BACTERIAL GENERA ASSOCIATED WITH NONSTICKY, MILDLY STICKY AND STICKY WESTERN COTTONS

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Abstract

A survey method is presented for the unbiased sampling and identification of bacterial genera associated with nonsticky, moderately sticky, and sticky cottons. The method utilizes the MIDI Microbial Identification System (MIS) which uses whole cell fatty acid analysis by gas chromatography rather than relying on colonial growth morphology and conventional biochemical testing. The method uncovers a greater diversity of bacterial species than has been reported. A gram-index concept is introduced which relates the 'gram-reaction character' of a cotton growing region. The nonsticky, moderately sticky, and sticky cottons all exhibited a high raw and relative gram-positive index. The corresponding raw and relative gram-negative index was very low. No difference was observed between the cottons. The predominant genera found were *Bacillus* spp. and *Staphylococcus* spp.; *Pseudomonas* spp. were surprisingly absent.

Introduction

Since the work of Prindle (1934a-c) and Clark et. al. (1947) on the association of bacteria and cotton lint, and the paper by Neal et. al. (1942) on the etiology of an acute illness among rural mattress makers who used low grade, stained cotton, which showed that the severity of the symptoms and physical manifestations were dependent on the presence and concentration of the 'cotton bacterium' or its products in cotton dust, researchers have had a keen interest in the bacterial population on cotton lint and dust. This interest to health professionals was heightened when it became known that decreases in pulmonary function were highly correlated with endotoxin level (Castellan et. al., 1984 & 1987; Rylander et. al., 1985) since endotoxin is a biological product of gram-negative bacteria. Of more current interest to the industry was a report by Wyatt and Heintz (1982) that associated capsule-producing coryneform bacteria with stickiness in cotton. Recent emphasis on relieving cotton stickiness by microbial decomposition of the insect honeydew has created a need for more precise identification of microorganisms on both normal and sticky cottons (Balasubramanya et. al., 1985; Heuer & Plaut, 1985; Perkins, Jr., 1993).

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The study of cotton bacteria has revolved almost entirely on determining the viable population of bacteria, with special interest on the proportion of gram-negative bacteria, on either the lint or dust (Simpson & Marsh , 1985; Borbon Reyes et. al., 1988; Chun, 1990; Chun and Perkins, Jr., 1991 & 1993) or on determining levels of endotoxin (Chun & Perkins, Jr., 1992; Fischer & Jacobs, 1988). Less emphasis has been placed on the actual make-up of the bacterial population. Studies where identifications have been made, have relied upon general surface morphology of the colonies and conventional biochemical methods (Akinwunmi et. al., 1989; Borbon Reyes et. al., 1988; Fischer & Foarde, 1989; Simpson et. al., 1988). Hundreds of colonies may appear on a plate, making it practically impossible to isolate and identify each colony. This study reports an unbiased method of selecting colonies for identification and utilizes the MIDI Microbial Identification System (MIS) which uses whole cell fatty acid analysis by gas chromatography as a means of taking a census of the bacteria common to cotton. In addition, a gram-index concept is introduced which takes into account the proportion of gram-negative or gram-positive bacterial species present and the total bacterial population.

Methods & Materials

Cotton

Ninety-five cotton samples from field experiments were sent by Dr. Eric Natwick (University of California, Cooperative Extension, 1050 E. Holton Road, Holtville, CA 92250-9615) to the Cotton Quality Research Station (CQRS; Clemson, SC) to be tested for stickiness. A precise history of the cottons was unavailable at the time of writing; however, the cottons are known to be from whitefly field control experiments during the 1994 season. The cottons were tested for stickiness on a thermodetector as described by Brushwood and Perkins (1993). The cottons were rated by thermodetector spots (TD) as described by Perkins and Brushwood (1995). Instead of using the four categories they recommended, the light stickiness and moderate stickiness categories were combined as moderate stickiness so that only 3 categories of stickiness were examined (less than 5 spots — nonsticky; 5-24 spots — light to moderate stickiness; and 25 spots and above — heavy stickiness). The cotton samples were sorted by TD into the three categories (Table 1). Ten 1-gm samples were chosen from each stickiness category; and from these 30 1-gm samples, ten samples were randomly chosen and processed at a time. Dry weights of the samples were determined.

