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GROWTH RESPONSES OF COLIFORM BACTERIA TO ANTIBODIES DIRECTED AGAINST FERRIC ENTEROBACTIN RECEPTOR FEPA

DISSEPTION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

The Ohio State University

1998

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ABSTRACT

The role of ferric enterobactin receptor FepA in the pathogenesis of bovine mastitis caused by coliform bacteria was investigated. All isolates of *E. coli* (n = 25), and *K. pneumoniae* (n = 25) were positive for siderophore production. Immunoblot technique in conjunction with rabbit anti-FepA serum showed that all isolates expressed 81-kDa FepA under iron-restricted condition. FepA is widely distributed and conserved among coliform isolates from naturally occurring bovine IMI.

The serum and milk IgG responses of lactating dairy cows were determined following immunization with purified FepA. Serum and whey IgG titers to FepA from cows vaccinated with either 100 or 500 µg FepA were significantly higher than those from cows immunized with either *E. coli* J5 bacterin or PBS. Serum and whey IgG titers to FepA were elevated by 14 d after primary immunization with FepA. Immunization with FepA elicited an immunological response in bovine serum and milk.

The abilities of purified bovine anti-FepA IgG and a murine monoclonal antibody directed against FepA to inhibit the growth of coliform bacteria were determined in vitro. Growth of all *E. coli* isolates was significantly inhibited by the addition of monoclonal antibody to synthetic medium containing apolactoferrin. Antigenic variation in the enterobactin binding site resulted in a low percentage of *K. pneumoniae* isolates that were
inhibited by the monoclonal antibody. Addition of 4 mg/ml of purified bovine IgG directed against FepA to synthetic medium resulted in significant growth inhibition for both *E. coli* and *K. pneumoniae* isolates. In dry cow secretion, growth of *E. coli* isolates was inhibited by addition of 4 mg/ml of bovine anti-FepA IgG. Less than half of *K. pneumoniae* isolates (43%) were inhibited by IgG from FepA immunized cows.

Supplementing 50 μM of ferric chloride to the synthetic medium and dry cow secretion completely reversed the inhibitory effects of the antibodies and lactoferrin.

Immunoglobulin G from cows immunized with FepA apparently inhibited the growth of coliform bacteria by interfering with the binding of ferric enterobactin complex to the cell surface receptor FepA.
Dedicated to my parents.
Jian Lin and Guoying Yuan
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proteins of three strains of infectious bursal disease virus. Academic Deliberation

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FIELD OF STUDY

Animal science
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INTRODUCTION

Bovine mastitis is the most costly disease of dairy cattle. Economic losses attributed to mastitis in U.S. were estimated to be approximately $185/cow/year or $1.8 billion per year (National Mastitis Council, 1996). The primary cause of mastitis is intramammary bacterial infection (Bramley et al., 1984; Dodd et al., 1969). Teat dipping and dry cow therapy are effective for mastitis control of contagious bacteria (Dodd et al., 1969; Neave et al., 1969) but ineffective against mastitis caused by environmental bacteria (Bramley et al., 1984; Eberhart et al., 1972; Smith et al., 1985b, 1993). The primary reservoir of environmental bacteria is the cows' environment rather than infected quarters (Eberhart et al., 1979; Smith et al., 1985b). Coliform bacteria are environmental bacteria frequently isolated from bovine IMI (Eberhart et al., 1979; Hogan et al., 1989b; Jasper et al., 1975; McDonald et al., 1970; Todhunter et al., 1991a). Currently, no uniform method exists for controlling coliform mastitis. Because elimination of coliform bacteria from the environment is not economically feasible, increasing cows' resistance against coliform bacteria would be a logical method to reduce coliform mastitis.

The nonlactating period is a critical time in the dynamics of IMI caused by coliform bacteria. Incidences of IMI caused by coliform bacteria was 3 to 4 times higher during the nonlactating period than during lactation (Smith et al., 1985a). Iron is an
essential element for survival and multiplication of many bacteria. However, most iron in 
mammary secretions of nonlactating cows is bound to lactoferrin; concentrations of 
which may reach 20 to 30 mg/ml (Bishop et al., 1976). Consequently, the amount of free 
iron is too low to support the growth of bacteria (Todhunter et al., 1990a). Coliform 
bacteria can overcome this limitation by producing efficient iron chelators (siderophores) 
and iron-regulated outer membrane proteins (Griffiths, E. 1987; Guerinot, 1994; 
Wooldridge, 1993). The iron-regulated outer membrane proteins of Gram-negative 
bacterial pathogens are often suggested as vaccine candidates for immunoprophylactic 
therapy (Byers, 1987; Gray-Owen et al., 1996; Johnson, 1991; Weinberg, 1989; Williams 

The general goal of this research was to determine the role of ferric enterobactin 
receptor FepA in the pathogenesis of bovine mastitis caused by coliform bacteria. 
Specific objectives were:

1) To determine the frequency distribution of siderophore and FepA expression of 
coliform bacteria isolated from naturally occurring bovine IMI.

2) To determine the growth responses of coliform isolates to a monoclonal 
   antibody specific for blocking the ferric enterobactin binding site of FepA.

3) To isolate FepA from coliform bacteria.

4) To determine humoral immune responses following vaccination with FepA.

5) To test the cross-reactivity of antiserum from cows immunized with FepA to 
coliform isolates from naturally occurring IMI.
6) To compare growth responses of coliform bacteria to highly purified bovine IgG from cows immunized with FepA in a chemically defined medium containing apolactoferrin and in a pooled source of dry cow secretion.
CHAPTER 1
REVIEW OF LITERATURE

Coliform Mastitis

Coliform mastitis is a term used to describe mastitis caused by Gram-negative bacteria which belong to the family Enterobacteriaceae (Eberhart et al., 1979; Howell, 1972; Murray, 1990; Smith et al., 1985b; Watts, 1988). Gram-negative bacteria most frequently associated with coliform mastitis are the coliform bacteria that ferment lactose with the production of acid and gas within 48 h at 37 °C (Eberhart et al., 1977, 1979; Murray, 1990). Genera classified as coliform bacteria are *Escherichia*, *Klebsiella*, and *Enterobacter* (Eberhart, 1977). *Escherichia coli* and *Klebsiella pneumoniae* are the coliform species most commonly isolated from bovine IMI (Smith et al. 1985b). In addition to the coliform bacteria, other Gram-negative bacteria such as species of *Serratia, Pseudomonas, Citrobacter, Proteus*, and *Salmonella* have also been isolated from mammary glands of cows with mastitis (Eberhart et al. 1972; Hogan et al., 1989b; Jasper et al., 1975; McDonald et al., 1970; Murphy et al., 1943; Smith et al. 1985b; Todhunter et al., 1991a, 1991b).

In a study of clinical mastitis on eight California dairies, Jasper et al. (1975) reported that *E. coli* accounted for 63% of all Gram-negative bacteria isolated from IMI.
McDonald et al. (1970) and Smith et al. (1985b) also suggested that *E. coli* were the primary Gram-negative bacteria isolated from clinical quarters. Other studies (Dewitte et al., 1980; Eberhart et al., 1972; Murphy et al. 1943; Oliver, 1988; Todhunter, 1991a) have found *Klebsiella* spp., in particular *K. pneumoniae*, as the most frequently isolated microorganism from bovine IMI. Todhunter et al. (1991a) reported that 39% of all cases were caused by *Klebsiella* spp.; 71% of which were identified as *K. pneumoniae*.

*Klebsiella pneumoniae* accounted for 43% of 84 Gram-negative IMI in a 2 year study (Eberhart, 1972). *Enterobacter* spp. have been isolated from IMI in several studies (Dewitte et al., 1980; Eberhart et al., 1972; Jasper et al., 1975; McDonald et al., 1970; Murphy et al., 1943; Smith et al., 1985b; Todhunter et al., 1991a). Approximately 60% of coliform IMI were caused by *Enterobacter aerogenes* in one study (Murphy et al., 1943). Other studies showed that *Enterobacter* spp. accounted for 1.5% (Smith et al., 1985b) to 23.8% (Eberhart et al., 1972) of Gram-negative IMI. Other less commonly isolated Gram-negative bacterial genera included *Serratia, Pseudomonas, Proteus, Citrobacter*, and *Salmonella* (Eberhart et al. 1972; Hogan et al., 1989b; Jasper et al., 1975; McDonald et al., 1970; Murphy et al., 1943; Smith et al. 1985b; Todhunter et al., 1991a, 1991b). Todhunter et al. (1991b) reported that *Serratia* species were isolated from 9% of IMI caused by Gram-negative bacteria. *Serratia marcescens* accounted for 60% of total *Serratia* IMI. *Pseudomonas* species have been isolated from 5% of Gram-negative IMI (Jasper et al., 1975). Three percent (Jasper et al., 1975) to 13% (McDonald et al., 1970) of Gram-negative IMI were caused by *Proteus* species. *Citrobacter* species accounted for as many as 17.1% of IMI in one study (McDonald et al., 1970).
Salmonella species have been isolated from 1% (Jasper et al., 1975) to 3% (McDonald et al., 1970) of coliform IMI.

Unlike IMI caused by contagious bacteria, coliform IMI are frequently associated with clinical signs (Eberhart et al., 1972; Hogan et al., 1989b; Jasper et al., 1975; Smith et al., 1985b). Approximately 80% to 90% of Gram-negative IMI result in clinical mastitis (Smith et al., 1985b). Culture data from several studies have shown that 52% to 85% of all Gram-negative bacterial IMI are associated with clinical signs (Eberhart et al., 1972; Murphy, 1943; Pankey et al., 1987). Most coliform clinical cases are mild with clots and flakes in the milk and moderate to no swelling of the infected mammary gland (Eberhart et al., 1979; Smith et al., 1985b). Jasper et al. (1975) reported that 85% of clinical mastitis cases in 8 commercial dairy herds in California were caused by coliform IMI. A study of clinical mastitis in nine well managed dairy herds (Hogan et al., 1989b) showed that 29.7% of clinical mastitis cases were caused by coliform IMI. Twenty-nine percent of coliform clinical cases (Hogan et al., 1989b) were acute with systemic signs. Although coliform bacteria are the major cause of peracute mastitis in dairy herds (Hazlett et al., 1984), perhaps less than 5% of all coliform IMI resulted in peracute clinical mastitis (Smith, 1983). Peracute cases require intensive therapy and can lead to death (Eberhart et al., 1979; Smith et al., 1985b; Ziv, 1992).

A characteristic of coliform IMI is the infections’ short duration with over half of these IMI lasting less than 10 d (Smith et al., 1985b; Todhunter et al., 1991a). Both experimentally induced (Carroll et al., 1973; Hill et al., 1978; Jain et al., 1971) and naturally occurring (Smith et al., 1985a, 1985b; Todhunter et al., 1991a) coliform IMI
were demonstrated to be of short duration. A high percentage of experimental infections by coliform bacteria were rapidly eliminated. Hill et al. (1978) reported that the majority of glands experimentally infected with serum resist strains of *E. coli* eliminated IMI within 3 to 4 d. Intramammary infusion with either *Klebsiella* spp. or *E. coli* demonstrated that these bacteria disappeared from most of the quarters within 2 weeks (Carroll et al., 1973). Similar results were reported following inoculating quarters with *E. aerogenes* (Jain et al., 1971). Murphy et al. (1943) reported that approximately 35% of naturally occurring coliform IMI that were first detected at calving and during lactation were eliminated from the gland within 14 d. One study (Smith et al., 1985a) reported that 61.5% of naturally occurring coliform IMI originating in the dry period and persisting into lactation were present for less than 8 d. Smith et al. (1985b) further reported that 56.7% of naturally occurring coliform IMI that originated during lactation had durations less than 10 d. Although 71.2% of *E. coli* IMI and 34.0% of *Klebsiella* IMI had a duration of less than 10 d, chronic infections occurred as 29.8% of *Klebsiella* IMI and 1.5% of *E. coli* IMI had a duration of greater than 100 d. Todhunter et al (1991a) reported that mean days infected by *E. coli* (23.6 d) was significantly lower than mean days infected by *Klebsiella* (124.4 d) and *Serratia* (162.1 d). Therefore, chronic *E. coli* infections are rare. Long duration (>200 d) Gram-negative IMI do occur and are likely associated with *Klebsiella* spp. or *Serratia* (Todhunter et al., 1991a). Prevalence of IMI is a function of rate of new infections and the duration of those infections (Bramley et al., 1984). Prevalence of IMI by coliform bacteria seldom exceeds 1-2 % of quarters at any one time in a dairy herd and coliform IMI are rarely the cause of high bulk milk SCC
The short duration of coliform IMI is a major factor determining the low prevalence of infected quarters (Smith et al., 1985b).

Four major physiological and environmental factors influence the coliform IMI patterns: 1) stage of lactation; 2) season of the year; 3) parity; and 4) housing and management. Rates of IMI are generally higher during the dry period than during lactation (Smith et al., 1985a). The dry period contributes significantly to the establishment of new coliform IMI in a dairy herd (Smith et al., 1985a). Eberhart et al. (1972) observed that 41.6% of new IMI occurred during the dry period or within days after calving. One study (Oliver et al., 1983) reported that 45.4% of IMI caused by coliform bacteria and streptococci other than Strep. agalactiae during the early dry period persisted to parturition. Rates of new coliform IMI were not constant throughout the dry period but elevated during the two weeks following drying-off and two weeks prior to calving (Smith et al., 1985a). Because involuting and fully involuted mammary glands are extremely resistant to new E. coli IMI and glands become susceptible during the periparturient period (McDonald et al., 1981; Todhunter et al., 1990a,b), new E. coli IMI during the early or mid dry period are very rare. The majority of E. coli IMI originating in the dry period and present in lactation occur during the 7 to 10 days prior to calving (Todhunter, 1991a). New IMI caused by Klebsiella spp. and other coliform bacteria appear to be evenly distributed between the early and late dry period (Smith et al., 1985a; Todhunter et al., 1991a). Rate of coliform IMI during lactation was highest early in lactation and decreased as lactation advanced (Eberhart et al., 1979; Smith et al., 1985b).
This decrease was reported to be more pronounced for IMI caused by *E. coli* than those by *Klebsiella* spp. (Todhunter, 1991a). Hogan et al. (1989b) reported that incidence of clinical cases caused by coliform bacteria was highest during the first month of lactation.

Season of the year influences the dynamic of coliform IMI during both the dry period and lactation (Smith et al., 1985b; Hogan et al., 1989b; Todhunter et al., 1991a). One study (Smith et al., 1985b) reported that rate of coliform IMI was 3 times higher during summer than other seasons, independent of stage of lactation. Hogan et al. (1989b) reported that the mean rates of clinical coliform mastitis were highest during summer (0.211 cases/305 cow-days) and lowest during spring (0.088 cases/305 cow-days) in 9 well managed commercial dairy herds. Todhunter et al. (1991a) reported that 46.9% of coliform IMI occurred in summer followed by 21.9% in fall, 20.0% in winter, and 11.2% in spring.

Parity has been reported to influence the rate of coliform IMI during both the dry period and lactation (Smith et al., 1985 a,b). Rate of coliform IMI during lactation increased from 0.0004 IMI/cow-day in first lactation animals to 0.0023 IMI/cow-day in sixth lactation or greater animals (Smith et al., 1985b). Smith et al. (1985a) reported that rate of coliform IMI during the dry period has also been shown to increase with age of animals. Cousins et al. (1980) suggested that increased IMI in older cows was possibly due to the relative ease by which bacteria penetrate a more patent teat canal. Conflicting results have indicated that a higher rate of clinical coliform IMI in first and second lactational animals than third or greater lactational animals (Hogan, 1989b).
The rate and prevalence of coliform IMI was higher in housed than pastured cows (Eberhart et al., 1979; Natzke et al., 1981; Smith et al., 1983, 1985a). Increased risk was related to exposure of teat ends to coliform bacteria (Bramley et al., 1984; Dodd et al., 1969). Increased number of bacteria on the teat end contribute significantly to the increased incidence of bovine IMI (DeHart et al., 1976; McDonald, et al., 1968).

Bedding materials have been regarded as the major source of coliform bacteria (Bramley et al., 1975; Hogan et al., 1989a, 1997a; Natzke et al., 1976; Newman et al., 1973; Rendos et al., 1975; Thomas et al., 1983). Bramely et al. (1975) observed that organic materials such as straw, corn fodder, and sawdust often contain > 10^6 cfu/g of coliform bacteria when used as bedding. A positive relationship was found between rate of new coliform IMI and coliform counts in bedding (Bramely et al., 1975). Bacteria counts also differ within organic beddings; wood products often contain the greatest number of coliform bacteria (Rendos et al., 1975). Newman et al. (1973) correlated a high incidence of *Klebsiella* IMI with isolation of *Klebsiella* spp. from wood product samples, such as sawdust and shavings. Similar strong correlation between *K. pneumoniae* counts in sawdust bedding and *K. pneumoniae* IMI also was reported (Thomas et al., 1983). Hogan et al. (1989a) reported that organic material (sawdust and chopped straw) had significantly higher bacterial counts than inorganic material (sand and crushed limestone). A recent study (Hogan et al., 1997a) showed that the addition of lime to sawdust significantly reduced bacteria counts in the bedding for 1 d. Daily replacement of bedding had a minimal effect on bacteria counts in bedding and on teat skin. The increased rate of coliform mastitis during summer months is thought to be associated with
increased numbers of coliform bacteria in bedding and increased exposure of teat ends (Hoagn et al., 1989a; Smith et al., 1985a,b). Poorly designed facilities and improper milking time hygiene and machine function can also contribute to teat end exposure and increased coliform IMI (Smith et al., 1993; Galton et al., 1986; Natzke, 1981; Pankey et al., 1987).

