Human α-amylase Present in Lower-Genital-Tract Mucosal Fluid Processes Glycogen to Support Vaginal Colonization by *Lactobacillus*

Gregory T. Spear,1 Audrey L. French,2 Douglas Gilbert,1 M. Reza Zariffard,1 Paria Mirmontsef,1 Thomas H. Sullivan,1 William W. Spear,1 Alan Landay,1 Sandra Micci,2 Byung-Hoo Lee,3 and Bruce R. Hamaker3

1Department of Immunology/Microbiology, and 2CORE Center of Cook County Health and Hospitals System, Rush University Medical Center, Chicago, Illinois; and 3Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, West Lafayette, Indiana

*Lactobacillus* colonization of the lower female genital tract provides protection from the acquisition of sexually transmitted diseases, including human immunodeficiency virus, and from adverse pregnancy outcomes. While glycogen in vaginal epithelium is thought to support *Lactobacillus* colonization in vivo, many *Lactobacillus* isolates cannot utilize glycogen in vitro. This study investigated how glycogen could be utilized by vaginal lactobacilli in the genital tract. Several *Lactobacillus* isolates were confirmed to not grow in glycogen, but did grow in glycogen-breakdown products, including maltose, maltotriose, maltopentaose, maltodextrins, and glycogen treated with salivary α-amylase. A temperature-dependent glycogen-degrading activity was detected in genital fluids that correlated with levels of α-amylase. Treatment of glycogen with genital fluids resulted in production of maltose, maltotriose, and maltotetraose, the major products of α-amylase digestion. These studies show that human α-amylase is present in the female lower genital tract and elucidates how epithelial glycogen can support *Lactobacillus* colonization in the genital tract.

**Keywords.** α-amylase; female genital tract; glycogen; *Lactobacillus*; maltose.

Colonization of the lower genital tract with *Lactobacillus* is an important component of the reproductive health of women. *Lactobacillus* inhibits other microbes in the genital tract, primarily by creating a low genital pH through the production of lactic acid [1–4]. This inhibition results in a genital microbiota that is predominantly composed of *Lactobacillus* in many women.

Factors that disrupt *Lactobacillus* colonization can lead to a shift of the genital microbiota to bacterial vaginosis (BV) in which *Lactobacillus* is no longer the predominant type of bacteria [1, 5–7]. Epidemiologic studies show that a genital microbiota dominated by *Lactobacillus* is protective from the acquisition of human immunodeficiency virus (HIV) when compared to BV (reviewed in [8]). In HIV-infected women, a *Lactobacillus*-dominated microbiota is also associated with decreased shedding of virus, decreased risk of heterosexual transmission to men, and vertical transmission to the fetus during birth [9–14]. Further, a genital microbiota dominated by *Lactobacillus* is associated with a lowered risk of acquiring other sexually transmitted diseases (STDs), such as herpes simplex virus type 2, chlamydia, and gonorrhea [15, 16]. Finally, *Lactobacillus* also protects against developing BV, pelvic inflammatory disease, and adverse fetal outcomes [17–21]. Certain species of *Lactobacillus* have been found to be more protective [4, 22].

Although *Lactobacillus* is recognized as important for genital tract health, the factors responsible for its colonization are not well understood. For example, while BV can be treated with antibiotics, *Lactobacillus* does not always become reestablished as the dominant bacteria type, and BV recurs in 60%–80% of cases after 1 year [23, 24]. However, a relationship between vaginal epithelial glycogen and *Lactobacillus* colonization in the female lower genital tract has been recognized for many decades. Thus, Cruickshank and others [25, 26]...
observed that at puberty, glycogen becomes expressed in the vaginal epithelium and at the same time colonization with *Lactobacillus* becomes apparent. Those studies showed that both glycogen expression and colonization by *Lactobacillus* persist through life until at menopause, when both *Lactobacillus* colonization and glycogen expression decline.

