CURRENT MICROBIOLOGY Vol. 20 (1990), pp. 47–52

Current Microbiology

Coaggregation of Urogenital Bacteria in Vitro and in Vivo

Gregor Reid,[1,2] Jacqueline A. McGroarty,[1] P. A. Gil Domingue,[3] Anthony W. Chow,[4] Andrew W. Bruce,[1] Andrea Eisen,[2] and J. William Costerton[3]

[1]Division of Urology, Department of Surgery, University of Toronto and Toronto General Hospital, Toronto; [2]Department of Microbiology, University of Toronto; [3]Department of Biological Sciences, University of Calgary; and [4]Division of Infectious Diseases, Department of Medicine, University of British Columbia, Canada

Abstract. The working hypotheses of the present study were that (1) bacterial coaggregates exist in the urogenital tract of healthy and infected women, and (2) coaggregation reactions can occur in vitro between members of the urogenital flora. Examination of urogenital specimens from 25 healthy women showed that lactobacilli were the dominant organisms colonizing the epithelia and coaggregating with other Gram-positive and Gram-negative bacteria. In vitro light and electron microscopic studies confirmed that members of the urogenital flora could coaggregate. An examination of specimens from 9 women with urinary tract infection showed the presence of autoaggregated uropathogens free-floating in the urine and attached to epithelial cells. The phenomenon of autoaggregation was also noted in vitro for various uropathogens, suggestive that this may represent a virulence factor. It is evident that bacterial cell-to-cell binding within a strain and among different genera occurs in the urogenital tract. Further studies of the mechanisms that maintain and disrupt these microbial interactions will help to improve our understanding of disease initiation.

The female genital tract is colonized by a dynamic array of aerobic and anaerobic microorganisms [8, 9, 16, 25]. Under certain conditions, organisms emerge from this ecosystem and cause infection in the urinary and vaginal areas [12]. Many factors, including trauma, hormones, and antibiotics [26, 27] can disrupt the normal flora of the vagina and cause disturbances within the bacterial ecosystem [13, 21]. The interaction of bacteria within the urogenital tract is poorly understood, and the present study was designed to examine aspects of this in relation to health and disease.

In parallel with the in vivo studies, it was decided to test our second working hypothesis that coaggregation reactions can occur in vitro. Similar studies have provided valuable information in relation to coaggregation in the oral cavity [5, 7]. The first report of this phenomenon in relation to the urogenital tract showed that lactobacilli coaggregated with a few uropathogens, mainly Gram-negatives [22]. The present study is an extension of these investigations.

Materials and Methods

In vivo studies. Twenty-five healthy premenopausal women consented to provide specimens for the study, authorized by the Ethics Committee of Vancouver General Hospital. None of the patients was receiving antimicrobial therapy. Specimens were collected by scraping material from the vulva, the posterior lateral third of the vaginal wall, and the exocervix with a sterile tongue depressor. The individual specimens were immediately cultured semi-quantitatively for aerobes and anaerobes by use of brain–heart infusion (BHI, Difco, Detroit, Michigan, USA) and Rogosa SL (RSL, Difco, Detroit) media. The specimens were incubated in air, 10% CO_2, and anaerobically (model 1024 anaerobic chamber, Caltec Ltd, Calgary). Full bacteriological (generic) identification was carried out with standard technology, in specimens from three patients, while the dominant organism alone was identified in the other 22 patients. Subsamples were prepared for transmission electron microscopy (TEM, as below). In addition, urine specimens from 9 female patients who presented with acute urinary tract infections were examined for the presence of bacterial aggregates. Their epithelial cells and unspun urine were examined microscopically and by culture.

In a separate study, bacterial clusters were observed in wet mounts of a vaginal specimen from a healthy woman. A Pasteur pipette, guided under a light microscope, was used to remove a cluster of organisms. The material was placed on a brain–heart

Address reprint requests to: Dr. Gregor Reid, Urology Research, Bell Wing G-631, Toronto General Hospital, 200 Elizabeth Street, Toronto, Ontario, Canada, M5G 2C4.