**Vaccination against Coliform Mastitis**

Mastitis control is achieved either by decreasing teat end exposure to pathogens or by increasing the resistance of cows against IMI (Smith et al., 1993). Teat end exposure to contagious pathogens occurred primarily during the milking process (Eberhart et al., 1979; Neave et al., 1969). However, teat end exposure to coliform bacteria can occur anytime because the primary reservoir of coliform bacteria is the dairy cows' environment (Smith et al., 1985b, 1993; Eberhart et al., 1979). Teat dipping and dry cow therapy for mastitis control are ineffective in controlling coliform mastitis (Bramley et al., 1984; Eberhart et al., 1972; Smith et al., 1985b, 1993). Control of coliform mastitis historically relied on reduced exposure to the coliform bacteria in the environment of the dairy cows (Bramley et al., 1984; Eberhart et al., 1979; Smith et al., 1983, 1985b, 1993). Exposure can be reduced by using inorganic bedding materials, using well designed barns that are properly ventilated, limiting exposure to muddy or manure covered areas, milking clean, dry teats and udders with properly functioning milking machines (Smith et al., 1993). Because elimination of coliform bacteria from environment is not economically feasible, increasing the resistance of cows against coliform bacteria would be a logical method to reduce coliform mastitis.
A number of problems are uniquely associated with vaccination of dairy cows for mastitis (Yancey, 1993). Therefore, immunization programs did not start to contribute to coliform mastitis control until the late 1980's (Gonzalez, 1989). *Escherichia coli* (O111:B4) J5 is a core antigen vaccine recently employed for controlling coliform mastitis. *Escherichia coli* J5 is an Rc rough mutant that is unable to attach the O-oligosaccharide side chain to the core oligosaccharide-lipid A complex (Elbein et al., 1965). Core polysaccharide regions of LPS possess structural and antigenic homology across strains, species, and genera of Gram-negative bacteria (Elbein et al., 1965; Mutharia et al., 1984; Woolcock, 1985). Studies in animal models (Fenwick et al., 1986; Mutharia et al., 1984; Tyler, 1991; Ziegler et al., 1973) reported that antibodies that are specific for epitopes at the core region of LPS were crossreactive and protective against a variety of heterologous Gram-negative bacteria.

Tyler et al. (1988) reported that dairy cows with low naturally occurring *E. coli* J5 titer had a 5.33-fold increased risk of developing clinical coliform mastitis. Based on the relationship of *E. coli* J5 antibody titer and risk of developing coliform mastitis, a series of field trials were conducted to determine the efficacy of *E. coli* J5 bacterin. Gonzalez et al. (1989) first demonstrated that vaccination with *E. coli* J5 bacterin was protective against natural challenge of coliform bacteria and significantly reduced the incidence of clinical coliform mastitis. The incidence of clinical coliform mastitis during the first 3 months of lactation was 2.6% in the J5 immunized group and 12.8% in the non-immunized control group. Subsequent studies by Cullor (1991) and Hogan et al. (1992b) confirmed the initial findings of Gonzalez et al. (1989). In contrast to the results of fields
trials (Gonzalez et al., 1989; Cullor, 1991; Hogan, 1992b), an experimental challenge trial (Hill, 1991) failed to demonstrate efficacy of J5 vaccine against coliform mastitis. Hill (1991) found that vaccination of cows with J5 failed to protect cows against experimental challenge with a virulent strain of *E. coli*. Hogan et al. (1992c) also observed that J5 vaccine did not prevent IMI in an experimental challenge model using a heterologous strain of *E. coli* from a naturally occurring bovine IMI. However, clinical signs in J5-vaccinated cows were less severe than that in unvaccinated control cows (Hogan, 1992c). Additionally, vaccinated cows had significantly lower bacterial numbers in milk and significantly higher milk production and feed intake than unvaccinated controls (Hogan, 1992c). Hogan et al. (1995) conducted another trial to test efficacy of *E. coli* J5 bacterin following intramammary challenge with a strain of *E. coli* previously shown to cause mild clinical mastitis. The results from this trial again illustrated that immunization with J5 bacterin did not prevent the establishment of IMI. However, immunization of cows reduced duration of IMI and local clinical signs of mastitis.

The mechanisms by which vaccination protects the mammary gland from clinical mastitis are not entirely understood. Previously proposed mechanisms of action for crossreactive immunity to Gram-negative bacteria infections include enhanced opsonization of bacterial cells and LPS, and detoxification of LPS by blocking active lipid epitopes (Hogan et al., 1992a; Tyler et al., 1990). Immunoglobulins specific for the LPS core antigen were thought to be involved in these protective mechanisms (Hogan et al., 1992a; Tyler et al., 1990). A high degree of anti-J5 LPS IgG crossreactivity with Gram-negative bacteria were reported in several studies (Tyler et al., 1991, 1992; Tomita
et al., 1995b). The core antigen of Gram-negative bacteria may be exposed when O-polysaccharide side chain formation is incomplete during log growth of bacteria (McCallus et al., 1987). Hogan et al. (1992a) reported that enhanced opsonization and phagocytosis of heterologous *E. coli* by bovine neutrophils coincided with high serum IgM titers to *E. coli* J5. A trend for enhanced opsonic activity of colostrum from vaccinated cows was noted (Hogan et al., 1992a). Colostrum and milk collected 21 d after calving from vaccinated cows had higher IgM titers to *E. coli* J5 than did mammary secretions from control cows. Hogan et al. (1992a) suggested that enhanced opsonization results in reduced bacterial numbers, less severe IMI, and subsequently a reduction in clinical cases of mastitis. In order to maximize the protection afforded by J5 bacterin, Hogan et al. (1997b) completed a trial to compare IgG and IgM titers of cows vaccinated with a conventional schedule for systemic immunization against *E. coli* J5 with those of cows vaccinated with a schedule including intramammary immunization during the early dry period. Intramammary immunization enhanced IgG titers in serum and whey at calving compared with subcutaneous immunization. IgM titers in whey and serum on d 21 of lactation were greater for cows that received intramammary immunization than for cows that were immunized by subcutaneous injections only. In another study (Tomita et al., 1995a), an *E. coli* J5 LPS conjugate vaccine was developed in an effort to increase specific antibody titer to LPS. Immunization of cows with LPS conjugate vaccine enhanced IgG and IgM titers to *E. coli* J5 whole-cell and J5 LPS antigen, and those titers were comparable with or higher than those of cows immunized with the J5 bacterin.
Another core antigen vaccine, Re-17 mutant *Salmonella typhimurium* bacterin toxoid, was also evaluated for its efficacy to control coliform mastitis (McClure et al., 1994). The Re-17 mutant *Salmonella typhimurium* bacterin toxoid provided cross-protection against coliform mastitis as incidence and severity of clinical coliform mastitis were significantly lowered during the first 5 months of lactation.

**Iron Uptake Mechanisms of Gram-negative Bacteria**

Iron is the most abundant transition metal in living organisms with a critical role in many diverse biological systems (Braun et al., 1998; Brock, 1989). Certain lactobacilli present the only known case of microbial life without requirement for iron (Archibald et al., 1983). All Gram-negative bacteria have an absolute requirement for iron in the range 0.4-4.0 μM (Weinberg, 1978). At high levels of environmental iron (>10 μM), such as in nutrient broth, Gram-negative bacteria utilize low affinity iron uptake systems that are responsible for passive iron uptake (Pollack et al., 1970b; Guerinot, 1994). Low affinity iron uptake systems are poorly understood; but low affinity systems appear to be nonspecific and do not require carriers (Guerinot, 1994). However, because of the low solubility of ferric iron (Neilands, 1981) and the need to avoid its participation in potentially damaging Harber-Weiss-Fenton chemistry (Flitter et al., 1983), higher organisms have evolved mechanisms for lowering the levels of free iron to well below those required for the growth of Gram-negative bacteria (Litwin et al., 1993; Otto et al., 1992). Most iron is located intracellularly in eucaryotic cells as ferritin or as heme-compounds. This iron is normally not available to invading Gram-negative bacteria (Litwin et al., 1993; Otto et al., 1992). The small amount of extracellular iron that appears
in body fluid is bound by the high-affinity iron-binding proteins, such as transferrin and lactoferrin in serum and mucosal secretions (Litwin et al., 1993; Otto et al., 1992). Therefore, to obtain sufficient iron for survival and multiplication, Gram-negative bacteria have evolved genetically controlled iron uptake systems. Several comprehensive reviews on iron uptake mechanisms of Gram-negative bacteria have been published (Braun, 1995, 1997a-b, 1998; Briat, 1992; Guerinot, 1994; Neilands, 1985, 1990; Otto et al., 1992; Payne S. M., 1990; Weinberg, 1995; Wooldridge, 1993). Four primary strategies for Gram-negative bacteria to acquire iron in an iron-restricted environment have been identified: 1) High-affinity iron uptake systems mediated by high-affinity iron chelators termed siderophores, such as enterobactin, aerobactin and ferrichrome (Guerinot, 1994; Neilands, 1984; Weinberg, 1995; Wooldridge, 1993); 2) Utilize naturally occurring organic acids as low-affinity iron carriers, such as citrate (Briat, 1992; Hussein et al., 1981; Litwin, 1993); 3) Directly use host iron compounds (such as heme-compounds, transferrin and lactoferrin) as iron source without utilization of siderophores or other chelators (Guerinot, 1994; Otto et al., 1992; Wooldridge, 1993; Weinberg, 1995), and 4) Secrete reductant to reduce ferric iron at the cytoplasmic membrane and subsequently transport ferrous iron into the cytoplasm (Cox et al., 1986).

One of the most common strategies evolved by Gram-negative bacteria is high affinity iron uptake systems which involve the synthesis of low molecular mass iron chelators, the expression of iron-regulated outer membrane proteins and enzymes to utilize the chelated iron (Guerinot, 1994; Neilands, 1985). The low molecular mass (500-1,000 daltons) iron chelators with a high affinity for ferric iron \( K_{\text{aff}}>10^{19} \) synthesized by
Bacteria have been collectively termed siderophores (Gr. "Iron bearer") (Neilands, 1993). Siderophores have highly divergent structures but can be classified into two main groups, hydroxamate and catechols, of which aerobactin and enterobactin (enterochelin) are representatives for Gram-negative bacteria, respectively (Briat, 1992; Neilands, 1990, 1993). Because siderophores are too large to pass through the porin channels of the Gram-negative bacterial outer membrane (Nikaido et al., 1981, 1983), siderophores require specific iron-regulated outer membrane receptor to enable their passage across the bacterial outer membrane and into the periplasm (Braun, 1995; Guerinot, 1994; Klebba et al., 1993; Wooldridge, 1993). In addition, some Gram-negative bacteria can also express outer membrane receptors for the siderophores produced by other microorganisms, such as fungal hydroxamates (ferrichrome, coprogen, rhodoturulate, ferrioxamines), and catecholes (2,3-dihydroxybenzoylserine and dihydroxybenzoate) (Braun, 1991; Guerinot, 1994; Neilands, 1981; Wooldridge, 1993;).

The enterobactin iron uptake system is involved in the synthesis of catechol siderophore enterobactin (Hollifield et al., 1978), a cyclic triester of 2,3-dihydroxy-N-benzoyl serine which has the highest affinity for ferric iron of any natural compounds tested (Raymond et al., 1979; Wooldridge, 1993). Enterobactin synthesis needs the products of gene entA,B,C to produce 2,3-hydroxybenzoic acid from chorismic acid, and the products of genes entD,E,F,G to catalyse production of one enterobactin molecule from three molecules of 2,3-hydroxybenzoic acid and L-serine (reviewed in Crosa, 1989; Earhart, 1987). Enterobactin is produced by virtually all wild strains of E. coli (Neilands, 1981; Rogers, 1977) and other enteric bacteria (Perry et al., 1979; Podschum et al., 1992;
Pollen et al., 1970a; Tarkkanen et al., 1992; Rutz et al., 1991), both pathogenic and commensal. An iron-regulated outer membrane receptor, FepA, specifically recognizes ferric enterobactin complex (reviewed in Earhart, 1987; Neilands, 1982). FepA also serves as a receptor for colicins B and D (Pugsley et al., 1976a, 1976b, 1977). Molecular mass and antigenic properties of FepA were highly conserved in different genera of Gram-negative bacteria (Chart et al., 1985; Rutz et al., 1991). The detailed mechanism of ferric enterobactin transport in Gram-negative bacteria was reviewed by Earhart et al. (1987) and Klebba et al. (1993).

A high-affinity iron uptake system independent of the enterobactin system was reported in E. coli strains harboring the plasmid ColV-K30 in 1979 (Williams, 1979). This system involved the synthesis of the hydroxamate siderophore aerobactin (Warner et al., 1981; Williams et al., 1980), a compound which had previously been identified in some strains of Klebsiella pneumoniae (Gibson et al., 1969). Although formation constant of aerobactin (10^{21}) is lower than that of enterobactin (10^{22}) (Bagg et al., 1987), an increased efficiency of aerobactin was found compared with enterobactin (Braun et al., 1984; Der Vartanian, 1988; Williams et al., 1986). Pathogenic E. coli excrete aerobactin more rapidly than enterobactin in response to iron stress in vitro (Der Vartanian et al., 1988). Aerobactin stimulated bacterial growth at concentrations some 500-fold lower than enterobactin (Williams et al., 1986), perhaps because aerobactin may be repeatedly recycled (Braun et al., 1984), while enterobactin molecules only deliver a single ferric ion and are subsequently degraded (O’Brien et al., 1971). The pColV-K30 genes (reviewed in Bagg et al., 1987; Crosa, 1989; Neilands, 1990; Waters et al., 1991) involved in the
biosynthesis of aerobactin are \textit{iucA}, \textit{iucB}, \textit{iucC}, and \textit{iucD}. These genes encode polypeptides of 63 kDa (synthetase), 33 kDa (acetylase), 62 kDa (synthetase), and 53 kDa (oxygenase), respectively. In addition, the operon includes the gene \textit{iutA}, which encodes a 74-kDa protein \textit{IutA} that acts as the receptor for ferric aerobactin (Williams, 1979). Genes for the aerobactin iron uptake system also have been found on the chromosome of \textit{E. coli} (Valvano et al., 1984, 1988). A high percentage of \textit{E. coli} clinical isolates from septicemia, pyelonephritis, and urinary tract infection were aerobactin positive when compared with fecal isolates (Carbonetti et al., 1986; Montgomerie et al., 1984; Orskov et al., 1988). Approximately 34\% of \textit{E. coli} fecal isolates from humans were aerobactin positive compared with 69\% isolated from septicemia and 75\% in pyelonephritis (Carbonetti et al., 1986). In cattle the presence of the aerobactin system in \textit{E. coli} was detected in 4\% of fecal strains, 85\% from septicemia, and 12\% from mastitis (Linggood et al., 1987). Approximately 67\% of \textit{Shigella} isolates produced aerobactin (Payne, 1989). Tarkkanen et al (1992) reported that all 39 strains of \textit{Klebsiella} species from urinary tract infections produced enterobactin but the aerobactin iron uptake system was not detected in any of the strains. Podschun et al. (1992) also reported that aerobactin production was observed among only six percent of \textit{K. pneumoniae} and 4\% of \textit{K. oxytoca} from different sources, but 99.4\% of \textit{Klebsiella} species excreted enterobactin. These findings suggest that aerobactin system does not represent a major iron uptake system in \textit{Klebsiella} species.

\textit{FhuA}, a 78 kDa protein encoded by \textit{fhuA} gene in \textit{E. coli} (Braun, 1976; Neilands, 1990), was the receptor for ferrichrome (Wayne et al., 1975), a cyclic trihydroxamate
siderophore produced by many fungal species including *Ustilago sphaerogena* and all *Penicillium* species (Hider, 1984). The closely related hydroxamate siderophores ferricrysin and ferricrosin, both produced by *Aspergillus* species, are also taken up through FhuA (Hider, 1984). FhuE is the receptor for the linear hydroxamate siderophores coprogen and rhodotorulic acid (Hantke, 1983) produced by fungal species (Hider, 1984). In addition, *E. coli* also utilized fugal catechols dihydroxybenzoic acid and dihydroxybenzoylserine (Hantke et al., 1990). Nelson et al. (1992) identified another receptor for fungal hydroxamate ferrioxamine B, FoxB, in *E. coli* K12.

The high affinity iron uptake systems described above are controlled by the product of the *fur* gene (ferric uptake regulation), an universal regulator for all siderophore biosynthesis and transport in *E. coli* and possibly other bacteria (reviewed in Crosa, 1989, 1997; Neilands, 1985, 1990, 1994). The *fur* gene has been sequenced and shown to encode a 17-kDa polypeptide rich in histidine (Hantke, 1982, 1984; Schaffer et al., 1985). Fur protein acts as a repressor, using ferrous ion as a cofactor by binding to the consensus operator sites, designated "iron box", of iron uptake genes. (Ernst, 1978; Hantke, 1984; Neilands, 1990, 1994). The consensus of the iron box sequence is 5'-GATAATGATAATCATTATC located near the -10 and -35 sites of the promoter (Crosa, 1989; Neilands, 1994).

Besides these highly specific siderophore systems, some Gram-negative bacteria have developed the ability to take up iron directly from naturally occurring organic iron-binding acids like citrate, isocitrate, pyruvate and malate (Archibald et al., 1980; Hussien et al., 1981). Of these organic acids, citrate-dependent iron uptake by *E. coli* K12 has
received much attention over the last 17 years (Hussien et al., 1981). The fec operon is responsible for iron-dicitrate uptake and has been cloned and sequenced (Staudenmaier et al., 1989). The ferric dicitrate system must be induced by both iron and citrate at low iron concentrations (Hancock et al., 1976; Hussein et al., 1981; Frost et al., 1973; Waggeg et al., 1981; Zimmermann et al., 1984). This system is also repressed at high iron concentration by the Fur protein (Pressler et al., 1988). The inducer Fe$^{3+}$-dicitrate does not need to enter the cytoplasm to induce the citrate system (Hussien et al., 1981). However, the components for transport across outer membrane, ferric dicitrate receptor FecA and TonB-ExbB-ExbD, are required for induction (Hussein et al., 1981; Killmann et al., 1993; Waggeg et al., 1981). The regulatory mechanism of ferric dicitrate system has recently been reviewed by Braun (1995, 1997b) and Crosa (1997). *Escherichia coli* are not the only bacteria that use citrate as a shuttle for iron uptake. *Mycobacterium smegmatis* (Messenger et al., 1982), *Neisseria meningitidis* (Archibald et al., 1980) and *Pseudomonas aeruginosa* (Harding et al., 1990) also can utilize this system.