Despite the evidence that glycogen promotes colonization by lactobacilli, most isolates of *Lactobacillus* do not grow in vitro in media containing glycogen as the carbohydrate source. Thus, Stewart-Tull [27] reported in 1964 that a number of isolates of *Lactobacillus* did not ferment glycogen. Later in that decade, Wylie and Henderson [28] showed that 39 of 42 isolates of *Lactobacillus* isolated from the genital tract of women. More recently, Martin [29] showed 45 strains of vaginal *Lactobacillus* could not use glycogen. The lack of *Lactobacillus* growth in vitro in the presence of glycogen leaves open the question of how glycogen could be utilized in vivo by *Lactobacillus*. The goal of this study was to investigate how glycogen could be utilized by genital lactobacilli in the environment of the lower female genital tract.

### MATERIALS AND METHODS

#### Subjects

Genital cervical-vaginal lavage (CVL) samples were obtained from women who had provided written informed consent. All procedures followed Department of Health and Human Service guidelines. The study was approved by the Cook County Health and Hospitals System Institutional Review Board. Samples were collected weekly over a 1–3 month period. All women reported not douching and not engaging in sex within the 48 hours before sample collection. Trichomonas, yeast, and BV were not detected at the time of sample collection as determined by w/v) was hy-

#### Bacterial Cultures

All reagents were purchased from Sigma (Sigma-Aldrich, St Louis, MO) unless noted. A basal de Man, Rogosa, and Sharpe (MRS) medium contained proteose peptone #3 (10.0 g, Remel, Thermo Fisher Scientific, Lenexa, KS), beef extract (10.0 g, Remel), yeast extract (5.0 g, Fisher), sorbitan monooleate (1.0 g), ammonium citrate (2.0 g), sodium acetate (5.0 g), MnSO₄·H₂O (0.05 g), and Na₂HPO₄ (2.0 g) per liter. Medium was supplemented with 2% glucose or other carbohydrates, including glycogen (from oyster), maltodextrins, maltose, maltotriose, and maltopentaose. In some experiments, normal saliva collected from 1 donor was immediately filtered (0.45 μm), and aliquots frozen. Upon thawing, saliva was added to glycogen-containing MRS medium immediately before the addition of bacteria to give concentrations of 2.5%, or 0.25% of the culture volume. Most cultures were in ambient air, except some run under anaerobic conditions using the Anaero Pack System (Mitsubishi Gas Chemical Co, Tokyo).

Bacteria tested included *Lactobacillus jensenii* (ATCC #25258), *L. gasseri* (ATCC #9857), and *L. johnsonii* (ATCC #33200). Cultures (4 mL) were initiated from frozen stocks with 50 μL phosphate-buffered saline–washed bacteria at time 0 and the optical density (OD) at 600 nm was taken at 24-hour intervals.

#### Glycogen Degradation by Genital Fluids

Genital fluid samples collected by CVL were centrifuged at 4°C in a microfuge for 10 minutes to pellet bacteria. The supernatants (25 μL) were added to oyster glycogen (75 μL of 5 mg/mL) dissolved in phenol-red free Roswell Park Memorial Institute 1640 medium. Aliquots were incubated at either 37°C or 4°C for 90 minutes and the amount of glycogen then measured using a colorimetric assay [31] by adding 50 μL of each of the following solutions: 10% potassium iodide, 0.01 N potassium iodate, and 1 N acetate. The mixture was incubated for 10 minutes and read at 565 nm. In some experiments, no extra glycogen was added to genital fluid samples so that degradation of endogenous glycogen could be observed.

#### Analysis of Oyster Glycogen Degradation Products

Oyster glycogen (20 mg/mL, weight per volume [w/v]) was hydrolyzed by adding saliva (see above) at 2.5% or 0.5% of the final volume for 10, 30, and 120 minutes at 37°C. Hydrolyzed samples were diluted 10× with deionized water. Molecular weight (Mₗ) distributions of salivary α-amylase-treated glycogen samples were obtained by high-pressure size exclusion chromatography (HPSEC) using a Varian model 9012 high-performance liquid chromatography pump system connected to a Varian Star 9040 refractive index detector (Agilent Technologies, Santa Clara, CA) at room temperature. Injected samples (100 μL, 0.45 μm filtered) were separated using Sephacryl TM S-500 HR gel filtration (GE Healthcare, Piscataway, NJ). The eluent was purified water (18.2 MΩ) with 0.02% sodium azide [32]. Pullulan standards (Polymer Laboratories Inc, Amherst, MA) were used as a standard curve.

