) onto Columbia agar (plates) and then in 200 ml of Todd-Hewitt broth (48 h, 37°C, in air) at 150 rpm. Bacteria were collected by centrifugation (4,000 x g, 15 min, 4°C) and resuspended in 10 mM of Tris-HCl, pH 7.4. Bacterial outer membranes were essentially extracted as previously described (Pannekoek *et al.*, 1992) with some modifications. Briefly, the bacteria were disrupted by sonication and the intact cells and insoluble debris were removed by centrifugation (12,000 x g, 5 min, 4°C). The supernatant was centrifuged (100,000 x g, 1 h, 4°C) to pellet the bacterial membranes. Outer membranes were isolated

after solubilization of the inner membrane in 10 mM of Tris-HCl (pH 7.4) containing 2% sodium *N*-lauroyl sarcosinate (1 h, 37°C). The outer membranes were collected by centrifugation (100,000 x g, 1 h, 4°C), resuspended in 10 mM of Tris-HCl and stored at –20°C awaiting further analysis.

Purification of lipopolysaccharide (LPS)

LPS was extracted and purified by hot phenol extraction according to Johnson and Perry (1976) with minor modifications. In brief, *ORT* was grown (37°C, 48 h, 150 rpm in air) in two liters of Todd-Hewitt broth and collected by centrifugation (7,000 x g, 20 min, 4°C). The bacteria were resuspended in 10 times of wet weight of distilled water and an equal volume of hot phenol (70°C) (Merck, Darmstadt, Germany) was added. The mixture was incubated (2 h, 70°C) in a water bath and vortexed for 1 min at 15 min intervals. After cooling on ice, the mixture was centrifuged (18,000 x g, 20 min, 4°C) and the waterphase (supernatant) was collected. Then, two volumes of acetone (-20°C) and 100 mg per gram bacterial wet weight of sodium-acetate were added to the waterphase. The mixture was centrifuged again, the pellet (with the LPS) washed in acetone (-20°C), again collected by centrifugation, and resuspended in distilled water. This solution was centrifuged (100,000 x g, 2 h, 4°C) to remove contaminating DNA. The pellet with the LPS was resuspended in distilled water and stored at -20°C until use. This procedure yielded 40 and 20 mg of pure LPS from two liters of *ORT* 30 and 41 cultures, respectively. The purity of the LPS was verified by SDS-PAGE and silver staining (Tsai and Frasch, 1982).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Tricine SDS-PAGE

Bacterial outer membrane protein analysis was performed using SDS-PAGE as described (Laemmli, 1970) using 4% (w/v) stacking gel and 10% (w/v) separating gels. Electrophoresis was performed at a constant voltage (100 V) in the Protean II electrophoresis cell (BioRad Laboratories, Richmond, CA). Separated proteins were visualized with 0.25% Coomassie Brilliant Blue R-250. Bacterial LPS was characterized using 16.5% tricine SDS-PAGE essentially as described (Schagger and von Jagow, 1987). LPS was visualized by silver staining according to the method of Tsai and Frasch (1982).

RESULTS

Kinetics of *ORT* adherence

The ability of *ORT* to adhere to eukaryotic cells was assessed by incubating cultured Chang epithelial cells in the presence of *ORT* strain 30 for various periods of time. Microscopic evaluation of infected epithelial cells indicated that strain 30 efficiently adhered to the cells in a time-dependent fashion with up to 40 adherent bacteria per cell at 6 h of infection (Fig. 1A). Microscopy showed bacteria apparently randomly distributed over the cell surface and that most of the cultured cells carried bacteria at their surface (Fig. 1B). No signs of cell toxicity were noted during the six-hour infection period. At prolonged infection (24 h), most epithelial cells had detached from the coverslips. This effect was not observed when cells were exposed to a 1/1 dilution of filtered (0.22 µm) culture supernatants collected from 24 h of infected cells, suggesting that the effect was probably not caused by a secreted toxin.

Further characterization of the bacterial adhesion showed that when different bacterial inocula ($5x10^6$-$5x10^9$) were applied to the cells, maximal adherence (approximately 100 bacteria per cell) was observed with an inoculum of $5x10^9$. At this inoculum about 10% of the added bacteria adhered to the cells. When 5×10^7 *ORT* was used as an inoculum, about 50% of the added bacteria adhered to the cells (Fig. 1C). These data indicate that the adherence event is a highly efficient and saturable process.

Variation in the composition of the culture media yielded similar bacteria adherence for experiments were performed in RMPI 1640 or DMEM tissue culture medium, while strongly reduced adherence was observed when experiments were performed in Dulbecco's phosphate- or HEPES-buffered salt solutions or with bacteria derived from solid phase media and liquid media (Fig. 1D).

This suggests that bacterial growth and/or specific components of the tissue culture media are required for *ORT* adhesion.

Immunogold-silver staining of the infected specimen that enables discrimination of extracellular and intracellular bacteria, revealed a few intracellular *ORT* at 3 h of infection (data not shown), suggesting that, at least under the conditions employed, *ORT* is not very efficient in gaining access to the host cell interior.

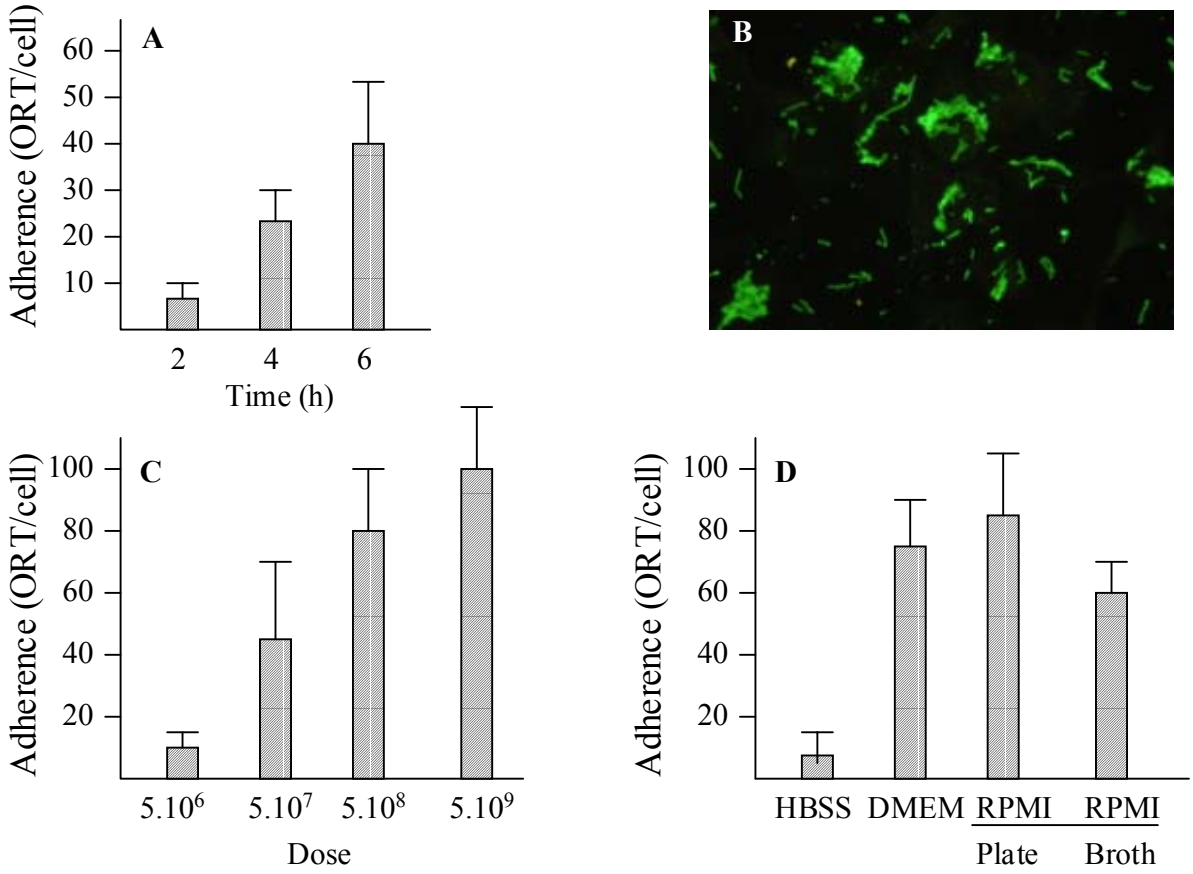

Fig. 1: Kinetics and characteristics of *ORT* adherence to epithelial cells.
(A) Time course of *ORT* adherence. Cultured Chang epithelial cells were incubated with plate-grown *ORT* strain 30 for 2, 4 and 6 h at 37°C. Adherence was scored by microscopy and presented as the number of *ORT* per cell. Data are the mean ± SEM of three experiments. **(B)** Adherence of *ORT* strain 30 as viewed by fluorescence microscopy at 1000x magnification. **(C)** Effect of inoculum size on *ORT* adherence. Chang cells were infected with the indicated numbers of *ORT* strain 30. Infection was stopped at 3 h of infection. Data represent the average number (± SEM) of adherent bacteria per cell of three experiments. **(D)** Effect of type of incubation medium on *ORT* adherence. *ORT* strain 30 grown onto Columbia agar plates for 48 h was added to Chang epithelial cells maintained in HEPES-buffered salt solution (HBSS), DMEM or RPMI without serum. As a comparison, Todd-Hewitt broth grown *ORT* strain 30 was added to epithelial cells maintained in RPMI. At 3 hours of incubation, infection was stopped and the number of cell-associated bacteria was determined. Data are the mean ± SEM of three experiments.

Inter-strain variability and cell type specificity of *ORT* adhesion

In order to ascertain that the adherence properties observed for *ORT* strain 30 (serotype D) represented a general feature of *ORT* strains, a number of other strains (grown on Columbia agar plates) was assessed for their ability to adhere to the cultured INT-407 epithelial cells. Strain 30 adhered to these cells as efficient as to Chang cells (Fig. 2). Infection of the cells with strain 29 (serotype D) and strain 7 (serotype A) also resulted in strong bacterial adherence when an inoculum of 5 x 10^7 was applied (Fig. 2). Similar experiments with strain 41 (serotype E) consistently yielded only 0-3 bacteria per cell under identical conditions (Fig. 2).

To further determine the cell type specificity of *ORT* adherence, adhesion of solid phase grown strains 29, 30 and 41 towards chicken red blood cells was assessed. The binding of *ORT* to red blood cells was explored in a haemagglutination assay with living and glutaraldehyde-fixed chicken erythrocytes. Incubation of the erythrocytes with various concentrations of *ORT* strains 29 and 30 gave virtually no haemagglutination. Similar experiments with strain 41 yielded strong haemagglutination of both the unfixed and glutaraldehyde-fixed erythrocytes derived from different animals (Fig. 3).

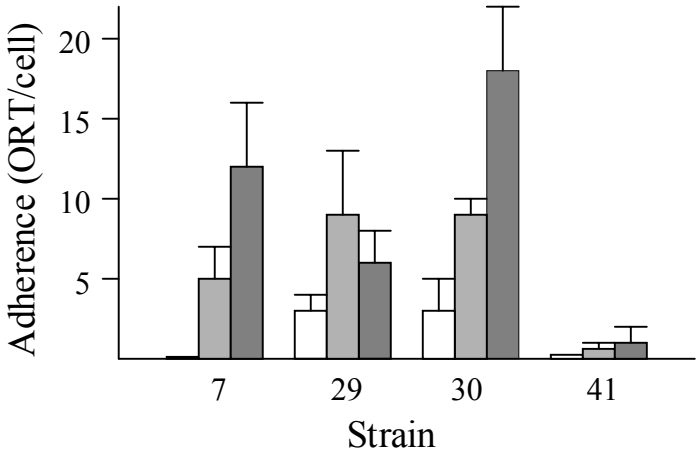

Fig. 2: Inter-strain variability in *ORT* adherence.
ORT strains 7, 29, 30 and 41 were added to cultured INT407 epithelial cells. Bacterial adherence (i.e. the average number of *ORT* per cell) was determined at 2 h (open bars), 4 h (light grey bars) and 6 h (dark grey bars) of infection. Results are the mean ± SEM of three independent experiments.

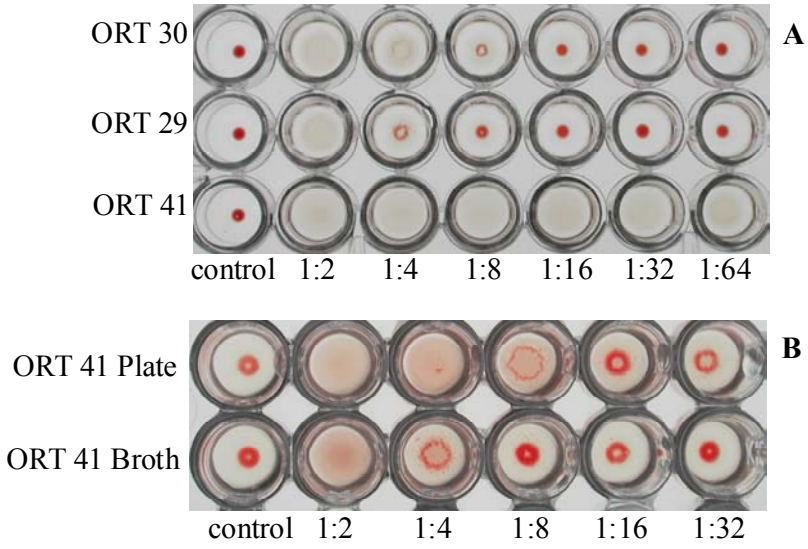

Fig. 3: Haemagglutination properties of *ORT*.
(A) Chicken red bloods cells (RBC) were mixed with serial dilutions of agar plate-grown *ORT* strains 29, 30 and 41 as indicated. After for 2 h of incubation (20°C), haemagglutination (HA) was scored. Note that *ORT* strain 41 showed strong HA activity in contrast to strains 29 and 30. **(B)** Comparison of HA activity of solid phase- and broth-derived *ORT* strain 41. The assay was carried as described under (A). Note the reduction in HA activity from broth cultures compared to solid-phase grown bacteria.

Growth-medium dependent switching between red blood cell and epithelial cell *ORT* adhesive phenotypes

The apparent cell type specificity of the strains 29 and 30 compared to strain 41 was further explored by direct comparison of the adherence of solid phase-grown and Todd-Hewitt broth grown bacteria to epithelial and red blood cells. When grown on Columbia (or Todd-Hewitt) agar plates prior to the infection assay, *ORT* strain 41 that only poorly adhered to the epithelial cells, effectively agglutinated chicken red blood cells (Fig. 3). Conversely, when grown in Todd-Hewitt broth prior to infection, strain 41 lost the ability to haemagglutinate the erythrocytes (Fig. 3), but gained the ability to adhere to the epithelial cells. In fact, strain 41 adhered as efficient to the epithelial cells as strain 29 and 30 with up to 55 bacteria per cell at 3 h of infection (Fig. 4). This switch was not observed when the bacteria were grown on Todd-Hewitt based agar plates (data not shown), suggesting that the transition in phenotype was caused by a change in growth phase rather than the composition of the medium. Together, these results strongly suggest that *ORT* strains carry different, cell type specific adhesins.

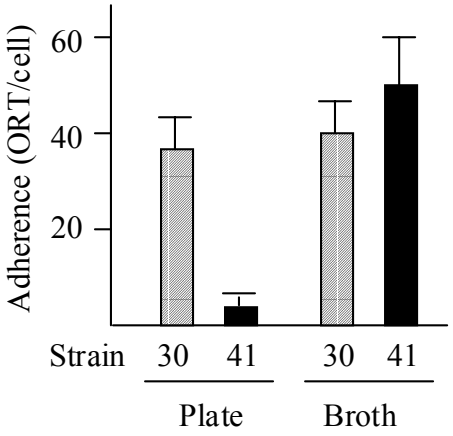

Fig. 4: Differential adherence of *ORT* strain 41.
Chang epithelial cells were inoculated with *ORT* strains 30 (hatched bars) or 41 (solid bars) derived from solid-phase (Plate) or liquid media (Broth). After 3 h of incubation, the infection was stopped. Data represent the average number of adherent *ORT* per cell. Note that the adherence properties of strain 41 but not of strain 30, varied with the growth media.

Comparison of outer membrane profiles of *ORT*

In order to investigate the difference in adherence properties of strain 41 compared to the other strains, we extracted outer membranes of the strains 29, 30 and 41 and compared their protein profiles by SDS-PAGE. As depicted in Fig. 5A virtually identical migration patterns were obtained for outer membrane proteins of strains 29 and 30. For strain 41 a different protein profile was observed particularly in the low molecular range (20-25 kDa).

To examine the lipopolysaccharide (LPS) characteristics of the strains, LPS was purified from strain 29, 30 and 41 with the hot-phenol method. Analysis of the purified LPS with tricine SDS-PAGE revealed unique LPS profiles for all strains, including the serotype D strains 29 and 30. The size and migration of the LPS bands suggest that the LPS of *ORT* consists of a heterogeneous set of oligosaccharide molecules with a molecular mass between 3 and 10 kDa (Fig. 5B). The absence of a ladder-like migration of LPS bands in the higher molecular weight range suggests that, at least under the conditions employed, the assembly of the LPS molecules into a smooth from of LPS built up of large repetitive oligosaccharide units, does not occur in this species.

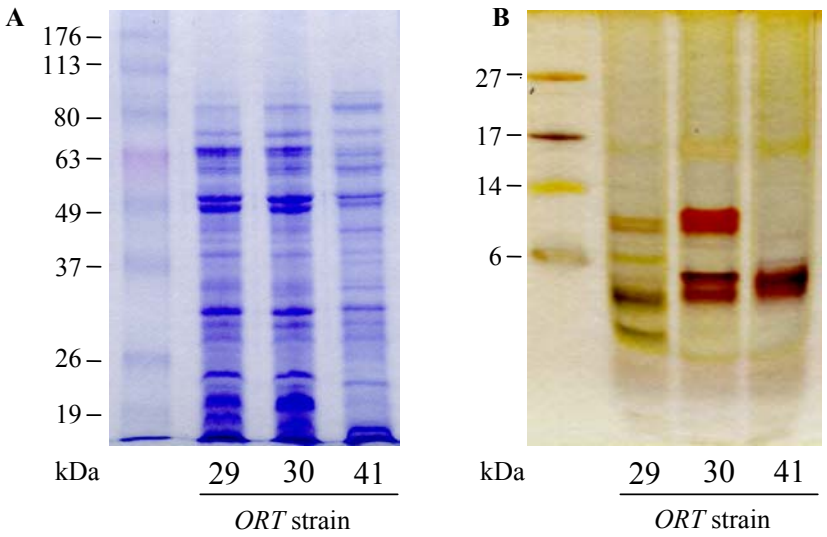

Fig. 5: Outer membrane profiles of different *ORT* strains.
(A) - SDS-PAGE profile of outer membranes proteins (OMP) isolated from *ORT* strains 29, 30 and 41. Proteins are visualized with Coommassie Brillant Blue. **(B)** - Tricine-SDS-PAGE profile of LPS purified from *ORT* strains 29, 30 and 41 as visualized with silver staining. Note the heterogeneity in LPS bands among the *ORT* strains.

Strain-specific and cell-type specific inhibition of *ORT* adherence by LPS

In an attempt to relate observed differences in outer membrane composition with the different adherence properties of the *ORT* strains, we tested the effect of purified LPS on the *ORT* adherence to the cultured epithelial cells. Infection of Chang epithelial cells with the *ORT* strains 29 and 30 in the absence and presence of various concentrations (1-100 µg/ml) of endogenous purified LPS showed that the LPS of strain 29 inhibited bacterial adherence of strain 29 up to 90% (Fig. 6). A similar effect

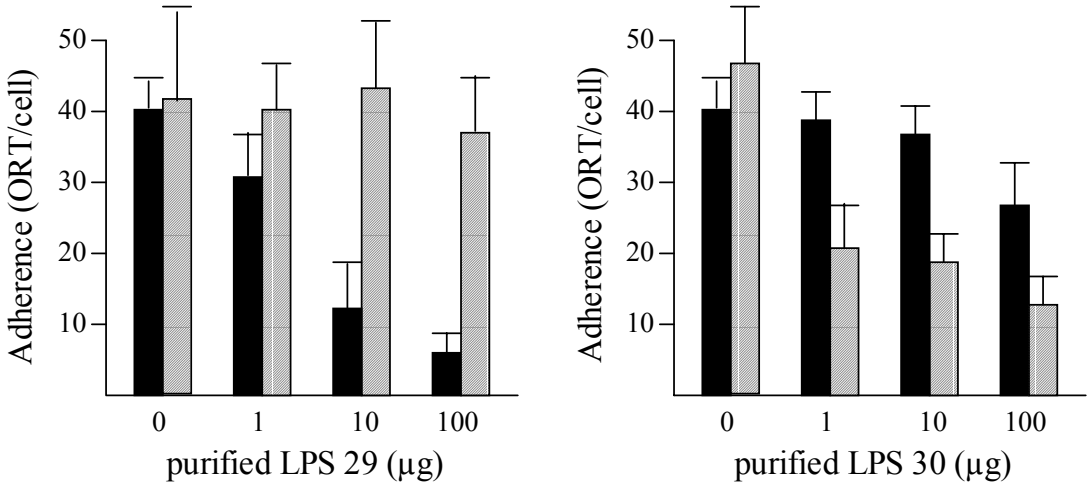

Fig. 6: Strain specific inhibition of *ORT* adherence by LPS.
Chang epithelial cells were infected with *ORT* strain 29 (solid bars) or strain 30 (hatched bars) in the absence and presence of the indicated amount of purified LPS derived from strain 29 (left panel) or strain 30 (right panel). Bacterial adherence was determined at 3 h of infection and indicated as average number of *ORT* per cell. Data are the mean ± SEM of three experiments. The inhibition of the bacterial adherence by LPS was statistically significant ($P < 0.01$) at concentrations ≥ 10 µg and ≥ 1 µg of LPS of strain 29 and 30, respectively.

was obtained for strain 30 when LPS of strain was added (Fig. 6). However, when heterologous LPS instead of homologous LPS was added to the infection assay, no inhibition of bacterial adherence was observed (Fig. 6). Thus, the LPS from strain 29 inhibited the adherence of strain 29 but not that of strain 30, while the LPS of strain 30 effectively blocked the adherence of strain 30 but not of strain 29.

To further ascertain the observed cell-type specificity in *ORT* adherence, the effect of purified homologous and heterologous purified LPS on the haemagglutination facilitated by *ORT* strain 41 was investigated. No haemagglutination inhibitory effect of the purified LPS was observed (not shown). These data strengthen the concept that the observed inhibition of the *ORT* adherence by LPS is a specific event and that *ORT* adherence to different cell types is conferred by strain-specific adhesins and/or host cell receptors.

DISCUSSION

Development of novel infection intervention and prevention strategies for the bacterial pathogen *O. rhinotracheale* requires more knowledge of the critical steps in the establishment of infection. Here we characterized the initial steps of the bacteria-host interaction, notably the adherence of *ORT* to eukaryotic cells. Our data indicate that *ORT* exhibits adhesive properties towards several types of epithelial cells as well as erythrocytes. The kinetics of the bacterial adhesion and the observed differential adhesion of different *ORT* strains to epithelial cells and erythrocytes, indicate that the binding is a specific and conferred by cell type-specific adhesions. The remarkable *ORT* strain-specific inhibition of the bacterial adhesion by purified LPS indicates that bacterial and/or host LPS receptors play a crucial role in the establishment of cellular *ORT* infection.