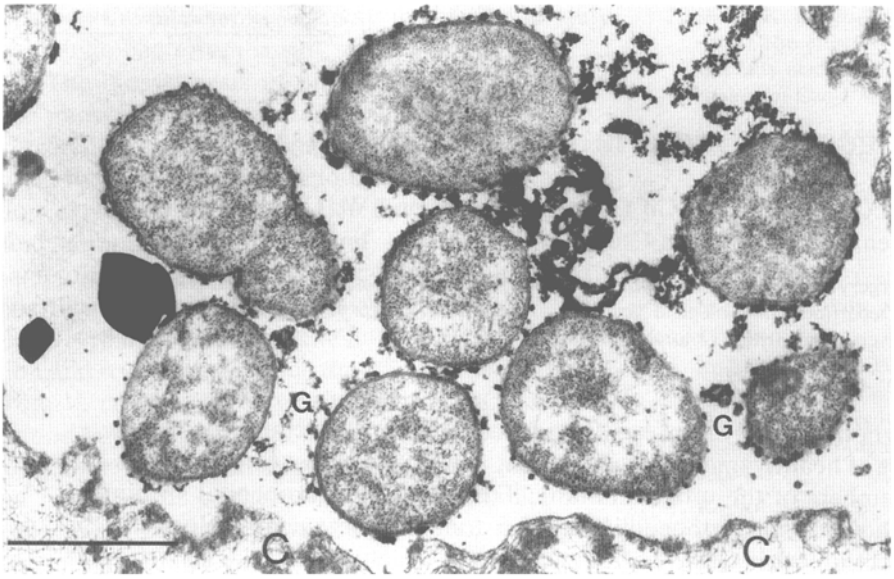

Fig. 1. TEM of a section of ruthenium red-stained preparations showing aggregating bacteria surrounded by fibrous glycocalyx material (G), in an adherent microcolony on the surface of an epithelial cell (C). Bar = 1.0 μm.

infusion–yeast extract–agar plate, gently disaggregated, and cultured aerobically overnight at 37°C.

Transmission electron microscopy. The specimens were prepared as described previously [4]. Briefly, the cells were fixed in 5% glutaraldehyde in cacodylate buffer (0.1 *M*, pH 7.2) with 0.15% ruthenium red for 2 h at room temperature before being post fixed in osmium tetroxide, dehydrated in acetone and propylene oxide, embedded in Spurr resin, sectioned, and stained with uranyl acetate and lead citrate.

Scanning electron microscopy was also carried out on selected specimens, as described previously [3].

Bacteria. The bacteria used for in vitro testing were selected for specific reasons. *Lactobacillus casei* GR-1 is a human distal urethral isolate, which has been extensively studied for adhesion to epithelial cells [20] and antagonism against uropathogens [14, 15, 22]; *L. acidophilus* strains 76 (ATCC 4357) and T-13, *L. brevis* 189, and *L. fermentum* A-60 and B-54 are representative of the most common vaginal isolates [24]. In addition, *L. fermentum* B-54 has been shown to have inhibitory activity against enterococci [15]. A beta-hemolytic streptococcus strain 6698 and a diphtheroid strain H (identified as a *Corynebacterium*) were freshly isolated from the vagina of two healthy women.

The uropathogens tested in the coaggregation studies comprised: *Escherichia coli* ATCC 25922 as a control, along with type 1 fimbriated *E. coli* strain 2239 (fimbriae detected by mannose-sensitive hemagglutination of horse red blood cells and by identification of the fimbriae by electron microscopy; 17). *Ent. faecalis* strain 29212 is an ATCC organism, strain 6696 came from the vagina of a healthy adult, and strains 1396, C1030, IC14, 1331 and 4b, along with *Staphylococcus saprophyticus* YA, were isolated from the urine of patients with urinary tract infections. *Staphylococcus epidermidis* 1938 is a catheter isolate from a patient with bacteriuria. The bacteria were grown in brain–heart infusion broth (Difco, Detroit) supplemented with 2% yeast extract (Difco, Detroit).

