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Biofilms, Thehypertextbook - Chapter 11 Lab Exercises

Section 21 Bacterial Coaggregation in Dental Biofilms

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Coaggregation Dental Biofilms

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Instructor Version (go to [Student Version](#))

Subject Area(s)	Microbiology, Allied Health, Microbial Ecology
Intended Audience	High school biology, independent study/science fair, introductory microbiology, advanced college microbiology
Type	Laboratory exercise
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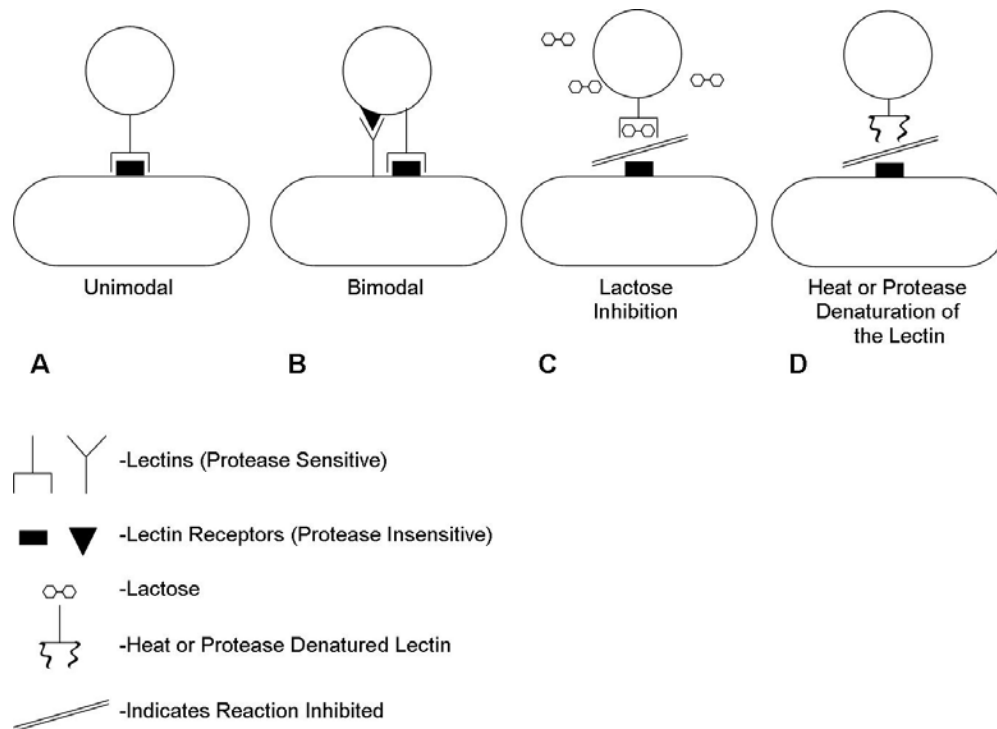
CONTENT

In 1970, Gibbons and Nygaard observed that when pure cultures of some dental plaque bacterial strains were mixed, the suspension rapidly cleared. For example, when broth cultures of *Actinomyces naeslundii* and certain strains of *Streptococcus sanguis* were combined a marked decrease in turbidity occurred within minutes¹. This phenomenon, now termed coaggregation, is of selective value to bacterial living in a flowing environment as any cells, which detach from the oral surface are washed away and swallowed. Kolenbrander and others working on this

phenomenon have found that coaggregation is exhibited by nearly all oral bacteria tested, a sample which includes more than seven hundred bacterial strains representing at least 18 genera^{2,3,4,5}.

It is now known that the dominant cell-cell interactions are between Lectin type (protein) adhesins on one of the cells and oligosaccharide moieties on the other. Coaggregation can be interrupted by denaturation of the lectin or, frequently, by the addition of sugar (e.g. lactose) which blocks the active lectin site (Figure 1).

Figure 1



This array of coaggregation associations is related to the succession by which dental plaque matures. Early colonizers predominantly Streptococci attach to a conditioning film. Subsequently, actinomycetes and Fusobacteria bind by coaggregation to these pioneer species. Depending largely upon the degree of dental hygiene, other organisms join the biofilm. These late arriving species have little ability to coaggregate with the pioneers, but do bind with the intermediates such as *Fusobacterium*. The fusobacteria are therefore called bridging species.