The first indication that *ORT* exhibits adhesive properties was obtained from the tight attachment of the bacteria to the epithelial cells in our *in vitro* infection model system. The time course, specificity and saturability of the adhesion process suggested that the binding was a specific event. This was further strengthened by the apparent lack of adhesion of strain 41 under conditions that allowed strains 7, 29 and 30 to strongly adhere to the host cells. The reason for the poor adherence of strain 41 is unknown but merits further investigation. Strain 41 acquired the ability to adhere to the epithelial cells when grown in Todd-Hewitt liquid medium instead of on Todd Hewitt-based agar plates despite its apparent normal growth on the agar plates compared to the adherent strains. The change in phenotype suggests that the strain has the intrinsic ability to adhere to epithelial cells but that this capacity is apparently influenced by the environmental conditions. It is currently unclear whether the transition of the strain from agar plates to liquid medium (and thus from a non-adherent to adherent phenotype) is accompanied by an unmasking of surface adhesins e.g. by a loss of a possible capsule or other surface molecules, or by altered expression of surface adhesins. A switching between adherent and non-adherent phenotypes however, has been observed before and is to be considered of major importance for other pathogens as it may enable bacteria to both adhere and spread to other niches with changing environmental conditions (Hertzig *et al.*, 2003).

Strong evidence for the existence of different cell-type specific adhesins on *ORT* was obtained from comparison of the *ORT* adherence to epithelial cells and erythrocytes. The apparent absence of (surface) haemagglutinins in strain 29 and 30 but their efficient adherence to epithelial cells clearly indicates the bacterial adhesins are host cell specific. Furthermore, the efficient haemagglutination of chicken erythrocytes by a phenotype of strain 41 that showed poor adherence to epithelial cells indicates the existence of multiple adhesins with different host cell specificity. Characterization of the outer membrane protein profiles of the strains 29, 30 and 41 revealed several differences between strain 41 and the strain 29 and 30. Whether these differences contribute to the different adherence properties of the strains remains to be investigated and awaits establishment of a system to genetically manipulate *ORT*. A similar point that remains to be addressed is the host specificity of the adhesins. While the haemagglutination assays were carried out with chicken erythrocytes, epithelial cell adherence was determined with a human epithelial cell infection model system. Thus, it is possible that additional avian specific adhesins will be identified when a suitable chicken epithelium model system has been established.

A striking finding from our work was the apparent heterogeneity in LPS molecules produced by the different strains. With the presence of relatively small molecular mass of the LPS molecules

(between 3 and 10 kDa) and the finding that a single strain appeared to produce multiple LPS molecules, the LPS of *ORT* mimics that of many other mucosal non-intestinal pathogens such as *Haemophilus influenzae* and *Neisseria* species. In these species, multiple lipooligosaccharides (LOS) molecules are produced simultaneously often reflecting natural variation in LOS biosynthesis (van Putten and Robertson, 1995). Whether the LPS of *ORT* is subject to variation is unknown and awaits the development of specific antibodies. The dissimilarity of LPS bands of strains 29 and 30 which both belong to *ORT* serotype D, suggests that the serotype classification of *ORT* strains is probably not based on differences in LPS.

The apparent unique LPS profile of the different *ORT* strains tested is consistent with observed apparent LPS sensitivity of the *ORT* adhesion to epithelial cells. In our infection experiments, purified LPS inhibited bacterial adhesion in a strain-specific fashion i.e. infection inhibition was only observed for the strain from which the LPS was purified. The finding that the adherence of strain 29 to the host cells was inhibited by LPS of strain 29 but not of strain 30, while the adherence of strain 30 was blocked by the LPS of strain 30 but not of strains 29 or 41 indicates that the inhibition was unlikely caused by impurity of the LPS. On the contrary, the data point to LPS-type specific mechanism of interaction between *ORT* and the host cells. At this point it would be of interest to investigate the repertoire of LPS molecules produced by *ORT* to gain more information about the extent of variation in bacterium-host cell interactions that has evolved in this species.

The mechanism via which LPS exerts its strain-specific infection inhibitory effect remains to be resolved. The data suggest that LPS-specific receptors on the bacteria and/or host cell are involved in the adherence process. It can be imagined that LPS acts as a receptor analog that binds to the bacterial adhesin thus preventing it to bind to the host cell receptors. In this scenario, the host cell receptors recognized by *ORT* should exhibit LPS mimicry. Candidate receptors with such properties would be host cell glycolipids. Host cell gangliosides have been implicated as receptors for other bacterial pathogens (Bitzan *et al.*, 1998). Whether LPS of *ORT* exhibits mimicry with host cell glycolipids awaits structural analysis of the molecule. An alternative explanation for the LPS inhibition of *ORT* infection is that the purified LPS inhibits bacterial adherence by acting as an analog of the *ORT* adhesin and thus occupies host cell receptors that are otherwise exploited by the bacterium. In this case, LPS itself would likely be the bacterial adhesion conferring molecule. Further analysis of the molecular basis and biological activity of the inhibitory effect may provide insight into the potential value of (parts of) this molecule in future infection intervention and/or prevention.

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CHAPTER 3

Interaction of the Avian Pathogen *Ornithobacterium rhinotracheale* with Chicken and Murine Macrophages

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Submitted for publication

ABSTRACT

The emerging bacterial pathogen *Ornithobacterium rhinotracheale* (*ORT*) causes respiratory infections in birds and is a major problem in the poultry industry. Pathology suggests that the bacteria infect macrophages and elicit an inflammatory response. To better understand the *ORT*-macrophage interaction, we infected HD-11 chicken macrophages with *ORT* strain 30. Microscopy showed a dose-dependent, non-opsonic adherence and uptake of *ORT* by the macrophages. Measurement of nitric oxide production as an inflammatory marker demonstrated that viable *ORT* stimulated NO production albeit much less than *E. coli* or *S.* Enteritidis. Priming of the macrophages with recombinant chicken gamma-interferon (rChIFNγ) slightly enhanced the NO production. The lipid A neutralizing agent polymyxin B did not inhibit NO production stimulated by *ORT*. This compound did effectively block the stimulation by the enteric bacteria. Addition of LPS purified from *ORT* to the HD-11 cells stimulated NO production in rChIFNγ primed HD-11 cells in a polymyxin B-sensitive fashion, suggesting that, in purified form, LPS of *ORT* exhibits pro-inflammatory activity. Infection experiments with rMoIFNγ stimulated murine macrophages (J774A.1 cells) showed that both viable *ORT* and purified LPS did not stimulate NO production. Together, the results indicate that *ORT* is rapidly ingested by macrophages and elicits an inflammatory response in chicken macrophages via a stimulus other than LPS. LPS of *ORT* is only bioactive in purified form. The observed differential responsiveness of chicken and mouse cells may contribute to the host specificity of the infection.

INTRODUCTION

In recent years, the Gram-negative bacterium *Ornithobacterium rhinotracheale* (*ORT*) has emerged as an important bacterial pathogen in turkeys and chickens (Sprenger *et al.*, 2000a; 2000b; van Veen *et al.*, 2000). Contact with this bacterium often results in clinical signs varying from mild symptoms of respiratory tract infection to systemic disease with arthritis, growth retardation and considerable mortality (van Empel and Hafez, 1999; van Veen *et al.*, 2000). Pathological examination of infected tissues at 2 days of *ORT* exposure showed infiltration of the respiratory epithelial mucosa with macrophages and polymononuclear leukocytes. At a later stage, granulomatous tissue was observed and a further accumulation of macrophages was seen at the site of infection (DeRosa *et al.*, 1996: van Empel *et al.*, 1999). The inflammatory response and the predominant association of *ORT* with macrophages (van Empel *et al.*, 1999) suggest that this cell type plays a pivotal role in the pathogenesis and/or outcome of *ORT* disease.

The molecular mechanisms via which *ORT* attracts and/or possibly evades killing by professional phagocytes have not been investigated. Chicken macrophages have been reported to be capable of killing both extra- and intracellular bacteria via production of nitric oxide (NO) (Karaca *et al.*, 1995; Lin *et al.*, 1996; Qureshi *et al.*, 1994; Sung and Dietert, 1994; Taylor *et al.*, 1992). This compound is a free radical generated from L-arginine by the inducible enzyme NO synthase (iNOS). NO is a potent inflammatory mediator that has anti-bacterial activity by generating highly reactive compounds via oxidation to nitrite and nitrate and by binding to bacterial metallo-enzymes (Lancaster, 1992; Moncade, 1991). Maximal iNOS expression typically requires the presence of specific combinations of lipopolysaccharide (LPS) and/or cytokines (Dil and Qureshi, 2002; Geller *et al.*, 1993; Kunz *et al.*, 1994; Lin *et al*., 1996; Tsujino *et al.*, 1994; Weisz, 1994). Known stimuli that induce iNOS in chicken macrophages are various immunological stimuli such as LPS and IFNγ and tumor necrosis factor-α (Karaca *et al.*, 1995; Lin *et al.*, 1996; Qureshi *et al.*, 1994; Sung and Dietert, 1994; Taylor *et al.*, 1992), although the response may vary with the genetic background of the chicken (Hussain and Qureshi, 1997; Kogut *et al.*, 2001; Qureshi *et al.*, 1994; Sekellick *et al.*, 1998). Once induced, iNOS remains active for sustained periods and produces large amounts of NO. Whether *ORT* induces or inhibits the production and/or resists the toxic activity of NO has not been investigated.

The present study was designed to unravel the cellular interaction between *ORT* and chicken macrophages. Using the chicken macrophage HD-11 cell line (Beug *et al.*, 1979) as a model system, the bacterial adherence and ingestion of *ORT* by macrophages was investigated. The development of an inflammatory response was assessed using NO production as marker. Results with *ORT* were compared with those obtained for the enteric pathogens *Escherichia coli* (*E. coli*) and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis). The host specificity of the effects was evaluated by comparing the interaction of *ORT* with chicken and murine macrophages.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

O. rhinotracheale strains 29, 30 and 41 were isolated from turkeys and kindly provided by Dr. P. van Empel (Intervet B.V., The Netherlands). Strains were grown on Columbia agar (Oxoid, Hampshire, England) at 37°C for 48 h in a humidified atmosphere of 5% CO_2 in air or in 45 ml of Todd-Hewitt (TH) broth (Oxoid, Hampshire, England) in 50 ml polypropylene tubes at 37°C for 48 h in air on an orbital shaker (150 rpm), unless indicated otherwise. *E. coli* strain DH5α and *S. enterica* serovar Enteritidis (*S.* Enteritidis) strain 706 were grown on LB agar plates or in LB broth.

Cell culture

The chicken macrophage cell line HD-11, generated by transformation of chicken hematopoietic cells with myelocytomatosis virus (MC-29) (Beug *et al.*, 1979), and the murine (Balb/C) macrophage cell line J774A.1 (ATCC TIB-67) (Ralph and Nakoinz, 1975) were grown in RPMI 1640 supplemented with 5% fetal bovine serum. For use in infection experiments, cells were seeded onto 12

mm circular glass coverslips in 24-well tissue culture plates (Costar, New York, USA) and grown for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

Infection experiments

Cellular infection was started by adding various numbers ($5x10^6$ - $5x10^9$) of bacteria suspended in Dulbecco's phosphate buffered saline (DPBS) to cultured macrophages ($\approx 10^5$ cells/well) maintained in 1 ml of serum-free RPMI. After various times of incubation (37°C, 5% CO_2), the infection was stopped by rinsing the cells three times with 1 ml of DPBS followed by fixation in 1 ml of 2% paraformaldehyde in DPBS for at least 1 h. The cells were stained with 0.002% crystal violet and viewed in an Olympus BH-2 microscope. At least 30 cells per well were randomly viewed to estimate the number of bacteria associated with the eukaryotic cells. Data are expressed as the mean ± SEM of at least three representative experiments. Statistical analysis was performed with the independent Student's *t*-test using SPSS 9.0 software.

Purification of lipopolysaccharide (LPS)

LPS was extracted and purified according to the hot phenol extraction procedure of Johnson and Perry (1976) with minor modifications. In brief, 2 liter cultures of bacteria grown (37°C, 150 rpm) in Todd-Hewitt broth in Erlenmeyer flasks for 48 h, were collected by centrifugation (Avanti J-20xp centrifuge, Beckman Coulter, USA) (6,800 x g, 20 min, 4°C). The bacterial yield was determined by weighing of the pellets. The bacteria were then resuspended in a volume of 10 times of bacterial wet weight of distilled water and an equal volume of hot phenol (70°C) was added. The mixture was incubated at 70°C in waterbath for 2 h with regular vortexing, cooled to 4°C, and centrifuged (18,000 x g, 20 min, 4°C). The waterphase (supernatant) was collected and two volumes of acetone (-20°C) plus 100 mg of sodium acetate per gram of bacterial wet weight were added. After another centrifugation step (18,000 x g, 30 min, -20°C), the pellet was washed in aceton (-20°C) and centrifuged again. Then the pellet was resuspended in distilled water and centrifuged at 100,000 x g (TL-100 Ultra centrifuge, Beckman) for 2 h at 4°C to remove DNA contamination. The pellet (i.e. the purified LPS) was dissolved in distilled water and stored at –20°C until use. The purity of the LPS was verified by SDS-PAGE and silverstaining (Tsai and Frasch, 1982). In the infection experiments, LPS from *Ornithobacterium rhinotrachele* (LPS-*ORT*), *Salmonella* Enteritidis (LPS-SE) or *Escherichia coli* (LPS-EC) was used at a concentration of 1 µg/ml, unless indicated otherwise.

Nitric oxide assay

Nitric oxide (NO) production in culture supernatants of infected macrophages was measured with the Griess assay (Green *et al.*, 1982). Supernatants were collected from cells grown in RPMI plus 5% FBS that had been incubated in the same medium with different concentrations of bacteria or purified LPS for various periods as indicated in results. In some experiments, 100 ng/ml of recombinant chicken gamma-interferon (rChIFN-γ, generally provided by Dr. E. Tijhaar, Utrecht University, The Netherlands) or 400 IU/ml recombinant mouse gamma-interferon (rMoIFNγ, BD Biosciences, Belgium) were added at 2 h before the addition of the bacteria or LPS. When appropriate, the lipid A neutralizing agent polymyxin B (PB, 20 or 60 µg/ml, Sigma) was added. The concentration of NO was determined by mixing 250 µl of culture supernatant (or dilutions of it) with an equal volume of freshly prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% phosphoric acid). After 10 min of incubation at 20°C, use of substrate was measured in a spectrophotometer at 550 nm. The concentration of nitrite in the medium was calculated from a standard curve generated by mixing 0 to 100 µM of sodium nitrite with Griess reagent. All experiments were done in triplicate. Data are presented as the mean ± SEM of at least three experiments and statistically analyzed with the independent Student's *t*-test with SPSS 9.0 software.

RESULTS

Adherence and uptake of *ORT* by chicken macrophages

The primary interaction between *ORT* and chicken macrophages was studied using the HD-11 cell line as a model system. Infection of HD-11 cells with *ORT* strains 29 and 30 (serotype D) for various periods showed that *ORT* adhered to and was ingested by the phagocytic cells. Microscopy on the infected specimen revealed about 50 cell-associated bacteria at 3 h of infection. These bacteria seemed to be located in large vacuoles inside the cells (Fig. 1A). Comparison of the effect of the inoculum size on the interaction indicated that the bacterial adherence and uptake steadily increased from 6 to 70 bacteria per cell with inoculum sizes of $5x10^6$ and $5x10^9$, respectively (Fig. 1B).

Infection experiments with *ORT* strain 41 (serotype E) yielded similar results but only when the inoculum was prepared from bacteria grown in Todd-Hewitt broth prior to infection. *ORT* strain 41 grown on solid phase media adhered much less to the HD-11 cells ($p<0.05$, Fig. 1C).

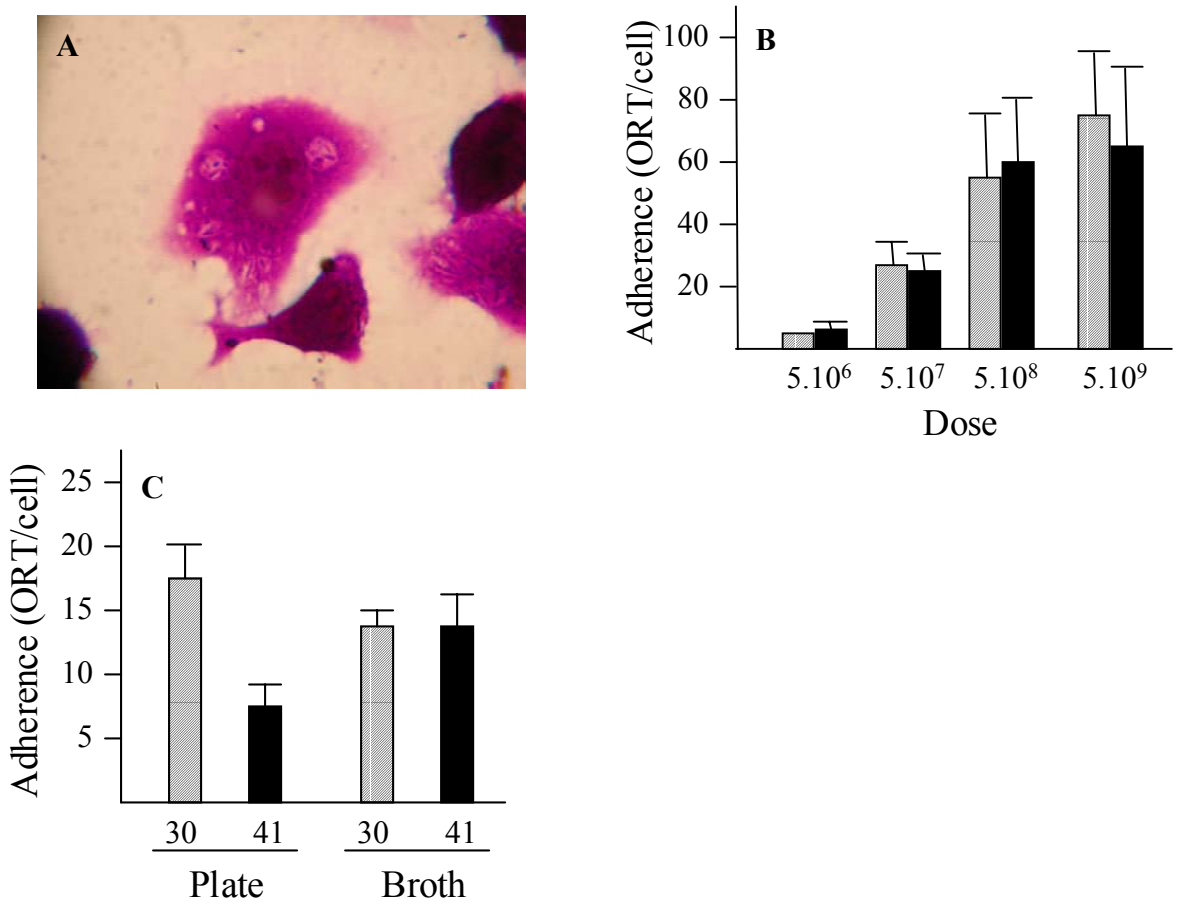

Fig. 1: Kinetics and characteristics of *ORT* adherence to macrophages.
(A) Micrograph of *ORT* infected macrophages (1000x magnification). Note the intracellular vacuoles containing *ORT*. **(B)** Effect of inoculum size on *ORT* adherence to macrophages. Chicken HD-11 macrophages (hatched bars) and murine J774A.1 macrophages (solid bars) were infected with the indicated doses of *ORT* strain 30. Infection was stopped at 3 h of infection. Data represent the average number (± SEM) of adherent *ORT* per cell of three experiments. **(C)** Effect of bacterial growth media on the *ORT*-macrophage interaction. HD-11 macrophages were infected with *ORT* strains 30 and 41 derived from solid phase (Plate) and liquid media (Broth). After 3 h of incubation, the infection was stopped and the average number of *ORT* per macrophage was determined. Data are the mean ± SEM of three experiments.

Inhibition of *ORT* infection of macrophages by LPS

The adhesion characteristics of the various *ORT* strains towards the HD-11 cells closely resembled those previously reported for the *ORT*-epithelial cell interaction (Chapter 2). In order to assess whether the adhesion to both cell type was conferred by the same mechanism, the effect of LPS on the cellular infection was tested as this molecule inhibits the *ORT* adhesion to epithelial cells in a strain-specific fashion. Infection assays with HD-11 cells and *ORT* strain 30 in the absence and presence of purified LPS derived from this strain or from strain 29 yielded no inhibition of adhesion (Fig. 2). Similar results were obtained for strain 29 (data not shown). These results strongly suggest that the *ORT* adhesion to macrophages and epithelial cells proceeds via different mechanisms.

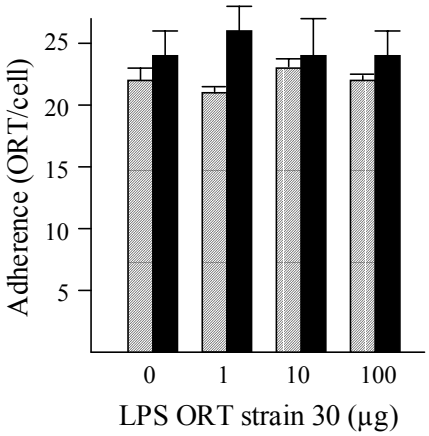

Fig. 2: Effect of purified LPS on the *ORT* adherence to macrophages.
HD-11 macrophages were infected with *ORT* strain 29 (solid bars) or strain 30 (hatched bars) in the absence and presence of the indicated amounts of purified LPS derived from strain 30. Bacterial adherence was determined at 3 h of infection and indicated as the average number of *ORT* per cell. Data are the mean ± SEM of three experiments. Note that purified LPS did not inhibit the *ORT* adherence.

Stimulation of NO production by *ORT*

Interaction of macrophages with bacterial pathogens often results in activation of the host defense including the production of the bactericidal molecule nitric oxide. To investigate the response of the HD-11 cells to *ORT* infection, the concentration of NO in the tissue culture supernatants was measured during a 24 h infection period. As shown in Fig. 3, *ORT* infection stimulated NO production. The concentration of NO steadily increased during the 24 h of infection and reached on average a concentration of 70.3 μM and 81.0 μM of NO per 10^5 cells for strains 30 and 41, respectively. Maximal NO levels were already obtained when $5x10^5$ bacteria were used as an inoculum. Similar results were obtained for strain 29 (data not shown). With inocula of 5 x 10^7 cfu/ml, a poor response was measured for strains 29 and 30, probably because of the detachment of cells that occurred at prolonged infection at this high inoculum. Infection with strain 41 did not cause cell damage and yielded high levels of NO production even after 24 h of infection with $5x10^7$ cfu/ml (Fig. 3).

As the production of the enzyme nitric oxide synthase (iNOS) is enhanced in the presence of gamma-interferon (IFNγ), we also measured NO production in HD-11 cells primed with recombinant chicken IFNγ (rChIFNγ, 100 ng/ml). Priming with rChIFNγ enhanced the *ORT* induced NO production by about 50-90% for all *ORT* strains tested (Fig. 4A). The specificity of the effect was demonstrated by the ineffectiveness of recombinant murine IFNγ (rMoIFNγ, 400 IU/ml) to enhance the NO response (data not shown).