Coaggregation assay. The coaggregation assay provides a measure of interaction between bacteria. The assay has been described in full elsewhere [22]. Briefly, 500 μl of bacterium A (10^9/ml PBS) was combined with 500 μl of bacterium B (10^9/ml) in a 24-well tissue culture tray (Costar, Canada). After being mixed, the bacteria were incubated at 37°C in an orbital shaker at 100 r.p.m. for 4 h. The suspensions were then scored for coaggregation according to the following scale: 0 = no aggregation, 1 = small aggregates comprising small visible clusters of bacteria, 2 = aggregates comprising larger numbers of bacteria, settling to the center of the well, 3 = macroscopically visible clumps comprising larger groups of bacteria which settle to the center of the well, 4 = maximum score allocated to describe a large, macroscopically visible clump in the center of the well. The aggregates were visualized using an inverted light microscope with a ×16 magnification lens. Two strains known to coaggregate, namely *L. casei* GR-1 and *E. coli* ATCC 25922, were employed as a positive control [22]. Auto-aggregation was assessed for each bacterial strain (500 μl bacterium A plus 500 μl PBS). The assay was performed in duplicate, and selected samples were examined by electron microscopy to assess the reactions more closely.

Results

An electron microscopic examination of urogenital specimens from 25 healthy premenopausal women demonstrated the presence of bacterial populations in planktonic form and adherent to epithelial surfaces. Representative illustrations are presented in Figures 1 and 2. The organisms were predominantly Gram-positive rods, identified by culture as lactobacilli, seen in microcolonies and in coaggregates with morphologically distinct Gram-positive and Gram-negative bacteria, (based on cell wall/envelope mor-

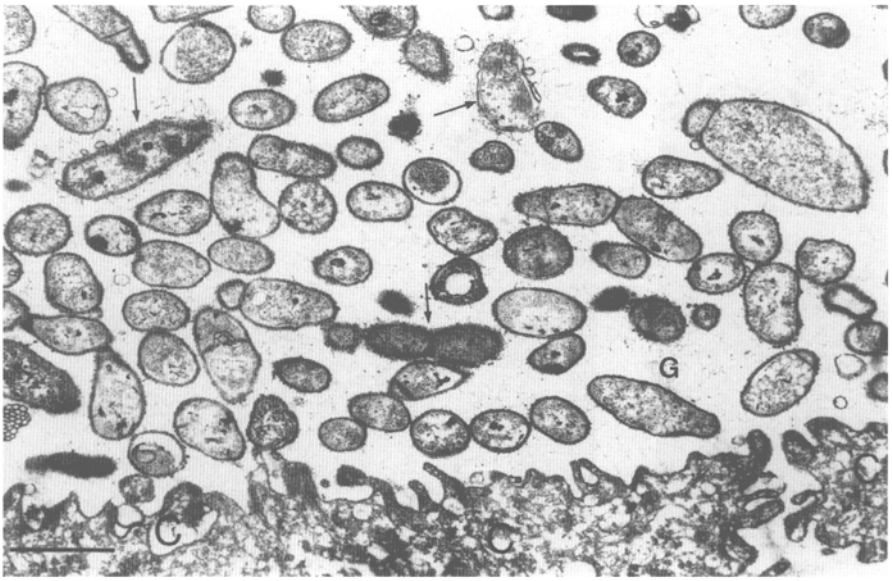

Fig. 2. TEM of a section of a ruthenium red-stained preparation scraped from the vaginal epithelium of a healthy woman, showing part of a very large adherent bacterial microcolony coaggregate on the cell surface (C) with glycocalyx material (G) around the cells. An extensive examination of epithelial cells from the urogenital tract showed that the majority of the bacterial cells were Gram-positive rods (confirmed as lactobacilli by culture), but some Gram-negative cells were also present, as highlighted by arrows in this micrograph. Bar = 1.0 μm.