#### Oligosaccharide Analysis by High-Performance Anion-Exchange Chromatography

Hydrolysis products from oyster glycogen treated with either the collected saliva or the vaginal fluids were determined by a high-performance anion-exchange chromatography (HPAEC) system equipped with an electrochemical detector ( Dionex, Sunnyvale, CA). The filtered (0.45 μm) samples were separated using a CarboPac PA-1 pellicular anion-exchange column ( Dionex) with gradient elution from 100% eluent A (150 mM NaOH) to 100% eluent B (600 mM NaOAc in 150 mM NaOH) [33].
Bacterial Polymerase Chain Reactions

Bacteria specific quantitative polymerase chain reaction (qPCR) assays were performed on isolated CVL genomic DNA [34]. Each 20 µL qPCR reaction contained Supermix (Bio-Rad Hercules, CA), primers, probes (IDT, Coralville, IA), and 1–10 ng template DNA. Primers and probes for the bacteria were described previously [34,35]. Known quantities of 16S rRNA plasmid targets were used as standards [34,35].

Enzyme Detection

Enzyme-linked immunosorbent assay (ELISA) was used to quantify human pancreatic α-amylase (AbCam, Cambridge, UK) and acidic α-glucosidase (MyBiosource, San Diego, CA).

RESULTS

Growth of Lactobacillus in Glucose, Glycogen, and Other Glucose Polymers

We first performed experiments to confirm previous reports that some genital Lactobacillus isolates do not grow in media with glycogen as the source of carbohydrate [27–29]. L. gasseri (ATCC #9857) was cultured in medium containing either 2% glucose, 2% glycogen, or no added carbohydrate (Figure 1). Over a 72-hour period, a relatively large increase in absorbance was observed for glucose cultures, while the ODs of cultures with either no carbohydrate or glycogen were similar. A similar lack of growth in glycogen when compared with no carbohydrate was observed with L. jensenii (ATCC #25258) and L. johnsonii (ATCC # 33200) (Figure 2). The lack of growth in glycogen was seen under both aerobic and anaerobic conditions (data not shown). Soluble starch (2% w/v) was also tested because its structure is similar to that of glycogen. No growth was observed using starch as the sole carbohydrate (Figure 1).

The lactobacilli tested above were able to utilize glucose for growth, but not glycogen, a high-molecular-weight polymer of glucose. Smaller polymers of glucose were also tested for their ability to support growth of lactobacilli. All 3 isolates of Lactobacillus were able to use 2% maltose for growth (Figure 2A–C). L. jensenii grew to relatively high levels by 48 hours in both maltotriose (polymer of 3 glucose units) and maltopentaose (5 glucose units) (Figure 2D).

Maltodextrins are derived from the partial hydrolysis of starch, and maltodextrins that are the least hydrolyzed have the lowest dextrose equivalent (DE) values. Growth of L. gasseri, L. jensenii, and L. johnsonii was also assessed in media containing 2% of 1 of 3 different maltodextrin preparations; DE 4–7, 13–17, and 16–19. All 3 of the species grew in the maltodextrins as a source of carbohydrates (Figure 2A–C), although the level of growth was generally less than that for glucose.

Growth of Lactobacilli in α-Amylase-Treated Glycogen

The above experiments indicated that some genital lactobacilli cannot utilize glycogen or starch for growth but can utilize smaller glucose polymers (dimers, trimers, pentamers). Therefore, the effect of glycogen breakdown by α-amylase on Lactobacillus growth over 72 hours was tested by adding a small amount of filtered saliva (2.5% or 0.25%) to the culture medium as a source of α-amylase. While L. gasseri did not grow in glycogen alone, it grew at a rate similar to cultures with glucose when cultured in glycogen with 2.5% filtered saliva added (Figure 3A). No growth above the control was seen with 2.5% saliva only. Similar rapid growth of L. gasseri was seen when 0.25% saliva was added to glycogen in cultures (data not shown).