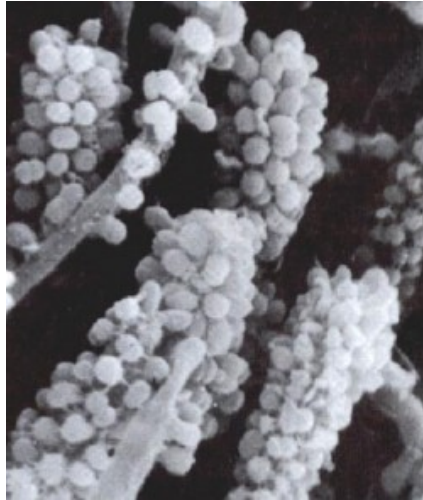
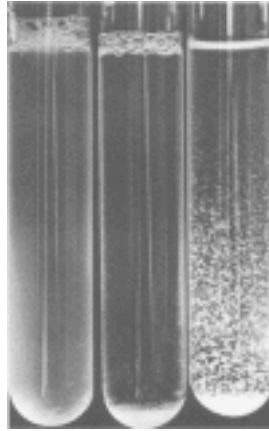


Figure 2. Corn-cob formations of a coccus coaggregating with a filamentous bacterium. Possibly a Streptococcus and a fusobacterium. Used with permission of SaneDentist - <http://www.sanedentist.com>

These exercises describe procedures for demonstrating the coaggregation of unrelated bacteria, an important event in the formation and succession of biofilms in the mouth in fresh water and in waste treatment plants. Specific adhesins on one member of a pair of organisms may bind to cognate compounds, often polysaccharides, on the other. These intergeneric pairings are important in the sequential formation of biofilms in many natural environments including plaque on teeth, and biofilms on rock surfaces in fresh water streams.

These protocols involve the mixing of specific pairs of bacteria in serological tubes. Some of the bacterial pairs coaggregate and some do not. Students may observe the clearing of the culture tube and quantify the strength and rate of the reaction by observing the degree of clearing. Other tubes contain lactose, which enables students to observe the effect of this coaggregation inhibitor.

A third protocol can be used to determine which of a pair of strains bears the lectin by denaturing the protein either by heating or by treatment with Protease K.



Tube 1 2 3

Figure 3

Results of a coaggregation assay. Tube one is a pure culture showing no coaggregation, tube 2 indicates a strong +4 reaction, while tube 3 should be read as a +2 reaction.

The coaggregation exercises may be used with entire classes in courses such as introductory microbiology, allied health microbiology or environmental microbiology. Alternatively they may be used as a jumping off point for students doing independent research searching for coaggregation among environmental isolates from the mouth, fresh or marine water environments or soil. In this, students will be exploring a hot contemporary field of research in which investigators are attempting to determine just how general this phenomenon of coaggregation is in nature.

PREREQUISITES

Students should be able to define a biofilm, describe the differences between biofilm (surface-attached) and planktonic (free-floating) bacteria, and describe why bacteria tend to grow on surfaces. The nature of adhesins should also be understood as well as the terms inter and intrageneric. Basic microbiological laboratory skills such as aseptic technique and transfer procedures should be mastered.

The order in which bacteria colonize the surface of teeth is, in large part, determined by the availability of bacterial species and the coaggregation pairings between them. In many instances, the mere availability of a particular strain is insufficient to guarantee its inclusion in the dental biofilm unless specific coaggregation partners have adhered previously. So biofilm formation in this instance is not random, but follows a specific pattern of succession. It remains to be seen in this phenomenon is entirely general or found in only certain

environments, but it has been demonstrated in the human gut, in streams.

The following exercises are designed to explore the interactions that exist between bacteria of the same genera (intrageneric coaggregations) and between bacteria of different genera (intergeneric coaggregations) in biofilms (Rickard et al. 2003, Kolenbrander et al. 2002, Kolenbrander 1989). The experiments are divided into three sections with the **first section** devoted to the exploration of coaggregations between three reference strains of oral bacteria. The **second protocol** enables students to block coaggregation with lactose a competitive inhibitor of many lectin carbohydrate interactions and the **third set of protocols** enables students to determine which of a pair of coaggregating bacteria possesses the lectin and which the cognate carbohydrate by using treatments (heat and protease enzymes) which denature the lectin protein.