Table 1. Percentage of bacteria present in urogenital specimens from three[a] healthy women

Percentage of isolated bacteria					
Vagina		Exocervix		Vulva	
Lactobacilli	27.3	Lactobacilli	27.4	Lactobacilli	22.6
Peptostrep.	14.7	Peptostrep.	15.1	Group B strep.	16.1
Group D strep.	13.9	Enterobact.	14.6	Peptostrep.	14.9
Enterobact.	13.8	Group D strep.	13.9	Group D strep.	13.5
Group B strep.	11.1	Group B strep.	12.4	Enterobact.	12.9
Corynebacterium	10.2	Coag. neg. staph	8.4	Coag. neg. staph	11.1
Coag. neg. staph	9.0	Corynebacterium	8.2	Corynebacterium	8.9

Peptostrep = peptostreptococci; strep = streptococci; enterobact = enterobacteriaceae; coag neg staph = coagulase-negative staphylococci.

[a] Lactobacilli were found to be the dominant organism in specimens from 22 other healthy women.

phology) (Fig. 2). This domination of lactobacilli occurred in specimens from all three sites, as verified by culture results, which showed secondary isolates to be peptostreptococci, group D and B streptococci, and enterobacteriaceae (Table 1). Anaerobic lactobacilli were more common than aerobic isolates in the vagina (15.9% versus 11.4%), exocervix (15.3% versus 12.1%), and on the vulva (13.9% versus 8.9%).

In certain specimens, the microcolonies were dense and surrounded by glycocalyx material in a dense biofilm. The bacteria isolated from the wet mount specimen were identified as *Lactobacillus* spp. and a coagulase-negative staphylococcus. After in vitro culture and testing, the lactobacillus (termed Lact 1) and staphylococcus (termed Staph 1) coaggregated on a net score of 1–2 (Table 2).

Infected urine specimens were found to contain autoaggregates of uropathogens (coliforms with autoaggregation scores 1–2) free floating and adherent to epithelial cells. When uropathogen strains 2239, 1331, YA, and 1938 were inoculated into fresh sterilized urine, they were found to autoaggregate, giving a score of 1–2.

In additional in vitro tests, lactobacilli coaggregated with other members of the normal flora, as well as with uropathogens (Table 2). There was a degree of coaggregation noted between lactobacilli and enterococci. Samples examined under scanning electron microscopy showed that the interaction

Table 2. Combinations of urogenital flora found to coaggregate in vitro

Coaggregating pairs			Score[a]
Control:			
L. casei GR-1	with	*E. coli* ATCC 25922	3
Bacteria isolated from wet mount of vaginal swab:			
Lact 1	with	Staph 1	1–2
Other in vitro tests:			
L. casei GR-1	with	*S. epidermidis* 1938	2
	with	*E. coli* 2239	2
	with	streptococcus 6698	1
	with	enterococcus 1331	0–1
	with	enterococcus 1396	0
	with	enterococcus C1030	0
	with	enterococcus IC14	1
	with	enterococcus 4b	1
	with	enterococcus ATCC 29212	0
	with	diphtheroid II	1
L. fermentum B-54	with	enterococcus 1396	0
	with	enterococcus C1030	0
	with	enterococcus IC14	0
	with	enterococcus ATCC 29212	0
	with	enterococcus 1331	0–1
	with	enterococcus 4b	0–1
L. acidophilus 76 (ATCC 4357)	with	*E. coli* 2239	1
L. acidophilus T-13	with	*S. saprophyticus* YA	1
L. brevis 189	with	*E. coli* 2239	1
L. fermentum A-60	with	*E. coli* 2239	4
Diphtheroid II	with	enterococcus 1331	2
	with	enterococcus 6696	4
Streptococcus 6698	with	*E. coli* 2239	1

[a] Scores from 0 = no coaggregation to maximum of 4.

took place within 1 h (Fig. 3), although coaggregates were difficult to detect by light microscopy after 3–4 h of incubation. *Ent. faecalis* 1331 appeared to bind to *L. casei* GR-1 in single cells (Fig. 3) and was only marginally seen under the light microscopy (score of 0–1), whereas *Ent. faecalis* 4b bound to *L. casei* GR-1 in large clusters (Fig. 4), giving a clearly visible light microscopy score of 1. The clustering effect was not due solely to autoaggregation and seemed to occur only around the lactobacillus cells. Furthermore, individual enterococci were seen in a non-autoaggregated state.