Viable Microbial Count & Culturing Method for Bacterial Identification

Viable total bacterial populations were determined for each of the 1-gm samples as described in Chun & Perkins, Jr., 1991; except that instead of pour plates, spread plates were used and the plates were cultured for 24 hours at $28^{\circ} \pm 0.5^{\circ}$C before being counted. In addition, prior to

counting the plates, bacterial cells from well isolated colonies were taken for identification.

After incubation, the best subjectively countable dilution plate (preferably, containing 50-250 colonies/plate) from each sample was placed on a circle drawn on a transparent sheet. The circle contained the same area as the petri plate bottoms used and was subdivided into 44 1-cm^2 locations or squares. Each location was numbered sequentially from right to left, top to bottom. To eliminate bias, ten locations were chosen randomly for each sample and an individual and well separated colony closest to the center of the square was touched with the end of a sterile toothpick. The bacterial cells adhering on the toothpick tip were then subcultured for 1 or more days on a fresh TSBA (trypticase soy broth agar) plate to amplify the starting inoculum. If no colonies were found in the randomly chosen location or the square was over run with overlapping colonies, that location was skipped and the next location was used until ten isolates were made.

The amplified inoculum was then spread over the plate surface as described by the MIDI system (see below) and cultured for 24 hours at $28^{\circ} \pm 0.5^{\circ}$C after which time the cells were harvested for fatty acid extraction.

Bacterial Identification

Bacterial identification was made using the MIDI Microbial Identification System (MIS; MIDI, Inc., Newark, Delaware) which uses whole cell fatty acid analysis by gas chromatography (Sasser, 1990; Sasser & Wichman, 1991). Fatty acid saponification, methylation, and extraction were performed as directed by the MIS protocol and analyzed using the MIDI MIS software (Sherlock system software, version 1.06: Version 3.8 of the Aerobic Method, and TSBA and CLIN libraries). The chromatographic unit used consisted of a Hewlett-Packard 5890E Series II Plus gas chromatograph with electronic pressure control, a 7673B automatic sampler (with injector, controller, and tray), and the Hewlett-Packard 3365 Series II ChemStation Software, version A.03.34 (Hewlett-Packard, Wilmington, DE). Column type, length, operating parameters were as prescribed by the MIS. Because of the overview nature of this study, the first recommended identification was used even when its similarity index (S.I.) was low or very close to the next recommended identification; and while the MIS reports bacterial identification to the species level, identification was sorted only to the genus level. 'No matches' were few and treated as a separate category.

Gram Index

A gram-index was calculated for each of the three sticky categories: a gram-negative index was calculated as the sum of the frequency of each gram-negative genera divided by the total frequency of the gram-negative plus the gram-positive genera; and a gram-positive index was calculated as the sum of the frequency of each gram-positive genera divided by the total frequency of the gram-negative plus the gram-positive genera. The 'No Match' category was not included in either index. To obtain a relative index, this raw index was multiplied by the log (base 10) of the average population of that region.

Statistical Analysis

Data was analyzed using release 6.08 of SAS (SAS, Statistical Analysis System; SAS System for Windows version 3.95: SAS Institute, Inc., Cary, NC USA) for making mean separations. Chi-square comparisons were made on the observed and expected distribution using the CHITEST function in Microsoft EXCEL for Windows 95 version 7.0 (Microsoft Corporation, USA).

Results & Discussion

The total bacterial population tended to increase with stickiness (Figure 1); however, total population was not significantly different for the three categories of sticky cotton (nonsticky = 2.9 x 10^4 cfu/gm, s.e. = 6.6 x 10^3 cfu/gm; moderately sticky = 3.6 x 10^4 cfu/gm, s.e. = 1.7 x 10^4 cfu/gm; and sticky = 7.6 x 10^4 cfu/gm, s.e. = 3.2 x 10^4 cfu/gm). The low bacterial population was consistent with California/Western cottons (Chun & Perkins, Jr., 1996).