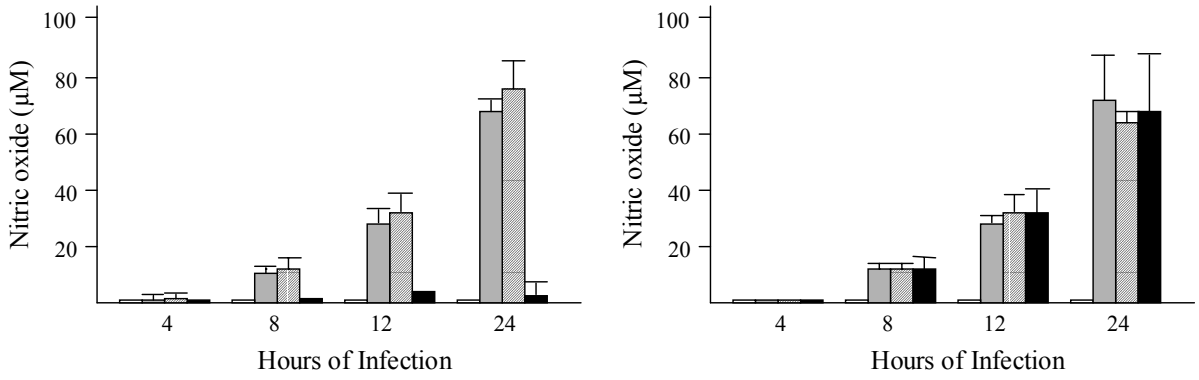

Fig. 3: Kinetics and characteristics of *ORT*-induced nitric oxide production.
HD-11 macrophages were infected with various amounts of *ORT* strain 30 (left panel) and strain 41 (right panel). After 4, 8, 12 and 24 hours of incubation, the infection was stopped and the number of macrophage-associated bacteria was determined. Data represent the mean number of *ORT* per cell. Inocula used were: 5 x 10^5 (grey bars), 5x10^6 (hatched bars) and 5x10^7 (solid bars). The amount of NO detected in the supernatant of uninfected cells served as control (open bars). Results are the mean ± SEM of three independent experiments.

Comparison of NO production induced by *ORT*, *E. coli* and *S.* Enteritidis in resting and IFNγ activated HD-11 cells

In order to be able to interpret the significance of the *ORT*-induced NO production in HD-11 cells, we compared the effect with that of the enteric bacteria *E. coli* and *S.* Enteritidis. Infection of HD-11 cells with these bacteria caused much more rapid NO production compared to *ORT* (Fig. 4B). For *E. coli*, NO was already detected at one h of infection, while at 2 h an average concentration of 80 µM of NO was measured in the medium. For *S.* Enteritidis a slightly delayed response was measured of 60 µM of NO at 3 h of infection (Fig. 4B). At this time, *ORT* did not induce a detectable response yet (Fig. 4A). When the infection experiments and NO assays were repeated after priming of the HD-11 cells with rChIFNγ, similar NO concentrations were measured in supernatants of *E. coli* and *S.* Enteritidis as for the absence of the cytokine (Fig. 4B), indicating that the NO response induced by these species is not enhanced by rChIFNγ in contrast to the response to *ORT* (Fig. 4).

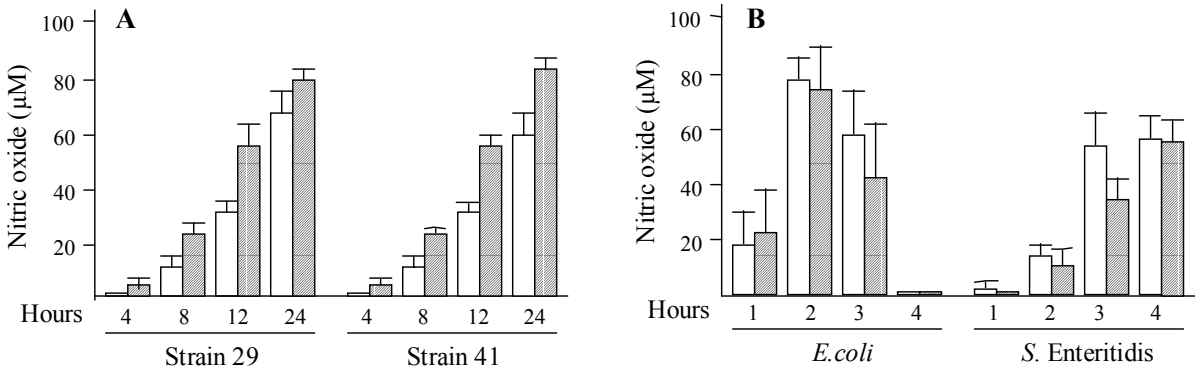

Fig. 4: Effect of IFNγ on bacteria-induced NO production.
(A) HD-11 cells were infected with 5x10^6 cfu/ml of *ORT* strains 29 and 41 and **(B)** *E. coli* and *S.* Enteritidis, in the absence (open bars) and presence (solid bars) of rChIFNγ. Infection was stopped at the indicated times and the concentration of NO present in the culture supernatants was determined. Data represent the mean concentrations ±SEM of three independent experiments. Note the difference in kinetics and IFN dependence of the NO production induced by *ORT* strains (Panel A) and the other species (Panel B).

Inhibition of *E. coli* and *S.* Enteritidis but not *ORT* induced NO production by polymyxin B

One of the primary stimuli of Gram-negative bacterial pathogens that trigger the host defense is lipopolysaccharide (LPS). In search for the nature of the bacterial stimulus that stimulated NO production, we tested the inhibitory effect of polymyxin B on the *ORT*, *E. coli* and *S.* Enteritidis induced NO production in HD-11 cells. Polymyxin B has been demonstrated to inhibit the biological activity of LPS through binding to the lipid A moiety of the molecule (Lasfargues *et al.*, 1989). Addition of polymyxin B to the HD-11 cells prior to infection completely prevented the induction of NO production by both *E. coli* and *S.* Enteritidis during the first 3 h of infection (Fig. 5A). In contrast, no inhibitory effect of polymyxin B on the *ORT* stimulation of NO production was observed (Fig. 5B). These results suggest that *E. coli* and *S.* Enteritidis stimulate NO production mainly via their LPS, while the *ORT* response is induced by a different stimulus.

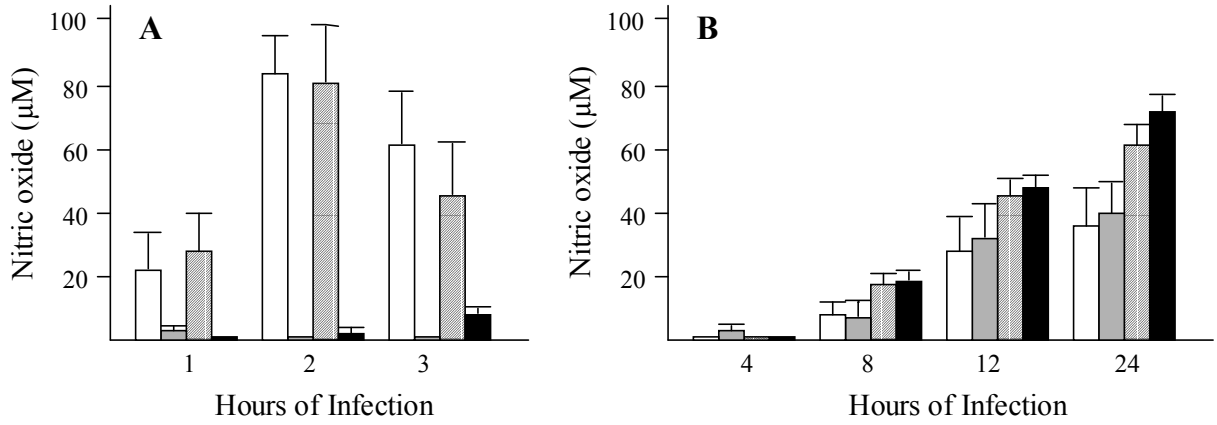

Fig. 5: Inter-species variability of the induced NO production by polymyxin B.
HD-11 cells were infected with **(A)** *E. coli* ($5x10^6$ cfu/ml) and **(B)** *ORT* strain 30 ($5x10^6$ cfu/ml) in the absence (open bars) and presence of rChIFNγ (hatched bars), polymyxin B (grey bars), or rChIFNγ plus polymyxin B (solid bars). At the indicated times, the infection was stopped and the concentrations of NO in the culture supernatants were determined. Values are the mean ± SEM of three assays. Note that NO production induced by the enteric bacteria is not enhanced by rChIFNγ but is blocked by polymyxin B, while the NO production induced by *ORT* is enhanced by rChIFNγ and not polymyxin B-sensitive.

Comparison of the bioactivity of purified LPS of *ORT*, *E. coli* and *S.* Enteritidis

To further investigate the apparent LPS-based difference in responsiveness of HD-11 cells towards the enteric bacteria and *ORT*, we tested the effect of purified LPS of these bacteria for its ability to stimulate NO production. For this purpose, LPS was purified by the hot-phenol method (Johnson and Perry, 1976). Addition of purified LPS of *E. coli* to the HD-11 cells elicited a strong NO response after 16 h of exposure, but only when the cells had been primed with rChIFNγ (Fig. 6). The response was prevented in the presence of polymyxin B demonstrating that it was mediated by lipid A (Fig. 6). For *S.* Enteritidis LPS, similar results were obtained, although in this case a moderate response was already observed in the absence of rChIFNγ (Fig. 6). Remarkably, the purified LPS derived from *ORT* strain 30 also stimulated NO production. This response was enhanced with cells that has been primed with rChIFNγ and was largely prevented by the addition of polymyxin B (Fig. 6). Similar results were obtained with LPS derived from *ORT* strains 29 and 41. These results suggest that LPS of *ORT* has the intrinsic ability to stimulate the NO response but is not biologically active in the context of the intact bacterium.

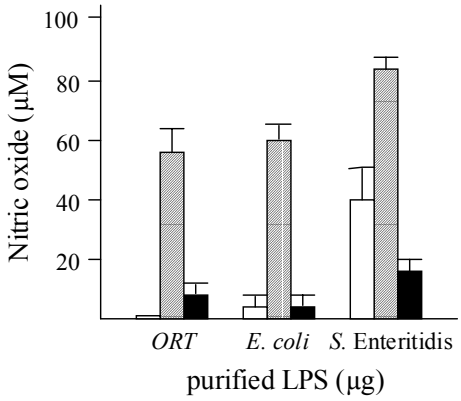

Fig. 6: Differential stimulation of NO production by LPS of different species.
LPS purified from *ORT* strain 30, *E. coli* and *S.* Enteritidis was added to HD-11 cells in the absence (open bars) and presence of rChIFNγ (hatched bars) or rChIFNγ plus polymyxin B (solid bars). After 24 h of incubation was maximum NO production levels were achieved, concentrations of NO in the culture supernatants were determined. Data are the mean ± SEM of three experiments. Note that, once purified, the LPS of all species (including *ORT*) stimulates NO production in the presence of rChIFNγ in a polymyxin B-sensitive fashion.

Differential effects of *ORT* and enteric pathogens on NO production in murine macrophages

The above results indicate that *ORT* stimulates NO production in HD-11 cells via a different stimulus than LPS. To further investigate the host specificity of this event, we assessed the ability of *ORT* to stimulate NO production in murine macrophages. Infection of J774A.1 cultured murine macrophages cells with *ORT* strains 30 and 41 resulted in adherence to and uptake of the bacteria by the eukaryotic cells (Fig. 2). Moreover, the kinetics of infection and optimal inoculum size resembled those determined for the chicken HD-11 cells. Measurement of NO production showed that *ORT* strain 30 did not induce any NO production during the 24 h infection period, irrespective of priming with 400 IU/ml of rMoIFNγ (Fig. 7). Similar results were obtained for strain 29 and 41 (data not shown). On the other hand, *E. coli* and *S.* Enteritidis were able to stimulate NO production with similar kinetics as determined for HD-11 cells and in a polymyxin-sensitive fashion (Fig. 7). The absence of an *ORT* response in the murine macrophages may indicate that the stimulus that induces the NO production in this cell type is species-specific.

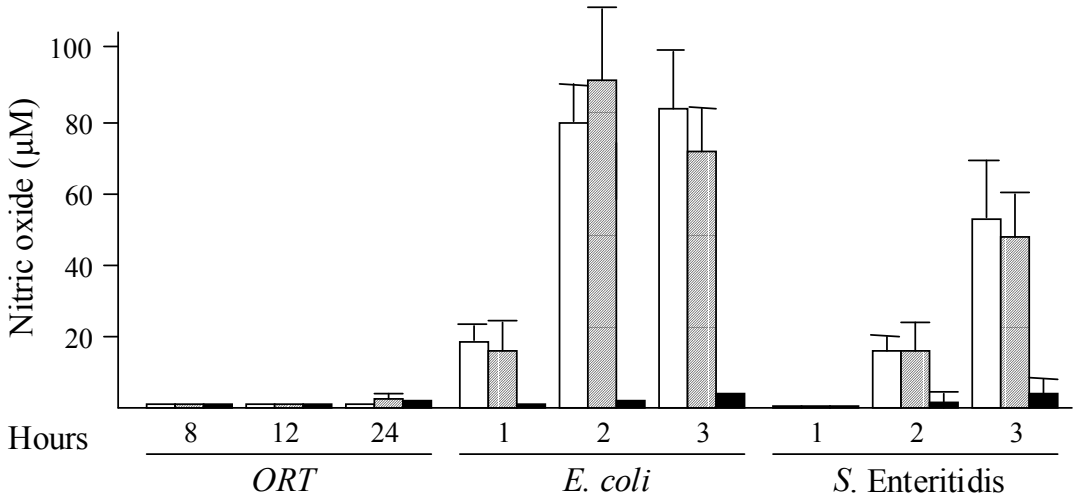

Fig. 7: Resistance of murine macrophages against *ORT*-stimulation of NO production.
Murine J774A.1 macrophages were infected with *ORT* strain 30, *E. coli* and *S.* Enteritidis ($5x10^6$ cfu/ml) in the absence (open bars) and presence of rMoIFNγ (hatched bars), and rMoIFNγ plus polymyxin B (solid bars). At the indicated times, the concentrations of NO in the culture supernatants were determined. Values are the mean ± SEM of three experiments. Note that unresponsiveness of the murine macrophages towards *ORT* compared to *E. coli* and *S.* Enteritidis.

DISCUSSION

Pathology studies suggest that macrophages play a dominant role in the establishment, pathology and/or persistence of *ORT* infections (van Empel *et al.*, 1999). From the present *in vitro* study we conclude that *ORT* adheres to and becomes ingested by both chicken HD-11 and murine J774A.1 macrophages. The bacterial components that contribute to the infection of macrophages remain to be defined, although they appear to be different than those conferring *ORT* adhesion to epithelial cells. In the chicken but not murine cells, the infection is accompanied by a stimulation of NO production via a rather slow LPS- and IFNγ-independent mechanism. In contrast, infection of the cells with *E. coli* and *S.* Enteritidis yields a much more rapid increase in NO production via an LPS-dependent mechanism. This stimulation was observed for both the chicken and murine macrophages. Comparison of the biological activities of LPS purified from *ORT*, *E. coli* and *S.* Enteritidis indicate that, in contrast to intact *ORT*, purified LPS of *ORT* is biological active and equally potent as *E. coli* and *S.* Enteritidis LPS. Overall, the data indicate that *ORT* infects chicken macrophages and stimulates NO production but via a different mechanism than *E. coli* and *S.* Enteritidis.

The finding that *ORT* adheres to and is internalized by chicken macrophages is consistent with the tight association of the bacteria observed during *in vivo* infection (van Empel *et al.*, 1999). Thus far, *ORT* has been isolated from domestic and wild birds suggesting that it has specificity for the avian host. Our data indicate that, at least under the conditions employed, *ORT* can infect murine macrophages. This suggests that if a host barrier exists for *ORT* and our data reflect the *in vivo* situation, this barrier is not located at the level of the macrophages. Alternatively, is can be imagined that *ORT* is able to colonize non-avian species but that this has not been noted because of the absence of associated pathology and/or unsuccessful isolation. The latter possibility is a serious option as our data suggest that *ORT* stimulates NO production in chicken cells only and isolation of *ORT* from colonized healthy animals is known to be difficult.

The observed stimulation of NO production by *ORT* may contribute to the pathology observed during the natural infection. At first sight, the apparent specificity of the stimulation for chicken cells may appear not as a surprise as different types of macrophages have been reported to vary in sensitivity towards with regard to NO stimulation (Bermudez, 1993; Chesrown, 1994; Denis, 1994; Nathan and Hibbs, 1991; Schneemann *et al*., 1993; Yan *et al*., 1993). However, the finding that *E. coli* and *S.* Enteritidis but not *ORT* induced a potent NO response in the murine macrophage cell line suggest that the cause of the lack of response in this cell type is located in the bacteria rather than macrophages. The finding that bacteria can stimulate NO production in the absence of IFNγ has been previously reported (Crippen *et al*., 2003) and indicates that cytokine induction of iNOS expression is not required for HD-11 cells. The increased response in the presence of rChIFNγ suggests that the cells still do respond to the cytokine and indicate that the effects of *ORT* on the host cells may vary with the presence of IFNγ at the site of infection.

The observed apparent different kinetics and mechanisms of the stimulation of NO production by *ORT*, *E. coli* and *S.* Enteritidis point to the existence of fundamental differences between the Gram-negative species. The differential polymyxin B sensitivity and increase in NO production observed for the various species indicate a different architecture and/or release of the LPS from the microorganisms. The ability of *E. coli* and *S.* Enteritidis to rapidly stimulate NO production via lipid A indicates that this molecule is presented in a biologically active form to the macrophages early in the infection. This does not hold for *ORT* as the NO response induced by *ORT* is not susceptible to inhibition by polymyxin B, suggesting that it is not mediated via lipid A. Further evidence that *ORT* exploits a different mechanism of macrophage activation is the apparent specificity of the effect for chicken macrophages which was not noted for *E. coli* and *S.* Enteritidis. The apparent absence of the LPS stimulus and the relative slow increase in NO production by *ORT* compared to *E. coli* and *S.* Enteritidis may at least partially explain why *ORT* seems to cause a comparatively moderate inflammatory reaction during the natural infection.

The natures of the *ORT* stimuli that drive the stimulation of NO production remain to be determined. The stimuli appear to activate chicken but not murine macrophages and thus show host specificity. The finding that *ORT* stimulates NO production in a polymyxin B-insensitive fashion without prior priming of the macrophages with rChIFNγ but that this response is enhanced in

rChIFNγ-activated macrophages, suggests either the existence of multiple *ORT* stimuli or a higher sensitivity of the cells for a distinct stimulus after IFNγ exposure. In this regard, it may be noteworthy that *ORT* pathology is generally increased following priming of the animals with e.g. NDV virus. On the basis of our results, a scenario can now be envisioned in which this increased pathology is based on a virus-induced, IFNγ-mediated activation of the macrophages that renders the cells more susceptible to stimulation by *ORT*. This may result in a more profound inflammatory response and associated pathology.

The structure of the LPS of *ORT* has thus far not been studied. We previously demonstrated that the LPS of *ORT* strains 29, 30 and 41 consist of a set of one of more oligosaccharides with molecular masses between 3 and 10 kDa when analyzed by tricine SDS-PAGE (Chansiripornchai and van Putten, unpublished results). The present data indicate no heterogeneity with regard to the biological activity of the LPS purified from these strains. Comparison of the biological activities of the purified LPS as estimated by the ability to stimulate NO production, suggests that the lipid A moiety of the *ORT* LPS is equally potent as the purified LPS of *E. coli* and *S. Enteritidis*. The significance of these observations however, appears limited as our data indicate that the biological activity of purified LPS gives no indication as to the activity of the LPS in the context of the intact bacterium and the kinetics of the stimulation of the NO production for the intact bacteria and the purified LPS are quite different.

Our data do indicate that the adhesion and bacterial uptake of *ORT* by the macrophages and the stimulation of NO production are separate phenomena as adherence was a rapid process without apparent host specificity, while NO production was detected much later and for in chicken macrophages only. A major future challenge is to identify the molecules involved in the interaction and the fate of the internalized bacteria. The latter awaits development of suitable methods as classical bacterial survival assays based on selective killing of extra- and intracellular bacteria (e.g. the gentamicin survival assay) cannot be applied for *ORT* due to its wide-spread resistance to most common antibiotics (Devriese *et al.*, 2001).

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CHAPTER 4

Differential Inhibition of *Ornithobacterium rhinotracheale* Infection of Epithelial cells and Macrophages by Serum Factors and LPS

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Submitted for publications

ABSTRACT

The avian bacterial pathogen *Ornithobacterium rhinotracheale* (*ORT*) adheres to epithelial cells and macrophages and induces of an inflammatory response. The molecular basis of these interactions is not known. Here we report that serum has *ORT* infection-inhibitory activity. Both bovine and chicken serum inhibited *ORT* attachment to cultured epithelial cells in a dose-dependent fashion, while the interaction with chicken macrophages was unaffected. Chemical and size fractionation of serum revealed that the cell-type specific infection inhibitory activity was predominantly present in the 10 - 100 kDa serum glycoprotein fraction and in the glycolipid fraction, suggesting that carbohydrates may exert the effect. Preincubation of *ORT* and host cells demonstrated that the infection-inhibition occurred via an effect on *ORT* rather than the host cells. Bindings assays with biotinylated isolated serum glycoproteins showed direct binding of several serum proteins to *ORT*. Comparison of serum and the previously identified LPS infection-inhibitory activity showed that the effect of serum was not strain specific in contrast to the effect of LPS. Differential inhibition by serum of *ORT* adherence to epithelial cells and red blood cells indicated the existence of cell-type specific *ORT* adhesins. The identified inhibitors are potentially powerful instruments to identify *ORT* adhesins and may have prophylactic potential.

INTRODUCTION

The bacterium *Ornithobacterium rhinotracheale* (*ORT*) is an emerging Gram-negative bacterium associated with respiratory tract infections in domesticated poultry (Vandamme *et al.*, 1994). The pathogen was likely introduced to poultry from wild bird populations. Once inhaled, *ORT* colonizes the respiratory tract and causes local infections that may disseminate to the joints and other body sites. The rapid spread of the infection and the associated loss of production cause major economic losses to the poultry industry. Several whole cell vaccines and a series of hygienic measures to control the pathogen have been developed but their efficacy is relatively poor. A logical approach to develop better vaccines or other forms of infection prevention is to try to identify the key players in the establishment of the infection as these may serve as suitable targets of novel infection prevention strategies.

We previously demonstrated that *ORT* adheres to several cell types and provokes an inflammatory response as indicated by the production of nitric oxide. Bacterial adherence to epithelial cells appears to involve strain specific elements as purified LPS was able to inhibit bacterial adhesion of the homologous strain but not of heterologous *ORT* strains (Chapter 2). *ORT* also adheres to and is ingested by chicken and mouse macrophages. These events are not inhibited by purified LPS, suggesting the existence of different adhesins and/or host cell receptors. Further diversity in the interaction between *ORT* strains and macrophages was observed for the stimulation of the production of the inflammatory mediator nitric oxide (NO). *ORT* stimulates NO production in chicken but not mouse macrophages suggesting the involvement of species signaling pathways. The bacterial stimulus of nitric oxide production has not been identified yet, but does not seem to be lipopolysaccharide (LPS) that in most other pathogens has a potent pro-inflammatory activity (Chapter 3).

In further unraveling of the molecular events underlying the *ORT*-host cell interaction, we tested the effect of variable environmental conditions on the establishment and course of *in vitro ORT* infection, including the effect of serum on the cellular infection. Serum has previously been demonstrated to contain a number of factors that may facilitate the establishment, alter the tissue tropism, or block the bacterial infection of eukaryotic cells (Duensing and van Putten, 1997; van Putten, 1993; van Putten *et al.*, 1997). Here we report that serum effectively inhibits *ORT* infection of epithelial cells but not macrophages. Serum fractionation and binding assays showed that a carbohydrate present on serum glycoproteins and glycolipids likely causes the inhibition. The inhibition was not strain-specific indicating that the inhibitory molecule may have therapeutic potential.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

O. rhinotracheale strains 29 and 30 (serotype D) and strain 41 (serotype E) were isolated from turkeys and generally provided by Dr. P. van Empel (Intervet B.V., The Netherlands). Bacteria were grown in Todd-Hewitt (TH) broth (Oxoid, Hampshire, England) or onto Columbia (CO) agar (Oxoid, Hampshire, England) for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell culture

Human Chang conjunctiva epithelial cells (ATCC CCL-20.2), INT 407 intestinal epithelial cells (ATCC CCL-6) and chicken HD-11 macrophages (Beug *et al.* 1979) were maintained in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 5% fetal bovine serum (FBS) (Bodinco B.V., Alkmaar, The Netherlands) and grown at 37°C in a humidified atmosphere of 5% CO_2 in air. For use in infection experiments, cells were grown onto circular (12 mm) glass coverslips placed into 24-well tissue culture plates in 1 ml of RPMI plus 5% FBS. Prior to infection, the medium was replaced by 1 ml of serum-free RPMI 1640, unless indicated otherwise.