A number of Gram-negative bacteria are able to directly utilize host iron compounds, such as transferrin, lactoferrin and heme compounds (reviewed in Cornelissen et al., 1994; Gueriot, 1994; Otto et al., 1992; Weinberg, 1995; Wooldrige, 1993). Pathogenic *Neisseria* species can directly utilize iron bound to transferrin (Tf) and lactoferrin (Lf) as sources of iron (McKenna et al., 1988; Mickelsen et al., 1981; Schryvers et al., 1989a; Simonson et al., 1982). Pathogenic strains of *Haemophilus influenzae* also utilize iron bound to Tf, but not Lf, as a source of iron (Herrington et al., 1985; Pidcock et al., 1988). In the absence of siderophores, the uptake of iron from Tf or
Lf requires direct contact between the host iron-binding protein and the bacterial cell surface. Biochemical techniques involving affinity purification have identified two Tf-binding receptors of *N. meningitidis* of 98 kDa and 70 kDa, termed transferrin binding proteins 1 and 2 (TBP1 and TBP2), respectively (Schryvers et al., 1989a). TBP1 appears to be highly conserved among clinical *N. meningitidis* isolates; but TBP2 displays considerable heterogeneity with respect to antigenicity and molecular mass ranging from 68 kDa to 85 kDa (Ferreiros et al., 1991; Griffiths et al., 1990; Schryvers et al., 1989a). The Lf-binding protein of *N. meningitidis* was identified as an iron-regulated outer membrane protein with a molecular mass of 105 kDa in both *N. meningitidis* and *N. gonorrhoeae* (Schryvers et al., 1989a). Similar TBP1 (100 kDa) and TBP2 (70 - 90 kDa) in *H. influenzae* were also identified (Holland et al., 1992; Schryvers et al., 1989b; Stevenson et al., 1992). Besides *Nesseriae* and *Haemophilus* species, at least 10 more species of Gram-negative bacteria have been reported to bind Tf or Lf. However, it has not been demonstrated in every system that iron can be extracted from Tf or Lf (Weinberg, 1995). Heme-compounds can also be an iron source for *E. coli* (Cavalieri et al., 1984), *Neisseria* species (Dyer et al., 1987), *Haemophilus* species (Lee, 1991; Pidcock et al., 1988), *Vibrio* species (Dai et al., 1992; Mazoy et al., 1991; Stoebner et al., 1988;), *Shegella* species (Payne et al., 1989), *Proteus* species (Welch et al., 1987), *Serratia marcescens* (Poole et al., 1988), *Yersinia* species (Sikkema et al., 1987; Stojiljkovic et al., 1992;) and *Pasteurella haemolytica* (Gentry et al., 1986). Outer membrane receptors for heme compounds were identified with molecular mass ranging from 39.5 kDa to 100 kDa in some Gram-negative bacteria (Wooldridge, 1993). Some
pathogenic bacteria are able to increase the availability of heme compounds in blood by secreting toxins capable of lysing erythrocytes (Cavaleri et al., 1984; Griffiths et al., 1995; Stoebner et al., 1988).

The fourth iron uptake mechanism in Gram-negative bacteria is the reduction of ferric iron at the cytoplasmic membrane and then transport ferrous iron into the cytoplasm (Cox, 1986). *Pseudomonas aeruginosa* strains can secret pyocyanin which is reduced to leukopyocyanin in the presence of oxidisable substrate or NADH. The reduced pyocyanin may reduce transferrin-bound ferric iron (Cox, 1986). *Escherichia coli* does not have such an extracellular iron reductase system, but if ferrous iron is available, the ferrous iron can be transported through a specific system induced by iron-restricted conditions (Hantke, 1987). The two genes involved in the ferrous iron uptake system have been cloned and sequenced; the predicated amino acid sequences of their products suggest that transport of ferrous iron is driven by ATP (Kammler et al., 1993).

**Enterobactin Iron Uptake System of Gram-negative Bacteria**

Enterobactin (enterochelin) was first purified and characterized from *Salmonella typhimurium* and *Escherichia coli* supernatants (O’Brien et al., 1971; Pollack et al., 1970a). Enterobactin, the prototype of the catechol-phenolate type of siderophore, is a cyclic triester of 2,3-dihydroxy-N-benzoyl serine which can bind one atom of ferric iron (Earhart, 1987). Enterobacin has the highest affinity for ferric iron of any natural compounds tested and can effectively capture iron from other iron complex in neutral and alkaline conditions (Earhart, 1987; Raymond et al., 1979; Wooldrige, 1993). The ferric-enterobactin complex is red, and, has a molecular weight of 719 Da and a net charge of
minus 3 at neutral pH (Earhart, 1987). Enterobactin, the indigenous siderophore, is transported and utilized by the vast majority of Gram-negative bacteria (Rutz et al., 1991). The ferric enterobactin transport pathway is summarized as follows: 1) Synthesis and excretion of enterobactin by iron-deficient bacteria, complexes Fe\(^{3+}\) in the extracellular milieu (Neilands, 1981); 2) Ferric enterobactin binds to its outer membrane receptor protein, FepA, at a site that is centrally located in its primary structure and is cell surface-exposed (Murphy et al., 1990); 3) FepA releases ferric enterobactin to an underlying hydrophilic channel that is open to the periplasm, in a TonB-dependent energy-dependent step (Rutz et al., 1992); 4) FepB, a periplasmic protein, binds ferric enterobactin and transports it to the Fep permease in the cytoplasmic membrane (Pierce et al., 1988); 5) The Fep permease complex, consisting of FepC, FepD, FepG, and P43 (Shea et al., 1991), and 6) Ferric-enterobactin is cleaved and reduced by Fes in the cytoplasm, releasing ferrous iron to Fur, which represses transcription of enterobactin biosynthesis and transport genes (de Lorenzo et al., 1988). Detailed descriptions of enterobactin iron uptake system are included in reviews by Neilands (1981, 1982, 1994), Earhart (1987), Crosa (1989), Klebba et al. (1993), and Braun (1995).

The enterobactin is synthesized in *E. coli* by a two-step process (Bryce et al., 1972; Greenwood et al., 1976, 1980; Woodrow et al., 1979). First, 2, 3-dihydroxybenzoic acid is produced from the aromatic amino acid precursor chorismic acid, and a subsequent conversion of 2, 3-dihydroxybenzoic acid and L-serine into active enterobactin. The initial step requires the products of three genes: entC, encoding isochorismate synthetase; entB, encoding 2,3-dihydro-2,3-dihydroxybenzoate synthetase;
and \textit{entA}, encoding 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase. The second step involves the synthesis of one molecule of enterobactin from three molecules each of 2, 3-dihydroxybenzoic acid and L-serine. A multienzyme complex composed of the products of the genes \textit{entD}, \textit{entE}, \textit{entF}, and \textit{entG} has been suggested as catalyst for this step (Greenwood et al., 1976, 1980). The enterobactin gene cluster encompasses approximately 22 kb at 13 min on the \textit{E. coli} chromosome (Earhart, 1987; Nahlik et al., 1989; Neilands, 1981, 1982; Ozenberger et al., 1989).

The ferric enterobactin is too big (719 Da) to traverse the outer membrane through the open channels of general porins (Nikaido et al., 1981, 1983). The membrane channels large enough to accommodate ferric enterobactin pose a threat to bacteria, because they would also allow the entry of detergents, antibiotics, and other noxious molecules (Jiang et al., 1997). Therefore, Gram-negative bacteria have evolved gated porins to solve this nutritional dilemma by elaborating ligand-binding surface loops that close their large channels (Jiang et al., 1997; Rutz et al., 1992). At least six genes are responsible for passage of ferric enterobactin through the cell envelope (Earhart, 1987; Klebba et al., 1982; Shea et al., 1991). One of these genes, \textit{fepA}, has been thoroughly studied and the product of the gene is the surface-exposed ferric enterobactin receptor, FepA (reviewed in Crosa, 1989; Earhart, 1987; Klebba et al., 1993; Neilands, 1982, 1994). FepA is a prototype of ligand-specific siderophore receptors for ferric enterobactin (Killmann et al., 1993; Liu et al., 1993; Murphy et al., 1989,1990; Rutz et al, 1992) and is also the receptor of colicins B and D (Guterman et al., 1973; Wayne et al., 1976). FepA protein is insoluble in water and requires at least 0.5% Triton X-100 or 0.1% SDS for solution.
The gene sequence suggests a mature protein of 723 amino acids, preceded by a single peptide (Lundrigan et al., 1986). The molecular mass of FepA was estimated at 81 kDa for Gram-negative bacteria (Chart et al., 1985; Neilands, 1982; Rutz et al., 1991). Chart et al. (1985) reported that *K. pneumoniae* may produce FepA with an approximate molecular mass of 83 kDa. When isolated and analyzed at low temperatures, FepA exists as a compact protein with a molecular weight of 63 kDa (Murphy et al., 1990). Murphy et al. (1990) perceived this structure as the native, monomeric receptor. Recent analysis of FepA in non-denaturing conditions reveals FepA as a high-molecular-weight oligomer in vivo (Liu et al., 1993). The relative mobility of FepA in lithium dodecyl sulfate-PAGE suggests that native FepA is a homotrimer. The structure of FepA was characterized by immunological mapping of epitopes with monoclonal antibodies (MAb) (Amstrong et al., 1995; Murphy et al., 1990; Rutz et al., 1991; Newton et al., 1997; Payne et al., 1997). The similar topological models of FepA were proposed by Amstrong et al. (1995) and Murphy et al. (1990). The ferric enterobactin-binding site has been localized to a central, surface exposed region of the FepA, bounded by residues 258 to 339 (Murphy et al., 1990). Excision of residues 202 to 340, which comprise the large surface loop, an additional surface loop, and the connecting transmembrane segments, convert FepA into a permanently open channel that no longer binds ferric enterobactin and colicins B and D (Rutz et al., 1992). To determine what residues within FepA contact the natural ferric enterobactin during transport through the outer membrane, Newton et al. (1997) used site-directed substitution mutagenesis to study ligand recognition by FepA. Comparison of the
primary structures of four FepA that have been cloned and sequenced (from *E. coli*
*Salmonella typhimurium, Pseudomonas aeruginosa, and Bordetella pertussis*) showed a
conservation of arginine residues in the central ligand binding region (Arg-286 and Arg-
316) (Newton et al., 1997). Double mutagenesis of these arginine residues (Newton et
al., 1997) revealed a very obvious effect on both ferric enterobactin and colicin binding to
FepA, as well as iron transport. The internalization of ferric enterobactin through FepA
involve conformational changes by the FepA (Jiang et al. 1997; Liu et al., 1994; Payne et
al., 1997; Rutz et al., 1992). Rutz et al. (1992) suggested that FepA is a gated porin and
that TonB acts as its gatekeeper by facilitating the entry of ferric enterobactin into the
FepA channel. The channel domain of the FepA was demonstrated by Rutz et al. (1992)
in vivo. Liu et al. (1993) reconstituted and characterized this channel domain in vitro.
Liu et al. (1994) then found small conformational changes occurred in the surface loops
of FepA upon ferric enterobactin binding. Dramatic loop motion was found to take place
during ligand entry into the FepA pore (Jiang et al, 1997). Jiang et al. (1997) further
demonstrated that FepA, a gated-porin channels open and close during membrane
transport in vivo. Payne et al. (1997) found ferric enterobactin binds to FepA in at least
two distinct steps, an initial rapid stage and a subsequent slower step, that presumably
establishes a transport-competent complex. Ferric enterobactin bound about 10-fold faster
than colicin B during the second step (Payne et al., 1997).

Ferric enterobactin crosses the outer membrane through FepA receptors in a
TonB-dependent, energy-dependent step (Rutz et al., 1992). TonB is a 26-kDa protein
containing a hydrophobic sequence at the N-terminal end with which TonB is anchored
in the cytoplasmic membrane (Postle et al., 1988). Previous data (Bradbeer, 1993; Woolridge et al., 1992) have led to the theory that TonB functions to transform the promotive force into a mechanical action or chemical reaction that energizes the operation of outer membrane receptor proteins including FepA. FepA and other iron-regulated outer membrane receptors contain a "TonB box", a pentapeptide motif close to N-terminal end (Neilands, 1994). The sequence of the "TonB box" is not strictly conserved but varies quite considerably in different receptors (Neilands, 1994). This region is required for a direct interaction between receptors and the TonB protein (Neilands, 1994). Some mutations in the TonB box strongly impair receptor activity and they can be suppressed by mutations in TonB (Bell, et al., 1990; Tuckman et al., 1992). The tonB mutants are also devoid the outer membrane receptor activities (Frost et al., 1975; Hantke et al., 1975; Williams et al., 1979). In addition, ExbB and ExbD protein, which are localized in cytoplasmic membrane, are important for normal TonB function (Braun, 1995). Therefore, transfer of energy from the cytoplasmic membrane into the outer membrane for iron uptake is through TonB-ExbB-ExbD-dependent receptors (reviewed in Braun, 1995, 1998).

**Virulence and Iron Uptake Systems in Gram-negative Bacteria**

The ability of Gram-negative bacteria to colonize and proliferate within a particular environmental niche in the host is essential for the initiation of an infection. Growth depends in part on the ability of a pathogen to uptake essential nutrients. Of the nutrients, iron plays a crucial role in the establishment and progression of an infection (Wooldrige, 1993). Because the levels of free iron in vivo are well below microbial
requirements, possession of iron uptake systems may be an important element in bacterial virulence (Payne, 1989, 1993; Weinberg, 1984; William, 1992).

Most iron uptake systems and their interactions with host have been analysed in vitro. However, the role of iron and microbial iron uptake systems in vivo is less easily determined. Several studies (Bullen et al., 1968, 1981, 1991; Keevil, 1989; Lee et al., 1979; Mofenson et al., 1987; Payne, 1989; Schryvers et al., 1989c) have demonstrated that excess iron enhances experimental infections with Gram-negative bacteria. This type of experimental design is difficult to interpret, however, since excess iron regulates the other virulence genes of bacteria in addition to influencing iron uptake systems of Gram-negative bacteria (Litwin et al., 1993). An alternative approach to studying the role of iron uptake systems in the virulence of Gram-negative bacteria has been to look for the evidence of expression of iron uptake systems in an infected host. There is considerable experimental evidence to demonstrate that siderophores and iron transport receptor proteins are produced by Gram-negative bacteria during infections. The enterobactin has been found in peritoneal fluid of guinea pigs infected with E. coli (Griffiths et al., 1980). Antibodies against this siderophore and its receptor have been detected in sera of humans and animals following infections with enterobactin-producing Gram-negative bacteria (Griffiths, 1985), indicating the expression of ferric enterobactin system in vivo. Rogers (1973, 1976) found that specific horse antiserum enhanced the bacteriostasis of transferrin of E. coli and addition of iron-binding catechols removed the bacteriostasis. Strains of Pseudomonas aeruginosa, V. cholera, K. pneumoniae, and Proteus have been demonstrated to express iron-regulated outer membrane proteins in vivo (Brown et al.,
Similarly, expression of Tf and Tf receptors by Gram-negative bacteria has also been demonstrated in vivo (Black et al., 1986; Ferreiros et al., 1994; Fohn et al., 1987; Gerlach et al., 1992; Gorringe et al., 1995; Holland et al., 1992; Johnson et al., 1997; Niven et al., 1989; Williams et al., 1992). Specific immunoglobulin directed against iron-regulated outer membrane proteins of Gram-negative bacteria were found in sera of some animals and humans (Chhibber et al., 1995; Confer et al., 1995; Gonzalez et al., 1987; McKenna et al., 1988; Niven et al., 1989; Sokol et al., 1986a; Todhunter et al., 1991c; Worse et al., 1996).

Evidence that iron uptake systems are important to the virulence of Gram-negative bacteria has also been obtained through the genetic manipulation of bacteria in the experimental infection models. Some Gram-negative mutants defective in iron-uptake, such as *E. coli* (Williams et al., 1980), *E. chrysanthemi* (Enard et al., 1988), *Shigella* species (Lawlor et al., 1987), *Vibrio* species (Crosa et al., 1980; Goldberg et al., 1990; Litwin et al., 1996), *Salmonella typhimurium* (Yancey et al., 1979), *Neisseria* species (Genco et al., 1991; Stojiljkovick et al., 1995), *Pseudomonas aeruginosa* (Woods et al., 1982), and *Yersinia* (Heesemann et al., 1993), showed reduced virulence or even loss of virulence in experimental infection models. Furthermore, the aerobactin system of plasmid ColV-K30 enhanced the virulence of *E. coli* and *K. pneumoniae* in mice (Jacobson et al., 1988; Nassif et al., 1986; Williams et al., 1979). Virulence of *Vibrio anguillarum* correlated with the presence of a 65-kDa plasmid is responsible for iron uptake (Crosa et al., 1977).
Based on the role of iron uptake systems on the virulence of Gram-negative bacteria, there are two different strategies to prevent Gram-negative bacterial infections: 1) to use iron-regulated outer membrane proteins of Gram-negative bacteria as vaccine; and 2) to transport antibacterial compounds (toxic metal ion or siderophore-drug conjugates) effectively into bacterial cell through iron uptake system. The iron-regulated outer membrane proteins of Gram-negative bacterial pathogens are often suggested as vaccine candidates for immunoprophylactic therapy (Byers, 1987; Gray-Owen et al., 1996; Johnson, 1991; Payne, 1993; Weinberg, 1989; Williams et al., 1992; Zollinger et al., 1991). Sokol et al. (1984) reported that antibodies directed against iron-regulated outer membrane proteins (IROMP) inhibited ferricyochelin binding to *Pseudomonos aeruginosa* cell envelope. Passive immunization with antibodies directed against the IROMP of *E. coli* and *P. aeruginosa* has indicated that such antibodies lowered mortality caused by these two Gram-negative bacteria in turkeys and mice, respectively (Bolin et al., 1987; Sokol et al., 1986a). Vaccine containing IROMP of *Pasteurella haemolytic* enhances protection against experimental pasterellosis in lambs and cattle (Confer et al., 1995; Gilmore et al., 1991). Polyclonal sera raised against the *E. coli* FhuA protein reduced ferrichrome binding to the receptor (Coulton et al., 1982). Polyclonal antibodies specific for aerobactin receptor IutA inhibited siderophore binding to its receptor (Roberts et al., 1989). Monoclonal antibodies against ferricyochelin-binding protein have been shown to block ferricyochelin uptake by *P. aeruginosa* (Sokol et al., 1986b). Murphy et al. (1990) observed that four anti-FepA monoclonal antibodies recognized the region that participates directly in ligand binding therefore inhibiting the uptake of ferric
enterobactin. Certain mouse monoclonal antibodies to the 70-kDa IROMP of *N. meningitidis* were bactericidal, but the activity was strain specific (Pettersson et al., 1990). Moreover, there is currently considerable interest in the vaccine potential of Tf-binding proteins. Immunization with recombinant TBP2 from *A. pleuropneumoniae* was protective against challenge with parental strain in pigs (Rossi-Campos et al., 1992). Meningococcal TBP2-specific antibodies were both bacteriostatic and bactericidal in vitro and protective against infections in mice (Ala’Aldeen et al., 1996; Lissolo et al., 1995). One potential limitation of the use of TBP2 as vaccine antigens is the antigenic heterogeneity present in these proteins (Gerlach et al., 1992; Rokbi et al., 1993). However, there is evidence for conserved epitopes among TBP2 (Stevenson et al., 1992; Holland et al., 1996), suggesting that TBP2 derivatives might induce a cross-reactive response.