Discussion

The present report has shown that the urogenital mucosa of healthy premenopausal women is colonized by microbial populations dominated by lactobacilli. This has been documented previously from an epidemiological perspective [16, 25]. The latest morphologic examination illustrates how bacterial coaggregates exist both in planktonic form and adherent to epithelial tissues. These autochthonous bacterial populations were found to contain a wide array of bacteria, tightly knit in coaggregates. The finding that coaggregated bacteria from the vaginal flora could be isolated and re-aggregated in vitro validates the use of the assay as a test for coaggregation reactions. Furthermore, it demonstrates that the coaggregation reactions can occur in phosphate-buffered saline and do not require the presence of urinary or mucus components.

Previous studies have shown that lactobacilli can exclude adherence of uropathogens [3, 4, 20] and prevent the onset of urinary tract infection in animals [19] and humans [2]. The recent in vivo findings showed that coaggregation of lactobacilli with normal flora and potential uropathogens (enterobacteriaceae, enterococci, and coagulase-negative staphylococci) occurs in healthy patients. This implies that an ecological balance exists in the presence of potential uropathogens. Therefore, complete exclusion of uropathogens from the urogenital mucosa does not appear to be essential for protec-

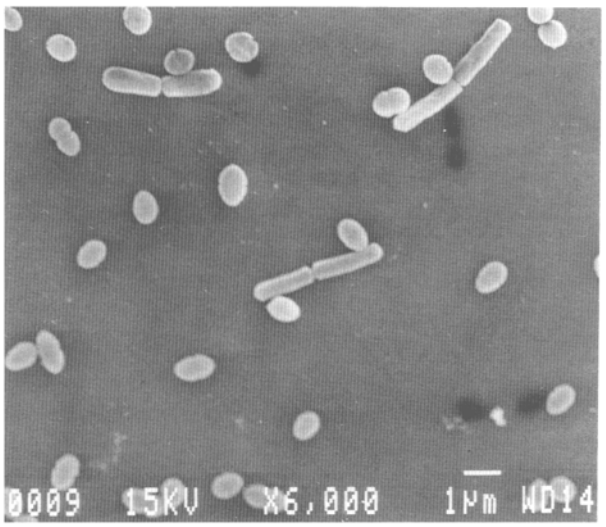

Fig. 3. Scanning electron micrograph of *L. casei* GR-1 coaggregating in single cells with *Ent. faecalis* 1331, following an in vitro assay.

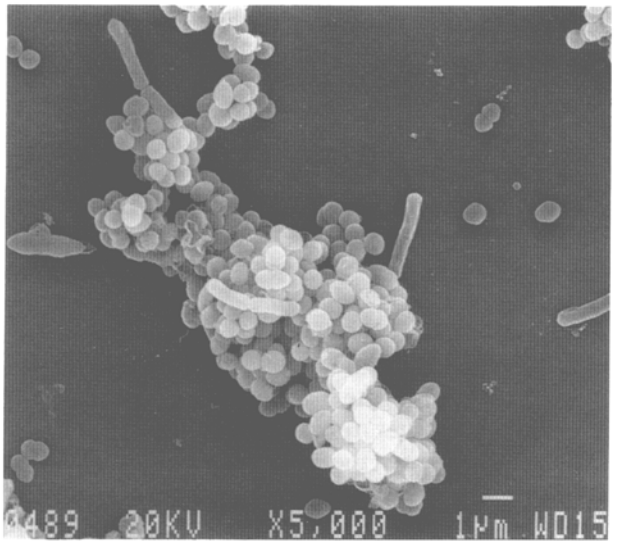

Fig. 4. Scanning electron micrograph of *L. casei* GR-1 coaggregating with clusters of *Ent. faecalis* 4b, following an in vitro assay.

tion of the host against infection. This is supported by a previous study that showed that lactobacilli and enterococci could coexist in the endocervical mucosa without detriment to the patient [23]. The latest findings also demonstrate the coexistence of lactobacilli with group D streptococci in the healthy urogenital tract and show that the two species can coaggregate in vitro. This interaction was observed to be in the form of single-cell and multiple-cell binding. Coaggregation of lactobacilli was also noted with *Escherichia coli*, organisms which attach to cells and uromucoid via type 1 fimbriae and which are the most common cause of urinary tract infections [18]. The interplay between coaggregation of lactobacilli with uropathogens and emergence of the latter to infect the bladder remains to be elucidated.