Infection assay

Infections were started out by the addition of a suspension of $5x10^7$ viable bacteria harvested from CO agar plates to $\approx 10^5$ cultured epithelial cells in absence of presence of various concentrations of FBS, fractions thereof, chicken serum (ChS), or purified bacterial lipopolysaccharide (LPS). After 3 h of incubation, the cells were rinsed three times with 1 ml of Dulbecco's PBS (DPBS) to remove for bacteria and the infection was stopped by fixing the cells in 1 ml of 2% paraformaldehyde in DPBS for at least 1 h. After staining of the cells with 0.002% (HD-11 cells) or 0.005% (epithelial cells) of crystal violet, bacterial interactions with the host cells were viewed in an Olympus BH-2 light microscope. Bacterial adherence was determined by counting the number of adherent bacteria on at least 30 randomly picked cells. Data are presented as the average + SEM of at least three experiments and were statistically analyzed with the independent Student's *t*-test using SPSS 9.0 software.

In some infection assays, the bacteria ($1x10^8$) or eukaryotic cells ($\approx 10^5$ cells per well) were preincubated in 1 ml of RPMI containing no or 20% of serum, isolated serum fractions, or LPS for 30 min. Before start of the infection, the bacteria were collected by centrifugation and washed twice with DPBS to remove all free serum or LPS components and resuspended in 1 ml of RPMI. After preincubation of the host cells (30 min), they were rinsed three times with 1 ml of DPBS. Infection experiments with the preincubated bacteria or eukaryotic cells were carried out as described above.

Extraction of glycoproteins and glycolipids from serum

Serum glycoproteins and glycolipids were extracted from FBS as described (Varki, 2002) with minor modifications. Briefly, 300 µl of FBS was mixed with methanol and chloroform at the ratio of 3:8:4. After 10 min of incubation on ice, the mixture was centrifuged (8,800 x g, 1 min, 4°C) and the supernatant was collected. The pellet was dissolved in 1 ml of 4:8:3 volumes of chloroform/methanol/water and the extraction procedure was repeated. The obtained supernatants from both rounds of extraction were pooled. The final pellet was extracted 3 times with 1:1 chloroform/methanol, 2:1 chloroform/methanol and 100% ethanol, respectively. The supernatants from these extractions were pooled with that from 1 and 2. The final pellet representing the serum glycoprotein fraction, was resuspended in distilled water and stored in siliconized tubes (Sigma-Aldrich, Steinheim,Germany) at -80 °C. The pooled supernatants were lyophilized and stored at –20 °C until further use. Size fractionation of the isolated glycoprotein fraction was carried out with Centricon C-10 and C-100 filtration devices (Millipore, Bedford, MA, USA) according the instructions of the manufacturer.

Biotinylation of serum glycoproteins

Serum glycoproteins (SGP) were biotinylated by mixing 1 mg of SGP with 2 mg of EZ-Sulfo-NHS-LC-Biotin (Pierce, Illinois, USA) in 1 ml of PBS. After 30 min of incubation (20^{o}C), 50 mM of Tris-HCl, pH 7.4 was added to quench the free aminogroups. The mixture was loaded onto a C-10 or C-100 Centricon filter devices to remove the free biotin. The biotinylation procedure was verified by SDS-PAGE and Western blotting using horseradish peroxidase (HRP) conjugated streptavidin (Sigma Chemical, St. Louis, USA) as a conjugate.

Protein binding assay

Binding of biotinylated serum glycoproteins to the bacteria was measured by incubating $5x10^7$ bacteria with 50 µg of biotinylated SGP in siliconized tubes. After 30 min the mixture was centrifuged (12,000 x g, 5 min, 20^{o}C) and the pellet was washed several times to remove unbound glycoprotein. Then, the pellet with the bound SGPs was dissolved in laemmli solution (Laemmli, 1970), boiled, and analyzed by SDS-PAGE and Western blotting.

SDS-PAGE and immunoblot analysis

Proteins were separated by SDS-PAGE (8% gels) and transferred onto nitrocellulose as described (Laemmli, 1970). Nitrocellulose membranes were blocked with 2% bovine serum albumin (BSA) in PBS for 30 min and then incubated with streptavidine-HRP diluted (1/10,000) in PBS supplemented with 0.5% BSA for 45 min. The blot was washed three times for 15 min with PBS to remove unbound conjugated before incubating with SuperSignal substrate working solution (Pierce, IL, USA) was added. Enzyme activity was monitored using Fuji medical X-ray film.

Haemagglutination assay

Chicken erythrocytes were isolated from heparinized chicken blood. In the haemagglutination assay (Eaves *et al.*, 1989) both viable and 1% glutaraldehyde fixed erythrocytes were used with essentially similar results. Agglutination of chicken red blood cells was assessed by mixing 50 µl of 1% erythrocytes with an equal volume of serial dilutions of *ORT* suspension ($5x10^7$ – $3.1x10^6$ bacteria per ml) in DPBS in 96-well microtiter plates. Haemagglutination was scored at 2 h and 24 h of incubation at 20°C.

RESULTS

Differential inhibition by serum of the interaction of *ORT* with epithelial cells and chicken macrophages

Infection of cultured epithelial cells with *ORT* strain 30 in the absence and presence of increasing concentrations of fetal bovine serum for 3 h revealed a dose-dependent inhibition of the *ORT* adherence by serum (Fig. 1). Half-maximal inhibition was obtained with approximately 1% serum, while virtually complete inhibition was observed at 5%. Similar effects were observed for strain 29 (Fig. 1).

To assess whether the inhibitory effect was specific for non-avian sera and thus perhaps explain the host specificity of infection, the effect of *ORT*-antibody free chicken serum on *ORT* infection was tested. Chicken sera even slightly more effectively inhibited the adherence of *ORT* strain 30 to the epithelial cells in a dose-dependent fashion (Fig. 1).

ORT has previously been demonstrated to adhere to and become ingested by chicken macrophages (Chapter 3). Analysis of the effect of serum on the interaction of *ORT* strain 30 with macrophages showed that this event was not affected by serum. Even in the presence of 10% serum, unaltered bacterial adherence was observed (Fig. 2). In contrast, serum inhibited epithelial cell adherence (both to Chang cells and INT-407 cells) for all *ORT* strains tested (Fig. 2). These data suggest that *ORT* interaction with epithelial cells and macrophages is mediated via different, cell type-specific adhesins and/or host cell receptors.

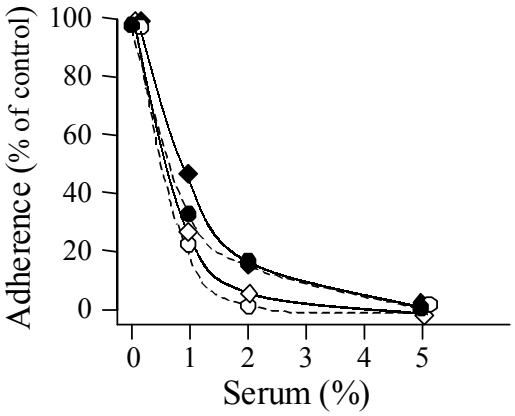

Fig. 1: Sensitivity of *ORT* epithelial cell adherence to serum factors.
Chang epithelial cell were infected with *ORT* strain 29 (diamonds) and 30 (circles) in the absence and presence of the indicated concentrations of fetal bovine serum (open symbols) or chicken serum (closed symbols). After 3 h of incubation, the infection was stopped and the number of bacteria that adhered to the cells was determined. Adherence is expressed as the average number of adherent *ORT* per cell. Values are from a representative infection assay.

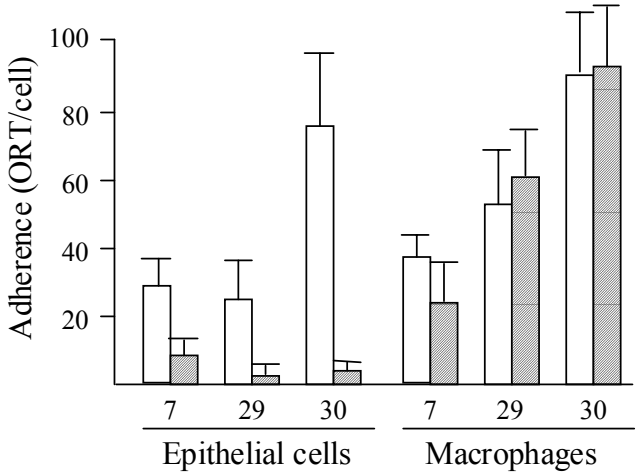

Fig. 2: Cell-type specificity of the infection-inhibitory effect of serum.
ORT strains 7, 29, 30 were added to cultured Chang epithelial cells and HD-11 chicken macrophages in the absence (open bars) and presence (filled bars) of 10% FBS. At 3 h of infection, the cells were washed to remove non-adherent bacteria and the number of cell-associated *ORT* was determined. Adherence is expressed as the average number of bacteria per cell. Values represent the mean ± SEM of three experiments. Note the differential sensitivity of the *ORT* adherence to epithelial cells and macrophages for all strains.

Serum blocks *ORT* adherence through an effect on the bacteria rather than the host cells

To locate the site of action of the infection inhibitory activity in serum, infection experiments were performed with *ORT* strain 30 in which either the bacteria or host cells were preincubated with serum. Following 30 min of preincubation, the bacteria or cells were washed to remove the unbound serum factors and the infection was started. This procedure demonstrated that the inhibitory activity of serum was caused by an effect on the bacteria rather than on the host cells. Preincubation of bacteria reduced bacterial adherence by 40% (Fig. 3), while preincubation of the host cells resulted in similar levels of adherence as in the absence of serum. This effect was observed for the adhesive strain 29 and 30 and thus did not exhibit strain specificity.

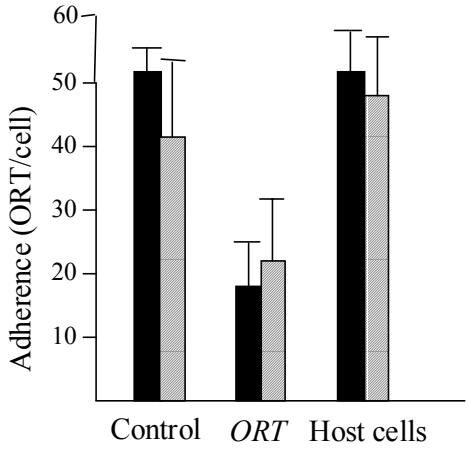

Fig. 3: Serum interferes with the functional properties of *ORT*.
The site of action of FBS was determined by preincubating either the *ORT* strains 29 (solid bars) and 30 (hatched bars) or the epithelial cells (Host cells) with 15% FBS for 30 min prior to infection. At 3 h of infection in the absence of serum, bacterial adherence was determined. Bacteria and cells not pre-incubated with serum served as control. Adherence is expressed as the average number of bacteria per cell. Data are the mean ± SEM of three experiments. Note that preincubation with serum of the bacteria but not the host cells, resulted in reduced adherence.

Characterization of the infection inhibiting factor in serum

The nature of the factor(s) in serum that blocked the *ORT* infection of epithelial cells was further investigated by testing the inhibitory activity of distinct serum fractions. For this purpose, glycoprotein and glycolipid fractions were isolated from bovine serum and tested for their effect on *ORT* infection. As shown in Fig. 4A, both the glycoprotein and glycolipid fractions of serum effectively inhibited *ORT* epithelial cell adherence. Maximal inhibition was observed at a concentration of 1 µg/ml for SGP and SGL for both strains 29 and 30. Again, the interaction of *ORT* with macrophages was not affected by the presence of the isolated serum fractions (Fig. 4B). Size fractionation of the serum glycoprotein fractions using filters with various pore size and use of the obtained fractions in infection assays, indicated that the infection inhibitory molecules were maintained in the 10-100 kDa fraction and, less prominent, in the >100 kDa fraction. No inhibition of adherence was observed for the <10 kDa fraction (data not shown).

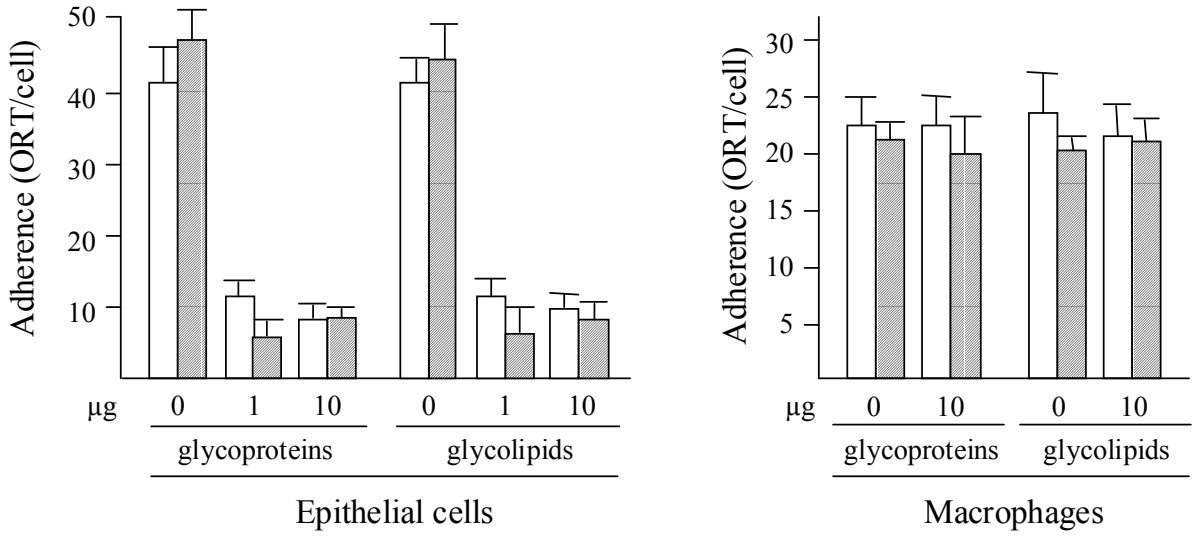

Fig. 4: Inhibition of epithelial cell adherence by serum glycoproteins and glycolipids.
Chang epithelial cells (*left panel*) and HD-11 chicken macrophages *(right panel*) were infected with *ORT* strains 29 (open bars) and 30 (hatched bars) preincubated for 30 min with the indicated amounts of the isolated serum glycoprotein or glycolipid fractions. At 3 h of infection in the absence of serum components, bacterial adherence was determined and expressed as the average number of bacteria per cell. Values are the mean ± SEM of three experiments. Note that both the serum glycoprotein and glycolipid fraction exhibited infection inhibitory activity towards epithelial cells but not macrophages.

Direct binding of serum glycoproteins to *ORT* strains 30 and 41

To further unravel the mechanism by which serum factors influence the binding properties of *ORT*, we measured the direct binding of the isolated size-fractionated serum glycoproteins to the bacteria. For this purpose, serum glycoproteins were biotinylated to enable assessment of their interaction with *ORT*. Glycoprotein binding was measured by incubation *ORT* strains 30 and 41 (10^8 bacteria/ml) with 50 µg of biotinylated glycoproteins for 30 min. Then, the bacteria were washed to remove unbound molecules. SDS-PAGE and Western blotting with streptavidin-horseradish peroxidase as a conjugate identified the biotinylated serum factors that had been bound by bacteria. These binding assays indicated direct binding of several biotinylated serum proteins of the 10-100 kDa fraction but not the >100 kDa fraction, to the bacterial cell surface (Fig. 5). Several proteins bound to strain 41 but not to strain 30 that differs in epithelial cell adherence and haemagglutination properties.

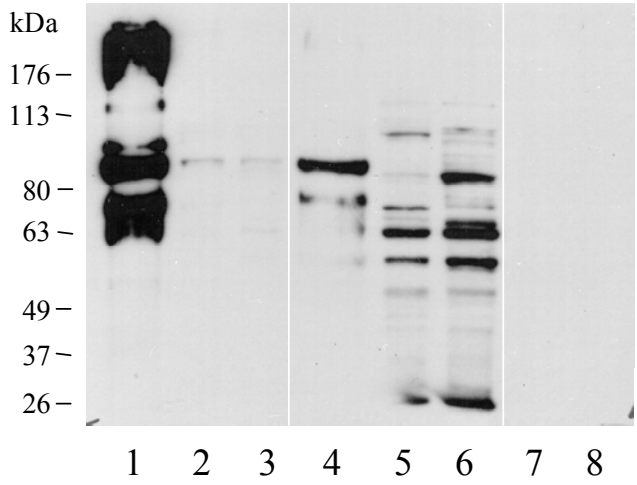

Fig. 5: Direct binding of serum glycoproteins to *ORT*.
ORT strains 30 (lanes 2, 5 and 7) and 41 (lanes 3, 6 and 8) were incubated without (lanes 7 and 8) or with biotinylated isolated serum glycoproteins with a molecular size of >100 kDa (lanes 2 and 3) or between 10-100 kDa (lanes 5 and 6). After removal of unbound material, the bacteria were subjected to SDS-PAGE and blotted onto nitrocellulose. Serum proteins bound to *ORT* were detected with horseradish peroxidase-conjugated streptavidin with the appropriate substrate. The >100 kDa and 10-100 kDa fractions of biotinylated serum glycoproteins that were added to the bacteria were run for comparison and are in lanes 1 and 4, respectively. Note that binding activity was observed for several proteins of the 10-100 kDa fraction with some differences between strain 30 and 41, which may be related to the different, but serum-sensitive adherence properties of these strains.

Comparison of the inhibitory effects of serum and LPS on *ORT* infection

We previously demonstrated that purified LPS of *ORT* was capable to inhibit the adherence of the homologous strain to cultured epithelial cells but not macrophages (Chapter 2 and 3). As the serum inhibition appears to show a similar cell type specificity and likely involves by carbohydrates residues, we tested whether the effect of LPS was also caused via binding of the (bacterial) glycolipid to *ORT*. Infection assays in which either the bacteria or epithelial cells were preincubated with LPS demonstrated that purified LPS derived from strain 30 was able to inhibit bacterial adherence of this strain when the bacteria but not the host cells were primed with LPS (Fig. 6A). This effect was not observed when LPS of strain 29 was used as an inhibitor. Similarly, the LPS of strain 29 inhibited the binding of strain 29 when the bacteria but not the host cells were primed with the LPS, while the LPS derived from strain 30 had no effect (Fig. 6A). Thus, although both serum and LPS block *ORT* adhesion to epithelial cells via an effect on the *ORT*, the effect of LPS but not serum exhibits strain specificity. This suggests that related but different carbohydrates may confer the infection inhibition by serum and LPS.

To further assess whether LPS and serum exhibited their effect through binding to the same or different bacterial ligands, a competitive binding assay was employed. *ORT* strains 29 was incubated with biotinylated purified serum protein with and without prior priming (15 min, 37^{o}C) with purified endogenous LPS or serum. SDS-PAGE and immunoblotting of these samples showed that serum but not LPS competed for the binding of the biotinylated serum glycoproteins (Fig. 6B). Similar results were obtained for strain 30. (not shown). These data are consistent with LPS and serum exerting their inhibitory effect via different mechanisms.

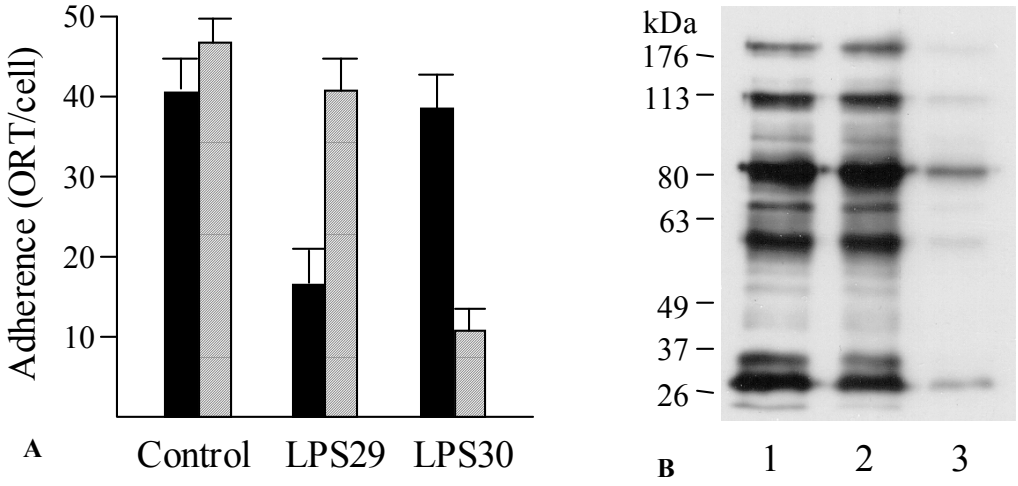

Fig. 6: Strain specific inhibition of *ORT* adherence by LPS.
(A) Chang epithelial cells were infected with *ORT* strain 29 (solid bars) or strain 30 (hatched bars) preincubated with buffer (control) or 10 µg of LPS derived from strain 29 (LPS29) or strain 30 (LPS30). Then, Chang epithelial cells were infected with these bacteria in the absence of LPS. Infection was stopped at 3 h of incubation and the number of adherent bacteria was determined. Data are expressed as the average number of bacteria per cell. Values are the mean ± SEM of three experiments. Note the strain specific inhibition of adherence by LPS. **(B)** *ORT* strain 29 was incubated with biotinylated isolated serum glycoproteins with a molecular size between 10-100 kDa in the absence (lane 1) and presence of 10 µg of purified LPS of strain 29 (lane 2) or 10% FBS (lane 3). After removal of unbound material, the bacteria were subjected to SDS-PAGE and blotted onto nitrocellulose. Bound were detected with horseradish peroxidase conjugated streptavidin with the appropriate substrate. Note that FBS but not LPS competed for binding of the biotinylated serum proteins to *ORT*.

Differential inhibitory effects of serum and LPS on epithelial cell haemagglutination and adhesion of *ORT* strain 41

When grown on Columbia agar plates, *ORT* strain 41 agglutinates chicken red blood cells but only poorly adheres to epithelial cells in contrast to *ORT* strains 29 and 30 (Chapter 2). To investigate whether the *ORT* induced haemagglutination was serum sensitive, haemagglutination assays with strain 41 in the absence and presence of 15% serum were performed. Serum effectively inhibited the *ORT*-mediated haemagglutination (Fig. 7). Similar experiments in the presence of LPS derived from strain 41 yielded unaltered haemagglutination compared to serum-free conditions.

When grown in Todd-Hewitt broth, strain 41 gains the ability to adhere to epithelial cells and loses its haemagglutination properties (Chapter 2). Epithelial cell adhesion assays with broth-grown strain 41 in the absence and presence of 15% serum showed that serum inhibited *ORT* infection of the epithelial cells (Fig. 8) thus resembling the effect on the haemagglutination observed for solid phase grown *ORT*. Assessment of the possible inhibitory effect of LPS on the epithelial cell adhesion showed that endogenous but not heterogeneous LPS inhibited *ORT* infection of the epithelial cells (Fig. 8), although the effects were much less profound that those obtained for strains 29 and 30. This data support the conclusion that serum and LPS inhibit epithelial cell infection via related but different mechanisms and implies that *ORT* strain 41 possesses at least two cell-type (epithelial and red blood cell) specific adhesins that are both sensitive to serum but differentially sensitive to LPS.