Scandium (Sc$^{3+}$) entered Gram-negative cell as analogue of Fe$^{3+}$ by the way of normal ferric enterobactin system (Plaha et al., 1983; Rogers et al., 1987) and was liberated intracellularly where Sc$^{3+}$ appeared to interfere with RNA synthesis (Plaha et al., 1984). The scandium complex of enterobactin was active in serum at micromolar concentration against *E. coli* (Rogers et al., 1982), *K. pneumoniae* (Rogers et al., 1980), and *P. aeruginosa* (Rogers et al., 1984). The approach to the design of siderophore-antibiotic may solve the problem of increasing resistance to antibiotics (Chin et al., 1994). The iron uptake systems of Gram-negative bacteria may also be used as a transport pathway of drugs into bacterial cells. Some investigators have shown that semisynthetic β-lactams having a terminal siderophore moiety have remarkably enhanced
bactericidal activity relative to other derivatives against various Gram-negative bacteria under iron-restricted conditions (Diarra et al., 1996; Gensberg et al., 1994; Miller et al., 1993; Smith, P.W., et al., 1995).

Summary

No uniform method currently exists for controlling coliform mastitis. Increasing cows' resistance against coliform bacteria through vaccination would be a logical method to reduce coliform mastitis due to the ubiquitous nature of coliform bacteria in the environment of the dairy cows. The commercial sale of *Escherichia coli* J5 (O111:B4) vaccines do not prevent coliform IMI. The need still exists for an effective vaccine to prevent IMI and control the growth of coliform bacteria in the bovine mammary gland, especially during the nonlactating period when the rate of new coliform IMI is fourfold higher than during lactation. The iron-regulated outer membrane proteins are often suggested as vaccine candidates for immunoprophylactic therapy. Ferric enterobactin receptor, FepA, is of particular interest in the pathogenesis of coliform bacteria because FepA protein is widely distributed, surface-exposed, and antigenically conserved among coliform bacteria. Immunization of cows with FepA protein may prevent coliform mastitis by blocking iron uptake of coliform bacteria. Therefore, complete information on the molecular interaction between FepA protein and anti-FepA antibodies during bacterial multiplication within iron deplete mammary gland will result in a novel vaccine for controlling coliform mastitis during the nonlactating period.
CHAPTER 2

EXPRESSION OF SIDEROPHORE AND FERRIC ENTEROBACTIN RECEPTOR BY GRAM-NEGATIVE BACTERIA ISOLATED FROM NATURALLY OCCURRING BOVINE IMTRAMAMMARY INFECTION

INTRODUCTION

The nonlactating period is a critical time in the dynamics of IMI caused by coliform bacteria. Incidences of IMI caused by coliform bacteria were 3 to 4 times higher during the nonlactating period than during lactation (Smith et al., 1985b). Iron is an essential growth factor for virtually all bacteria. Most extracellular iron in mammary gland secretions of nonlactating cows is bound to lactoferrin. Consequently, the amount of free iron is too low to support the growth of most bacteria (Todhunter et al., 1990a). Gram-negative bacteria can overcome this limitation by utilizing as many as four high affinity iron acquisition systems that are aerobactin, enterobactin, citrate, and ferrichrome systems. These systems involve the synthesis of iron chelators (siderophores) with a low molecular mass, the expression of iron-regulated outer membrane proteins (IROMP) and enzymes to utilize the chelated iron (Griffiths, 1987; Guerinot, 1994; Neilands, 1985, 1990; Wooldrige, 1993). Iron-siderophore complexes are bound by outer membrane receptors of bacteria, and the iron is internalized. Iron uptake mediated by enterobactin is of particular interest in bacterial pathogenicity, because enterobactin has the highest
affinity among all siderophores (Bagg et al., 1987) and because the enterobactin system
is common in coliforms isolated from a variety of sources (Neilands, 1981; Perry, 1979;
Podschun et al., 1992; Rutz et al., 1991).

The ferric enterobactin receptor FepA has been thoroughly characterized
(Armstrong, 1995; Klebba, 1982, 1993; Jiang et al., 1997; Murphy et al., 1990; Rutz et al.
1992). The region of FepA that is exposed to the surface and is responsible for the
binding of ferric enterobactin also has been identified (Murphy, 1990; Newton et al..
1997). Chart (1985) reported that the molecular mass and antigenic properties of FepA
are highly conserved among members of the family Enterobacteriaceae. Rutz et al.
(1991) found that FepA appeared to be conserved in 18 different genera of Gram-
negative bacteria tested. However, little information exist concerning the role of
siderophore production and FepA expression in the pathogenesis of IMI caused by
coliform bacteria. Therefore, the objective of this study was to determine the frequency
distribution of siderophore and ferric enterobactin receptor of coliform bacteria isolated
from naturally occurring IMI.

MATERIALS AND METHODS

Bacterial Isolates

The isolates tested were Escherichia coli (n = 25), Klebsiella pneumoniae (n =
25) and Serratia marcescens (n = 3) from bovine IMI in five herds. Escherichia coli
strain AN193B,I, (entA') was kindly provided by Mark Coy (University of California,
Berkeley). Escherichia coli AN193B,I, lacks the ability to produce enterobactin. All
coliform bacteria were stored on trypticase soy agar slants ( BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD ) at 25°C prior to use.

Screening for Siderophore Production

Bacterial isolates were tested by the chrome azurol sulfonate (CAS) assay for detection of siderophore production. Procedures were described by Payne (1994) using modified M9 growth medium. Isolates were streaked onto CAS agar plates and incubated aerobically at 37°C for 18 h.

Isolation and Electrophoresis of Outer Membrane Proteins

Bacteria were grown in trypticase soy broth (TSB) or TSB containing 300 μM α-α'-dipyridyl ( Sigma Chemical Co., St. Louis, MO ). Cultures were incubated at 37°C for 18 h on a rotary shaker (200 rpm) in all growth media.

Outer membrane proteins were extracted as described by Todhunter (1991c). Outer membrane proteins were separated by SDS-PAGE as described by Todhunter (1991c). Molecular mass standards (Bio-Rad, Richmond, CA) were rabbit muscle phosphorylase b, 97,400 Da; bovine serum albumin, 66,200 Da; ovalbumin, 42,700 Da; bovine carbonic anhydrase, 31,000 Da; soybean trypsin inhibitor, 21,500 Da; and lysozyme, 14,400 Da. Protein bands were stained by Coomassie brilliant blue R.

Detection of FepA by Western blots

After SDS-PAGE, the gels were equilibrated for 30 min in transfer buffer (0.025 M Tris base and 0.192 M glycine with 20% methanol, pH 8.3) and electrotransferred onto nitrocellulose sheets at 70 V of constant voltage for 2 h (Towbin, 1979). After transfer, the nitrocellulose sheets were blocked in PBS plus 5% instant
nonfat dry milk for 1 h at 25°C. The nitrocellulose sheets were washed in PBS plus 0.05% Tween 20 (PBS-Tween) and then incubated for 1 h at 25°C in diluted rabbit anti-FepA serum (FepA from *E. coli* K12 was used as an antigen to produce antiserum; kindly provided by Phil Klebba, University of Oklahoma, Norman) (Murphy, 1989). Nitrocellulose sheets were rinsed three times in PBS-Tween and incubated in a 1:16,000 (vol/vol) dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (whole molecule) for 1 h at 25°C. Nitrocellulose sheets were washed as described previously and incubated with a substrate solution containing 50 mg of 3, 3'-diaminobenzidine dissolved in 100 ml of PBS and 0.1 ml of 30% H₂O₂ (vol/vol). The reaction was stopped after 10 min by washing the nitrocellulose with distilled water. All antibodies were diluted in PBS plus 5% instant nonfat dry milk.

RESULTS

CAS Assay

All isolates produced siderophores under iron-restricted condition as displayed by small orange halos around the colonies on the CAS agar plate (Figure 1). Compared with Gram-negative isolates from IMI, the mutant AN193B₁,1 (entA⁺) did not display a halo and grew poorly on the CAS agar plate. Size of halos around colonies varied among isolates. Figure 2 shows that halos around *E. coli* 727 colonies are much larger than that around *E. coli* 471.
Electrophoresis and Immunoblots

All Gram-negative bacteria expressed IROMP when grown in TSB plus α-α'-dipyridyl. The outer membrane profiles of isolates of typical *E. coli, K. pneumoniae,* and *S. marcescens* are shown in Figure 3. Proteins with high molecular mass ranging from 75 to 85-kDa were induced when bacteria were grown in an iron-restricted medium. The IROMP were not observed in bacteria that were grown in TSB replete with iron. However, there was variation among different Gram-negative bacteria species with respect to the number and quantity of IROMP expressed.

The proteins that underwent electrophoresis were reacted with the polyclonal antiserum that was specific for purified 81-kDa FepA from *E. coli K12*. Under iron-restricted conditions, all isolates expressed large quantities of FepA that reacted with the antiserum. Figure 4 shows the immunoblot of typical isolates of *E. coli, K. pneumoniae,* and *S. marcescens*. The rabbit anti-FepA antibodies recognized the FepA protein from *E. coli* 471, *K. pneumoniae* 525 and *S. marcescens* 155. The molecular mass of FepA protein was approximately 81-kDa. Both *E. coli* 471 and *K. pneumoniae* 525 grown in an iron replete culture expressed FepA at a very low level, and expression of FepA by *S. marcescens* grown in an iron sufficient medium was not detected.

DISCUSSION

The CAS technique is a universally applicable method to detect siderophore (Schwyn et al., 1987). A total of 53 isolates *E. coli, K. pneumoniae* and *S. marcescens*
bacteria from five different herds was examined for siderophore production. Results of previous trials indicated that *E. coli, Klebsiella* spp., and *Serratia* spp. were the most frequently isolated coliform bacteria during the dry period and accounted for 32.6, 28.6 and 11.1%, respectively, of all Gram-negative bacterial IMI (Todhunter, 1989). Screening for siderophore production by CAS assay revealed that all isolates produced siderophores. To obtain iron in this assay, the bacterium has to express the high affinity uptake system, but only to the level that satisfies its requirements, resulting in relatively small orange halos around the colonies on the CAS agar plate. The mutant *E. coli* AN193B.I, was negative for siderophore production because of the deficiency in production of the intermediate 2,3-dihydroxybenzoic acid for enterobactin synthesis (Earhart, 1987). The different levels of siderophore production are likely attributable to several mechanisms. For example, a mutation in the ferric uptake regulation gene *fur* resulted in constitutive depression of the high affinity uptake system and the mutant overproduced siderophore (Schwyn, 1987). A strain carrying a mutation in one of the transport genes (*fepB*) was unable to take up ferric enterobactin from the environment, and the accumulation of siderophore also resulted in large halos around colonies (Schwyn, 1987).

Although all isolates were positive for siderophore production on CAS plates, this assay cannot determine which high affinity iron acquisition system plays a major role in siderophore production. Previous research indicated that enterobactin is produced by virtually all wild strains of *E. coli* (Neilands, 1981; Rogers, 1977) and other enteric bacteria (Perry et al., 1979; Podschum, 1992; Tarkkanen, 1992; Pollack, 1970a; Rutz, 1970).
1991), both pathogenic and commensal. The aerobactin system was detected in only 12% of *E. coli* isolated from mastitis (Linggoood, 1987). Tarkkanen (1992) recently reported that all 39 strains of *Klebsiella* spp. from urinary tract infections produced enterobactin, but the aerobactin iron-sequesting system was not detected in any of the strains. Podschun (1992) also reported that 99.4% of *Klebsiella* spp. from different sources excreted enterobactin, and aerobactin production was observed among only 6% of *K. pneumoniae* and 4% of *K. oxytoca*. Results of electrophoresis and immunoblots in the current study also indicated the wide expression of the enterobactin iron acquisition system among Gram-negative bacteria isolated from naturally occurring bovine IMI. All isolates in the current study expressed large quantities of outer membrane proteins that reacted with anti-FepA antiserum under iron-restricted conditions. Low expression of FepA by *E. coli* in an environment that was sufficient in iron may be due to the fact that FepA acts as the cognate outer membrane receptor for colicin B and D (Pugsley, 1976a). FepA that specifically adsorbs ferric enterobactin has been thoroughly studied. The molecular mass of the receptor is estimated to be approximately 81 kDa in *E. coli* and 83 kDa in *Klebsiella* spp. (Chart, 1985). Using Western blots with monoclonal antibodies, Rutz et al. (1991) surveyed a panel of Gram-negative bacteria to identify IROMP that were structurally related to the *E. coli* K12 FepA. This research found that FepA, or some significant structural remnant of FepA, appears to be conserved in all 18 different genera of Gram-negative bacteria. In the current study, we investigated 53 Gram-negative bacterial isolates to determine IROMP that were structurally related to the *E. coli* K12 FepA. Results of our immunoblot assay also strongly suggested that high
antigenic and molecular homology of FepA exists among all coliform pathogens isolated from naturally occurring bovine IMI.

IROMPs are attractive candidates for vaccines because they are exposed to the surface, antigenic, and may induce antibodies that block the essential iron uptake of the bacteria (Todhunter, 1991c; Zollinger et al., 1991). Passive immunization with antibodies that are directed against the IROMP of *Escherichia coli* and *Pseudomonas aeruginosa* has indicated that such antibodies can lower mortality caused by these two Gram-negative organisms in turkeys and mice, respectively (Bolin, 1987; Sokol, 1986). Certain mouse monoclonal antibodies to the 70-kDa IROMP of *Neisseria meningitidis* were bactericidal, but the activity was strain specific (Pettersson, 1990). Recent research (Confer, 1995) suggested that antibodies from cattle to IROMP in conjugation with antibodies to other surface antigens enhance immunity to a challenge with *Pasteurella haemolytica*. Murphy (1990) reported that four anti-FepA monoclonal antibodies recognized the region that participates directly in ligand binding, and, therefore, inhibited the uptake of ferric enterobactin. Because the enterobactin system is distributed widely among coliform bacteria and FepA is antigenic and is conserved, the FepA could be used as a novel vaccine to control mastitis caused by coliform bacteria during nonlactating period. The FepA may induce antibodies against the FepA binding site. These antibodies may block the essential iron uptake function of the bacteria and inhibit the growth of coliforms.
Figure 1. Typical growth responses of *Escherichia coli*, *Klebsiella pneumoniae*, and *Serratia marcescens* isolates on chrome azurol sulfonate agar plates. One synthesis mutant (entA-) of enterobactin system is compared with *E. coli* 471, *K. pneumoniae* 525, and *S. marcescens* 155.
Figure 2. Growth responses of *Escherichia coli* 471 and *Escherichia coli* 727 isolates on chrome azurol sulfonate agar plates. Both isolates are from naturally occurring bovine IMI.
Figure 3. Typical outer membrane proteins profiles of Escherichia coli, Klebsiella pneumoniae, and Serratia marcescens isolates separated by SDS-PAGE and stained with Comassie blue. Protein per lane was 20 µg. Bacteria were grown in trypticase soy broth (TSB) or TSB plus iron chelator α-α’-dipryridyl (TSB-Fe). Lane contents were 1) molecular mass (×10^3) standards; 2) E. coli 471, TSB; 3) E. coli 471, TSB-Fe; 4) K. pneumoniae 525, TSB; 5) K. pneumoniae 525, TSB-Fe; 6) S. marcescens 155, TSB; and 7) S. marcescens 155, TSB-Fe. Approximate position of iron-regulated outer membrane protein is indicated (IROMP).
Figure 4. Immunoblots of the separated iron-regulated outer membrane proteins of typical isolates of *Escherichia coli*, *Klebsiella pneumoniae* and *Serratia marcescens* reacted with the rabbit antiserum directed against the 81-kDa protein of *E. coli* K12. Protein per lane was 20μg. The bacteria were grown in trypticase soy broth (TSB) or TSB plus iron chelator α-α'-dipyridyl (TSB-Fe). The serum was diluted 1:400 (vol/vol). Lane contents were 1) molecular mass (×10^3) standards; 2) *E. coli* 471, TSB; 3) *E. coli* 471, TSB-Fe; 4) *K. pneumoniae* 525, TSB; 5) *K. pneumoniae* 525, TSB-Fe; 6) *S. marcescens* 155, TSB; and 7) *S. marcescens* 155, TSB-Fe. Approximate position of FepA and molecular mass are indicated.
CHAPTER 3

IN VITRO GROWTH INHIBITION OF COLIFORM BACTERIA BY A MONOCLONAL ANTIBODY DIRECTED AGAINST FERRIC ENTEROBACTIN RECEPTOR

INTRODUCTION

Iron is an essential element for survival and multiplication of many bacteria, and efficient iron assimilation seems to be an important virulence factor for many pathogens (Griffiths, 1985, 1987; Nassif, 1986; Weinberg, 1984). In the secretions from the mammary gland of nonlactating cows, most iron is bound to lactoferrin (Lf). the concentration of which may reach 20 to 30 mg/ml (Bishop et al., 1976). Consequently, the amount of free iron is too low to support the growth of most bacteria (Todhunter, 1990a). In response to iron deficiency, coliform bacteria produce efficient bacterial iron chelators (siderophores) which capture iron from Lf and express iron-regulated outer membrane proteins (IROMP) that recognize ferric siderophore complexes and transport them into the bacterial cells (Guerinot, 1994; Neilands, 1985, 1990; Weinberg, 1995).