Another question worth posing is what influences the ecological balance? Previous studies have indicated that certain lactobacilli produce inhibitory substances that can reduce *E. coli* and enterococcal numbers [14, 15]. This activity is believed to have a role in the maintenance of a healthy status. The importance of inhibitor production is further supported by the clinical evidence that enterococci, resistant to inhibitor action, can coexist with lactobacilli and emerge to infect the urinary tract [2]. It is possible that the ability of uropathogens to resist inhibitory activity, to coaggregate with normal flora, and to flourish under conditions of low pH represents a virulence factor(s) of these organisms.

In a series of experiments, various bacterial strains were found to interact in a similar fashion to the in vivo situation. The actual mechanisms of coaggregation remain to be elucidated, as does the significance of high scoring reactions, such as those found for *Lactobacillus fermentum* A-69 with *E. coli* 2239, and for Diphtheroid II with *Ent. faecalis* 6696. By following lines of investigation similar to previous in vitro work related to the oral cavity, it should be possible to investigate multigeneric pairings and to examine their mechanism of interaction [7, 10, 11]. One point of caution is that, although the coaggregation assay used by ourselves and others can demonstrate reactions, it may not be sensitive enough to detect all interbacterial binding. The system utilizes visual assessment under light microscopy, but additional techniques such as electron microscopy may be required to detect weak coaggregation reactions that cannot be otherwise visualized. In addition, if autoaggregation occurs, it can be more readily distinguished from coaggregation by electron microscopy, as can the binding of multiple organisms to a single bacterium.

The autoaggregation phenomenon was noted for uropathogens in infected urine and was confirmed by in vitro testing. Similar associations with aggregation have been reported in relation to intra-abdominal sepsis [1] and peritonitis (Reid et al., submitted). On prosthetic surfaces, bacterial aggregation leading to biofilm formation has been associated with resistance to host defenses and antimi-

crobial activity [6]. The latter point was not investigated here, but autoaggregation was noted among uropathogens. In addition, coaggregation was seen between streptococci and *E. coli*, suggestive that this may be an initial stage in biofilm build-up. The types of organisms that coaggregate on a surface and in free-floating form will likely determine whether the outcome for the patient is an infectious or a healthy one.

In conclusion, there appears to be a relation between a lactobacilli-dominated urogenital microflora and a healthy patient status. The ability of lactobacilli to coaggregate with other bacteria probably influences the structure and stability of the urogenital flora. However, the ecological balance can be altered by microbial factors, possibly including autoaggregation. Further studies on coaggregation will increase our understanding of the microflora that inhabit the urogenital mucosa in infected and healthy patients.

ACKNOWLEDGMENTS

This work was funded by the Medical Research Council of Canada and in part by The Physicians Services Incorporated Foundation and The Kidney Foundation of Canada. Additional thanks go to The University Research Incentive Fund and Merck Frosst Canada for assisting with equipment purchase. Dr. Reid is an Ontario Ministry of Health Career Scientist.

We are grateful to Dr. Roger L. Cook, Carol Preston, Rosanne Angotti, and Dale Autry for technical assistance, and to Joyce Nelligan for helping with electron microscopy. The support of the Microbiology Departments at Vancouver General and Toronto General Hospitals was appreciated.