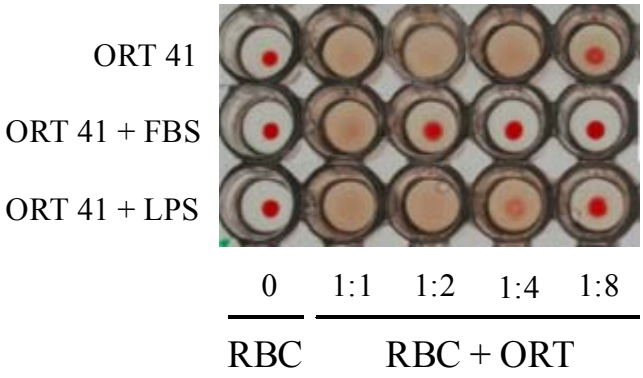

Fig. 7: Sensitivity of *ORT*-mediated haemagglutination to serum but not LPS.
Chicken red bloods cells (RBC) were mixed with serial dilutions of plate grown *ORT* strain 41 (*ORT*) in the absence or presence of 10 µg of isolated serum glycoproteins or 10 µg of LPS purified from strain 41. After for 2 h of incubation (20°C), haemagglutination (HA) was scored. Note that the HA mediated by strain 41 was inhibited by the serum components but not by LPS.

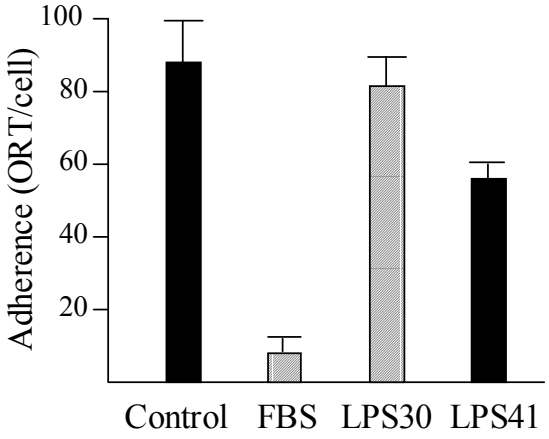

Fig. 8: Effects of serum and LPS on *ORT* strain 41 mediated epithelial cell adherence.
Chang epithelial cells were infected with liquid media grown *ORT* strain 41 in the absence (control) and presence of 10% FBS, or 10 µg of purified LPS derived from strains 30 or 41. At 3 h of infection, bacterial adherence was determined and expressed as average number of bacteria per cell. Data are the mean ± SEM of three infection assays. Note that the epithelial cell adherence of strain 41 is sensitive to FBS and, to a lesser extent, to endogenous but not heterologous LPS.

DISCUSSION

Adhesion of bacterial pathogens to eukaryotic cells is a key step in the establishment of most mucosal infections and thus an attractive target of infection intervention and prevention. Here we report that the emerging avian pathogen *ORT* carries multiple adhesins that confer cell-type specific interactions with epithelial cells, macrophages and red blood cells. The existence of various mechanisms of adhesion became apparent via the differential inhibitory effects of serum and LPS on the *ORT*-host cell interaction. The discovery that both serum factors and LPS inhibit the adhesion of *ORT* through binding to the pathogen may be instrumental in the identification of the nature of the adhesins. In addition, the infection inhibitors may have prophylactic potential by preventing the initial colonization of the epithelium.

The first indication of the existence of multiple mechanisms of *ORT* adhesion to eukaryotic cells came from the differential inhibition by serum of the bacterial adhesion to epithelial cells and chicken macrophages. The finding that serum factors block *ORT* adhesion to epithelial cells but not macrophages indicate the existence of multiple bacterial surface adhesins. This conclusion is further supported by the differential inhibitory effect of LPS on the adhesion of *ORT* to epithelial cells and macrophages. Identification of the nature of the adhesins awaits further investigation. Our data indicate that purified LPS and the factors in serum that bind to the bacteria may aid in the identification process.

Another major challenge is to determine the nature of the inhibitory molecules in the serum. Our results indicate that both the serum glycoprotein and glycolipid fraction contain infection inhibitory activity. This may point to the involvement of carbohydrates. Carbohydrates frequently serve as receptors for bacterial pathogens (Heumann and Roger, 2002) and it can be imagined that serum factors carry carbohydrates that show mimicry with the epithelial cells receptors exploited by *ORT* for bacterial adherence. The finding that serum exerts its infection inhibitory activity through direct binding to the bacterial surface is consistent with this hypothesis. Our attempts to identify the serum glycoproteins through analysis of the isolated biotinylated proteins to the bacterial surface were not conclusive as at least eight different proteins exhibited binding activity. At this time, it is uncertain whether these molecules share common carbohydrate moieties, bind to different receptors, or bind via a common intermediate molecule analogous to the glycosaminoglycan bridge recruitment mechanism (Duensing *et al*., 1999). Because of the observed cell type specificity, we currently favor the scenario that the serum inhibition is caused through binding to bacterial factors involved in the adhesion process rather than a non-specific steric hindrance of the adhesion event.

The striking resemblance in cell-type specificity and nature of inhibitory compounds in serum with those of LPS led us to hypothesize that serum and LPS may exert their effect via the same mechanism. Indeed, LPS was found to inhibit bacterial adhesion by influencing the adhesive properties of the bacteria rather than influencing the host cells. Several lines of evidence however, suggest that serum and LPS act via different mechanisms. First, the binding of biotinylated serum glycoproteins was effectively blocked by serum but not by purified LPS, and second, the inhibitory effect of LPS showed remarkable strain specificity in contrast to serum, suggesting the involvement of different molecules. The differential sensitivity of haemagglutination conferred by *ORT* strain 41 to serum and LPS support this conclusion, although it can be argued that haemagglutination and epithelial cell adhesion may be separate phenomena mediated via different bacterial factors. Support from this concept comes from the apparent switching of *ORT* strain 41 between a haemagglutination-positive and epithelial cell adhesive phenotype, dependent on the growth conditions. These phenotypes are both sensitive to serum but only the epithelial cell adhesion is blocked by LPS. This indicates cell type specificity and the involvement of different bacterial factors.

The molecular mechanism underlying the transition between the haemagglutination-positive and epithelial cell adhesive phenotype of *ORT* strain 41 is not known. The finding that on solid media the bacteria are able to cause haemagglutination but that this property is replaced by an epithelial cell adhesive phenotype when the bacteria are grown in liquid media may indicate different expression of bacterial adhesions. Alternatively, it can be imagined that strain 41 produces a capsule when grown on solid media, which is lost when during growth in suspension. In this scenario, the epithelial cell adhesion may already be present but hampered in function by the capsule. In this case, the capsule may be responsible for the haemagglutination phenotype. Thus far, attempts to identify a capsular structure for *ORT* have not been successful.

Perhaps the most intriguing and important aspect from our work is the potential application of the infection inhibitory factors in serum. It can be imagined that the active serum factors, once identified, can be applied to prevent initial *ORT* colonization and thus have prophylactic potential. In addition, the serum factors and also LPS may be applied to identify the bacterial factors that drive the colonization. These molecules may have great vaccine potential.

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CHAPTER 5

Characterization of Plasmid pOR1 from *Ornithobacterium rhinotracheale* and Construction of a Shuttle Plasmid

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ABSTRACT

The bacterium *O. rhinotracheale* has been recognized as an emerging pathogen for poultry about ten years ago. Knowledge of this bacterium and its mechanisms of virulence are still very limited. Here we report the development of a transformation system that enables genetic modification of *O. rhinotracheale*. The system is based on a cryptic plasmid, pOR1, that was derived from an *O. rhinotracheale* strain of serotype K. Sequencing indicated that the plasmid consisted of 14,787 nucleotides. Sequence analysis revealed one replication origin and several *rep* genes that control plasmid replication and copy number, respectively. In addition, pOR1 contains genes with similarity to a heavy metal-transporting ATPase, a TonB linked siderophore receptor, and a laccase. RT-PCR demonstrated that these genes were transcribed. Other putative open reading frames exhibited similarities with a virulence associated protein in *Actinobacillus actinomycetemcomitans* and a number of genes coding for proteins with unknown function. An *E. coli* - *O. rhinotracheale* shuttle plasmid (pOREC1) was constructed by cloning the replication origin and *rep* genes from pOR1 and the *cfxA* gene from *Bacteroides vulgatus,* that codes for resistance to the antibiotic cefoxitin, into plasmid pGEM7 using *E.coli* as a host. pOREC1 was electroporated into *O. rhinotracheale* and yielded cefoxitin resistant transformants. The pOREC1 isolated from these transformants was reintroduced into *E. coli*, demonstrating that pOREC1 acts as an independent replicon, both in *E.coli* and *O. rhinotracheale,* fulfilling the criteria for a shuttle plasmid that can be used for transformation, targeted mutagenesis and the construction of defined attenuated vaccine strains.

INTRODUCTION

The Gram-negative bacterium *Ornithobacterium rhinotracheale* was identified and recognized as a pathogen for birds in 1991 by DuPreez (van Beek *et al.*, 1994). The bacterium normally causes infection of the respiratory tract, but may disseminate to other body sites resulting in osteitis, meningitis and joint-infections (van Empel *et al.*, 1999). *O. rhinotracheale* infections in flocks occur worldwide and are increasingly recognized as a health problem in chicken and turkey farming. The major economic losses due to *O. rhinotracheale* infection result from the rejection of carcasses for consumption, growth retardation and mortality. *O. rhinotracheale* is a member of the *Flavobacteriaceae* that belong to the *Cytophaga-Flavobacterium-Bacteroides* phylum (Vandamme *et al.*, 1994). Related pathogens are the bird pathogens *Riemerella anatipestifer* and *Coenonia anatina* (Vandamme *et al.*, 1999).

Infections with *O. rhinotracheale* can be successfully treated with antibiotics, but the bacterium rapidly develops antibiotic resistances (Devriese *et al.*, 1995). Infections of *O. rhinotracheale* can spread both horizontally by aerosols and vertically via infected eggs. Vaccination against *O. rhinotracheale* infections with a bacterin has been found suitable for the prevention of infections by the vertical route, but to be effective the bacterin has to be administered with an oily adjuvant (van Empel and van den Bosch, 1998). To prevent horizontal transfer of the bacterium, vaccination with a live avirulent strain seems to be effective (van Empel and Hafez, 1999). The construction of genetically well-defined and attenuated strains however, is seriously hampered by the lack of tools to genetically manipulate the pathogen. For the related *Flavobacteriaeceae* a few tools have been developed (McBride and Baker, 1996) but these plasmids have been found not to be suitable for transformation of *O. rhinotracheale* (unpublished data).

An attractive approach for the development of a plasmid that can be exploited for genetic manipulation of *O. rhinotracheale* isolates is to modify an endogenous *O. rhinotracheale* plasmid. One strain of *O. rhinotracheale* has been reported to carry such a plasmid (Back *et al.*, 1997). Here the isolation and characterization of the native plasmid pOR1 from *O. rhinotracheale* and the construction of a shuttle vector that can replicate both in *E. coli* and *O. rhinotracheale* is reported. This shuttle vector paves the way for genetic modification and characterization of the *O. rhinotracheale* genome and for the development of genetically defined vaccine strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains of *O. rhinotracheale* used in this study (Table I) were kindly provided by Dr. P. van Empel (Intervet International, Boxmeer, The Netherlands). The characteristics of the strains have previously been described (van Empel *et al.*, 1998). *O. rhinotracheale* strains were grown at 37°C in an atmosphere of 5% CO_2 on Columbia agar plates (Oxoid, Basingstoke, England) or in Todd-Hewitt broth (Oxoid). The broth cultures were incubated on an orbital shaker (120 rpm) at 37°C in a tightly sealed 50 ml Falcon tube.

Table I. Characteristics and origin of the *O. rhinotracheale* strains used in this study.

strain	serotype	bird	country
7	A	guinea fowl	France
11	B	turkey	Germany
12	B	turkey	the Netherlands
18	B	turkey	Germany
35	D	turkey	France
45	E	turkey	Germany
99-0118	K	turkey	USA (Minnesota)
215	K	turkey	South Africa
97-0321	K	turkey	USA (Iowa)

DNA isolation, hybridization, cloning and sequencing of pOR1

Total genomic DNA from bacterial strains was isolated using the High Pure PCR template kit (Roche diagnostics, Nederland B.V., Almere, The Netherlands). Southern blot hybridization was done using Hybond N membranes (Amersham Biosciences, Roosendaal, The Netherlands). The probes were labeled with ^{32}P dATP using the Random primer labeling kit (Boehringer Mannheim, Germany). The hybridizations were done at high stringent conditions with a final wash of 0.5 X SSPE at 65°C.

Cloning and sequencing of pOR1

The plasmid pOR1 was isolated from total genomic DNA from strain 99-0118 by CsCl gradient centrifugation (Sambrook *et al.*, 1989) in a TL-100 ultracentrifuge (Beckman Palo Alto, Calif., USA). The full length pOR1 was cloned with its unique *Bam*HI site into lambda EMBL3 phage (Promega corp., Madison, WI., USA) with *E. coli* JM109 as a host. The pOR1 DNA was subcloned as *Hin*dIII fragments into the pGEM7Zf(+) cloning vector (Promega corp., Madison, WI, USA) using *E. coli* DH5α as a host. Serial deletion clones for sequencing purposes were made using the Erase-a-base system (Promega corp., Madison, WI., USA). The DNA sequence was determined using the Big-Dye terminator system on a 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Foster city, Calif., USA).

Expression studies on pOR1 genes

Total RNA was isolated from *O. rhinotracheale* cultured for two days on a blood agar plates. RNA was isolated using RNasol B (Tel-Test, Friendswood, TX, USA) according to the manufacturers conditions. Residual DNA was removed from the RNA preparation using RQ1 RNase-free DNase (Promega). The reverse transcription (RT) of the RNA was done using the Access RT-PCR system (Promega). The RT reaction was done at 37°C during 60 minutes using the downstream primers 2, 4, or 6 (Table II). An aliquot of the RT reaction mixture was used in the PCR. The 35 PCR cycles were run at the following conditions; annealing at 55°C for 30 seconds, extension at 74°C for 1 minute, and melting at 94°C for 30 seconds. The primers that were used in the RT reactions are listed in Table II.

Table II. PCR and RT-PCR primers used in this study

PCR-primer	Position in pOR1	Sequence	Target gene
16S-F		GAGAATTAATTTACGGATTAAG	16SRNA
16S-R		TTCGCTTGGTCTCCGAAGAT	16SRNA
rep		GAAAGGATTTAAGCGGAC	*rep2*
M13U		CACGACGTTGTAAAACGACGGCCAG	
RT-primer			
1	4599-4615	CGTGATAGATTACGGAG	siderophore receptor
2	4832-4849	ATGATTGGACATTTAGCA	
3	6759-6776	CCAATGTAAGAGGGAAAG	Cd resistance
4	7164-7181	GTATAGCAATGGGTGGAT	
5	10575-10592	CAACCGAAGACAGGACAC	Laccase
6	11210-11227	GAGGAAGGTGATTGGTTC	
7	EFGPup	ACCCTCGTGACCACCCTGACCTAC	*efgP*
8	EFGPdown	GACCATGTGATCGCGCTTCTCGTT	*efg*P

Heavy metal resistance

Resistance to heavy metal ions was determined on Columbia agar plates with the heavy-metal salts $CdCl_2$, $CoSO_4$, $CuSO_4$, and $HgCl_2$ (Merck, Darmstadt, Germany) at concentrations of 0.01, 0.1, 1, or 10 mM. The plates were inoculated with the *O. rhinotracheale* strains and after two days of incubation the colonies were scored on a scale from 0 to 3 (0 = no colonies, 1 = visible colonies, 2 =

intermediate size colonies, 3 = similar colonies as the control). The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration at which scale 2 colonies were obtained.

Construction of pOREC1

The shuttle vector pOREC1 consists of pGEM7 with the replication origin of pOR1 and the *cfx*A gene of *Bacteroides vulgatus* (EMBL/Genbank accession number U38243). Construction of pOREC1 was carried out in two steps. First, the replication origin of pOR1 was cloned as a *Bam*HI/*Nde*I fragment of 2681 bp into the *Bam*HI and *Eco*RI sites of pGEM7Zf(+). Prior to the ligation the *Nde*I and *Eco*RI sites were filled in by the Klenow fragment of DNA polymerase to obtain blunt ends. In the second step, the *cfx*A gene was cut from pCP29 as a *Bam*HI/*Pst*I fragment of 2.1 kbp (kindly provided by Dr. Sarika Agarwal, University of Wisconsin) and ligated into the *Bam*HI/*Nsi*I sites of the former construct.

Electroporation of *O. rhinotracheale*

Transformation of *O. rhinotracheale* was done by electroporation using a GenePulser (BioRad, Uppsala, Sweden). Electrocompetent bacteria were prepared from bacteria grown for 2-days in 10 ml of Todd-Hewitt broth in 50 ml Falcon tubes on an orbital shaker (120 rpm, 37°C). The OD_{600} of the cultures was between 0.3 and 0.4. The cultures were washed two times with 25 ml of sterile distilled water and finally washed with 10% glycerol. The cell pellet was resuspended in approximately 100 µl of 10% glycerol resulting in a suspension of 10^9 to 10^{10} colony forming units per ml (cfu). Electroporation was done in disposable cuvettes with an electrode distance of 2 mm, using 40 µl of cell suspension and 5 µl of DNA solution containing approximately 1 mg/ml of DNA. All manipulations of the bacteria were done at room temperature. The settings of the GenePulser were: 25 µF capacity, 2500 V and 800 Ohm parallel resistor. Directly after the pulse 960 µl of Todd-Hewitt broth was added and the bacteria were recovered for at least 90 min at 37°C. After recovery the bacteria were plated on selective plates (cefoxitin 5 µg/ml) and incubated for at least 3 days at 37°C and 5% CO_2.

The impact of the electric pulse was measured by the survival rate of the bacterial cells before and after the pulse. The survival rate was measured by comparing the cfu of serial dilutions of the cell suspension before and after the electric pulse. It was observed that the survival rate was close to 100%, which implicates that the *O. rhinotracheale* cell wall is resistant to electric pulse and probably difficult to be made permeable.

PCR and RT-primers

The primers used in a PCR to identify *O. rhinotracheale* on its 16S rRNA genes were: primer: 16S-F and primer 16S-R. These primers amplify a 784 bp fragment of the 16S rRNA gene of *O. rhinotracheale* (accession number L19156). The primers used to identify the presence of pOREC1 in transformants were primer rep, which is located in the *rep*2 gene of pOR1 and primer M13U that is located in pGEM7Zf(+). The sequence of these primers and those of the primers used in the RT-PCR to demonstrate expression of pOR1 genes and EFGP are listed in Table II.

Assembly and annotation of pOR1 sequence

The nucleotide sequence of pOR1 was determined using a combination of subcloning and primer walking. The full length sequence of pOR1 was assembled from the single read sequences using the SeqMan program of the Lasergene package (DNAStar inc., Madison, WI, USA). The whole pOR1 sequence was covered at least three times by single reads and each base was identified with a reliability of at least 95% (Q value larger than 20).

The annotation of potential coding sequences was done by GeneMark.hmm at website http://dixie.biology.gatech.edu/GeneMark/heuristic.cgi (Besemer and Borodovsky, 1999) and with BlastX at website http://www.ncbi.nlm.nih.gov/blast/blast.cgi of the National Institute of Health. To predict the potential function of the genes, the encoded proteins were compared with the proteins of the EMBL/Genbank database using BlastP. Repetitive sequences and AT contents were determined using the GeneQuest program of Lasergene. The nucleotide sequence of the pOR1 plasmid is deposited in the EMBL/Genbank database under accession number AY513488.

RESULTS

Cloning and prevalence of pOR1 among *O. rhinotracheale* isolates

Plasmid pOR1 was isolated by CsCl density gradient centrifugation. The plasmid banded at a higher density than genomic DNA, indicating that pOR1 is circular and supercoiled. The entire plasmid pOR1 was cloned into lambda phage EMBL3, using its unique *Bam*HI site.

The occurrence of pOR1 among *O. rhinotracheale* isolates was investigated by screening total DNA of a panel of *O. rhinotracheale* isolates by dot-blot DNA hybridization using the full length pOR1 clone in lambda EMBL3 phage as a probe. The *O. rhinotracheale* strain 99-0118 that carries pOR1 originates from Minnesota and has the rare serotype K. The panel consisted of two other isolates that have serotype K, 34 non-K serotype isolates that originated in Minnesota and 20 isolates that were isolated from different bird species from Europe and Africa. In one of the other serotype K strains (97-0321) that originate in Minnesota, pOR1 sequences were detected. All other isolates including the serotype K isolate (215) from South Africa yielded no positive signals under the conditions employed. Southern blot analysis on the *Hind*III digested DNA from isolates 99-0118 and 97-0321 using pOR1 as a probe yielded identical patterns for both strains (data not shown). This indicates that both strains carry pOR1.

Determination and annotation of pOR1 sequences

The nucleotide sequence or pOR1 comprised 14,787 bp. The plasmid has a GC content of 35.7%, which is similar to the 37 to 39% reported for the whole genome of *O. rhinotracheale* (Vandamme 1994). The AT and GC nucleotides are unevenly distributed on the plasmid. Part of the plasmid, encompassing the putative genes 4 to 9 (see below) have a higher GC content (38.8%) than the remainder of the plasmid (31.2%), which might indicate that pOR1 has a mosaic composition.

The putative genes on the plasmid were identified using the GeneMark.hmm program (Besemer and Borodovsky, 1999). Thirteen regions that start with an initiation codon with the potential to encode a protein of more than 100 amino acids were found on pOR1. A map of the putative genes of pOR1 is presented in Fig. 1. Genes encoding proteins less than 100 amino acids were only included when they had significant homology to a protein sequence in the database. The *vapD* gene is therefore included in Fig.1 and Table III, whereas two other genes encoding proteins less than 100 amino acids are excluded. To attribute a function to the putative genes, the encoded proteins were compared with the EMBL/Genbank database using BlastP. The genes from the database with the best match to the putative genes are listed in Table III.

Table III. Characteristics of putative genes of pOR1.

Orf	Gene	Similar gene product	Probable function	e-value	PID	Species
1	*repA*	replication protein A	plasmid replication	3e-72	g1075495	*Bacteroides vulgatus*
2	*rep2*	replication protein 2		3e-24	g10956802	*Riemerella anatipestifer*
3	*trans*	transposase	transposition	1e-41	g16082872	*Yersinia pestis*
4		no similarity				
5		tonB-linked outer membrane receptor	iron uptake	2e-33	g29345912	*Bacteroides thetaiotamicron*
6	*yvgW*	heavy metalion-transporting ATPase	cadmium efflux	1e-159	g21398552	*Cytophaga hutchinsonii*
7		no similarity				n.a
8	*merP*	periplasmic transport protein	mercury efflux	6e-04	g2935549	*Pseudomonas alcaligenes*
9	*mlpB*	putative lipoprotein	unknown	2e-19	g4467968	*Myxococcus xanthus*
10	*pcoA*	copper resistance protein	copper binding	1e-72	g13632794	*Escherichia coli*
11		chromosome assembly protein homologue	chromosome assembly	0.002	g15606061	*Aquifex aeolicus*
12	*mbpB*	mobilization protein	plasmid mobilization	2e-10	g915338	*Bacteroides fragilis*
13		no similarity				
14	*vapD*	virulence associated protein	unknown	2e-24	g6136169	*A.actinomycetemcomitans*

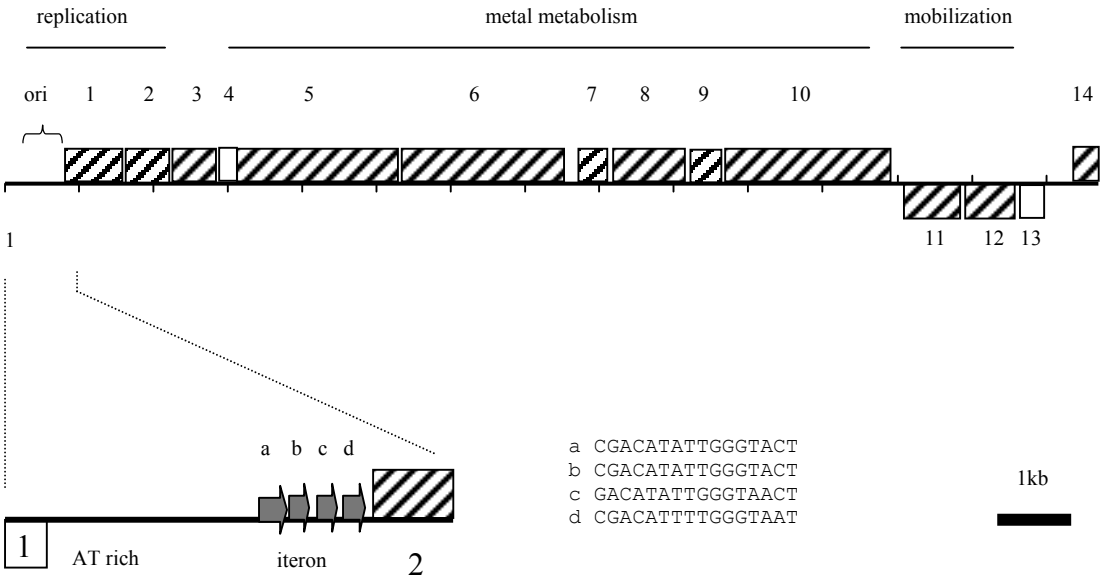

Fig. 1. Linear presentation of pOR1. In the upper panel the pOR1 sequence of 14,787 bp is indicated by the solid line. The open reading frames (orf) of pOR1 are indicated by boxes. The orfs on the direct strand are positioned above and those on the reverse strand below the sequence line. Dashed orfs have orthologs in the EMBL/Genbank database. In the lower panel the replication region of pOR1 is enlarged. Indicated are the four direct repeats of the iteron. The sequences of the interon are presented.