The enterobactin iron acquisition system is widely distributed among Gram-negative bacteria (Neilands, 1981; Rutz, 1991). This high affinity iron acquisition system is composed of the various enzymes required for the synthesis and secretion of 1) the siderophore enterobactin; 2) an IROMP, FepA, that specifically adsorbs ferric
enterobactin; and 3) a variety of other cell envelope proteins that act in the transportation and deferration of ferric enterobactin (Earhart et al., 1987; Klebba et al., 1993). The FepA plays an important role in iron assimilation. The region of FepA that is exposed to the surface and that is responsible for the binding of ferric enterobactin has been identified and thoroughly characterized (Armstrong et al., 1995; Liu et al., 1993; Murphy et al., 1990; Rutz et al., 1992). Previous research indicated that the molecular mass and antigenic properties of FepA were highly conserved in different genera of Gram-negative bacteria (Chart, 1985; Rutz et al., 1991). Studies on the expression of siderophore and FepA by Gram-negative bacteria isolated from naturally occurring bovine IMI demonstrated that FepA was widely distributed and highly conserved among Gram-negative isolates from different herds (Chapter 2). These characteristics make FepA a possible vaccine for controlling mastitis caused by coliform bacteria during the nonlactating period. Antibodies against IROMP, some of which are siderophore receptors, could prevent infection in animal models of Gram-negative infection (Bolin, 1987; Confer et al., 1985; Sokol, 1986) or inhibit the growth of bacteria in vitro (Murphy, 1990; Pettersson et al., 1990). However, the ability of these antibodies to alter the growth of coliform pathogens is unknown. Therefore, to investigate the function of the 81-kDa protein FepA and to assess its potential as a subunit vaccine, we determined the growth responses of coliform isolates to a monoclonal antibody (MAb) specific for blocking the ferric enterobactin binding site of FepA in synthetic medium containing apolactoferrin (apoLf).
MATERIALS AND METHODS

Bacterial Isolates and Culture Conditions

Isolates tested were Escherichia coli (n = 25) and Klebsiella pneumoniae (n = 25) from bovine IMI in five herds. A mutant strain of Escherichia coli, AN193B1, (entA'). was kindly provided by Mark Coy (University of California, Berkeley). The mutant can not produce enterobactin and lacks FepA outer membrane receptor activity. All coliform bacteria were stored on trypticase soy agar slants (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) at 25°C prior to use. For preparation of outer membrane proteins, isolates were grown in trypticase soy broth (TSB) or TSB containing 300 μM α-α’-dipyridyl (Sigma Chemical Co., St. Louis, MO). Cultures were incubated at 37°C for 18 h on a rotary shaker (200 rpm) in all growth media. For the in vitro growth assay, bacteria were cultured in synthetic medium (Bacto-Synthetic Broth (BSB); Difco Laboratories, Detroit, MI). The iron-restricted medium was BSB supplemented with 0.5 mg/ml apoLf. Purified MAb and apoLf were sterilized by membrane filtration (pore size 0.45 μM; Millipore Corp., Bedford, MA).

MAb

Monoclonal antibody 35 ascitic fluid was kindly provided by Phil Klebba (University of Oklahoma, Norman) (Murphy, 1990). MAb 35 was directed against the region of FepA of E. coli K12 that was exposed to the surface and inhibited the uptake of ferric enterobactin by blocking the binding site of FepA (Murphy, 1990). For the bacterial in vitro growth assay, MAb 35 was isolated from ascitic fluid by ammonium sulfate precipitation (Coligan et al., 1991).
Electrophoresis and Immunoblots

Bacterial outer membrane proteins were prepared by the method of Todhunter (1991c). Electrophoresis and Western immunoblots were performed as described in Chapter 2 with slight modification. Proteins were transferred to nitrocellulose sheets and blocked in PBS plus 5% instant nonfat dry milk for 1 h at 25°C. The nitrocellulose sheets were washed in PBS plus 0.05% Tween 20 (PBS-Tween) and then incubated with the diluted MAb 35 ascitic fluid (1:5000, vol/vol). Nitrocellulose sheets were rinsed three times in PBS-Tween and incubated in a 1:5000 (vol/vol) dilution of horse radish peroxidase-conjugated goat anti-mouse IgG (Fab specific) for 1 h at 25°C. Nitrocellulose sheets were washed as described previously and developed in a substrate solution containing 50 mg of 3, 3'-diaminobenzidine dissolved in 100 ml of PBS and 0.1 ml of 30% H₂O₂. The reaction was stopped after 10 min by washing the nitrocellulose with distilled water. All antibodies were diluted in PBS containing 5% instant nonfat dry milk.

In Vitro Bacterial Growth Assay

In vitro growth of coliform bacteria was as described by Todhunter (Todhunter, 1990a) with the following exceptions. Prior to in vitro growth assays, coliform bacteria were grown for 6 h at 37°C in 5 ml of TSB containing 300 μM α-α'-dipyridyl. Cultures were washed twice in PBS and serially diluted in PBS to achieve approximately 10⁶ cfu/ml. In vitro growth assays were performed in 96-well microtiter plates (Sartedt, Newton, NC) with a total volume of 260 μl, consisting of 250 μl of medium inoculated with 10 μl of diluted coliform bacteria. Cultures were aerobically incubated in a humidified atmosphere for 12 h at 37°C. In vitro microcultures were in duplicate.
Following incubation, cultures were serially diluted in PBS, and four 10-μl spots of
diluted and undiluted cultures were delivered onto the surface of a MacConkey agar plate
(Difco Laboratories). MacConkey agar plates were incubated for 12 h at 37°C, bacterial
colonies were counted, and in vitro growth was expressed as log_{10} colony-forming units
per milliliter. Control medium for optimal growth of coliform bacteria was BSB. The
iron-restricted medium was BSB containing 0.5 mg/ml apoLf. Sterile MAb was diluted
with iron-restricted medium to a final concentration of 0.8 mg/ml.

*Escherichia coli* 471 and *Klebsiella pneumoniae* 531 were used to test the effects
of incubation time and MAb 35 dose on bacterial growth. Sterile MAb 35 was diluted
with iron-restricted medium to final concentrations of 800, 400, 100, and 50 μg/ml.
Cultures were assayed as described previously at 6, 12, and 24 h. All assays were
performed in duplicate, and the means are reported.

**Statistical Analysis**

The difference between bacterial species in the frequency of MAb 35 reacted with
FepA was analyzed by Chi-square analysis (Daniel, 1995). Differences between bacterial
species in different media were analyzed by least squares analysis of covariance. Growth
in control medium was the covariant. Mean comparisons among different media within
species were tested by ANOVA. Multiple comparisons of means of unequal sample size
were by the Tukey-Kramer method (Daniel, 1995). All statistical analyses were
performed using MINITAB software.
RESULTS

MAb 35 Against FepA

Figure 5 shows an immunoblot of MAb 35 reacted with IROMP from coliform bacteria grown with or without iron. *Escherichia coli* 471 and *Klebsiella pneumoniae* 531 were typical of the isolates tested. The MAb 35 recognized an 81-kDa band in the outer membrane protein profiles of *E. coli* 471 and an 83-kDa band in those of *K. pneumoniae* 531. In the culture that was replete with iron, FepA was undetected for both *E. coli* and *K. pneumoniae* isolates. Each *E. coli* isolate produced FepA that reacted with MAb 35. However, only 4 of 25 *K. pneumoniae* isolates produced FepA that reacted with MAb 35. Frequency of expressed FepA that reacted with MAb 35 was different (*P < 0.05*) between *E. coli* and *K. pneumoniae* (Table 1).

In Vitro Growth Responses to MAb 35

Effect of MAb 35 on the in vitro growth of coliform bacteria was initially tested by growing the isolates in the synthetic medium BSB containing 0.5 mg/ml of apoLf and 0.8 mg/ml of MAb35 (Table 2). Compared with the optimal growth in BSB, both *E. coli* and *K. pneumoniae* isolates had significant (*P < 0.05*) growth reduction in the presence of 0.5 mg/ml of apoLf. The control strain *E. coli* AN193BJ, had the greatest (*P < 0.05*) reduction, which was approximately 3.86 log₁₀ units. The addition of MAb 35 in synthetic medium BSB supplemented with apoLf resulted in significant growth inhibition (*P < 0.05*) of *E. coli* isolates. In contrast, in vitro growth of *E. coli* AN193BJ, in the presence of MAb 35 was greater (*P < 0.05*) than growth in BSB plus apoLf. Growth of *K. pneumoniae* in the presence of MAb 35 was not different (*P > 0.05*) from
growth in BSB plus apoLf. The inhibitory effect of MAb35 was greater than 0.5 cfu log$_{10}$/ml for each *E. coli* isolate. However, only 4 of 25 *K. pneumoniae* isolates (16%) experienced more than 0.5 cfu log$_{10}$/ml of growth reduction in the presence of MAb 35. These four *K. pneumoniae* isolates were the same isolates that reacted with MAb 35 in the immunoblot assay.

**Effects of Incubation Time and MAb 35 Dose on Bacterial Growth**

Bacterial growth was inhibited in a dose-dependent and time-dependent fashion by MAb 35. As little as 50 µg/ml of MAb 35 significantly inhibited the growth of *E. coli* 471 and *K. pneumoniae* 531 in iron-restricted medium (Figure 6) after 12 h of incubation. Growth of both isolates was significantly reduced at higher concentrations of MAb 35 after 6 h of incubation. Growth reduction by MAb 35 was greatest after 12 h compared with after 6 h and 24 h incubation. High doses of MAb 35 still inhibited bacterial growth at 24 h of incubation. Growth of *E. coli* 471 and *K. pneumoniae* 531 in BSB supplemented with apoLf alone after 6 and 12 h of incubation was reduced when compared with growth in BSB. However, after 24 h of incubation, inhibitory effect of apoLf on bacterial growth had disappeared. Growth in BSB plus apoLf was not different from that in BSB.

**DISCUSSION**

In the current trial, MAb 35 produced a distinctive pattern of reactivity with FepA expressed by *E. coli* and *K. pneumoniae* isolates from bovine IMI. Antigenic variation in
the enterobactin ligand binding site resulted in a very low percentage of *K. pneumoniae* isolates that expressed FepA that was recognized by MAb 35. Results from Chapter 2 demonstrated that polyclonal antiserum specific for purified FepA from *E. coli* K12 recognized FepA expressed by each *E. coli* and *K. pneumoniae* isolate. Consequently, FepA appears to be widely distributed among coliform isolates from various sources, with both molecular mass and the antigenic properties of FepA being highly conserved. Unlike polyclonal antiserum that is expected to contain a large amount of different antibodies that may recognize various epitopes, MAb is an epitope-specific antibody. Monoclonal antibodies that bind the region of FepA that is exposed to the surface between residues 290 and 339 inhibited ferric enterobactin uptake (Murphy, 1990). The MAb 35 recognized the FepA amino acid residues from position 314 to 339 (Murphy, 1990). Rutz et al. (1991) used immunoblots with 28 MAb to survey a panel of Gram-negative bacteria to identify IROMP that were structurally related to the *E. coli* K12 FepA. Although FepA appeared to be conserved in all 18 different genera of gram-negative bacteria, MAb 35 reacted with FepA from *E. coli* but was not reactive with FepA produced by one strain of *K. pneumoniae* (Rutz, 1991). In general, epitopes of FepA buried in the outer membrane bilayer were more conserved among Gram-negative bacteria than epitopes exposed on the bacterial cell surface (Rutz, 1991). The polypeptide region of FepA of *E. coli* K12 responsible for ferric enterobactin binding and its topological structure have been identified (Murphy, 1990; Newton, 1997; Rutz et al., 1992). However, little information exists concerning the amino acid sequence and epitopes of the *K. pneumoniae* FepA.
Bacterial multiplication in secretion from involuted mammary glands was dependent on iron availability in the medium (Todhunter, 1990a). Mammary secretions from nonlactating glands have little available iron because of the high concentration of Lf, the proteins that bind with iron, and a decrease in the concentration of citrate (Bishop et al., 1976). Concentrations of Lf in bovine mammary secretion are approximately 20 to 30 mg/ml in the involuted gland (Bishop et al., 1976) compared with 4 mg/ml in human milk (Masson, 1971) and 0.25 mg/ml in normal bovine milk (Smith, 1977). In vitro growth responses of \( E. coli \) and \( K. pneumoniae \) were negatively correlated with the concentration of Lf in synthetic medium BSB (Todhunter, 1990a). Growth responses of the isolates in synthetic medium containing Lf were similar to those in mammary secretions (Todhunter, 1990a). Therefore, BSB supplemented with apoLf was used as an iron-restricted medium for coliform in vitro growth assay in the current study.

Lactoferrin inhibited the in vitro growth of coliform bacteria when unsaturated with iron (Bishop, 1976; Rainard, 1986). Data from the current trial indicated that low concentrations of apoLf (0.5 mg/ml) alone resulted in relatively large reductions (>2 log\(_{10}\) reduction) for many of the \( E. coli \) and \( K. pneumoniae \) isolates tested. When grown in the iron-restricted medium, coliform bacteria from IMI could utilize iron via the enteroobactin iron uptake system and expressed ferric enterobactin receptor FepA (chapter 2). Therefore, after MAb 35 was added in synthetic medium BSB plus apoLf, MAb 35 could bind the region of FepA that is responsible for binding ferric enterobactin and prevent the ferric enterobactin complex from being internalized. The growth of each \( E. coli \) isolate was greatly inhibited compared with growth in BSB plus apoLf. Inhibitory
effects were not significant for the growth of *K. pneumoniae*. Only those four *K. pneumoniae* isolates that reacted with MAb 35 had reduced growth (>0.5 log₁₀ units) in the presence of MAb 35. This finding further indicated that the ligand binding site of FepA exhibits antigenic variability in different coliform species. However, MAb 35 recognizes only one specific epitope. A polyclonal antiserum may contain a large quantity of different antibodies that recognize additional epitopes on the ferric enterobactin binding site. *Klebsiella pneumoniae* and *E. coli* may share one or more identical epitopes in the ligand binding site. Sequence data and epitope mapping for *K. pneumoniae* are necessary for a more complete understanding of different epitopes.

*Escherichia coli* AN193B, was used as a control strain in which *fepA* and *entA* genes have been rendered nonfunctional by a specific mutation. This strain provided the reference point for growth in the presence of MAb 35. Because of the inability to capture iron from apoLf, growth of *E. coli* AN193B, was greatly inhibited (3.86 log₁₀ cfu) in the medium BSB plus apoLf compared with optimal growth in BSB. Growth pattern of this mutant suggested that no other high affinity iron uptake system was able to compensate for the loss of the enterobactin system. The addition of MAb 35 to BSB containing apoLf did not inhibit the growth of this mutant. On the contrary, growth was enhanced compared with growth in BSB plus apoLf. Perhaps iron carryover in the purified MAb 35 partly overcome the inhibitory effect caused by apoLf.

Effects of MAb dose and incubation time on coliform growth revealed that very low concentrations (50 µg/ml) of MAb specific for blocking enterobactin binding caused significant growth reduction in vitro. Higher concentrations of MAb 35 could more
efficiently compete with ferric enterobactin to bind FepA, resulting in greater growth reduction. Immunoglobulins in mammary secretion are derived from blood or are produced locally by plasma cells in the subepithelia connective tissue (Bulter, 1974; Lascelles, 1974). During the nonlactating period, the number of plasma cells that produce immunoglobulin increased gradually from drying off and reached peak concentrations at 2 wk prepartum (Sordillo et al., 1988). If cows immunized with FepA could produce antibodies directed against the epitopes at the ligand binding site of FepA, even relatively low concentrations of secreted antibodies may block the essential iron uptake function and inhibit the growth of coliforms during the nonlactating period.

Although *E. coli* lipopolysaccharide core and O-antigen sugars obscure most of surface epitopes of FepA, ferric enterobactin-binding domains in region 290 to 339 are free of lipopolysaccharide steric hindrance (Murphy et al., 1990). In addition, the core antigen of Gram-negative bacteria might be most exposed when O-polysaccharide side chain formation was incomplete during log growth of bacteria (McCallus et al., 1987). These characteristics enables antibodies to bind the regions of FepA that are responsible for binding ferric enterobactin.

Specific immunoglobulin directed against IROMP of Gram-negative bacteria were found in serum of animals and humans (Griffiths et al., 1980, 1985; Sokol et al., 1986a; Worse et al., 1996). Bovine antibodies directed against IROMPs were also detected in mammary secretions from involuted glands, milk, colostrum, and serum (Chhibber and Bajaj, 1995; Confer et al., 1995; Todhunter et al., 1991c). Antibodies directed against siderophore receptor have been shown to block bacterial iron assimilation (Coulton et al., 1991a).
1982; Roberts et al., 1989; Sokol et al., 1986b). The ability of anti-siderophore receptor antibodies to block iron uptake was thoroughly investigated in enterobactin system for *E. coli* K12 (Murphy et al., 1990; Rutz et al., 1991). In the current trial, a monoclonal antibody specific for the binding site of FepA inhibited the growth of coliform mastitis pathogens in chemically defined medium containing apoLf.
Figure 5. Immunoblots of the separated iron-regulated outer membrane proteins of typical isolates of *Escherichia coli* and *Klebsiella pneumoniae* reacted with the monoclonal antibody 35 directed against the 81-kDa protein of *E. coli* K12. The bacteria were grown in trypticase soy both (TSB) or TSB plus iron chelator α-α' -dipyridyl (TSB-Fe). Lane contents were 1) Molecular mass (x10^3) standards; 2) *E. coli* 471, TSB-Fe; 3) *E. coli* 471, TSB; 4) *K. pneumoniae* 531, TSB-Fe; and 5) *K. pneumoniae* 531, TSB. Approximate position of FepA and molecular mass are indicated.
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Total(^1)</th>
<th>Reactivity with MAb35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n(^2))</td>
<td>(%)(^3)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100(^a)</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16(^b)</td>
</tr>
</tbody>
</table>

TABLE 1. Frequency of MAb35 reactive FepA among *Escherichia coli* and *Klebsiella pneumoniae* isolated from naturally occurring bovine IMI.

\(^{a,b}\) Chi-square analysis of the difference between *Escherichia coli* and *Klebsiella pneumoniae* in the frequency distribution of MAb35 reactive FepA. Percentage followed by different lettered superscripts differ (\(P<0.05\)).

\(^1\)Total number of tested isolates.

\(^2\)Number of the isolates that reacted with MAb35.