CULTURES, MATERIALS AND EQUIPMENT

ORAL BACTERIAL REFERENCE STRAINS

Table 1

Bacterium	Strain	Coaggregation Group
<i>Actinomyces naeslundii</i>	PK606 (ATCC 51655)	D
<i>Streptococcus gordonii</i>	Challis (ATCC 35105)	1
<i>Streptococcus oralis</i>	34	3
<i>Streptococcus gordonii</i>	PK488 (ATCC 51656)	6

Note: These strains are available from D. Clemans, Department of Biology, Eastern Michigan University, Ypsalanti MI, <daniel.clemans@emich.edu>

MATERIALS AND EQUIPMENT

Per student or student group	Item
20 - 30	10 X 75mm or 12 X 75mm glass test tubes
10 ml	Each of the reference strains in Table 1
4 ml	300 mM lactose
1 each	P200, P20, and P1000 pipettor with appropriate tips
4 ml	Protease K solution (Sigma-Aldrich) 0.5 mg/ml

Per Lab	Item
1	Refrigerated centrifuge
1	Spectrophotometer
1	85 ° waterbath or dry heat block
1	50 ° waterbath or dry heat block

INSTRUCTIONAL PROCEDURES

Prior to the laboratory period

1. Cultivation of Bacteria. All reference oral bacterial strains used in this study are listed in Table 1. Streptococci and actinomyces are cultured in Todd-Hewitt (TH) broth (BBL Microbiology Systems, Cockeysville, Md.) or Brain Heart Infusion (BHI) broth (BBL Microbiology Systems, Cockeysville, Md.) optimally at 37°C under anaerobic conditions with the GasPak system (BBL Microbiology Systems, Cockeysville, Md.). These strains, however, do grow well when cultured aerobically at 37°C on TH or BHI media.

2. Preparation of Bacteria for Coaggregation Assay. Bacterial cells used for coaggregation assays are pelleted by centrifugation at 10,000 X *g* for 10 min at 4°C. The supernatant is discarded and the cells are resuspended in coaggregation buffer. This wash step is repeated twice more (total of 3 washes). Coaggregation buffer consists of (1 mM Tris [pH 8.0], 0.1 mM CaCl₂, 0.1 mM MgCl₂, 150 mM NaCl, and 0.02% NaN₃).

Adjust the bacterial suspensions to an A₆₀₀ of approximately 1.0 (~10⁹ bacteria/ml).

The cell suspensions thus prepared, are stored at 4°C until the laboratory period^{6,7,8}.

Safety Note: if the suspensions are to be used within a few weeks of the time of preparation, the sodium azide may be omitted.

During the laboratory period – Section one, measuring auto and coaggregation.

- 1) Each group of students is provided with:
 - 10 ml of each reference bacterial strain listed in Table 1.
 - 8 ml of 300 mM lactose (Other sugars e.g. N acetyl-glucosamine or N acetyl-galactosamine may be provided as well, Kolenbrander et al. 1990)
 - 20-30 glass tubes for coaggregation assays.
 - 1 P200 and 1 P20 pipettor per group with 1-2 boxes of sterile yellow tips. 1 P1000 pipettor with a box of blue sterile tips.
 - An 85°C water bath or dry-heat block, and a 50°C water bath or dry-heat block.

- 2) Each individual strain should be tested for autoaggregation by placing 400 µl of the bacterium in a test tube. The tube is vortex mixed for 5 seconds and then rocked back and forth while observing aggregation formation. Any degree of settling or clearing of the tube indicates autoaggregation and this number (from the assessment scale) should be subtracted from the value measured for coaggregation.

- 3) Pairs of organisms are tested for coaggregation by mixing 200 µl of each strain in a test tube. The tube is vortex mixed (~5 seconds) and then rocked back and forth until aggregates form. The degree of coaggregation is assessed by comparing the results with Table 2. Visually score the coaggregates using the following 4-point scale (Kolenbrander, 1988).