Literature Cited

1. Blake M, Rotstein O, Llano M, Girotti MJ, Reid G (1989) Aggregation of fragilis and non-fragilis Bacteroides strains. J Med Microbiol 28:9–14
2. Bruce AW, Reid G (1988) Intravaginal instillation of lactobacilli for prevention of recurrent urinary tract infections. Can J Microbiol 34:339–343
3. Chan RCY, Bruce AW, Reid G (1984) Adherence of cervical, vaginal and distal urethral normal microbial flora to human uroepithelial cells and the inhibition of adherence of gram-negative uropathogens by competitive exclusion. J Urol 131:596–601
4. Chan RCY, Reid G, Irvin RT, Bruce AW, Costerton JW (1985) Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. Infect Immun 47:84–89
5. Cisar JO (1982) Coaggregation reactions between oral bacteria: studies of specific cell-to-cell adherence mediated by microbial lectins. In: Genco RJ, Mergenhagen SE (eds) Host-parasite interactions in periodontal diseases. Washington, DC: ASM Publications, pp 121–131
6. Costerton JW, Cheng K-J, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464
7. Ellen RP, Schwarz-Faulkner S, Grove DA (1988) Coaggregation among periodontal pathogens, emphasizing *Bacteroides gingivalis-Actinomyces viscosus* cohesion on a saliva-coated mineral surface. Can J Microbiol 34:299–306
8. Fair WR, Timothy MM, Millar MA, Stamey TA (1970) Bacteriologic and hormonal observations of the urethra and vaginal vestibule in normal, premenopausal women. J Urol 104:426–431
9. Gorbach SL, Menda KB, Thadepalli H, Keith L (1973) Anaerobic microflora of the cervix in healthy women. Am J Obstet Gynecol 117:1053–1055
10. Kolenbrander PE, Anderson RN (1986) Multigeneric aggregation among oral bacteria: a network of independent cell-to-cell interactions. J Bacteriol 168:851–859
11. Komiyama K, Gibbons RJ (1984) Interbacterial adherence between *Actinomyces viscosus* and strains of *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Pseudomonas aeruginosa*. Infect Immun 44:86–90
12. Larsen B, Galask RP (1980) Vaginal microbial flora: practical and theoretical relevance. Obstet Gynecol Suppl 55:S100–113
13. Mackowiak PA (1982) The normal microbial flora. N Engl J Med 307:83–93
14. McGroarty JA, Reid G (1988) Detection of a lactobacillus substance which inhibits *Escherichia coli*. Can J Microbiol 34:974–978
15. McGroarty JA, Reid G (1988) Inhibition of enterococci by *Lactobacillus* species *in vitro*. Microb Ecol Health Dis 1:215–219
16. Marrie TJ, Swantee CA, Hartlen M (1980) Aerobic and anaerobic urethral flora of healthy females in various physiological age groups and of females with urinary tract infections. J Clin Microbiol 11:654–659
17. Reid G, Brooks HJL (1984) *In vitro* attachment of *Escherichia coli* to human epithelial cells. N Z Med J 97:439–442
18. Reid G, Sobel JD (1987) Bacterial adherence in the pathogenesis of urinary tract infections: a review. Rev Infect Dis 9:470–487
19. Reid G, Chan RCY, Bruce AW, Costerton JW (1985) Prevention of urinary tract infection in rats with an indigenous *Lactobacillus casei* strain. Infect Immun. 49:320–324
20. Reid G, Cook RL, Bruce AW (1987) Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. J Urol 138:330–335
21. Reid G, Bruce AW, Beheshti M (1988) Effect of antibiotic treatment on receptivity of uroepithelial cells to uropathogens. Can J Microbiol 34:327–331
22. Reid G, McGroarty JA, Angotti R, Cook RL (1988) Lactobacillus inhibitor production against *E. coli* and coaggregation ability with uropathogens. Can J Microbiol 34:344–351
23. Reid G, Hawthorn LA, Mandatori R, Cook RL, Beg HS (1988) Adhesion of lactobacilli to polymer surfaces *in vivo* and *in vitro*. Microb Ecol 16:241–251
24. Rogosa M, Sharpe ME (1960) Species differentiation of human vaginal lactobacilli. J Gen Microbiol 23:197–201
25. Sautter RL, Brown WJ (1980) Sequential vaginal cultures from normal young women. J Clin Microbiol 11:479–484
26. Sprunt K, Leidy G (1988) The use of bacterial interference to prevent infection. Can J Microbiol 34:332–338
27. Sprunt K, Redman W (1968) Evidence suggesting importance of role of interbacterial inhibition in maintaining balance of normal flora. Ann Int Med 68:579–590