\(^3\)Percentage of MAb35 reactive isolates within genera.
Figure 6. Growth inhibition of *Escherichia coli* 471 (A) and *Klebsiella pneumoniae* 531 (B) in the presence of various concentrations of purified monoclonal antibody 35 (MAb 35): Bacto Synthetic Broth (BSB, Difco Laboratories, Detroit, MI) (○), iron-restricted medium BSB + apolactoferrin (apoLf) (◇), BSB + apoLf + 50 µg/ml of MAb35 (▽), BSB + apoLf + 100 µg/ml MAb35 (▲), BSB + apoLf + 400 µg/ml of MAb35 (☆), BSB + apoLf + 800 µg/ml of MAb35 (◇). Each point is the mean of four independent measurements; standard error is indicated by error bars.
| Culture conditions$^1$ | Bacterial type | \( \log_{10}\text{cfu/ml} \) | | | |
|------------------------|---------------|----------------|----------------|----------------|
|                        | \( E.\ coli \) & \( K.\ pneumoniae \) & \( E.\ coli\ AN193B13 \) |
|                        | \( (N = 25)^2 \) & \( (N = 25)^2 \) & \( \) |
|                        | \( \bar{x} \) & \( \bar{x} \) & \( \bar{x} \) |
|                        | SE & SE & SE |
| BSB                    | 8.97$^{a,x}$ & 0.04 & 9.03$^{a,x}$ & 0.03 & 8.60$^{a,y}$ & 0.13 |
| BSB + apoLf            | 6.85$^{b,y}$ & 0.12 & 7.27$^{b,x}$ & 0.12 & 4.74$^{b,z}$ & 0.06 |
| BSB + apoLf + MAb35    | 5.04$^{c,y}$ & 0.19 & 7.09$^{b,x}$ & 0.16 & 5.22$^{c,y}$ & 0.05 |

**TABLE 2.** Effects of anti-FepA monoclonal antibody 35 (MAb35) on the growth of *Escherichia coli* and *Klebsiella pneumoniae* in chemically defined medium.

$^{a,b,c}$ Means within a column followed by different superscripts differ \( (P < 0.05) \).

$^{x,y,z}$ Means within a row followed by different superscripts differ \( (P < 0.05) \).

$^1$BSB = Bacto synthetic Broth (Difco Laboratories, Detroit, MI); apoLf = apolactoferrin. Concentration of apoLf = 0.5mg/ml; concentration of MAb 35 = 0.8 mg/ml.

$^2$Number of tested isolates from five herds.

$^3$Results are averaged from eight independent determinations.
CHAPTER 4

IMMUNIZATION OF COWS WITH FERRIC ENTEROBACTIN RECEPTOR FROM COLIFORM BACTERIA

INTRODUCTION

Iron is an essential growth factor for virtually all bacteria (Griffith et al., 1987). Most iron in mammary gland secretions of nonlactating cows is bound to lactoferrin; concentrations may reach 20 to 30 mg/ml (Bishop et al., 1976). Consequently, the amount of free iron is too low to support the growth of bacteria (Todhunter et al., 1990a). Coliform bacteria can overcome this iron deficiency by producing efficient iron chelators (siderophores) and iron-regulated outer membrane proteins (IROMP) (Griffiths et al., 1987; Guerinot, 1994; Weinberg, 1995). Some of the IROMP recognize ferric siderophore complexes and transport iron-siderophore complexes into the bacterial cell (Griffiths et al., 1987; Guerinot, 1994; Weinberg, 1995). The iron uptake system mediated by enterobactin is of particular interest in bacterial pathogenicity because enterobactin has the highest affinity for iron among all siderophores (Bagg et al., 1987) and because the enterobactin system is common among coliform genera that are isolated from a variety of sources (Rutz et al., 1991). The ferric enterobactin receptor FepA plays an important role in this system. The surface-exposed region of FepA is responsible for
the binding of ferric enterobactin (Klebba et al., 1993). Previous research (Chart et al., 1985; Rutz et al., 1991) has indicated that the molecular mass and antigenic properties of FepA are highly conserved in different genera of Gram-negative bacteria. Experiments on the expression of siderophore and FepA by Gram-negative bacteria that have been isolated from naturally occurring bovine IMI also demonstrated that FepA was widely distributed among Gram-negative isolates from different sources and was highly conserved (Chapter 2). These characteristics make FepA a possible antigen choice for the formulation of a vaccine to control mastitis caused by coliform bacteria during the nonlactating period.

Previous research (Todhunter et al., 1990a) has shown that dry cow secretion inhibits the growth of most coliform bacteria. This growth inhibition has been attributed in part to antibodies and the iron-binding protein lactoferrin. Antibodies enhance the growth inhibitory properties of lactoferrin (Todhunter et al., 1990a). Antibodies against IROMP prevented infection in animal models of Gram-negative infection (Bolin et al., 1987; Confer et al., 1995; Sokol et al., 1986a) or inhibited the growth of bacteria in vitro (Murphy et al., 1990; Petterson et al., 1990; Sokol et al., 1986b). Results from Chapter 3 further demonstrated that antibody that is specific for blocking FepA binding sites inhibited the in vitro growth of coliform bacteria isolated from bovine IMI. Demonstration of an immunological response to E. coli FepA would be critical for subsequent testing of a possible vaccine for the control of mastitis caused by coliform bacteria during the nonlactating period. Therefore, the objectives of this study were 1) to isolate FepA from coliform bacteria, 2) to determine humoral immune responses
following vaccination with FepA and 3) to test the crossreactivity of antiserum from cows immunized with FepA to coliform isolates from naturally occurring IMI.

**MATERIALS AND METHODS**

**Purification of FepA Protein**

FepA was derived from *E. coli* 471 by the method of Fiss et al.(1982) with slight modification. Bacteria were grown in trypticase soy broth (TSB) containing 300 μM α-α’-dipyridyl (Sigma Chemical Co., St. Louis, MO). Cultures were incubated at 37°C for 18 h on a rotary shaker (200 rpm). Following incubation, bacteria were harvested by centrifugation at 2500 × g for 30 min at 4°C and washed three times in 0.15 M NaCl. Cells were suspended in deionized, distilled water and disrupted by sonication for 10 min. Sonicated bacteria were centrifuged at 5000 × g for 10 min at 4°C. N-Lauroylsarcosine sodium salt (Sigma Chemical Co.) was added to the supernatant at a final concentration of 2% and incubated for 30 min at 25°C. Outer membranes were collected by centrifugation at 50,000 × g for 60 min at 4°C and washed twice in deionized, distilled water. Outer membranes were solubilized with 2% Triton X-100, 10 mM Tris (pH 8.0), and 5 mM EDTA. The suspension was centrifuged at 50,000 × g for 60 min, and pellets were discarded. The supernatant was then dialyzed against 2% Triton X-100, 50 mM Tris (pH 7.2), and 5 mM EDTA and applied to a column (DE-52; Whatman Inc., Clifton, NJ) equilibrated with the same buffer. The ratio of bed volumes to total applied protein was 0.3 ml/mg. Flow rate was 0.6 ml/min. The column was washed with four to six bed volumes of buffer, followed with six bed volumes of a salt gradient of 0-0.1 M NaCl in
2% Triton X-100, 50 mM Tris (pH 7.2), and 5 mM EDTA. The aliquots from the DE-52 column were analyzed by the bicinchonic acid protein assay reagent (Pierce Chemical Co., Rockford, IL). FepA in aliquots was further determined by 12% SDS-PAGE, utilizing the discontinuous buffer system of Laemmli (1970). Concentrations of FepA were measured by the bicinchonic acid protein assay reagent with BSA as a standard.

**Immunization and Sample Collection**

Twenty multiparous cows, ranging from 160 to 230 DIM, were assigned to four treatment groups of five cows blocked by breed and DIM. Treatment groups were vaccinated with 100 μg of FepA, 500 μg of FepA, *Escherichia coli* J5 (5 × 10⁹ boiled cells/ml), or sterile PBS. FepA protein and *E. coli* J5 preparations were suspended in 5 ml of sterile PBS and emulsified with 5 ml of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI). Control immunization was 5 ml of sterile PBS emulsified with 5 ml of Freund's incomplete adjuvant. Cows were injected subcutaneously posterior to the scapula on approximately 200 DIM and received booster immunizations 14 and 28 d later. Cows were observed for adverse reactions at the injection site, and rectal temperature was taken at 0, 12, 24, 48, 72 h postimmunization. Serum (25 ml of blood) and milk samples (50 ml) were collected from all cows immediately prior to the primary immunization and on d 14, 28, 35, 42, and 49 after the primary immunization.

**ELISA**

The coating antigen for ELISA was FepA suspended in 0.01 M ammonium acetate and 0.01 M ammonium carbonate (pH 8.2) to a final concentration of 300 ng/ml. A 100-μl sample of each suspension was dispensed into the wells of polystyrene
microtiter plates (Nunc-Immuno Plates; Nunc, Inc., Roskilde, Denmark), and the volatile salts were evaporated by drying the plates at 37°C for 18 h. The wells were blocked with PBS containing 0.1% Tween 20 for 30 min at 37°C. Serum or whey samples were serially diluted in assay plates and incubated for 1 h at 37°C. The wells were washed three times with PBS containing 0.05% Tween 20, and 100 µl of goat anti-bovine IgG-peroxidase (30 ng/ml of PBS; Kirkegaard and Perry Laboratories, Gaithersburg, MD) were added to each well. After 1 h of incubation at 37°C, the plates were washed as described previously, and 100 µl of 2, 2'-azinodi-ethylbenzothiazolinesulfonic acid peroxidase substrate (Kirkegaard and Perry Laboratories) were added and incubated at 25°C for 10 min. The reaction was stopped by the addition of an equal volume of 1% SDS, and the optical density of each well at 410 nm was determined. End point titer was defined as the last dilution at which the optical density of sample wells exceeded the mean optical density of four control wells plus 0.05. Titer was expressed as the reciprocal of the end point dilution log₂.

**Reactivity of bovine antiserum against FepA**

Reactivity of bovine antiserum against FepA was determined by SDS-PAGE and immunoblot. SDS-PAGE and Western immunoblot were performed as described in Chapter 3 with slight modification. After grown in TSB + 300 µM α-α'-dipyridyl at 37°C for 18 h, both *E. coli* 471 and *K. pneumoniae* 525 cells were harvested and whole bacteria (~3×10⁹/ml) were solubilized by boiling for 5 min in SDS-PAGE sample buffer (Laemmli, 1970), and approximately 10⁸ bacteria were applied to a well in the gel. For immunoblot assay, both preimmunization (d 0) and postimmunization (d28) bovine
serum from cows vaccinated with 500 µg FepA were diluted 1:400 (vol/vol) with PBS containing 0.1% Tween 20.

**Bovine IgG Purification**

Postimmunization (d 35) serum from cows immunized with FepA protein or PBS was pooled for purification of the IgG fraction. The IgG fraction of pooled serum samples was precipitated by the method of ammonium sulfate precipitation (Harlow, 1988). Precipitated IgG was further purified by protein G affinity chromatography. The affinity system was Protein G Sepharose®4 Fast Flow (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The affinity column (1.5 x 5 cm) equilibrated with the binding buffer (20 mM sodium phosphate, pH 7.0) was prepared as described by the manufacture. The IgG sample was dialyzed against binding buffer and was applied to the column. The ratio of total applied protein to bed volumes was 20 mg/ml. Flow rate was 0.8 ml/min. The column was washed with 10 bed volumes of binding buffer. The bound bovine IgG was eluted by 10 bed volumes of elution buffer (0.1 M glycine-HCl, pH 2.7). 100 µl/ml eluate of 1 M Tris-HCl (pH 9.0) was placed in the fraction collector tubes prior to use. The purification profile of bovine IgG was monitored at 280 nm. The protein-containing fractions were pooled and dialyzed against PBS at 4°C. Purity of IgG was determined by 12% SDS-PAGE utilizing the discontinuous buffer system of Laemmli (1970). Purified bovine IgG was stored at -20°C prior to use. The IgG concentration was measured by the Bicinchonic acid protein assay reagent (Pierce Chemical Co., Rockford, IL).
Crossreactivity Assay

Crossreactivity of purified bovine IgG was determined by ELISA as described by Tomita et al. (1995b) with slight modification. After cultured in TSB containing 300 μM α-α'-dipyridyl at 37°C for 18 h, bacterial cells were harvested and washed three times in PBS. Wells of microtiter plates (Immunolon-1®; Dynatech Laboratories, Chantilly, Chantilly, VA) were coated with 100 μl of the heat-killed bacterial suspension, the optical density of which was 15% transmission at 610 nm. The isolates tested were E. coli (n=20) and K. pneumoniae (n=20) that were isolated from naturally occurring bovine IMI. Escherichia coli strain UT5600 which lacks FepA production was kindly provided by Dr. Dick van der Helm (The University of Oklahoma, Department of Chemistry and Biochemistry, Norman, OK). Escherichia coli UT5600 whole cells were tested as negative control for IgG crossreactivity. The wells coated with E. coli 471 whole cells were tested as a positive control. A total of 100 μl (4 μg/ml) of purified IgG was added to each well. Assay scores were read on a spectrophotometer (Minireader II®; Dynatech Laboratories, Alexandria, VA) set at a wavelength of 410 nm (A410). The degree of crossreactivity was expressed as percentage of crossreactivity [(mean A410 of tested antigen)/(mean A410 of E. coli 471 antigen)].

Statistical Analysis

Differences in serum and whey IgG titers among treatment groups were analyzed by least squares analysis of covariance with the day titer as the covariant. Main effects were day of sample collection, treatment, and block. Comparisons of serum and whey IgG titer within treatment groups across time were tested by ANOVA; main effects were
day of sample collection and block. Crossreactivity data for IgG were analyzed among
and within treatment groups by ANOVA; main effects were treatment group and test
antigen. All statistical analyses were performed using MINITAB® software.

RESULTS

Purification of 81-kDa FepA

Figure 7 illustrates the stepwise purification scheme utilized to isolate 81-kDa
FepA. FepA protein was induced under the iron deficient environment (lane 3-6).
Lauroylsarcosine extraction (lane 4) and Triton X-100 extraction (lane 5) successively
remove high molecular mass proteins (> 100 kDa) and low molecular mass proteins (<31
kDa), respectively. Chromatography using the DE-52 column (lane 6) finally resulted in
substantial purification of the FepA receptor. However, a protein band with approximate
molecular mass of 74-kDa could not be separated from FepA by chromatography using
the DE-52 column. Figure 8 showed that proteins in second peak (peak B) from DE-52
column were mainly composed of FepA proteins.

Immunological Responses to FepA Protein

Subcutaneous injection with FepA, *E. coli* J5 bacterin, or PBS did not induce
systemic reactions. Rectal temperature was not changed after each vaccination. Swelling
was observed at injection sites for cows immunized with FepA antigen and J5 bacterin,and the diameter of nodes were approximately 5 to 6 cm. The swelling persisted for the
duration of study. Immunization with PBS resulted in slight swelling (2 to 3 cm).
Antibody titer responses of serum and whey (Figures 9 and 10, respectively) to FepA were similar. Cows immunized with FepA had higher ($P < 0.05$) IgG titer to FepA on d 14, 28, 35, 42, and 49 than did cows immunized with J5 bacterin or PBS (Figures 9 and 10). Serum and whey IgG titer to FepA did not differ ($P > 0.05$) between cows immunized with different doses of FepA (Figures 9 and 10). After initial FepA immunization, both serum and whey IgG titers to FepA increased dramatically ($P < 0.05$) by d 14 and were maintained through d 49 (Figures 9 and 10).

**Antisera Against the 81-kDa FepA**

Serum from cows that were vaccinated with FepA proteins reacted with FepA that was expressed by both *E. coli* 471 and *K. pneumoniae* 525 (Figure 11). Preimmunization serum reacted with FepA very weakly at the same dilution as the postimmunization serum. Both preimmunization and postimmunization sera recognized several minor protein bands produced by *E. coli* 471 and *K. pneumoniae* 525.

**Bovine IgG Purification**

The method of ammonium sulfate precipitation combined with Protein G affinity chromatography resulted in highly purified bovine IgG (Figure 12 and 13). The majority of albumin was removed by ammonium sulfate precipitation (Figure 13). The ammonium sulfate precipitated bovine IgG were loaded onto the Protein G Sepharose® 4 Fast Flow column for further purification (Figure 12). Two protein parts (peak A and peak B) were eluted from column successively (Figure 12). The proteins in peak B which was eluted
from column by lowering the pH of mobile phase is highly purified bovine IgG (Figure 13). The SDS-PAGE was run under reducing conditions resulting in typical IgG profile appearing as its constituent "heavy" chains (~55 kDa) and "light" chains (~25 kDa).

**Crossreactivity Assay**

The crossreactivity of purified IgG from FepA immunized cows with *E. coli* isolates was higher (*P*<0.05) than that with *K. pneumoniae* isolates (Table 3). Control isolate *E. coli* UT5600 had lower (*P*<0.05) crossreactivity value than both *E. coli* and *K. pneumoniae* isolates (Table 3). Frequency distribution of crossreactivity of purified IgG from FepA immunized cows with coliform isolates is shown in Figure 14. *Escherichia coli* isolates had a degree of crossreactivity ranging from 50 to 100 while *K. pneumoniae* isolates had lower degree of crossreactivity ranging from 30 to 70.

Figure 15 showed optical density values for the reactivity of different sources of bovine IgG to coliform isolates. The pattern of optical density values was consistent within bacterial species isolated from IMI. Within both species, purified IgG from FepA immunized cows had higher (*P*<0.05) reactivity than the IgG from cows immunized with *E. coli J5* or PBS. The reactivity of IgG from cows immunized with *E. coli J5* was not different (*P*>0.05) from the reactivity of IgG from PBS immunized cows. Reactivity of IgG from FepA immunized cows to both *E. coli* and *K. pneumoniae* bacteria isolated from IMI was higher (*P*<0.05) than that to the FepA negative mutant, *E. coli* UT5600.
DISCUSSION

The IROMP play an important role in bacterial survival and multiplication. Specific immunoglobulin directed against IROMP of Gram-negative bacteria were found in the serum of animals and humans (Chhibber et al., 1995; Confer et al., 1995; Sokol et al., 1986a; Worse et al., 1996). Bovine antibodies directed against IROMP were also detected in serum, colostrum, milk, and mammary secretions from involuted glands (Chhibber et al., 1995; Confer et al., 1995; Todhunter et al., 1991c). In the current study, immunization with FepA resulted in a significantly elevated IgG titer to FepA in both serum and whey. A substantial increase was elicited in the serum IgG titer (16-fold) and whey IgG titer (32-fold) to FepA in cows immunized with FepA compared with that in cows immunized with PBS. The crossreactivity of IgG to whole cells of each isolate showed that IgG from FepA immunized cows reacted with coliform isolates more frequently than IgG from cows immunized with PBS or \textit{E. coli J5}. This finding suggested that the titer of anti-FepA IgG with high crossreactivity was increased greatly in bovine serum following immunization with FepA.