Note: Good lighting is essential for viewing the extent of coaggregation. We have found that the surface of an overhead projector works well.

Table 2

<u>Coaggregation score</u>	<u>Description</u>
0	Evenly turbid suspension of bacteria
1	Finely dispersed clumps in a turbid background
2	Definite clumps of bacteria are easily seen but do not settle immediately and remain in a

	turbid background
3	Clumps settle immediately with a slight turbid background
4	Clumps settle immediately and the supernatant is completely clear

- 4) Rescore each assay. An easy way to keep track of each coaggregation assay is to set up a simple table with each strain represented on each axis (see Kolenbrander et al. 1990, or Clemans and Kolenbrander, 1995 for examples). Score each assay 0 - 4 using the scale indicated above for the initial coaggregation score. Next, add lactose to each assay, vortex briefly, and read assay. Indicate the coaggregation score with lactose as a superscript to the original score without lactose.
- 5) Study the heat- and protease-sensitivity of bacterial coaggregates. Incubate 1 – 2 ml of each partner separately in 85°C water bath or with proteinase K (Sigma-Aldrich) in a 50°C water bath as indicated above. Perform coaggregation assays with treated and untreated pairs as in step 2. Record the coaggregation assay before and after the addition of lactose.
- 6) For the laboratory report, identify any coaggregation pairs from the list of reference oral bacteria from Table 1. Identify which partner organism carries the protein adhesin, and which organism carries the carbohydrate receptor.

Section Two – Reversal of coaggregation with Lactose

1. Many Lectin carbohydrate interactions may be reversed by competitive inhibition with sugars such as lactose.
2. 200 µl of a 300 mM stock added to the 400 µl coaggregation assays from one of the existing coaggregation assays carried out in Section 1. This produces a final concentration of 60 mM lactose.
3. The tubes are again vortex mixed for 5 seconds and once again rocked while observing for coaggregation. If desired, other sugars can be added instead to a final concentration of 15 mM (Kolenbrander et al. 1990).
4. An easy way to keep track of each coaggregation assay is to set up a simple table with each strain represented on each axis (see Kolenbrander et al. 1990, or Clemans and Kolenbrander, 1995 for examples).

Section Three – Assessment of the Nature of the Interactions Between Bacterial Strains

1. To determine which partner contains the protein adhesin, incubate 0.5 ml of each bacterium at 85°C for 30 min, and then perform coaggregation as directed above.
2. Coaggregation assays should be performed using both heated and unheated cells of the partner strains.
3. Those bacterial strains that lose the ability, after heat treatment, to coaggregate with their partners contain the protein adhesin.
4. Those treated bacterial strains that still coaggregate with their partners contain the carbohydrate receptor.
5. Confirmation of the adhesin-containing strain can be performed by incubating the 0.5 ml of the bacterial suspension in a final concentration of 0.5 mg/ml of proteinase K at 50°C for 60 min prior to performing the coaggregation assay.

ASSESSMENT / EVALUATION

Assessment may be made by the instructor through visual evaluation of each student's coaggregation tube results as compared with a list of the known reactions for the microorganism pairs used.

FOLLOW-UP ACTIVITIES

1) Coaggregation has been studied most intensively in dental plaque and to a lesser extent in fresh water environments. The field of investigation is wide open to explore coaggregation in any other natural (or human influenced) ecosystem. Organisms isolated from soil, sink drains, shower curtains, and any other habitat are potential materials for investigations of coaggregation.

2) Exploration of bacterial coaggregation occurring in laboratory teaching strains of streptococci and other bacteria. Many teaching laboratories use stock strains for teaching purposes. The coaggregation properties of these strains, especially streptococci, can be assessed using the procedure followed in Section I.

- 1) Culture the bacterial strain in an appropriate growth medium and wash the bacterial cells in coaggregation buffer as indicated above. Resuspend the bacterial suspension to a final cell density of $A_{600} 1.0$ ($\sim 10^9$ bacterial cells/ml).
- 2) Assess the coaggregation properties as in Sections 2 and 3. Determine which partners contain the protein adhesin, and which strains contain the carbohydrate receptor.

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Appendix