Three functional regions can be distinguished on pOR1. These regions are involved in plasmid replication (orfs 1 and 2), heavy metal metabolism (orfs 5 to 10) and mobilization (orfs 11 and 12). The open reading frames 1 and 2 are homologous to the replication genes *repA* and *rep2* of *Bacteroides vulgatus* and *R. anatipestifer* plasmids, respectively. These Rep proteins are usually found in plasmids with a theta-form replication mode (Chattoraj, 2000). In front of the *repA* gene four imperfect repeats can be recognized that may function as the iteron and regulates plasmid replication (Fig. 1). No other iteron sequences are present elsewhere on the plasmid. The replication origin of the plasmid is most likely located in front of the iteron (del Solar *et al.*, 1998). The region of 262 bp before the iteron has a high AT content (76%) characteristic of plasmid replication origins.

Orf 3 encodes a protein of 201 amino acid residues. This protein is similar to the proximal half of a large number of bacterial transposases and encodes about 400 amino acid residues. Therefore, orf 3 is most likely the truncated transposase of an IS element.

Orf 5 encodes a protein that is highly similar to TonB linked siderophore receptors. Siderophore receptors allow the bacteria to recruit iron from the environment.

The proteins encoded by orfs 6 to 10 show similarities to proteins that are involved in resistance to heavy metal ions. Orf 6 is highly similar to numerous bacterial heavy metal ion-transporting ATPases of the CPx-type ATPase family. These proteins facilitate efflux of heavy metal ions such as cadmium, cobalt and copper across the bacterial cell envelope (Williams and Pittman, 2000). The small orf 7 has no significant similarity to any proteins of the databases. The 291 amino acid residues encoded by orf 8 have similarity to the MerP protein that is involved in mercury resistance. However, it is unlikely that orf 8 codes for a MerP protein as these proteins are much smaller than the orf 9 protein. Metallothioneins are approximately 90 amino acid residues in length. In addition, the *merP* genes are part of an operon encoding proteins that are involved in sensing, transport and reduction of mercury ions. The orf 9 protein is similar to a single protein MlpB of *Myxococcus xanthus*. No function was attributed to MlpB that renders the function of the orf 9 protein enigmatic.

The protein encoded by orf 10 has a high similarity to two groups of proteins. The highest similarity (e^{-154}) was found with bacterial proteins involved in copper resistance such as *PcoA* of *E. coli* and *CopA* of *Xanthomonas campestris* and Pseudomonas species. A lower, but very significant similarity ($4e^{-37}$) was found with the large family of the laccases. Laccases are enzymes (EC 1.10.3.2) that are widely distributed among bacteria and fungi (Liu *et al.*, 1999). The laccases require copper

binding for activity. The orf 10 protein has the four copper binding domains that are found in all laccases (see annotation at EMBL/Genbank AY513488 and at website www.llu.edu/llu/medicine/micro/laccase/), which strongly suggests that the orf 10 protein is a laccase.

The proteins encoded by the orfs 11 and 12 may form a functional unit that is involved in plasmid mobilization. The functional entity of these genes is also reflected by their reverse orientation with respect to the other genes of pOR1. The protein encoded by orf 11 has a weak similarity to a putative chromosome assembly protein of *A. aeolicus*, but orf 12 shows a clear similarity to the plasmid mobilization proteins MbpB of *B. fragilis*.

The protein encoded by orf 14 is similar to the Virulence Associated protein D (vapD) that is present in at least thirteen bacterial species among which are some known bird pathogens like *Riemerella anatipestifer*. Although, the name of these proteins suggests involvement in bacterial virulence, no function has been attributed to these proteins.

Transformation of *O. rhinotracheale* and construction of a shuttle vector

To establish a transformation system for *O. rhinotracheale*, an *E. coli-O. rhinotracheale* shuttle vector was constructed. This shuttle vector pOREC1 was made by cloning the putative replication origin, the iteron and the *repA* and *rep2* genes (orfs 2 and 3) needed for replication in *O. rhinotracheale* into pGEM7. As a selection marker the cefoxitin resistance gene *cfxA* from *Bacteroides vulgatus* was included. A map of the shuttle vector pOREC1 is presented in Fig 2.

Transformation of *O. rhinotracheale* was achieved by electroporation with 0.5 μg pOREC1 that was isolated from *E. coli*. Several *O. rhinotracheale* strains were subjected to electroporation with pOREC1 and cefoxitin resistant colonies were found after four days incubation. The efficiency of the transformation was approximately 400 recombinants per μg of plasmid DNA. No strain differences were found in the efficiency of transformation. The presence of pOREC1 in the recombinant *O. rhinotracheale* was confirmed by PCR using primers M13U and primer rep (Fig. 2). In addition, recombinants were verified as *O. rhinotracheale* by a species specific PCR on the 16S rRNA gene with primers OR16S-F and OR16S-R. Transformation of *E. coli* with total DNA of the *O. rhinotracheale* recombinants again resulted in transformed *E. coli* with pOREC1. This indicates that pOREC1 was present in *O. rhinotracheale* as an independent replicon. The transformation of both *O. rhinotracheale* and *E. coli* with pOREC1 demonstrates the functionality of the replication origin and rep genes of pOR1.

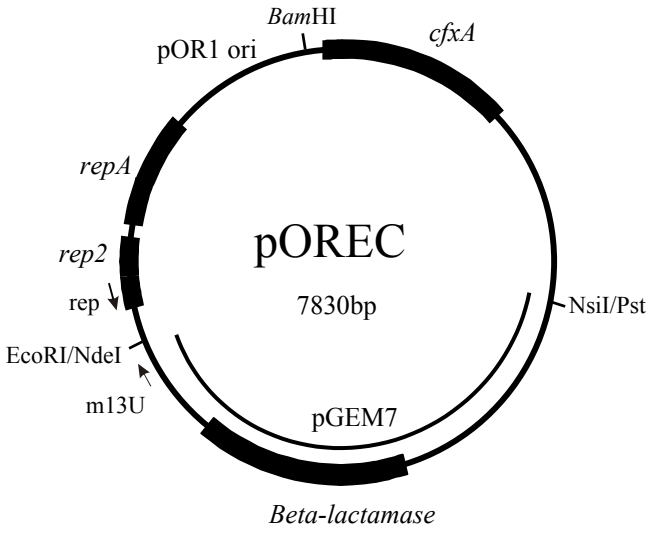

Fig. 2. Organization of the shuttle plasmid pOREC1. Boxes represent the genes that are involved in replication and selection in *O. rhinotracheale* (repA, rep2 and cfxA) and *E. coli* (beta-lactamase). The restriction sites that were used in the construction of pOREC1 are indicated.

Expression of heavy metal resistance encoding genes of pOR1

RT-PCR was used to test the expression of three of the functionally related genes of pOR1. The three genes are orf 5 encoding the putative TonB linked siderophore receptor, orf 6 encoding the

putative heavy metal-transporting ATPase, and orf 10 encoding the putative copper resistance or the putative laccase. RT-PCR was carried out on total RNA from strain 99-0118 grown on agar plates for two days. RT-PCR products were obtained for the all three genes, indicative of gene transcription (Fig. 3).

The functionality of the putative heavy metal metal-resistance protein encoded by orf 6 was tested on its ability to protect *O. rhinotracheale* from toxic heavy metal ions. Strains 99-0118 and 97-0321 that carry pOR1 were exposed to various concentrations of the heavy metal ions cobalt, chromium, cadmium, mercury, and copper. Strain 215 of serotype K, strain 7 of serotype A, and strains 11 and 18 of serotype B, that all lack pOR1, served as control in these experiments. The growth of the six strains was fully inhibited by mercury (0.05 mM), while cobalt and chromium were not inhibitory to the *O. rhinotracheale* strains at a concentration of 1 mM. Cadmium inhibited growth of the three serotype K strains at 0.05 mM, while the three non-serotype K strains were inhibited at the lower concentration of 0.01 mM cadmium. Copper inhibited the growth of the four strains that do not carry pOR1 at concentrations of 0.5 mM, while the two pOR1 carrying strains were inhibited at concentrations ten times higher (5 mM). Mercury ions inhibited growth of all strains at concentrations of 0.05 mM. These data indicate that the putative heavy metal ATPase of pOR1 hardly protects the bacteria from heavy metal toxicity. Only a slightly higher copper resistance is correlated with the presence of pOR1. The higher cadmium resistance of the plasmid carrying stains is unlikely correlated with the presence of the plasmid, since strain 215 that has the same serotype K but not pOR1 exhibited similar levels of resistance to cadmium.

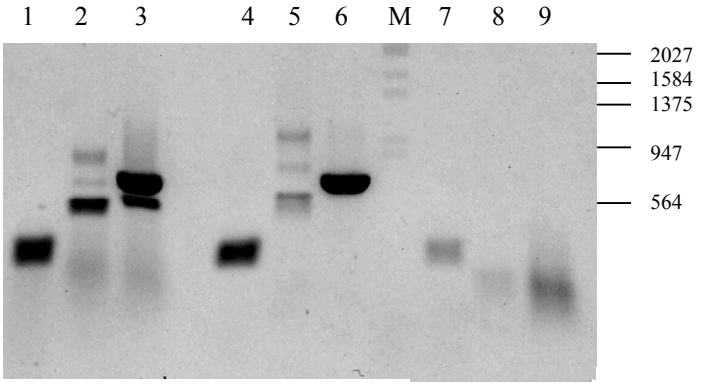

Fig. 3. Transcription of pOR1 genes. Ethidium bromide stained agarose gel of DNA fragments amplified by RT-PCR of genes located on pOR1 in *O. rhinotracheale* strain 99-0118. Lanes 1, 2, and 3, RT-PCR products of the putative siderophore receptor, Cd resistance gene and the laccase respectively. Lanes 4, 5, and 6 PCR on the cloned pOR1 sequences. Lanes 7, 8, and 9, RT-PCR on the RNase treated RNA preparation. The molecular weightmarker (M) was *Eco*RI and *Hin*dIII digested phage lambda DNA. The fragment sizes of the marker in bp are indicated.

Expression of enhanced green fluorescent protein (EGFP) on pOREC1

The expression of the EGFP by *O. rhinotracheale* can be a useful tool to monitor the tissue localization of the bacteria after experimental infection of cultured cells and animals. The gene encoding the EGFP protein was cloned into pOREC1 between the replication origin of pOR1 and the pGEM7 part of the shuttle vector (Fig. 2). The EGFP gene was placed under the control of the *lac* promoter. It was demonstrated by RT-PCR using EGFP specific primers that transcripts of the EGFP gene were present in *O. rhinotracheale* transformed with the pOREC1-EGFP construct, demonstrating that the *lac* promoter is active in *O. rhinotracheale*. However, the bacteria that transcribed the EGFP gene did not show any fluorescence. The absence of fluorescence likely resulted from inefficient translation of the mRNA possibly because the codon usage of EGFP is not suitable for *O. rhinotracheale*. The codon usage of the genes from plasmid pOR1 differs strongly from the codon usage of EGFP. Most notable is that only 3.6 percent of the EGFP codons have an A or T residue at the first position while the codons of the native *O. rhinotracheale* genes have 58% A or T at the first position.

DISCUSSION

In this report the successful transformation of *O. rhinotracheale* using a newly constructed plasmid shuttle vector pOREC1 is described. The transformation of *O. rhinotracheale* for the first time paves the way to genetic manipulation of this bacterium and will aid in a better understanding of the virulence of this bird pathogen. The genetic manipulation of *O. rhinotracheale* also may aid in the development of a vaccine strain by directed mutagenesis of *O. rhinotracheale* leading to defined attenuated vaccine strains.

The bacterium *O. rhinotracheale* has been subjected to numerous fruitless attempt for transformation using shuttle vectors from other *Flavobacteriaceae* and wide host range plasmids (R Jansen, unpublished data). Most likely this lack of success can be attributed to the failure of the introduced plasmids to replicate within *O. rhinotracheale*, since for these other vectors the same transformation protocol and the same selection marker, the *cfxA* gene, as for pOREC1, were employed.

In the construction of the functional shuttle plasmid pOREC1, the replication origin, iteron and the two putative replication genes *repA* and *rep2* of pOR1 were used. The presence of two replication genes, *repA* and *rep2* is uncommon for plasmids with theta-like replication. Most of these plasmids have a single *repA* gene. A second replication gene similar to *rep2* is also present on pCFC1 of *Riemerella anatipestifer* and on pUCL287 of *Tetragenococcus halophilus* (Benachour *et al*., 1997; Weng *et al.*, 1999). In *T. halophilus* the Rep2 protein is not essential for replication, but involved in copy-number control and segregational stability (Benachour *et al.*, 1997). It can be imagined that rep2 of *O. rhinotracheale* has a similar function.

The plasmid pOR1 was detected in only two isolates of the numerous *O. rhinotracheale* strains that were analyzed. These two isolates might represent the same strain, since both isolates originate from the same geographical region and have the rare serotype K. The apparent rare occurrence of pOR1 suggests that the plasmid was introduced into *O. rhinotracheale* on a single recent occasion and has not spread in the population. The selective advantage for the bacterium of carrying pOR1 is unclear. Apart from the replication genes, for three genes the advantage for the bacterium can be speculated on. Firstly, the putative laccase might be a virulence factor for *O. rhinotracheale* as was suggested for the pathogenic fungus *Cryptococcus neoformans* (Liu *et al.*, 1999). The laccase of *C. neoformans* possibly is involved in protection of the pathogen against killing by alveolar macrophages. The laccase prevents the production of hydroxyl radicals by oxidizing Fe(II) of Fe(III) in the macrophage. However, attempts to demonstrate laccase activity in *O. rhinotracheale*, using the polymerization activity of the laccase in pigment formation failed (R. Jansen unpublished data). The activity of other bacterial laccases also is enigmatic and the assays that were developed for plant and fungal laccases are not suitable for the bacterial family of laccases.

The second gene that may be beneficial for *O. rhinotracheale* is the heavy metal-transporting ATPase. This enzyme may aid the bacterium to scavenge for copper, an essential component of the laccase. Our experiments did not reveal such a function as inferred from the equal sensitivity of the various strains to the heavy metals cadmium and mercury. The slightly higher resistance to copper that was observed is unlikely to have physiological significance considering the extremely high concentrations (0.5 mM) at which the effect became apparent.

The third gene that might be advantageous to the bacterium is the TonB linked siderophore receptor encoding gene. The corresponding protein might aid the bacterium to scavenge for iron, which is essential for bacterial growth or, alternatively, for copper to aid the laccase activity.

Clearly, the most important conclusion from the present work is that transformation of *O. rhinotracheale* is feasible and that the developed transformation system can be exploited to genetically modify the bacterium. The efficiency of transformation with the shuttle vector pOREC1 is still rather low and needs improvement for efficient use in, for instance, transposon mutagenesis or DNA displacement by homologous recombination. The low efficiency of transformation is likely caused by to the rather poor permeabilization of the *O. rhinotracheale* membrane during electroporation and/or by restriction of the entering plasmid DNA. Optimization of the electroporation protocol may be achieved by growing the bacteria under conditions that weakens the cell wall.

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CHAPTER 6

GENERAL DISCUSSION

1. Context and aim of the study

The first steps following the discovery of a novel bacterial pathogen usually focus on the microbiological characterization of the infectious agent, the description of the associated pathology, the development of diagnostic tools, and simple measures to contain the spread of the pathogen. *Ornithobacterium rhinotracheale* (*ORT*) has emerged as a pathogen in turkeys and chickens about 20 years ago and since then indeed all research efforts have been directed at developing new diagnostics and on the treatment and prevention of the highly contagious *ORT* infections. Nowadays *ORT* causes considerable economic losses to the poultry industry and the excessive use of anti-microbial drugs to contain *ORT* has resulted in a widespread resistance to many commonly used antibiotics. Several rather ill defined vaccines have been developed that show moderate efficacy but hold promise that vaccination may be an effective tool to provide full protection against *ORT* infection. The work described in this thesis was initiated to better understand the pathogenesis of *ORT* infection in the belief that knowledge of the bacterial and host determinants that contribute to the establishment of *ORT* infections would provide a solid basis for the development of well-defined and effective vaccines and possibly novel infection intervention methods.

At the start of the work described in this thesis, no information regarding any aspect of the pathogenesis of *ORT* infections was available. Pathological examination of infected animals suggested that the bacteria colonized the respiratory epithelium (Back *et al.*, 1998; van Empel *et al.*, 1999) and interacted with macrophages (van Empel *et al.*, 1999). The specific objectives of the present study were to better understand this interaction of *ORT* with epithelial cells and macrophages, to access the host cell response, and to develop a genetic tool that would enable genetic manipulation of the pathogen. The latter is essential to facilitate targeted mutagenesis and for the construction of attenuated strains with vaccine potential.

2. Interaction of *ORT* with epithelial cells

Bacterial adherence to mucosal epithelium is the initial and most important step in the establishment of most mucosal infections. The interaction of *ORT* with epithelial cells was investigated using cultured epithelial cells as a model system. This approach is attractive as it simplifies the infection to the direct interaction of the pathogen with the target cells. At the same time this approach is flawed as it neglects the complexity of the natural setting of the infection. Nevertheless, the focus on the cellular infection revealed potentially important information regarding the kinetics of the infection, the molecules involved, and the variability among *ORT* strains.

A primary finding was the rapid and efficient adherence of *ORT* to several types of cultured epithelial cells (Chapter 2). This for the first time indicated that *ORT* has the intrinsic machinery to adhere to eukaryotic cells. The fact that *ORT* adherence was established with epithelial cells of human origin may indicate that *ORT* does not show host specificity with regard to its initial epithelial cell adherence. The adherence properties of *ORT* towards epithelial cells derived from poultry could not be investigated due to a lack of existing cell lines. If indeed *ORT* epithelial cell adherence does not show host specificity this may imply that the bacterium may colonize other species than birds. This has not been seriously investigated and may easily be missed when colonization is not associated with pathology.

The nature of the bacterial and host molecules involved in the cellular infection of epithelial cells remains to be defined. In this fishing expedition, the observation that not all *ORT* strain were capable to adhere to the epithelial cells (Chapter 2) may proof to be of value. The finding that *ORT* strain 41 only poorly adhered to the epithelial cells when grown on agar plates but gained this trait when grown in suspension suggests that the bacterial adhesin may not be invariably exposed at the bacterial cell surface. At this time it is uncertain whether the observed growth environment-dependent adhesive properties are caused by a variable expression of the bacterial adhesin or a masking by other surface structures such as a capsule. For *Pasteurella multocida*, which belongs to the same rRNA V superfamily, capsular hyaluronic acid appears to act as an adhesin (Lee *et al.*, 1994). This bacterial polysaccharide confers adherence via

binding to a specific host cell glycoprotein receptor (Pruimboom *et al.*, 1996). For other bacterial pathogen however, polysaccharide capsules have been reported to prevent efficient bacterial adherence by masking bacterial surface adhesins (de Vries *et al.*, 1998). Detailed comparison of the surface characteristics of *ORT* strain 41 grown under various environmental may provide more information which mechanism account for the variable adhesion of this strain.

An important observation that provided more information about the factors that confer *ORT* adhesion to epithelial cells was the inhibition of the event by endogenous but not heterologous *ORT* LPS (Chapter 2). These data point to the involvement of carbohydrates in the adhesion process. The effect of LPS was mediated through an interaction of LPS with the bacteria rather than the host cells, which makes it tempting to speculate that LPS of *ORT* acts as a receptor analogue preventing the interaction of the bacterial adhesin with the epithelial cell receptor. A problem with this attractive scenario is that it implies that each of the *ORT* strains tested interacts with a different host receptor as the observed inhibitory effects of LPS were found to be strain-specific. Clearly, resolving the mechanism by which LPS inhibits *ORT* adhesion may provide important information about the nature of bacterial adhesins and the corresponding host cell receptors.

In many bacterial mucosal infections, bacterial adherence is followed by entry and transcytosis or survival of the microorganism inside the host cell. Based on microscopy observations (Chapter 2), *ORT* appears not very efficient in inducing its own uptake into epithelial cells, at least under the conditions employed. Measurement of intracellular survival of *ORT* was seriously hampered by the lack of an appropriate survival assay. The commonly used gentamicin killing assay, based on the ability of the antibiotic to kill extracellular but not intracellular bacteria, could not be applied as all *ORT* strains tested were resistant towards the antibiotic and no alternative drugs were found suitable. The possibly relatively moderate ability or *ORT* to breach the mucosal barrier may explain why *ORT* infections are often found in combination with other viral or bacterial infections. It can be imagined that these microorganisms or the associated inflammatory response, generate cell damage that facilitate *ORT* penetration of the mucosa, although alternative mechanisms such as an up-regulation of relevant host cell receptors, can be hypothesized. Co-infection experiments in which epithelial cells are primed with e.g. ND virus, prior to the addition of *ORT* may shed more light at this key issue.

3. Interaction of *ORT* with macrophages

Consistent with the pathology observations, *ORT* adhered to and became ingested by macrophages (Chapter 3). Although at first sight the interaction with the macrophages may seem to mimic that described for epithelial cells, the infection of both cell types appears to involve separate mechanisms. The main difference between the infections of two cell lines is their sensitivity towards LPS and serum factors. In contrast to *ORT* infection of epithelial cells, infection of macrophages was not inhibited by LPS or serum (Chapters 3 and 4). This strongly suggest that the infection of macrophages is mediated via different bacterial adhesins and / or host cell receptors. Again it should be emphasized that these results were obtained for established macrophage cell lines and thus do not necessarily hold for the diverse set of macrophages present at the various body sites. However, the fact that similar observations were made for chicken and mouse macrophages suggests that, at least with regard to the adhesion and uptake, the interaction with macrophages may follow the general principles described above. The intriguing question as to what is the fate of the *ORT* ingested by the macrophages could again not be addressed because of the lack of an appropriate intracellular survival assay.