Growth of L. johnsonii in glycogen with 2.5% saliva was also similar to growth in glucose (Figure 3B). In contrast, L. jensenii grew slowly in glycogen with saliva for the first 48 hours when compared to glucose, but growth increased by 72 hours to a level similar to glucose (Figure 3C).

The characteristics of the saliva-treated and control-treated glycogen were analyzed (Figure 4). HPSEC showed a peak at approximately 45 minutes that corresponded to undegraded glycogen. As little as 10 minutes of incubation with 2.5% saliva resulted in the appearance of a peak at 69 minutes, which was not present in untreated glycogen (Figure 4A). Longer incubation times (30 minutes, 120 minutes) led to an increase in the size of this peak. Analysis of the smaller carbohydrates in the 120-minute tube by HPAEC (Figure 4B) showed the appearance of peaks corresponding to maltose (G2) and maltotriose (G3).
with some maltotetraose (G4), which are known major products of the α-amylase reaction [36, 37].

**Vaginal Fluid From Normal Women Contains an Activity That Degrades Glycogen**

The above experiments showed that salivary α-amylase could process glycogen into products that could be used by vaginal lactobacilli for growth. Experiments were next performed to determine if any α-amylase-like activity was present in the genital fluid of women [31]. Samples were obtained from women that were all premenopausal with ages ranging from 34 to 48. Ten were African American and the race of 1 classified as “other.” Aliquots of 2 genital mucosal fluid samples (collected by lavage) with detectable glycogen were incubated at either 4°C or 37°C for 90 minutes, and a colorimetric iodine assay was then used to detect glycogen [31]. Sample A had more detected glycogen than sample B after incubation at 4°C (Figure 5A). For both of the samples, less glycogen was detected in the aliquot incubated at 37°C than in the portion incubated at 4°C, indicating temperature-dependent degradation of glycogen.

Several other genital-fluid samples were tested (not shown) for glycogen breakdown in a similar manner. However, many of the samples had very low or no detectable glycogen and so glycogen degradation could not be assessed. Therefore, in further experiments, exogenous glycogen was added to genital fluids collected from women to ensure that degradation could be detected. Figure 5B shows the results from incubating genital fluid from 4 different individuals with exogenous glycogen at...
4°C and 37°C. Less glycogen was detected for all 4 samples after incubation at 37°C compared to incubation at 4°C, with sample F exhibiting the most glycogen degradation and C having the least. Twenty-one samples from a total of 11 women were tested in this manner and the percent degradation was calculated using the following formula: 1 − glycogen 37/glycogen 4 × 100. The median degradation for the 21 samples was 73%, with only 3 samples having values less than 20% (Figure 5).

To investigate whether the source of the glycogen-degrading activity could be bacterial, PCR was used to quantify several types of bacteria in the same samples. No significant relationships were observed between either L. crispatus, L. iners, or Gardnerella vaginalis and the glycogen degradation activity in women (P > .05, Spearman rank correlation, Figure 6A–C). Similarly, no relationships were observed between either L. jensenii, Mycoplasma hominis, or Sneathia (not shown). Further, the vaginal pH that was measured for diagnosing bacterial vaginosis was not significantly associated with α-amylase activity (P > .05, Figure 6D). These experiments therefore showed no obvious relationship between the types of microbiota in genital tract samples and the levels of the glycogen degradation activity.

To investigate a possible human source of the glycogen degradation activity, the genital fluid samples were tested for human α-amylase using an ELISA for pancreatic α-amylase. All tested samples had ELISA-detectable α-amylase, and the median α-amylase detected in genital fluids was 1.86 mU/mL (range, 0.02–12.7 mU/mL). The pancreatic α-amylase levels were strongly associated (Spearman r = 0.73; P = .0002) with the amount of glycogen degradation (Figure 6E). Acidic α-glucosidase was also detected in some of the samples (Figure 6F). However, its presence was not significantly associated with glycogen degradation (P > .05).