\textit{Escherchia coli} 471 was selected as the source of FepA antigen for two reasons: 1) \textit{E. coli} 471 was isolated from naturally occurring bovine IMI (Chapter 2) and 2) \textit{E. coli} 471 produces a large quantity of FepA under iron-restricted conditions. The porin proteins account for 35% of outer membrane proteins (Fiss et al., 1982). Separation of porins from FepA by chromatography using the DE-52 column resulted in substantial purification of the FepA receptor. However, a 74-kDa protein copurified with 81-kDa FepA on the DE-52 column. A similar finding has been reported by Fiss et al. (1982).
Two different doses of FepA (100 µg and 500 µg) were tested in the current study. Immune responses did not differ between FepA doses. Thus, there appeared to be no advantage to the larger dose. Cows immunized with 100 µg of FepA per dose successfully induced elevated IgG titer to FepA. The immunization dose of previous subunit vaccines for cattle varied from 3.1 µg of bovine herpesvirus-1 glycoproteins (Hurk et al., 1990) to 10 mg of outer membrane proteins from *Pasterurella haemolytica* per dose (Sreevastan et al., 1996).

Crossreactivity assay conducted in the current study demonstrated that FepA immunization in cows generated antibodies that reacted to various *E. coli* and *K. pneumoniae* isolates from naturally occurring bovine IMI. *Escherichia coli* UT5600 was used as a negative control because this mutant lacks FepA production. No recognition of *E. coli* UT5600 by anti-FepA IgG would be observed theoretically. Because the cows immunized with FepA appeared to have experienced natural exposure to Gram-negative bacteria, the purified IgG from FepA immunized cows also contains small amount of IgG directed against surface-exposed and conserved antigen of Gram-negative bacteria, which resulted in weak crossreactivity of IgG from cows immunized with FepA to *E. coli* UT5600. Therefore, using percentage of crossreactivity of *E. coli* UT5600 as a control value, the enhanced reaction to both *E. coli* and *K. pneumoniae* isolates was attributed to the high crossreactivity of IgG from cows immunized with FepA. *Klebsiella pneumoniae* isolates had lower crossreactivity than *E. coli* isolates. The presence of a capsule surrounding *K. pneumoniae* prevented access of antibodies to its target, the bacterial outer membrane (Williams, P. et al., 1988). Consequently, this
mechanism may reduce the recognition of anti-FepA IgG by *K. pneumoniae* in ELISA test. However, McCallus et al. (1987) proposed that the core antigen of Gram-negative bacteria might be most exposed when O-polysaccharide side chain formation was incomplete during log growth of bacteria. Anti-FepA IgG may bind to FepA receptor of *K. pneumoniae* during rapid growth phase when capsule polysaccharide is also not synthesized completely, thereby provide higher degree of crossreactivity than that reported in this ELISA study.

FepA is an IROMP responsible for the binding of ferric enterobactin (Klebba et al., 1993) and was demonstrated to be distributed widely among different Gram-negative isolates from bovine IMI in chapter 2. Results from Chapter 2 also demonstrated that both molecular mass and the antigenic properties of FepA are highly conserved among coliform isolates from bovine IMI. One murine monoclonal antibody that blocks the binding of FepA inhibited the in vitro growth of coliform bacteria derived from bovine IMI (Chapter 3). In the current study, immunization of cows with purified FepA protein elicited a significant IgG immune response to FepA. Purified IgG from cows immunized with FepA was crossreactive to *E. coli* and *K. pneumoniae* isolates from naturally occurring bovine IMI. Consequently, cows immunized with FepA may produce antibodies that are directed against the epitopes at the ligand binding site of FepA. After antibodies migrate into the mammary gland, these anti-FepA antibodies may prevent iron uptake by heterologous coliforms by blocking FepA receptor. Interference of iron uptake may reduce the ability of coliform to replicate and cause IMI during the nonlactating period.
Figure 7. SDS-PAGE of purified ferric enterobactin outer membrane receptor FepA from *Escherichia coli* 471. *Escherichia coli* 471 was grown in trypticase soy broth (lane 2) or trypticase soy broth plus iron chelator α-α'-dipyridyl (lane 3-6). Lane contents were 1) molecular mass (x10^3) standard; 2) outer membranes; 3) whole membranes; 4) lauroylsarcosine insoluble outer membranes; 5) Triton-EDTA-solubilized outer membranes; 6) pooled fractions from DE-52 column. Approximate position of FepA and molecular mass are indicated.
Figure 8. DEAE-cellulose chromatography of FepA receptor. (A) Chromatography of Triton-solubilized outer membrane proteins (20 mg) with a Whatman DE-52 column (1.5 x 25 cm) equilibrated with 2% Triton X-100-50mM Tris (pH 7.2)-5mM EDTA. (B) Protein composition of the DE-52 column peak fractions. Aliquots from the DE-52 column were analyzed by 12% SDS-PAGE. Approximate position of FepA and molecular mass are indicated.
Figure 9. Serum IgG titer to *Escherichia coli* 471 FepA antigen. Cows were immunized with 500 μg FepA (▼), 100 μg FepA (V), *E. coli* J5 bacterin (O), and PBS (●) on d0, d14 and d28. Values are expressed as least squares means of log₂ titer. Each point is the average of five measurements from five cows respectively with standard error indicated by error bars.
Figure 10. Whey IgG titer to *Escherichia coli* 471 FepA antigen. Cows were immunized with on 500 µg FepA (▼), 100 µg FepA (▼), *E. coli* J5 bacterin (○), and PBS (●) d 0, d 14 and d 28. Values are expressed as least squares means of log₂ titer. Each point is the average of five measurements with standard error indicated by error bars.
Figure 11. Immunoblots of separated outer membrane proteins of *Escherichia coli* 471 (lane 2,4) and *Klebsiella pneumoniae* 525 (lane 3,5) reacted with preimmunization (PRE) and postimmunization (POST) bovine serum against FepA protein. Both sera are diluted 1:400 (vol/vol). The bacteria were grown in trypticase soy broth plus iron chelator α-α'-dipyridyl. Lane 1 is molecular mass ($\times 10^3$) standard. Approximate position of FepA and molecular weight are indicated.
Figure 12. Purification of bovine IgG by protein G affinity chromatography. The ammonium sulfate precipitated bovine IgG (200 mg) was loaded onto the Protein G Sepharose® 4 Fast Flow column (1.5 x 5 cm; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The majority of the protein (peak A) eluted in the binding buffer (20 mM sodium phosphate, pH 7.0). The bound bovine IgG (peak B) was eluted by low pH of elution buffer (0.1 M glycine-HCL, pH 2.7).
Figure 13. Analysis of the purification of IgG from bovine serum by SDS-PAGE. Protein per lane was 20 μg. Proteins were stained with 0.1% Comassie blue. Lane contents were 1) molecular mass standard (× 10^3); 2) crude bovine serum, 1:100 dilution in PBS; 3) ammonium sulfate precipitated IgG from bovine serum; 4) unbound protein which was eluted in the protein G column flowthrough (peak A in Figure 10); 5) purified bovine IgG which was eluted from protein G column by lowering the pH of mobile phase (peak B in Figure 10); and 6) standard affinity-purified bovine IgG (Sigma Chemical Co., St. Louis, MO).
Table 3. Crossreactivity of purified IgG from FepA immunized cows with coliform bacteria from naturally occurring bovine IMI.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>N</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>20</td>
<td>68.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.43</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>20</td>
<td>53.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.65</td>
</tr>
<tr>
<td><em>Escherichia coli</em> UT5600</td>
<td>6</td>
<td>41.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.63</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Means within a column followed by different superscript differ (P < 0.05).

<sup>1</sup>The degree of crossreactivity was expressed as mean percentage of crossreactivity \[ \left( \frac{\text{mean } A_{410} \text{ of tested whole cell antigen}}{\text{mean } A_{410} \text{ of } E. \ coli \ 471 \text{ whole cell antigen}} \right) \times 100. \]

<sup>2</sup>Number of isolates.

<sup>3</sup>Number of independent determinations.
Figure 14. Frequency distribution of crossreactivity of purified IgG from FepA immunized cows with *Escherichia coli* (n = 20) (diagonal bars) and *Klebsiella pneumoniae* (n = 20) (solid bars) isolates from naturally occurring bovine mastitis. The degree of crossreactivity was expressed as percentage of crossreactivity [(mean $A_{410}$ of tested antigen)/(mean $A_{410}$ of *E. coli* 471 antigen)] × 100.
Figure 15. ELISA reactivity of *Escherichia coli* (n = 20) and *Klebsiella pneumoniae* (n = 20) isolates from naturally occurring bovine mastitis to purified IgG from cows immunized with FepA, cows immunized with *Escherichia coli J5*, and cows immunized with PBS. Results of control isolate *E. coli* UT5600 were averaged from 6 independent determinations. Concentration of IgG from each pooled bovine serum was 4 µg/ml. Values are expressed as mean optical density; bars indicated standard error. Legend: IgG from cows immunized with FepA (solid bar); IgG from cows immunized with *E. coli J5* (open bar); and IgG from cows immunized with PBS (diagonal bar).
INTRODUCTION

Under the normal physiological environment of low iron availability, Gram-negative bacteria synthesize and secrete a group of low molecular mass siderophores that bind iron with high affinity (Neilands, 1984). The siderophores are too large (600-1,200 daltons) to pass through the porin channels of the bacterial outer membrane (Nikaido et al., 1981, 1983). Therefore, the siderophores require specific iron-regulated outer membrane proteins (IROMP) to enable their passage across the bacterial outer membrane and into the periplasma (Braun, 1994; Guerinot, 1994; Klebba et al., 1993; Wooldridge, 1993). The ability to assimilate iron through these receptors can be an important virulence factor (Griffiths et al., 1980; Heesemann et al., 1993; Jacobson et al., 1988; Nassif et al., 1986; Williams et al., 1979; Yancey et al., 1979). Consequently, the IROMP are often suggested as vaccine components to control bacterial infections (Johnson et al., 1991; Weinberg, 1989; Williams, 1992; Zollinger, 1991).

Enterobactin is the primary siderophore of Escherichia coli and of many other Gram-negative bacteria (Rutz et al., 1991). The FepA, an IROMP, plays a crucial role in
the enterobactin-mediated iron uptake system by specifically binding ferric enterobactin (Murphy et al., 1990). The molecular mass and antigenic properties of FepA were highly conserved among different genera of Gram-negative bacteria (Chart et al., 1985; Rutz et al., 1991) including coliform isolates from naturally occurring bovine IMI (Chapter 2). These characteristics make FepA a possible antigen choice for vaccine formulation to control coliform mastitis during the nonlactating period when most iron in bovine mammary gland secretions is bound to lactoferrin (Lf) (Bishop et al., 1976). Results from Chapter 3 indicated that a murine monoclonal antibody (MAb) specific for blocking FepA binding site inhibited the growth of coliform bacteria isolated from bovine IMI. Studies in Chapter 4 demonstrated that immunization with native FepA successfully elicited an immunological response in bovine serum and milk. However, the growth responses of coliform bacteria to antisera from cows immunized with purified FepA are unknown. The objective of this study was to compare growth responses of coliform bacteria to highly purified bovine IgG from cows immunized with FepA in chemically defined medium containing apolactoferrin (apoLf) and in a pooled source of dry cow secretion.

**MATERIALS AND METHODS**

**Bacterial Isolates**

Isolates tested were *Escherichia coli* (n = 21) and *Klebsiella pneumoniae* (n = 21) from bovine IMI in five herds. A mutant strain of *Escherichia coli*, AN193BJ, (entA'). was kindly provided by Mark Coy (University of California, Berkeley). The mutant can
not produce enterobactin and lacks FepA outer membrane receptor activity. All coliform bacteria were stored on trypticase soy agar slants (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) at 25°C prior to use.

**Deferration of Labware**

All glassware was washed in 0.1 N of concentrated nitric acid for 4 h and rinsed three times in distilled, deionized water. Water for buffer and media was distilled and subjected to NANOpure system (Barnstead Thermolyne, Dubuque, IA) with the conductivity tested prior to use. Dialysis tubing was treated with sodium carbonate and EDTA as described previously (Cox, 1994).

**Purification of Bovine IgG**

Bovine IgG was purified from serum by protein G affinity chromatography as described previously (Chapter 3). In order to get desired serum, 15 multiparous cows, ranging from 160 to 230 DIM, were assigned to three treatment groups of five cows blocked by breed and DIM. Treatment groups were vaccinated with 100 μg or 500 μg of FepA, or *Escherichia coli J5* (5 × 10⁹ boiled cells/ml). Cows were injected subcutaneously posterior to the scapula on approximately 200 DIM and received booster immunizations 14 and 28 d later. Serum (25 ml of blood) was collected from all cows on d 35 after the primary immunization. The sera from FepA vaccinated cows were pooled for IgG purification because IgG responses did not differ between immunization doses (chapter 3). The purified IgG was sterilized by membrane filtration. Filtered IgG was stored at -20°C prior to use.
Standard Dry Cow Secretion

Standard dry cow secretion (SDCS) was prepared from mammary secretion obtained from 20 cows at 21 d of the dry period. Secretion from all animals was pooled and centrifuged at $48,000 \times g$ at $4^\circ C$ for 60 min. The fat layer was removed and the supernatant decanted. The cell-free, fat-free supernatant was sterilized by filtration through a series of membrane filters (Schleicher & Schuell, Keene, NH): prefilter; 12.0 μm; 5.0 μm; 1.2 μm; 0.8 μm; and 0.45 μm. Filtered secretion was stored at -20°C prior to use.

In Vitro Growth of Coliform Bacteria

In vitro growth of coliform bacteria was as described by Todhunter (Todhunter. 1990a) with the following exceptions. Prior to in vitro growth assays, coliform bacteria were grown for 6 h at $37^\circ C$ in 5 ml of trypticase soy broth containing 300 μM α-α'-dipyridyl. Cultures were washed twice in PBS and serially diluted in PBS to achieve approximately $10^6$ cfu/ml. All in vitro growth assays were performed in 96-well microtiter plates (Sarstedt, Newton, NC) with a total volume of 260 μl consisting of 250 μl of synthetic medium inoculated with 10 μl of diluted coliform bacteria. Growth assay in SDCS consisted of 240 μl of SDCS inoculated with 20 μl mixture of diluted bacteria plus IgG. Equal volume (10 μl:10 μl) of diluted coliform bacteria and concentrated IgG were mixed and incubated at $37^\circ C$ for 30 min prior to use. Cultures were aerobically incubated in a humidified atmosphere at $37^\circ C$ for 10 h for growth assay in synthetic medium or 18 h for growth assay in SDCS. All in vitro microcultures were in duplicate. Following incubation, cultures were serially diluted in PBS, and four 10-μl spots of
diluted and undiluted cultures were delivered onto the surface of a MacConkey agar plate (Difco Laboratories). MacConkey agar plates were incubated for 12 h at 37°C, bacterial colonies were counted, and in vitro growth was expressed as log_{10} colony-forming units per milliliter. Minimum detection limit of assay was 1.3979 cfu log_{10}/ml. Control medium for optimal growth of coliform bacteria was Bacto Synthetic Broth® (Difco Laboratories, Detroit, MI) (BSB). The iron-restricted medium was SDCS or BSB containing 0.5 mg/ml apoLf. Final concentration of sterile bovine IgG in iron-restricted synthetic medium or SDCS was 4 mg/ml. Ferric chloride (50 μM) was added in medium to determine if exogenous iron would override the inhibitory effects of antibodies.

**Statistical Analysis**

Differences between bacterial species in different media were analyzed by least squares analysis of covariance; growth in control medium (BSB for growth in synthetic medium and SDCS for growth in SDCS) was the covariant. Mean comparisons among different media within species were tested by ANOVA. Multiple comparisons of means of unequal sample size were by the Tukey-Kramer method. All statistical analyses were performed using MINITAB® software.
RESULTS

Growth Responses to Bovine IgG in BSB

Effects of purified IgG on the in vitro growth of coliform bacteria was initially tested by culturing the isolates in the synthetic medium BSB containing 0.5 mg/ml of apoLf and 4 mg/ml of IgG from cows immunized with FepA or Escherichia coli J5 (Table 4). Compared with the optimal growth in BSB plus dextrose, both E. coli and K. pneumoniae isolates had significant ($P < 0.05$) growth reduction in the presence of 0.5 mg/ml of apoLf. The addition of 4 mg/ml of purified bovine IgG directed against FepA in BSB supplemented with apoLf resulted in additional growth inhibition ($P < 0.05$) of both E. coli and K. pneumoniae isolates. Supplementation of BSB with bovine anti-FepA IgG resulted in greater growth reduction ($P < 0.05$) of E. coli than that of K. pneumoniae. Purified IgG from cows immunized with E. coli J5 had no inhibitory effect on the growth of E. coli isolates ($P > 0.05$) but had slight inhibitory effect ($P < 0.05$) on the growth of K. pneumoniae isolates. In vitro growth of E. coli AN193B2I, in the presence of IgG from cow immunized with FepA or E. coli J5 did not differ ($P > 0.05$) from the growth in BSB plus apoLf. Supplementation of 50 μM of ferric chloride to the medium completely reversed the inhibitory effects of lactoferrin and antibodies.

Growth Responses to Bovine IgG in SDCS

Table 5 showed the effects of purified IgG from cows immunized with FepA or E. coli J5 on the growth of E. coli and K. pneumoniae in SDCS. The addition of 4 mg/ml of purified anti-FepA IgG in SDCS resulted in significant ($P < 0.05$) growth reduction of E. coli isolates. Unlike the growth in synthetic medium, the growth of K. pneumoniae was
not significantly \((P > 0.05)\) inhibited by addition of purified IgG from cows immunized with FepA. Only 9 of 21 \(K.\) pneumoniae isolates (43%) experienced > 0.2 cfu log_{10}/ml of growth reduction in the presence of anti-FepA IgG in SDCS. Supplementation of 50 \(\mu M\) of ferric chloride to SDCS completely reversed the inhibitory effects of the antibodies. Purified IgG from cows immunized with \(E.\) coli J5 has no inhibitory effect \((P > 0.05)\) on the growth of \(E.\) coli isolates; but had an enhancing effect \((P < 0.05)\) on the growth of \(K.\) pneumoniae. The enhancing effect of anti-J5 IgG was > 0.2 cfu log_{10}/ml for 14 of 21 \(K.\) pneumoniae isolates in SDCS. Growth of only two \(E.\) coli isolates was enhanced ( > 0.2 cfu log_{10}/ml ) by addition of IgG from \(E.\) coli J5 immunized cows.