Macrophages play an important role in the host defense against invading microorganisms and part of their defense is based on the production of nitric oxide (NO) (Mellouk *et al.*, 1994). As described in Chapter 3, infection of chicken macrophages with *ORT* was followed by an increased production of NO, although the response was much slower than was observed for *E. coli* and *Salmonella* Enteritidis. This slower response is consistent with the apparently rather moderate inflammatory response observed during *ORT* infection compared to e.g. *E. coli* infection of the respiratory tract. The differences in NO response between *ORT* and the other bacteria is likely based on the difference in stimuli that induce the stimulation

of NO production. In contrast to the effects of *E. coli* and *S.* Enteritidis, the stimulation of NO production by *ORT* was not inhibited by the lipid A neutralizing agent polymixin B. This suggests that it was not mediated by LPS but by another unknown stimulus. The lack of responsiveness towards *ORT* LPS seems not related to the structure of the LPS as purified LPS was capable to induce NO production. A likely explanation for the difference in LPS bioactivity among the different species may be the variable exposure of the LPS. The data suggest that in *ORT* the LPS remains firmly embedded within the outer membrane, while *E. coli* and *S.* Enteritidis may release membrane material into the environment.

In contrast to the adherence of *ORT*, stimulation of NO production showed dramatic differences between chicken HD-11 cells and murine J774A.1 macrophages (Chapter 3). This finding suggests that bacterial adherence and stimulation of NO production are independent phenomena. The difference in NO responsiveness between macrophage lines is important as it may translate to the natural situation. It has been reported that LPS stimulated macrophages obtained from chickens with a different genetic background showed different levels of expression of the enzyme nitric oxide synthase as well as NO production (Hussain and Qureshi, 1997). Translated to *ORT* infection, the genetic differences in macrophage responsiveness may manifest as variation in pathology. It would be of interest to learn more about the variation in *ORT* associated pathology between chicken lines.

4. Infection inhibitory activity of serum

The importance of taking the complexity of the natural infection into account in studies on the pathogenesis of bacterial infections is illustrated once more by the observation that serum exhibits infection inhibitory activity towards *ORT* infection of epithelial cells (Chapter 4). This important finding may proof to be particularly valuable in the further identification of the bacterial adhesins and/or receptors that drive the initial colonization of *ORT* as well as in the prevention of colonization. The specificity of the serum inhibition for epithelial cells compared to macrophages clearly indicates that *ORT* carries multiple cell-type specific adhesins.

The exact nature of the inhibitory compounds and the mechanism via which serum inhibits the infection remain to be resolved. The properties of the infection inhibitory activity were explored by biochemical analysis and binding assays with labeled purified molecules. This revealed that the activity co-purified with the serum glycoprotein and serum glycolipid fraction, suggesting that it may be contained in the attached carbohydrate moieties. Importantly, the serum compound(s) exerted their effect by influencing the adhesive properties of the bacteria rather than the host cells. Thus the mechanism appears to resemble that noted for the inhibitory effect of bacterial LPS. The inhibition by serum however, showed no strain specificity in contrast to LPS, and the binding of biotinylated isolated serum protein to the bacteria was blocked by serum but not LPS. This suggests that serum and LPS act through possibly related but different mechanisms. It may be noteworthy that a similar cell type-specific inhibition of infection by serum has recently been reported for the bacterial pathogen *Listeria monocytogenes* (Hertzig *et al.* 2003). In this study, *L. monocytogenes* invasion to the epithelial cells was reduced in the presence of human serum, while no effect was observed towards the infection of macrophages. The serum compounds responsible for this effect have not yet been resolved either.

Serum also inhibited the haemagglutination caused by solid phase grown *ORT* strain 41. The molecular basis for this phenomena is uncertain, although it seem to be different than for the inhibition of the *ORT* - epithelial cell interaction as the haemagglutination was not blocked by LPS and the haemagglutination activity was lost when the bacteria gained their ability to adhere to epithelial cells (Chapter 4). The apparent presence of a different serum-sensitive epithelial cell adhesin and haemagglutinin, and of a serum-resistant adhesin that confers the interaction of *ORT* with macrophages indicates the existence of at least three different cell-type specific *ORT* adhesins. It is currently unknown what conditions promote the haemagglutination activity and whether all strain have the intrinsic capability promote haemagglutination. The occurrence of haemagglutination during the natural infection may contribute to the observed obstruction of the capillaries at the advanced stages of pulmonary infection.

5. Plasmids and the potential use of the *E. coli* - *ORT* shuttle vector

Bacterial pathogenicity strongly depends on the repertoire of virulence genes carried by the pathogen. Knowledge of these genes and of the regulatory elements that control their transcription are crucial to understand the emergence of novel pathogens and to further delineate the bacterial strategies exploited to establish an infection. To accommodate this need, genetic tools to manipulate the bacterium are extremely helpful. For *ORT*, no such tools were available. Development of a system to introduce foreign DNA into *ORT* encountered major obstacles as plasmids derived from close or more distantly related bacterial species could not be introduced and propagated in *ORT*. A breakthrough in developing a genetic transformation system for *ORT* was achieved when parts of a rare cryptic plasmid (pOR1) of *ORT* were exploited as vector (Chapter 5).

Use of parts of pOR1 as part of an *E. coli* - *ORT* shuttle vector required sequencing and extensive analysis of pOR1. The plasmid which could be identified in only two isolates among the numerous *ORT* strains that were analyzed, comprised 14,787 bp, and contained 14 open reading frames that encoded proteins of at least 100 amino acids (Chapter 5). The fact that the plasmid could not be detected in most clinical isolates suggests that it does not encode critical virulence determinants. Sequence analysis revealed that the plasmid may have a function in the transport of heavy metals but whether this is sufficient beneficiary to *ORT* to maintain can be questioned.

The most interesting and useful part of pOR1 was the replication region that obviously contained all information to enable replication of the plasmid within *ORT*. Introduction of the putative replication origin, iteron, *repA* and *rep2* genes into the vector pGEM7 yielded the shuttle vector pOREC1. This vector replicated in *E. coli* and could be transferred to various *ORT* strains by electrotransformation (Chapter 5). The introduction of the cefoxitin resistance gene (*cfxA*) and the selection of *ORT* transformants on the basis of this resistance demonstrated the potential of the shuttle vector as a vehicle to introduce foreign genes into *ORT*.

To fully exploit the potential of the shuttle vector e.g. in allelic recombination experiments, the problem of the rather low efficiency of transformation has to be addressed. The low frequency of transformation is probably caused by the rigidity of the bacterial membrane as survival assays following electroporation demonstrated limited bacterial killing indicative of a poor permeabilization of the membrane. This problem may be solved by weakening the cell wall prior to transformation e.g. with the help of lysozyme or antibiotics. Whether additional restriction barriers between *E. coli* and *ORT* also contribute to the low transformation efficiency remains to be determined.

A second problem that may arise in the utilization of pOREC1 is the poor translation of foreign proteins. This is likely caused by the abnormal codon usage of *ORT* compared to many other bacteria. This problem became apparent after the failed construction of fluorescent *ORT* via the introduction pOREC1 carrying the EGFP gene. Although the gene was transcribed and thus contained a functional promoter, no protein could be detected. Obviously, this problem does not exist for use of the shuttle vector for targeted mutagenesis purposes or complementation studies in which mutated strain are complemented *in trans* with an intact copy of the disrupted *ORT* gene.

Despite its limitations that likely can be solved in the near future, the developed *E. coli* - *ORT* shuttle vector has great potential. The vector can be exploited for random and targeted mutagenesis and complementation studies, and thus nicely complements the functional approach followed in the most part of the present work. In addition, the vector may be used to construct attenuated strains that can be used as vaccines.

6. Implications for infection intervention and prevention

ORT causes disease in chickens and turkeys with considerable economic losses due to lower production, growth retardation and high mortality rates that range from 2 to 11% in chickens (Chin and Droual, 1997; Hafez, 1996) and to sometimes even up to 50% in older turkeys (DeRosa *et al.*, 1996;

Tahseen, 1997). The rapid emergence of antibiotic resistance as illustrated by the 80-100% resistance of *ORT* strains collected from 45 different broiler farms (Devriese *et al.*, 2001), indicates that future containment of *ORT* via the use of antibiotics is no option. The only way to control the disease seems to be vaccination. Vaccination of broiler breeders has been demonstrated to induce long-standing immunity that protected the progeny against experimental challenge with *ORT* for 30 days (van Empel and van den Bosch, 1998). Similarly, live or killed *ORT* vaccines seem to protected susceptible turkeys against development of pathologic lesions (Bock *et al.*, 1997; Sprenger *et al.*, 2000). In a novel approach, Lopes *et al.* (2002a; 2002b) selected a temperature-sensitive (Ts) mutant of *ORT* for use as a live vaccine. This vaccine is based on the ability of the mutant to colonize the upper respiratory tract but not the lower respiratory tract and other organs of the birds. The Ts vaccine elicits a secretory immune response (IgA) and protects against challenge. Despite these promising results the current vaccines appear to provide protection against a limited window of strains. In addition, their composition and mechanism of action are ill-defined, leaving room for the emergence of vaccine resistant strains.

The research approach and results described in this thesis are the first steps towards better understanding of the molecular events that attribute to the establishment of an infection. This work should provide a solid basis to guide future vaccine development by targeting key molecules of infection. Our data suggest that *ORT* carries at least three different adhesins that confer cell-type specific interaction with epithelial cells, macrophages and red blood cells, respectively. For vaccine purposes, identification of the adhesion that confers adherence to epithelial cells seems of most interest. The finding that this interaction can be inhibited by purified *ORT* LPS and serum factors provides the optimal setting to identify and purify the adhesin. Subsequent *ORT* challenge studies should demonstrate the vaccine potential of the adhesin. The function of the molecule can further be demonstrated via analysis of the behavior of defined *ORT* mutants that can be constructed with the developed *ORT* transformation system. When the mutants are compromised in their ability to colonize birds, the attenuated strain may be tested for its vaccine potential. At the same time, a more random genetic approach may be followed to identify additional vaccine candidates that e.g. do not infect macrophages or colonize without causing pathology.

Complementary to the vaccine approach, it seems appropriate to further elucidate the mechanisms via which serum and LPS inhibit epithelial cell infections as these properties can be exploited to prevent *ORT* infection. As the infection inhibiting serum factors are a natural compound and target the bacteria rather than the host cell, they likely exert their effect without toxicity. The greatest challenge to fulfill this dream is probably to deliver the compound(s), once identified, at sufficient high concentration at the appropriate niche.

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SUMMARY

Summary

Since its discovery, about 10 years ago, the bacterium *Ornithobacterium rhinotracheale* (*ORT*) has emerged as a serious pathogen of turkeys and chickens. The bacterium possibly made its transition from wild birds to domesticated poultry about twenty years ago. In poultry, *ORT* has been associated with clinical signs of respiratory distress, as well as more systemic infections, including arthritis and meningitis. Nowadays *ORT* causes considerable economic loss to the poultry industry and much effort is given to treat and prevent the disease. The diagnosis of *ORT* infection is based on relatively non-specific clinical signs, in combination with the isolation of the bacterium and/or serology but many infections are probably frequently missed. Treatment of the disease is complicated by wide spread resistance of *ORT* to a number of antibiotics. Several vaccines have been developed, which seem to provide protection against a limited number of serotypes. The present study was designed to better understand the pathogenesis of the infection, in the belief that, in the long run, solid scientific knowledge of the mechanisms that contribute to the establishment of an infection could be the best basis for the development of novel infection intervention and prevention strategies.

In Chapter 2, the primary interaction of *ORT* with epithelial cells was studied at the cellular level. *ORT* efficiently adhered to epithelial cells in a dose-dependent fashion, reaching levels of up to 80 bacteria per cell. Comparison of the adhesive properties of different strains indicated that strain 41, virtually, lacked the ability to infect epithelial cells. This strain gained an adhesive phenotype when it was grown in suspension rather than on a solid phase medium. Comparison of the outer membrane composition of the various strains by polyacrylamide gel electrophoresis (SDS-PAGE), revealed that the lipopolysaccharide (LPS) of *ORT* is composed of a heterogeneous set of small oligosaccharides. Strain 41 exhibited a different type of lipopolysaccharide from the other strains that were analyzed. Infection assays in the absence and presence of purified LPS, demonstrated that LPS inhibits the adherence of *ORT* to epithelial cells. This effect was strain specific, i.e. it was only seen when endogenous LPS was used. LPS exerted its infection inhibitory activity by influencing the adhesive properties of the bacteria rather than the host cell. This suggests that it may act as a receptor analogue.

In Chapter 3, the interaction of *ORT* with macrophages was studied. This topic of investigation was chosen as pathological examination of *ORT* infected animals indicated a close association of the bacterium with macrophages. *ORT* efficiently adhered to and became ingested by both chicken and murine macrophages. After prolonged infection the bacteria appeared to be located inside intracellular vacuoles. LPS did not block the interaction of *ORT* with the macrophages in contrast to the *ORT* adhesion to epithelial cells. This finding led to the conclusion that *ORT* carries multiple cell-type specific adhesins.

The macrophage response to *ORT* was investigated using the production of nitric oxide as an inflammatory marker. NO exhibits bactericidal activity but it is also a regulator of the intracellular inflammatory signaling cascade and thus is a central molecule in innate host defenses. As described in Chapter 3, *ORT* is able to induce the production of NO by macrophages. This stimulation did not require but was enhanced by rChIFNγ. This cytokine is thought to enhance NO production by increasing the activity of the key enzyme nitric oxide synthase (iNOS). The NO response stimulated by *ORT* differed from that induced by the Gram-negative bacteria *E. coli* and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis). The *ORT* response was slower and was not inhibited by the lipid A neutralizing agent polymixin B. This implies that the effect was not caused by LPS. In purified form, LPS derived from *ORT* did stimulate NO production. This indicates that the LPS of *ORT* is bioactive but apparently not in the context of the intact bacterium. Comparison of the NO response in chicken and mouse macrophages demonstrated that the stimulation of NO production by *ORT* was specific for chicken macrophages. Mouse macrophages were not susceptible to *ORT* but did respond to *E. coli* and *S.* Enteriditis. This differential responsiveness towards *ORT* may contribute to the host specificity of the *ORT* associated pathology, although it should be noted that large differences between macrophage populations exist.

Although the unraveling of bacteria-host cell interaction, with aid of *in vitro* infection model systems, is almost indispensable to unravel key determinants of infection, it does not take into account the significance of the complexity of natural infection. In Chapter 4, we addressed this issue and investigated the effect of serum on the interaction of *ORT* with various kinds of host cells. Both bovine and chicken serum inhibited *ORT* infection of epithelial cells in, a dose-dependent fashion. Further analysis demonstrated that the inhibition was caused via an effect of serum factors on the adhesive properties of the bacteria rather than on the host cells. Binding assays, using biotinylated isolated serum glycoproteins showed direct binding of multiple serum glycoproteins to the bacteria. Serum inhibition was not observed in the interaction of *ORT* with macrophages, supporting the idea that *ORT* carries multiple cell-type specific adhesins.

The striking resemblance between the inhibitory effect of serum and LPS on the *ORT*-host cell interaction, led us to test the hypothesis that LPS and serum exerted their inhibitory effects through interaction with the same bacterial ligand. Competitive binding assays, in which the bacteria were first incubated with LPS or serum and subsequently with biotinylated isolated serum glycoproteins that bound to the bacteria, proved that the serum but not LPS competitively inhibited the binding of the biotinylated serum proteins to *ORT*. The difference between the effect of serum and LPS was confirmed by the difference in strain specificity of the inhibition and the differential inhibition of *ORT*-mediated haemagglutination. Based on these data it was concluded that serum and LPS inhibit *ORT* infection of epithelial cells via related but different mechanisms (Chapter 4).

An additional noteworthy finding was the variable ability among *ORT* strains to cause haemagglutination of chicken red blood cells. One strain of *ORT* (strain 41) was unable to adhere to epithelial cells but did cause haemagglutination when the bacteria were grown onto solid phase media. When the bacteria were grown in suspension, opposite binding characteristics were observed. The apparent transition between phenotypes with different cell-type specificities (erythrocytes versus epithelial cells), dependent on the media growth conditions, may indicate the existence of a regulatory mechanism that enables the bacteria to adapt to various environmental niches encountered in the host.

In Chapter 5, a transformation system for *ORT* is introduced that for the first time enables genetic modification of *ORT*. This is a major step as genetic manipulation of bacterial pathogens is nowadays an important factor in the identification and mutagenesis of putative virulence determinants and their regulatory components and in the development of attenuated strains with vaccine potential. The developed transformation system is based on a cryptic plasmid that is found in very few *ORT* isolates. This plasmid was completely sequenced and characterized. It contained up to 14 different open reading frames of which some appear to encode the proteins involved the transport of heavy metals across the membrane. Introduction of the origin of replication and several regulatory sequences of this plasmid (designated pOR1) into the vector pGEM7, yielded a shuttle vector that replicated as an independent unit, in both *E. coli* and *ORT*. Introduction of an antibiotic resistance gene *cfxA* from *Bacteroides vulgatus* into the plasmid and the transfer of this plasmid to *ORT* rendered *ORT* resistant to the antibiotic. This confirmed that the plasmid could be utilized for bacterial genetics.

As discussed in Chapter 6, the data presented in this thesis can be considered as a first, but major step in the unraveling of the pathogenesis of *ORT* infection. Identification of the multiple *ORT* adhesins and their corresponding host cell receptors, the mechanism by which LPS inhibit the infection in a strain-specific fashion and the nature of the inhibitory compounds in serum, are major future challenges that should provide a solid basis for the development of novel vaccines and complementary infection prevention strategies.

SAMENVATTING

Samenvatting

De bacterie *Ornithobacterium rhinotracheale* (*ORT*) is een belangrijke oorzaak van luchtweginfecties infectieziekten bij pluimvee. Onderzoek achteraf heeft aangetoond dat de bacterie waarschijnlijk afkomstig is van wilde vogels en ongeveer 20 jaar geleden voor het eerst problemen gaf in pluimvee. In deze dieren wordt de aanwezigheid van *ORT* niet alleen in verband gebracht met luchtweginfecties, maar ook met meer systemische infecties waaronder gewrichtsontstekingen en hersenvliesontsteking. Heden ten dage brengen *ORT* infecties aanzienlijke schade toe aan de pluimvee industrie. Bestrijding en preventie van de ziekte heeft dan ook grote prioriteit. Het bestaan van een infectie als gevolg van *ORT* wordt vastgesteld op basis van de aanwezigheid van niet-specifieke klinische symptomen in combinatie met isolatie van de bacterie en/of een positieve serologie. Behandeling van de ziekte wordt in toenemende mate bemoeilijkt door een wijd verbreide verworven resistentie van *ORT* tegen diverse antimicrobiële middelen. Er zijn intussen verschillende vaccins ontwikkeld. Deze beschermen echter slechts tegen een beperkt aantal serotypes. Het onderzoek beschreven in dit proefschrift is opgezet om de pathogenese van de infectie beter te begrijpen ervan uitgaande dat, op de lange termijn, gedegen wetenschappelijke kennis van de mechanismen die bijdragen tot het onstaan van een infectie de beste basis is voor het ontwikkelen van nieuwe vormen van infectie-interventie en preventie.

Een belangrijke stap in het onstaan van de meeste infecties is de hechting van de bacteriën aan het slijmvliesepitheel. In hoofstuk 2 wordt de initiële interactie van *ORT* met epitheelcellen beschreven. *ORT* heeft het vermogen om op een snelle en efficiënte wijze aan epitheelcellen te hechten. Afhankelijk van de toegediende dosis kunnen tot 80 gehechte bacteriën per epitheelcel worden waargenomen. Vergelijking van de hechtingseigenchappen van verschillende bacteriestammen toonde aan dat *ORT* stam 41 vrijwel niet in staat was om aan de epitheelcellen te hechten in tegenstelling tot de andere onderzochte stammen. Goede aanhechting van stam 41 kon echter worden geïnduceerd door de bacteriestam te groeien in vloeibare media in plaats van op agarplaten. Vergelijking van de samenstelling van geïsoleerde buitenmembranen van de verschillende bacteriestammen met behulp van SDS-electroforese toonde aan dat het lipopolysaccharide (LPS) van *ORT* bestaat uit een heterogene set van kleine oligosacchariden en dat stam 41 een ander LPS patroon heeft dan de andere stammen. *In vitro* infectie-experimenten met gekweekte epitheelcellen in de aan- en afwezigheid van gezuiverd LPS toonde aan dat LPS de hechting van *ORT* aan epitheelcellen remt. Dit effect was stam-specifiek, d.w.z. er trad alleen remming van de hechting op met LPS afkomstig van de eigen stam. Deze remming kwam tot stand door een effect van LPS op de bacterie; Preincubatie van de epitheelcellen met LPS gaf geen remming van de hechting. Deze bevindingen zouden erop kunnen wijzen dat LPS zich gedraagt als een receptor analoog en op deze wijze voorkomt dat bacteriële hechtingseiwitten aan LPS-achtige moleculen op de gastheercel kunnen binden.

In hoofstuk 3 is het onderzoek naar de interactie tussen *ORT* en macrofagen beschreven. Pathologisch onderzoek van met *ORT* besmette dieren heeft aangetoond dat de bacteria zich ten tijde van een natuurlijke infectie veelvuldig in de nabijheid of wellicht zelfs in macrofagen bevindt. De resultaten beschreven in hoofdstuk 3 tonen aan dat *ORT* inderdaad hecht en wordt opgenomen door zowel gekweekte kippen- als muizenmacrofagen. De interactie van *ORT* met macrofagen werd niet geremd door het toevoegen van gezuiverd LPS. Dit in tegenstelling tot de interactie met de epitheelcellen. Deze bevinding wijst erop dat *ORT* verschillende cel-type specifieke hechtingseiwitten bezit.

De reactie van macrofagen op het contact met *ORT* werd onderzocht aan de hand van de productie van nitric oxide (NO). NO is een zeer belangrijke molecuul bij het in gang zetten van de afweer van de gastheer en heeft bovendien bacterie-dodende eigenschappen. Zoals beschreven in hoofdstuk 3 leidt infectie met *ORT* tot een verhoogde productie van NO door de macrofagen. Dit effect vond reeds plaats in afwezigheid van recombinant kippen gamma-interferon (rChIFNγ), hoewel een krachtiger productie van NO werd gevonden in aanwezigheid van dit cytokine. rChIFNγ wordt gedacht de productie van NO te versterken door de activiteit van het sleutelenzym in de productie van NO, nitric oxide synthase (iNOS), te verhogen. De kinetiek van de inductie van NO door *ORT* was wezenlijk anders dan die welke werd

waargenomen bij de gram-negatieve bacteriële species *E. coli* and *Salmonella enterica* serovar Enteridis (*S.* Enteritidis). De toename in NO productie geïnduceerd door *ORT* was veel langzamer en niet geremd door het lipid A neutralizerende polimixin B. Dit in tegenstelling tot de stimulatie van de NO productie door de andere species. Deze vondst wijst erop dat de stimulatie van de NO productie door *ORT* niet wordt veroorzaakt door LPS. Toegediend in gezuiverde vorm was LPS echter wel in staat de productie van NO door macrofagen te verhogen. Dit wijst erop dat het LPS van *ORT* biologisch actief is, maar niet in de vorm zoals die aanwezig is in de intacte bacterie. Vergelijking van de NO productie door kippen- en muizenmacrofagen toonde aan dat de stimulatie van de NO productie door *ORT* specifiek was voor kippenmacrofagen. Muizenmacrofagen gaven geen toename in NO productie te zien, hoewel deze cellen wel reageerden op *E. coli* and *S.* Enteriditis. Het verschil in reactie van kippen- en muizenmacrofagen op *ORT* zou kunnen bijdragen aan de gastheerspecifieke pathologie van *ORT* infecties. Deze conclusie moet echter nog verder worden bevestigd aangezien de resultaten werden verkregen met gekweekte macrofagen en er *in vivo* waarschijnlijk grote verschillen bestaan tussen verschillende populaties van macrofagen.