Figure 3. Growth of lactobacilli in salivary α-amylase-treated glycogen. L. gasseri (A), L. jensenii (B), or L. johnsonii (C) were cultured in MRS broth that contained either no added carbohydrates (No Carb), 2% glucose, 2% glycogen, 2.5% saliva alone, or glycogen plus saliva for 72 hours. The OD at 600 nm was read each day. Abbreviations: MRS, de Man, Rogosa, and Sharpe; OD, optical density.

Figure 4. Degradation of glycogen by salivary α-amylase. Glycogen (2% oyster) was incubated for 10 minutes with no saliva (Oyster) or for 10, 30, or 120 minutes with 2.5% saliva. The size of the degraded glycogen was determined by HPSEC (A) and the products analyzed by HPAEC (B). Abbreviations: HPAEC, high-performance anion-exchange chromatography; HPSEC, high-pressure size exclusion chromatography.
Glycogen Breakdown Products Generated by Genital Fluids

Because the above experiments suggested that glycogen produced by the genital epithelium could be degraded into smaller glucose polymers such as maltose and maltotriose in genital fluids, genital fluids from several women were analyzed by HPAEC for small carbohydrates. However, only trace amounts of small sugars were detected, and these levels were too low to determine the characteristics of these sugars. Therefore, in further experiments, exogenous glycogen was added to genital fluids and the types of carbohydrates that were generated were analyzed by HPAEC. The glycogen used for this experiment had only trace peaks corresponding to glucose, maltose, maltotriose, and maltotetraose (Figure 7A). After incubation for 48 hours in buffer with no added genital fluids, there was essentially no change in these peaks (Figure 7B). However, incubation for 48 hours with genital fluid from 2 different subjects resulted in substantial increases in peaks corresponding to maltose (G2), maltotriose (G3), and maltotetraose (G4), the major known α-amylase digestion products (Figure 7C and 7D). Trace peaks corresponding to glucose and maltopentaose were also observed. Similar results were observed after 24 hours of incubation, although the size of each of the peaks was reduced when compared with the 48-hour peaks (not shown). No peaks were detected in genital fluids with no added glycogen (not shown).

DISCUSSION

Genital colonization with Lactobacillus has been associated with protection from STDs, BV, and adverse pregnancy outcomes [1, 5, 6, 8–21]. However, the factors that promote and maintain Lactobacillus colonization are not well understood. Glycogen is
Glycogen is synthesized in the vaginal epithelium and has long been postulated to provide an energy source for genital Lactobacillus [25, 26]. However, it has not been clear how Lactobacillus uses glycogen because most genital isolates do not grow on glycogen in vitro [27, 28]. This study shows that (1) isolates of Lactobacillus that cannot use glycogen can grow in glycogen breakdown products, including maltose and maltotriose; (2) an activity that breaks down glycogen to maltose and maltotriose is present in the lower-genital-tract fluid of women; (3) human α-amylase is present in the genital fluids of women; (4) the glycogen breakdown activity in genital fluid correlates with genital levels of α-amylase; and (5) incubation of genital fluids with glycogen generated products such as maltose, maltotriose, and maltotetraose. This study therefore supports a model where α-amylase present in the genital tract breaks down glycogen into smaller polymers, such as maltose and maltotriose, that are then utilized by Lactobacillus to aid its colonization.

Glycogen is composed mainly of α1,4-linked chains of glucose but also has α1,6-linked branches. Human α-amylase (both salivary and pancreatic) preferentially processes glycogen to α1,4-linked multimers, such as maltose and maltotriose, but also results in production of α-dextrins that contain α1,6 branches and α1,4 linkages [36, 37]. This study shows that genital lactobacilli can grow well in the small unbranched glucose polymers. It is not clear, however, if lactobacilli can utilize branched carbohydrates.