**DISCUSSION**

The purpose of the in vitro growth assays in BSB and in SDCS was to investigate the interaction between coliforms and host defenses during bacterial multiplication within iron-restricted environments. The current study showed that IgG from cows immunized with FepA successfully inhibited both \(E.\) coli and \(K.\) pneumoniae isolates in synthetic medium. These results differ from an earlier finding in which a MAb directed against FepA inhibited the growth of \(E.\) coli but had no significant effect on the growth of \(K.\) pneumoniae due to antigenic variation in the enterobactin ligand binding site (Chapter 3). Unlike polyclonal antiserum that is expected to contain a large quantity of different antibodies that recognize various epitopes, MAb is an epitope-specific antibody. The cows immunized with FepA may produce antibodies directed against the
epitopes at the ligand binding site or in the other regions of FepA that are important for FepA conformation. In the current study, anti-FepA IgG was demonstrated to inhibit growth of all coliform isolates in vitro. Supplementation of exogenous iron reversed the inhibitory effects of the antibodies. Therefore, bovine IgG directed against FepA apparently inhibited the growth of coliform bacteria by interfering with the binding of ferric enetrobactin complex to its cell surface receptor FepA because Gram-negative bacteria can use low affinity iron acquisition system when free iron is available in medium (Guerinot, 1994). Consequently, after antibodies migrate into the mammary gland, these anti-FepA antibodies may prevent iron uptake of heterologous coliforms by blocking FepA, thereby reduce coliforms’ ability to replicate and cause IMI during the nonlactating period.

The mechanism by which immunization with LPS core-antigen vaccines protect the mammary gland from clinical mastitis is not entirely understood. Previously proposed mechanisms of action for crossreactive immunity to Gram-negative bacterial infections include enhanced opsonization of bacterial cells and lipopolysaccharide (LPS), and detoxification of LPS by blocking active lipid A epitopes (Hogan et al., 1992a; Tyler et al., 1990). However, the ability of dry cow secretion to support phagocytosis is decreased during involution of the mammary gland (Paape et al., 1992). The decreased ability of opsonization was due to the formation of immune complexes that blocked Fc receptor on PMN (Targowski et al., 1986). The high concentration of Lf in the involuted mammary glands could account in part for the decrease in opsonic ability of dry cow secretion (Sordillo et al., 1987). Therefore, vaccines such as E. coli J5 bacterin may not
effectively prevent coliform IMI during the nonlactating period. The IROMP of Gram-
negative pathogens are often suggested as candidate immunogens for immunoprophylactic
therapy (Johnson et al., 1991; Weinberg, 1989; Williams, 1992; Yancey et al., 1979).
Antibodies raised against IROMP inhibited iron-uptake of some Gram-negative bacteria
(Roberts et al., 1989; Sokol et al., 1984, 1986a-b) and enhanced host immunity against
Gram-negative bacteria (Confer et al., 1995; Gilmore et al., 1991). Results from Chapter 3
and the current investigation demonstrated that monoclonal antibody and bovine
polyclonal antibodies against ferric enterobactin receptor FepA inhibited coliform
bacterial growth in vitro. Furthermore, considerable potential for FepA crossreactivity
was also observed in coliform bacteria from naturally occurring IMI (Chapter 3).

The complicated physical factors and their dramatic change in the secretion from
involuted mammary gland (Nonnecke et al., 1984) resulted in greater diversity of
bacterial growth in SDCS compared with growth in synthetic medium. Although the
growth of *K. pneumoniae* isolates was significantly inhibited in the presence of bovine
anti-FepA IgG in BSB, a low percentage of *K. pneumoniae* isolates were inhibited by
anti-FepA IgG in SDCS. Three possibilities may explain this finding. Murphy et al.
(1990) reported that ferric enterobactin-binding domains of FepA of *E. coli* strain are
free of LPS steric hindrance. No information is available now concerning the molecular
structure of FepA expressed by *K. pneumoniae* strain. Therefore, the polysaccharide
moiety of LPS may shield FepA of some *K. pneumoniae* isolates from the antibodies
interaction and concomitantly decreased the antibacterial effect of purified IgG from
cows immunized with FepA, especially in the presence of high concentration of Lf in
SDCS. In addition, the presence of a capsule surrounded *K. pneumoniae* in vivo (Williams et al., 1988) may prevent access of antibodies to their target, the bacterial outer membrane in SDCS. Consequently, anti-FepA IgG cannot easily bind to FepA receptor in SDCS. The third possibility is that some *K. pneumoniae* isolates possibly use other iron-uptake systems besides ferric enetrebactin system, such as iron-uptake system mediated by aerobactin. The aerobactin is more efficient than enterobactin as an in vivo iron scavengers (Wooldridge and Williams, 1993).

The concentration of Lf, an iron-binding protein, dramatically increased from 0.1 to 0.3 mg/ml in bovine milk to 30 mg/ml in dry cow secretion (Bishop et al., 1976). The high concentration of Lf attributed in part to the enhanced bacteriostatic properties of secretion from involuted bovine mammary gland (Bishop et al., 1976). Lf has bacteriostatic properties due to its ability to chelate iron (Weinberg et al., 1984). In addition, Lf could bind to the bacterial surface and directly kill bacteria by damaging the outer bacterial membrane, with subsequent change of its permeability (Ellison et al., 1988; Yamauchi et al., 1993). The bactericidal membrane damage involves binding of Lf to LPS molecules and releasing them from the outer membrane of Gram-negative bacteria (Yamauchi et al., 1993). More recently, Naidu et al. (1993) showed a correlation between Lf binding to porins and Lf-mediated bactericidal effect. Erdei et al. (1994) further showed that the binding of Lf to porins caused permeability changes of *E. coli*. In the current study, two different media were used to determine bacterial growth. In SDCS, bacterial growth was strongly inhibited compared with growth in BSB plus apoLf. The inhibitory property of SDCS was contributed to Lf, immunoglobulins, and other
unknown components (Todhunter et al., 1990a). Bactericidal function of Lf may play a
dominant bacteriostatic role in SDCS for some isolates because the Lf concentration in
the growth media was critical for the antibacterial effect (Naidu et al., 1993).
Alternatively, the low concentration of apoLf used in synthetic medium BSB was
sufficient to create an iron-restricted condition but possibly not enough to induce strong
bactericidal effect.

A surprising finding in the SDCS assay system was the growth of *K. pneumoniae*
being enhanced in the presence of purified IgG from cows immunized with *E. coli J5.*
The possibility of iron carryover in purified IgG that could overcome the inhibitory effect
caused by Lf was minimized. In order to exclude iron carryover in growth assay, affinity
purified bovine IgG was used. The small amount of extracellular iron in serum is bound
to transferrin (Otto, 1992). Most Gram-negative bacteria could utilize iron from
transferrin and heme compounds effectively (Weinberg, 1995). Protein G affinity
chromatography resulted in highly purified bovine IgG (Chapter 4) that apparently did
not contain any iron-binding protein. Therefore, some other mechanisms may attribute to
the enhancing growth of *K. pneumoniae* in the presence of anti-J5 IgG in SDCS. The
antiserum from cows immunized with *E. coli J5* contains elevated IgG titer against LPS
core antigen and against porins because porins are a group of molecules common in the
outer membrane surface of Gram-negative bacteria (Benz et al., 1988). The binding of
antibodies to LPS core antigen and porins may have prevented the bactericidal effect of
Lf. This finding suggested that *E. coli J5* bacteria may not effectively prevent coliform
IMI during the nonlactating period. The serum from cows immunized with *E. coli J5* also
contains a small amount of anti-FepA IgG because the cows immunized with *E. coli J5* appeared to have experienced natural exposure to Gram-negative bacteria (unpublished data). For *K. pneumoniae* isolates, the inhibitory effect of anti-FepA IgG in the purified IgG from J5 immunized cows may be completely shadowed by the enhancing effect of anti-LPS IgG and anti-porins IgG. Since a low percentage of *K. pneumoniae* isolates were inhibited by addition of purified IgG from FepA immunized cows in SDCS. Contrary to the growth of *K. pneumoniae*, the growth of *E. coli* isolates was slightly inhibited by addition of purified IgG from *E. coli J5* immunized cows in SDCS. This finding can be explained by the possibility that presence of a small amount of anti-FepA IgG in the purified IgG from cows immunized with *E. coli J5* may successfully override the growth-enhancing effect of anti-LPS IgG and anti-porins IgG in SDCS because anti-FepA IgG has stronger inhibitory effect on the growth of *E. coli* than that of *K. pneumoniae* in SDCS.
<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Bacterial type</th>
<th>E. coli (N = 25)</th>
<th>E. coli AN193B2l3</th>
<th>K. pneumoniae (N = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Log$_{10}$cfu/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSB</td>
<td>8.91$^{a,x}$</td>
<td>0.04</td>
<td>9.00$^{a,x}$</td>
</tr>
<tr>
<td></td>
<td>BSB + apoLf</td>
<td>6.98$^{b,x}$</td>
<td>0.16</td>
<td>7.53$^{b,y}$</td>
</tr>
<tr>
<td></td>
<td>BSB + apoLf + α-FepA</td>
<td>6.14$^{c,x}$</td>
<td>0.19</td>
<td>6.99$^{c,y}$</td>
</tr>
<tr>
<td></td>
<td>BSB + apoLf + α-J5</td>
<td>6.85$^{b,x}$</td>
<td>0.19</td>
<td>7.17$^{c,x}$</td>
</tr>
<tr>
<td></td>
<td>BSB + apoLf + α-FepA + FeCl$_3$</td>
<td>8.95$^{a,x,y}$</td>
<td>0.03</td>
<td>9.07$^{a,x}$</td>
</tr>
<tr>
<td></td>
<td>BSB + apoLf + α-J5 + FeCl$_3$</td>
<td>8.92$^{a,x,y}$</td>
<td>0.03</td>
<td>9.03$^{a,x}$</td>
</tr>
</tbody>
</table>

**TABLE 4.** Effects of purified IgG from cows immunized with FepA or *Escherichia coli* J5 on the growth of *Escherichia coli* and *Klebsiella pneumoniae* in chemically defined medium.

$a,b,c$ Means within a column followed by no common superscript differ ($P < 0.05$).
$x,y,z$ Means within a row followed by no common superscript differ ($P < 0.05$).

1BSB = Bacto synthetic broth® (Difco Laboratories, Detroit, MI); apoLf = apolactoferrin; α-FepA = purified IgG from cows immunized with FepA; α-J5 = purified IgG from cows immunized with *E. coli* J5. Concentration of apoLf = 0.5 mg/ml; concentration of α-FepA = 4 mg/ml; concentration of α-J5 = 4 mg/ml; concentration of ferric chloride (FeCl$_3$) = 50 μM.

2Number of isolates.

3Results are averaged from 6 independent determinations.
<table>
<thead>
<tr>
<th>Culture conditions¹</th>
<th>Bacterial type</th>
<th>E. coli (N = 20)²</th>
<th>K. pneumoniae (N = 21)²</th>
<th>E. coli AN193B_J₃³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \bar{x} )</td>
<td>SE</td>
<td>( \bar{x} )</td>
</tr>
<tr>
<td>SDCS</td>
<td>E. coli</td>
<td>3.92&lt;sup&gt;a,x&lt;/sup&gt;</td>
<td>0.32</td>
<td>5.35&lt;sup&gt;a,c,y&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>K. pneumoniae</td>
<td>5.23&lt;sup&gt;a,y&lt;/sup&gt;</td>
<td>0.20</td>
<td>3.58&lt;sup&gt;a,z&lt;/sup&gt;</td>
</tr>
<tr>
<td>SDCS + α-FepA</td>
<td>E. coli</td>
<td>3.17&lt;sup&gt;b,x&lt;/sup&gt;</td>
<td>0.37</td>
<td>5.67&lt;sup&gt;b,c,y&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>K. pneumoniae</td>
<td>5.23&lt;sup&gt;b,y&lt;/sup&gt;</td>
<td>0.20</td>
<td>3.58&lt;sup&gt;b,z&lt;/sup&gt;</td>
</tr>
<tr>
<td>SDCS + α-J₅</td>
<td>E. coli</td>
<td>3.69&lt;sup&gt;a,x&lt;/sup&gt;</td>
<td>0.32</td>
<td>8.44&lt;sup&gt;c,x&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>K. pneumoniae</td>
<td>5.67&lt;sup&gt;b,c,y&lt;/sup&gt;</td>
<td>0.23</td>
<td>8.75&lt;sup&gt;d,y&lt;/sup&gt;</td>
</tr>
<tr>
<td>SDCS + FeCl₃</td>
<td>E. coli</td>
<td>8.41&lt;sup&gt;c,x&lt;/sup&gt;</td>
<td>0.12</td>
<td>8.78&lt;sup&gt;d,y&lt;/sup&gt;</td>
</tr>
<tr>
<td>SDCS + α-FepA + FeCl₃</td>
<td>E. coli</td>
<td>8.59&lt;sup&gt;c,x&lt;/sup&gt;</td>
<td>0.08</td>
<td>8.78&lt;sup&gt;d,y&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

TABLE 5. Effects of purified IgG from cows immunized with FepA or *Escherichia coli* J5 on the growth of *Escherichia coli* and *Klebsiella pneumoniae* in standard dry cow secretion.

²Means within a column followed by no common superscript differ (\( P < 0.05 \)).
²Means within a row followed by no common superscript differ (\( P < 0.05 \)).
¹SDCS = standard dry cow secretion; α-FepA = purified IgG from cows immunized with FepA; α-J5 = purified IgG from cows immunized with *E. coli* J5. Concentration of α-FepA = 4 mg/ml; concentration of α-J5 = 4 mg/ml; concentration of ferric chloride (FeCl₃) = 50 μM.
²Number of isolates.
³Results are averaged from 6 independent determinations.
CHAPTER 6
SUMMARY AND CONCLUSIONS

The area of coliform mastitis control with the greatest advances in recent years is vaccination (reviewed in Yancey, 1993). The commercial sale of *Escherichia coli J5* (O111:B4) vaccines provides dairy producers with a management tool to reduce the severity and duration of clinical signs following a coliform IMI (Gonzalez et al., 1989; Hogan et al., 1992b, 1992c, 1995; Hill et al., 1991). The physiological process that *E. coli J5* vaccines protect the mammary gland is by increasing antibodies to coliform bacteria for neutrophils to more effectively kill the bacteria once an infection has established (Hogan et al., 1992a; Tyler et al., 1990). However, *E. coli J5* vaccines do not prevent IMI and the influx of protective antibodies into the gland begins 12 to 24 h after bacterial populations began to increase (Hogan et al., 1992b, 1992c, 1995). In addition, the ability of dry cow secretion to support phagocytosis is decreased during involution of the mammary gland (Pappe et al., 1992). Therefore, the need still exists for an effective vaccine to prevent IMI and control the growth of coliform bacteria in the bovine mammary gland, especially during the nonlactating period when the rate of new coliform IMI is fourfold higher than during lactation.

This study was an unique approach to achieve potential control of coliform mastitis during the nonlactating period. The hypothesis was that coliform bacterial
growth can be controlled by preventing the uptake of essential nutrients, such as iron.

Each coliform bacteria isolated from naturally occurring IMI produced siderophore and expressed iron-regulated outer membrane proteins under an iron-restricted environment (Chapter 2). The ferric enterobactin receptor, FepA, was distributed widely among gram-negative isolates from different sources. In addition, both the molecular mass and the antigenic properties of FepA were highly conserved.

The iron-regulated outer membrane proteins of Gram-negative bacterial pathogens are often suggested as vaccine candidate for immunoprophylactic therapy (Byers, 1987; Gray-Owen et al., 1996; Johnson, 1991; Weinberg, 1989; Williams et al., 1992; Zollinger et al., 1991). Results from in vitro growth studies using synthetic medium (Chapter 3) demonstrated that monoclonal antibody specific for blocking ferric enterobactin binding site of FepA inhibited the growth of *E. coli* in vitro. Antigenic variation in the enterobactin ligand binding site resulted in a low percentage of *K. pneumoniae* isolates that were inhibited by the monoclonal antibody. Low concentrations of antibody against the FepA epitope had an inhibitory effect on coliform growth in vitro.

The anti-FepA antibodies used in Chapter 2 and Chapter 3 are from lab animals. Antigenicity of FepA protein in cows was investigated in chapter 4. FepA protein was successively extracted from *Escherichia coli* 471 by N-lauroylsarcosine sodium salt and Triton X-100 and ion-exchange chromatography resulted in substantial purification of FepA. Immunization with FepA elicited an immunological response in serum and milk. Serum and whey IgG titers to FepA proteins from cows immunized with FepA were significantly higher than those from cows immunized with either *Escherichia coli J5* or
PBS. ELISA tests showed that purified IgG from cows immunized with FepA was crossreactive to *E. coli* and *K. pneumoniae* isolates from naturally occurring bovine IMI.

The growth responses of coliform bacteria in chemically defined medium containing apoLf to purified IgG from cows immunized with FepA (Chapter 5) demonstrated that those antibodies inhibited the growth of each coliform isolate derived from naturally occurring bovine IMI. In SDCS, all *E. coli* isolates and 43% of *K. pneumoniae* isolates were inhibited by addition of anti-FepA IgG. Supplementation of exogenous iron to the medium completely reversed the inhibitory effects of antibodies. This suggested that bovine IgG directed against FepA inhibited the growth of coliform bacteria by interfering with the binding of ferric enterobactin complex to its cell surface receptor FepA.

The results of these studies suggest that FepA protein may be used as an effective vaccine component to optimize current vaccination programs against coliform IMI. Clinical trials are needed to determine whether the FepA vaccine would prevent coliform IMI and further reduce severity and clinical episodes of coliform mastitis during the nonlactating period compared with currently available whole-cell bacteria.
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