Hoewel voor het ontrafelen van de bacterie-gastheer interactie het gebruik van *in vitro* infectiemodel systemen in de praktijk de meest succesvolle strategie is gebleken, gaat deze aanpak voorbij aan de complexiteit van de natuurlijke infectie. Het belang hiervan blijkt uit de resultaten van de experimenten beschreven in hoofdstuk 4. In dit hoofdstuk werd het effect van serum op de interactie van *ORT* met verschillende typen gastheercellen onderzocht. Er werd aangetoond dat zowel runder- als kippenserum in staat is de hechting van *ORT* aan epitheelcellen volledig te remmen. Nader onderzoek toonde aan dat dit effect werd veroorzaakt door een interactie van serumcomponenten met de bacterie. Bindingsexperimenten waarbij gebruik werd gemaakt van gezuiverde gebiotinyleerde serum glycoproteinen toonde directe binding van serumeiwitten aan de bacteria aan. De remming van de hechting door serum werd niet gevonden voor de interactie van *ORT* met macrofagen. Dit ondersteunt de bovengenoemde hypothese dat *ORT* beschikt over verschillende typen hechtingseiwitten.

Op grond van de opvallende overeenkomst tussen de remmende effecten van serum en LPS op de hechting van *ORT* aan epitheelcellen werd de hypothese ontwikkeld dat LPS en serum hun remmende effect wellicht zouden kunnen bewerkstelligen door een interactie met hetzelfde bacteriële ligand. Competitieve bindingsexperimenten waarin de bacterie eerst werd gepreincubeerd met LPS of serum en vervolgens met gezuiverde gebiotinyleerde serumeiwitten toonde echter aan dat serum, maar niet LPS de binding van de serumeitwitten aan de bacterie remde. Het bestaan van een verschil in de mechanismen via welke serum en LPS de hechting aan epitheelcellen remmen, werd verder bevestigd door het verschil in stam-specificiteit van de remming: LPS remde alleen de aanhechting van de eigen stam, terwijl de remming door serum niet stam-specfiek was. Op grond van deze resultaten werd geconcludeerd dat serum en LPS de *ORT* infectie van epitheelcellen remmen via wellicht verwante, maar toch verschillende mechanismen (Hoofdstuk 4).

Een andere opvallende bevinding beschreven in hoofdstuk 4 was de variatie in het vermogen van verschillende *ORT* stammen om kippenerythrocyten te agglutineren. Een van de stammen (stam 41) die, na kweek op agarplaten, nauwelijks het vermogen bezat om aan epitheelcellen te hechten vertoonde sterke haemagglutinatie activiteit. Echter wanneer de bacteriestam werd gegroeid in vloeibaar medium, werden geheel tegenovergestelde eigenschappen gevonden, d.w.z. de bacteriën hechtten nu sterk aan epitheelcellen maar hadden hun vermogen tot haemagglutinatie nagenoeg verloren. Deze klaarblijkelijke overgang tussen fenotypen met een verschillende celtype specificiteit (erythrocyt versus epitheelcel) afhankelijk van de groeiomstandigheden, zou kunnen wijzen op het bestaan van een regulatiemechanisme dat de bacteriën in staat stelt zich aan te passen aan de verschillende milieu's zoals die bestaan in de gastheer.

In hoofdstuk 5 wordt voor het eerst een methode om *ORT* genetisch te transformeren beschreven. Deze resultaten zijn van groot belang aangezien genetische manipulatie van bacteriën hoe langer hoe meer van belang blijkt te zijn voor het identificeren van virulentie-eigenschappen van bacteriën en voor de ontwikkeling van vaccins. De ontwikkelde methode van genetische transformatie is gebaseerd op een cryptisch plasmide dat aanwezig bleek in enkele *ORT* stammen. De volledige DNA volgorde van dit plasmide (pOR1) werd bepaald en geanalyseerd. Het plasmide bleek 14 open reading frames te bezitten

waarvan een aantal lijken te coderen voor eitwitten die betrokken zijn bij het transport van zware metalen over de bacteriemembraan. Van groter belang is echter dat dit plasmide kon worden gebruikt voor het ontwikkelen van een shuttle vector. Door introductie van de plasmide replicatie oorsprong en enkele regulerende sequenties van (pOR1) in de vector pGEM7 onstond een shuttle vector die zich vermeerderde in zowel *E. coli* als *ORT*. Introductie van het antibiotica resistentie gen *cfxA*, afkomstig van *Bacteroides vulgatus,* in de shuttle vector en overdracht van deze vector van *E. coli* naar in *ORT*, resulteerde in cefoxitine resistente *ORT*. Dit bevestigde dat de ontwikkelde shuttle vector geschikt is voor genetische modificatie van *ORT*.

Zoals aangegeven in hoofdstuk 6, kunnen de resultaten beschreven in dit proefschrift worden beschouwd als een eerste, maar belangrijke stap in het ontrafelen van het onstaan van *ORT* infecties. De identificatie van de verschillende bacteriële hechtingseiwitten en hun receptoren op de gastheercellen, van het mechanisme via welk LPS de hechting kan remmen, en van de aard van de infectie-remmende stoffen in serum zijn belangrijke uitdagingen voor toekomstig onderzoek. Kennis van deze factoren zal hopelijk een stevige basis zijn voor het onwikkelen van nieuwe vaccins en andere vormen van infectie-preventie.

บทสรุป

บทสรุป

ตั้งแต่มีการค้นพบแบคทีเรีย *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์ (Ornithobacterium rhinotracheale)* เมื่อ ๑๐ ปีที่ผ่านมาพบว่าแบคทีเรียชนิดนี้ สามารถก่อโรคได้ในไก่และไก่งวง โดยแบคทีเรียชนิดนี้ปรับตัวจากการก่อโรคในนกป่ามายังไก่เมื่อประมาณ ๒๐ ปีที่ผ่านมา ในไก่ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* สามารถก่อให้เกิดอาการผิดปกติของระบบทางเดินหายใจและการติดเชื้อตามระบบ รวมทั้งข้ออักเสบและสมองอักเสบ ปัจจุบัน *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* เป็นสาเหตุให้เกิดความสูญเสียทางเศรษฐกิจอย่างมากต่ออุตสาหกรรมการเลี้ยงสัตว์ปีกจึงมีความพยายามในการควบคุมและป้องกันโรค การวินิจฉัยการติดเชื้อ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* มักอาศัยอาการทางคลินิกซึ่งไม่เฉพาะเจาะจงกับโรค ร่วมกับการแยกเชื้อแบคทีเรีย และ/หรือ ร่วมกับการใช้วิธีทดสอบทางซีรั่มวิทยา แต่ตัวเชื้อก็ยังมีโอกาสหลุดรอดจากการวินิจฉัยได้บ่อยครั้ง การรักษาโรคค่อนข้างมีความซับซ้อน เนื่องจากเชื้อแบคทีเรียชนิดนี้มีความสามารถในการต้านทานยาปฏิชีวนะได้อย่างกว้างขวาง แม้มีการพัฒนาวัคซีนชนิดต่างๆ ขึ้นมาก็พบว่ามีความสามารถในการป้องกันโรคได้อย่างจำกัดเฉพาะซีโรไทป์ การศึกษาในปัจจุบันออกแบบเพื่อให้เกิดความเข้าใจ ในพยาธิกำเนิดของการติดเชื้อ เนื่องด้วยความเชื่อที่ว่า ความรู้ทางวิทยาศาสตร์ที่เข้มแข็งในกลไกของการติดเชื้อ เป็นพื้นฐานที่สำคัญที่สุดในการพัฒนานวัตกรรมของกลวิธีในการป้องกันและควบคุมโรคในระยะยาว

ในบทที่ ๒ เป็นการศึกษาในระดับเซลล์ของปฏิสัมพันธ์ปฐมภูมิของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* กับเซลล์เยื่อบุ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* สามารถเกาะยึดกับเซลล์เยื่อบุได้อย่างมีประสิทธิภาพ ซึ่งแปรผันตามปริมาณของเชื้อ โดยพบจำนวนแบคทีเรียสูงถึง ๘๐ แบคทีเรียต่อเซลล์ การเปรียบเทียบคุณสมบัติการเกาะยึดเซลล์ของแบคทีเรียเสตรนต่างๆ พบว่าเสตรน ๔๑ ขาดความสามารถในการติดเชื้อต่อเซลล์เยื่อบุ เสตรนนี้มีลักษณะฟีโนไทป์ในการเกาะยึดเมื่อได้รับการเพาะเชื้อในอาหารเลี้ยงเชื้อชนิดเหลวแทนการเพาะเชื้อบนผิวอาหารวุ้น การเปรียบเทียบส่วนประกอบของเปลือกชั้นนอกของเยื่อหุ้มเซลล์ของแบคทีเรียเสตรนต่างๆ โดยวิธี sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) พบว่า lipopolysaccharide (LPS) ของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ประกอบด้วยกลุ่ม oligosaccharides เล็กๆ ที่มีขนาดแตกต่างกัน เสตรน ๔๑ แสดง LPS ที่แตกต่างไปจากเสตรนอื่นที่ทำการวิเคราะห์ การทดลองการติดเชื้อที่มีและไม่มี LPS แสดงให้เห็นว่า LPS ยับยั้งการเกาะยึดของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ต่อเซลล์เยื่อบุ จากการทดลองนี้แสดงลักษณะความเฉพาะของเสตรน คือ การยับยั้งการเกาะยึดของแบคทีเรียต่อเซลล์ โดยพบเฉพาะ LPS ที่แยกจากแบคทีเรียเสตรนเดียวกัน และพบว่า LPS มีอิทธิพลต่อแบคทีเรียมากกว่าเซลล์เยื่อบุ ซึ่งแสดงให้เห็นว่าแบคทีเรียน่าจะมีตัวรับต่อ LPS

ในบทที่ ๓ เป็นการศึกษาปฏิสัมพันธ์ของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* และมาโครฟาจ การศึกษาในบทนี้สืบเนื่องมาจากผลทางพยาธิวิทยาของสัตว์ที่ติดเชื้อ มักพบความสัมพันธ์ร่วมกันระหว่างแบคทีเรียและมาโครฟาจ โดยพบว่า *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* มีประสิทธิภาพในการเกาะยึดและถูกกลืนทำลายโดยมาโครฟาจ ทั้งที่มีต้นกำเนิดจากสัตว์ปีกและหนู และเมื่อเพิ่มระยะเวลาในการทดลองการติดเชื้อจะพบแบคทีเรียอยู่ภายในแวคิวโอลส์ภายในเซลล์มาโครฟาจ ปฏิสัมพันธ์ระหว่าง *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* และมาโครฟาจไม่ถูกยับยั้งโดย LPS ซึ่งตรงข้ามกับการติดเชื้อของเซลล์เยื่อบุ การค้นพบนี้นำไปสู่ข้อสรุปที่ว่า *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* มีแอดฮีซินเฉพาะกับเซลล์หลายชนิด การศึกษาการตอบสนองของมาโครฟาจต่อ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* โดยอาศัยผลผลิตของไนตริก ออกไซด์ (Nitric Oxide) ในฐานะที่เป็นตัวบ่งชี้ (marker) ของการอักเสบ ไนตริก ออกไซด์มีความสามารถในการทำลายแบคทีเรีย และยังมีความสามารถในการเป็นตัวควบคุม (regulator) ของลำดับสัญญาณ

(signaling cascade) ของการอักเสบอีกทั้ง ไนตริก ออกไซด์ ยังเป็นโมเลกุลหลักของการป้องกันโดยกำเนิดของเซลล์เจ้าบ้าน ตามที่ได้อธิบายในบทที่ ๓ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* สามารถเหนี่ยวนำการผลิต ไนตริก ออกไซด์จากมาโครฟาจ การกระตุ้นนี้ไม่ต้องการอินเตอร์เฟียรอนแกมม่าสังเคราะห์ของไก่ (recombinant chicken inteferon gamma, rChIFNγ) แต่ rChIFNγ สามารถเพิ่มผลผลิต ไนตริก ออกไซด์ ได้ การที่ไซโตไคน์ช่วยเพิ่มการผลิต ไนตริก ออกไซด์ อาจเนื่องจากการไปเพิ่มกิจกรรมของเอนซัยม์ตัวสำคัญ คือ ไนตริก ออกไซด์ ซินเทส (nitric oxide synthase) การตอบสนองของ ไนตริก ออกไซด์ ต่อการกระตุ้นโดย *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* แตกต่างจากการเหนี่ยวนำด้วยแบคทีเรียแกรมลบชนิดอื่น *คือ อี. โคไล* และ *ซัลโมเนลล่า เอ็นเตอริก้า* ซีโรวาร์ เอ็นเตอร์รัยติดิส (*S.* Enteritidis) โดยการตอบสนองของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ลดลงและไม่ถูกยับยั้งโดยโพลีมิกซิน บี ซึ่งมีคุณสมบัติในการหักล้างฤทธิ์ของลิปิด เอ จากผลการทดลองนี้ แสดงให้เห็นว่าการกระตุ้นการผลิต ไนตริก ออกไซด์ไม่ได้เกิดจาก LPS ของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* เพียงลำพัง โดยพบว่า LPS บริสุทธิ์ที่สกัดได้จาก *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* กระตุ้นการผลิต ไนตริก ออกไซด์ ซึ่งแสดงให้เห็นว่า LPS ของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* มีฤทธิ์ทางชีวภาพ เปรียบเทียบจากการตอบสนองการผลิต ไนตริก ออกไซด์ ในมาโครฟาจจากไก่และ-หนู แสดงให้เห็นว่าการกระตุ้นการผลิต ไนตริก ออกไซด์ โดย *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* มีความเฉพาะต่อมาโครฟาจจากไก่ โดยพบว่า มาโครฟาจจากหนูไม่ตอบสนองต่อการกระตุ้นด้วย *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* แต่ตอบสนองต่อการกระตุ้นด้วย *อี. โคไล* และ *S.* Enteritidis การตอบสนองที่แตกต่างกันของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* นี้อาจเกิดเนื่องจากความเฉพาะเจาะจงของเซลล์เจ้าบ้านร่วมกับการเกิดพยาธิสภาพ อย่างไรก็ตาม ควรตั้งข้อสังเกตว่า มีความแตกต่างอย่างมากระหว่างมาโครฟาจชนิดต่างๆ ในการตอบสนองการกระตุ้นด้วย *ออร์นิโธ-แบคทีเรียม ไรโนเทรเคียอัลเล่ย์*

แม้เป็นที่แน่ใจได้ว่าปฏิสัมพันธ์ระหว่างเซลล์เจ้าบ้านและแบคทีเรีย ช่วยในการเสริมความเข้าใจในรูปแบบการติดเชื้อในหลอดทดลอง ซึ่งมีความจำเป็นในการค้นพบกุญแจสำคัญในการติดเชื้อ แต่การติดเชื้อในหลอดทดลองอาจไม่สัมพันธ์กับการติดเชื้อในธรรมชาติ ในบทที่ ๔ ได้กล่าวถึงความสำคัญในประเด็นนี้และทำการศึกษาผลของซีรั่มต่อปฏิสัมพันธ์ของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* กับเซลล์เจ้าบ้านชนิดต่างๆ พบว่าซีรั่มจากโคและไก่สามารถยับยั้งการติดเชื้อ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ของเซลล์เยื่อบุ โดยการยับยั้งนี้มีความสัมพันธ์กับความระดับเข้มข้นของซีรั่ม การวิเคราะห์ต่อมาแสดงให้เห็นว่า การยับยั้งนี้มีสาเหตุจากผลของปัจจัยในซีรั่มต่อคุณสมบัติในการเกาะยึดของแบคทีเรียมากกว่าผลที่มาจากเซลล์เจ้าบ้าน การเชื่อมไบโอตินเข้ากับซีรั่มไกลโคโปรตีน ได้แสดงให้เห็นถึงการจับกัน โดยตรงระหว่างซีรั่มไกลโคโปรตีนต่างๆ กับแบคทีเรีย การยับยั้งโดยซีรั่มนี้ไม่พบในปฏิสัมพันธ์ระหว่าง *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* กับมาโครฟาจ ซึ่งเป็นการสนับสนุนความคิดที่ว่า *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ประกอบด้วยความสามารถในการเกาะยึดที่เฉพาะต่อเซลล์ชนิดต่างๆ

ความคล้ายคลึงที่น่าสนใจระหว่างผลของการยับยั้งโดยซีรั่มและ LPS ต่อปฏิสัมพันธ์ระหว่าง *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* กับเซลล์เจ้าบ้าน ส่งผลต่อการทดสอบสมมติฐานว่า LPS และซีรั่มอาศัยผลในการยับยั้งการเกาะยึดโดยผ่านไลแกน (ligand) ของแบคทีเรียชนิดเดียวกันหรือไม่ วิธีการแข่งขันการเกาะยึด โดยการบ่ม (incubate) แบคทีเรียกับ LPS หรือซีรั่ม และต่อมาบ่มแบคทีเรียด้วยไบโอตินเชื่อมกับซีรั่มไกลโคโปรตีนซึ่งเกาะกับผิวแบคทีเรีย ความแตกต่างระหว่างผลของซีรั่มและ LPS ถูกยืนยันโดยอาศัยความแตกต่างของความเฉพาะของเสตรนของการยับยั้ง และการยับยั้งที่แตกต่างของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ด้วยวิธีการตกตะกอนกับเม็ดเลือดแดง (haemagglutination) จากข้อมูลเหล่านี้ สามารถสรุปได้ว่า ซีรั่มและ LPS ยับยั้ง การติดเชื้อ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ต่อเซลล์เยื่อบุ ด้วยกลไกที่แตกต่างกัน (บทที่ ๔)

การค้นพบอื่นจากการศึกษานี้ คือ ความแปรผันระหว่างเสตรนต่างๆ ของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ต่อการตกตะกอนกับเม็ดเลือดแดงของไก่ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* เสตรน ๔๑ ไม่สามารถเกาะยึดกับเซลล์เยื่อบุ แต่สามารถตกตะกอนกับเม็ดเลือดแดงเมื่อทำการเพาะเชื้อแบคทีเรียบนผิวอาหารวุ้น แต่เมื่อทำการเพาะเชื้อแบคทีเรียในอาหารเหลวสามารถสังเกตพบลักษณะตรงข้ามที่กล่าวมา โดยพบการเปลี่ยนของฟีโนไทป์กับความเฉพาะของชนิดเซลล์ที่แตกต่างระหว่างเม็ดเลือดแดงกับเซลล์เยื่อบุ โดยขึ้นกับสภาพในการเพาะเลี้ยงเชื้อแบคทีเรียบ่งชี้ถึงการปรากฏของกลไกในการควบคุม ซึ่งส่งเสริมให้แบคทีเรียปรับตัวต่อการเปลี่ยนแปลงของสภาพแวดล้อมต่อเซลล์เจ้าบ้าน

ในบทที่ ๕ เป็นการศึกษาระบบการส่งผ่านสารพันธุกรรม (transformation system) ของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ซึ่งเป็นการศึกษาการเปลี่ยนแปลงทางพันธุกรรมของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* เป็นครั้งแรก ถือเป็นก้าวย่างที่สำคัญของการจัดการทางพันธุกรรมของแบคทีเรียก่อโรค ซึ่งปัจจุบันพบว่ามีความสำคัญในการพิสูจน์และการเปลี่ยนแปลงของตัวกำหนดความรุนแรงที่เป็นที่ยอมรับและเป็น ส่วนประกอบในการควบคุมการก่อโรคของแบคทีเรีย และการพัฒนาเสตรนแบคทีเรียเพื่อศักยภาพในการนำไปใช้เป็นวัคซีน การพัฒนาระบบการส่งผ่านสารพันธุกรรมนี้ โดยการศึกษาพลาสมิด (plasmid) ซึ่งพบในไม่กี่เสตรนของ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* พลาสมิดนี้ได้ทำการสืบหาลำดับเบส (sequence) ที่สมบรูณ์พร้อมกับจำแนกยีนชนิดต่างๆ โดยพบว่าพลาสมิดนี้ประกอบด้วยกรอบการอ่านยีน (open reading frames) ๑๔ จุดที่แตกต่างกัน โดยบางส่วนนั้นได้ให้รหัส (encode) ของโปรตีนซึ่งเกี่ยวพันกับการขนส่งโลหะหนักข้ามเยื่อหุ้มเซลล์ การนำบางส่วนของลำดับเบสที่เกี่ยวข้องกับการเพิ่มจำนวนและการควบคุมการเพิ่มจำนวนของพลาสมิดนี้ใส่ในเวคเตอร์ pGEM7 ทำให้ได้เวคเตอร์กระสวย (shuttle vector) ซึ่งให้ชื่อว่า pOR1 ซึ่งสามารถเพิ่มจำนวนในฐานะที่เป็นหน่วยอิสระทั้งในเซลล์ *อี. โคไล* และ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* การนำยีนที่ควบคุมการต่อต้านยาปฏิชีวนะ *cfxA* จาก *แบคทีรอยเดส วาลกาตัส* (*Bacteroides vulgatus*) ไปใส่ในพลาสมิดช่วยทำให้พลาสมิดนี้มีความต้านทานต่อยาปฏิชีวนะ ซึ่งยืนยันว่าพลาสมิดนี้สามารถใช้สำหรับการศึกษาพันธุกรรมของแบคทีเรียได้

ในบทที่ ๖ เป็นบทวิจารณ์ โดยข้อมูลที่ปรากฏในวิทยานิพนธ์นี้ ถือได้ว่าเป็นก้าวแรกแต่เป็นก้าวสำคัญ ในการศึกษาพยาธิกำเนิดของการติดเชื้อ *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* ซึ่งไม่สามารถโต้แย้งได้ การพิสูจน์พบสารเกาะยึด *ออร์นิโธแบคทีเรียม ไรโนเทรเคียอัลเล่ย์* หลายชนิดและความสื่อสารกับตัวรับของเซลล์เจ้าบ้าน กลไกที่ LPS ยับยั้งการติดเชื้อในรูปแบบเฉพาะต่อเสตรน และธรรมชาติของส่วนประกอบการยับยั้งในซีรั่มเป็นการท้าทายที่สำคัญในอนาคตว่าสามารถใช้ความรู้เหล่านี้ในการผลิตวัคซีนชนิดใหม่ที่มีประสิทธิภาพในการป้องกันโรค

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Utrecht, April 2004

Niwat Chansiripornchai

CURRICULUM VITAE

Niwat Chansiripornchai was born on 2nd June 1969 in Bangkok, Thailand. His primary and secondary school education took place at two local schools, Rajvinij Prathombangkae and Wat Nualnoradit schools. After finishing high school, in June 1987 he passed the general entrance examination to commence his undergraduate studies at the Faculty of Veterinary Science, Chulalongkorn University. During his time there, he received annual awards for top ten academic performance as a veterinary student. In March 1993, he earned the degree of Doctor of Veterinary Medicine. In April, he became an instructor at the Division of Avian Medicine, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. His work was in the field of avian medicine, epidemiology and disease prevention and control. After 5 years of teaching and research, he was promoted to assistant professor, in April 1998. In September 1998, he obtained a scholarship from the Swedish Foundation for International Co-operation in Research and Higher Education (STINT), to study Master Science in the field of Molecular Microbiology, at the Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden. He was given his Master Science degree in March 2000. During this time, he obtained a fellowship from the Utrecht Scholarship Program and the Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. In May 2000, he started his Ph.D. study at the Division of Bacteriology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University. His research topic was the Molecular Interaction of *Ornithobacterium rhinotracheale* with Eukaryotic cells under the supervision of Prof. Dr. Jos van Putten. The results of his work are described in this thesis, which will open in public discussion on 1st April 2004. After graduation, he will return to the Division of Avian Medicine, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, as an Assistant Professor.

LIST OF PUBLICATIONS (not included in this thesis)

1. **Chansiripornchai, N.** and Sasipreeyajan, J. 2002. Efficacy of sarafloxacin in broilers after experimental infection with *Escherichia coli*. Vet. Res. Comm. 26:255-262.

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