There are some previous studies of utilization of maltose by Lactobacillus in the food and beverage industries, such as L. casei, and L. reuteri [38, 39]. However, those Lactobacillus species are different than the ones found in the genital tract, including L. crispatus, L. jensenii, L. iners, and L. gasseri [4, 20, 40, 41]. There is a paucity of studies on the utilization of small glucose polymers by genital tract lactobacilli. This study suggests that the small glucose polymers can support growth by genital Lactobacillus in vivo, and the consequent acid production suppresses growth of nonlactobacilli. The low genital pH found may vary somewhat depending on the species of Lactobacillus that is dominant [4]. The pH can be neutralized by factors such as sexual activity due to semen or by douching [42–44]. During periods of increased pH, other bacteria may utilize the glycogen breakdown products, and due to competition for this food source and production of pH buffering amines [45], prevent Lactobacillus from acidifying the genital environment.
Starch and glycogen have similar structures and there are reports of nongenital lactobacilli, such as *L. fermentum* and *L. plantarum*, degrading starch [46]. While there have been some reports of genital *Lactobacillus* isolates breaking down glycogen, this was later discovered in some cases to have been due to serum added to the culture medium that likely was a source of α-amylase [27, 47]. However, some genital *Lactobacillus* isolates can directly utilize glycogen [28].

We observed in this study that α-amylase and *Lactobacillus* levels were not correlated (Figure 6). However, we recently observed that there are variable levels of glycogen in the genital fluids of different women and that the levels of glycogen correlate strongly with the level of colonization by *Lactobacillus* (manuscript in preparation). Based on that observation, we postulate that women must have 2 conditions met to be dominantly colonized by *Lactobacillus*: (1) an adequate amount of glycogen in genital fluid; and (2) α-amylase to cleave the glycogen into smaller carbohydrates. If glycogen is low, then *Lactobacillus* levels will also be low. Potentially, if α-amylase levels were low but glycogen high, this could also lead to low *Lactobacillus*. Our studies do not rule out the possibility that other enzymes, including bacteria-derived enzymes, could process glycogen in some women. Also, because these experiments were conducted with women consisting mostly of 1 ethnic group, it is possible that women from other groups could have differing genital amylase activity.

This study has implications for the measurement of glycogen in genital secretions because α-amylase acts on glycogen, resulting in a mixture of glycogen and smaller glucose polymers. Glycogen is made in the epithelium, but it has not been clearly established how glycogen in epithelial cells becomes available for *Lactobacillus* utilization. Factors such as the amount of glycogen in cells, the rate of cell release, and the rate of glycogen breakdown are unknown. In our study, samples were collected on ice so that glycogen breakdown was arrested relatively quickly, but even so, many women had glycogen levels too low to measure with the iodine method. Further, glycogen breakdown products were too low to measure, possibly due to rapid uptake by bacteria.

While this study showed that human α-amylase was in genital fluid, the source for it was not established. As mentioned above, α-amylase is produced in both the pancreas and salivary glands, and essentially all of the extracellular α-amylase activity in the body is produced by those 2 glands [47, 48]. Both
pancreatic and salivary α-amylase are in serum and urine at relatively high levels that can become elevated during certain clinical conditions such as alcoholism or trauma. The salivary and pancreatic enzymes are closely related, with only about 3% difference in the protein sequence [47]. Because the protein sequences are so similar, the ELISA for pancreatic α-amylase may have cross-reactivity with salivary α-amylase. Therefore, it is possible that the α-amylase in genital fluids is from 1 or both of these glands.

In conclusion, this study provides evidence that human α-amylase in the genital tract processes epithelial glycogen. This, in turn, suggests a mechanism for how glycogen can support genital colonization by strains of Lactobacillus that cannot directly use glycogen. Further studies are needed to clarify the source of the enzyme and whether too much or too little α-amylase could affect levels of Lactobacillus.

Notes

Financial support. This work was supported by National Institutes of Health (NIH) grants P01 AI082879 and P30 AI 082151.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

2. O’Hanlon DE, Moench TR, Cone RA. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. PLOS ONE 2013; 8: e80074.