For the nonsticky, moderately sticky and sticky cottons, 90, 87 and 87 species were identified, respectively. Even though only a few morphologically different colonial types appeared on the spread plates from which colonies for isolation were taken, when the different species were sorted to bacterial genera, 19 different genera, including the 'No Match' category, were obtained, which was 6 more genera than found in a concurrent study looking at three cotton growing regions (Chun & Perkins, Jr., 1996). This represents a far greater number of genera and species than reported by others using just the morphological character of colonies and conventional biochemical tests (Akinwunmi et. al., 1989; Borbon Reyes et. al., 1988; Fischer & Foarde, 1989; Simpson et. al., 1988). Of these 19 different genera categories, 10 genera made up less than 2% of the total species identified (Figure 2). The number of species found in these genera were low; and chi-square testing did not appear warranted.

On the other hand, chi-square tests on genera containing 2% or more of the species identified did have a highly significant probability of being unevenly distributed between the three categories (Figure 3). The 'No Match' samples fell in this group. Most of the individual genera did not show significant distribution differences. However, the 16 species of *Staphylococcus* spp. were found significantly more often in the nonsticky cottons than in either the moderately sticky and sticky cottons, or the moderately and sticky cottons together ($P < 0.001$). In a concurrent study looking at regional differences, *Pseudomonas* spp. was the most common genus found in the Mississippi cottons (51), followed by Texas cottons (29), and least often in the California cottons (11) (Chun &

Perkins, Jr., 1996). In this study, *Pseudomonas* spp. were not found! Also, only a single *Corynebacterium* sp. was found and it was found in the nonsticky cottons which argues against bacteria being associated with stickiness (Wyatt & Heintz, 1982). The genera with the largest number of species identified was *Bacillus* spp. When the chi-square was applied, no significant difference could be found between the three categories of stickiness. However, the average occurrence of *Bacillus* spp. was significantly more common than the other genera (Table 2). What I find interesting is that while the percentage of *Bacillus* spp. is high, it is considerably higher than what was observed in the concurrent study involving three cotton growing regions (Chun & Perkins, Jr., 1996), where the percentage of *Bacillus* spp. in the California cotton from the regional study was only 26.7% while in this study it averaged 67%.

This difference was more pronounced when the raw gram-indexes were compared. In Table 3, the average of the raw gram-positive index for the sticky cottons (nonsticky, moderately sticky and sticky cottons) was 0.9, whereas, the raw gram-positive index for California cotton from the regional study was 0.3 (Chun & Perkins, Jr., 1996). In addition, no *Pseudomonas* spp. was found in this study while in the California region cottons, *Pseudomonas* spp. made up 9.2% of the genera. The raw relative gram-negative index values ranged from 0.0 to 0.1 for the nonsticky, mildly sticky and sticky cottons (Table 3); whereas the raw gram-negative index for California cottons from the regional study was 0.7. The gram-index follows trends of other studies which suggest that California is low in endotoxin levels and that Mississippi is high in endotoxin (Fischer and Foarde, 1989; Fischer and Jacobs, 1988; Simpson & Marsh, 1985). To verify the usefulness of this index would require additional studies to determine whether a correlation exists between increased gram-negative index with increased endotoxin levels. On speculation, a rational explanation might be that the cottons in this study were sent direct to CQRS from researchers in the field while the cottons used in the three cotton growing region study were processed commercially before reaching CQRS and may have spent time stored in cotton modules before being ginned. Conceivably the time in storage may have contributed to the higher proportion of gram-negative bacteria. Morey et. al (1982) have suggested that fiber yellowness was significantly and positively correlated with endotoxin content. Since endotoxin content is generally highly correlated with gram-negative bacteria (Fischer & Jacobs, 1988), the effect of module storage should be examined very closely as it relates to cotton color and quality.

Summary

A survey method is presented for the unbiased sampling and identification of bacterial species. The method utilizes a randomized selection process and the MIDI Microbial Identification System (MIS) which uses whole cell fatty acid analysis by gas chromatography rather than relying on colonial growth morphology and conventional biochemical testing. The method uncovers a greater diversity of bacterial species than has been reported. No significant differences between populations were observed in the nonsticky, moderately sticky and sticky cottons. *Bacillus* spp. was the major genera observed in these cottons. No *Pseudomonas* spp. was found. A gram-index concept is introduced which relates the 'gram-reaction character' of a cotton growing region.

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Disclaimer

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References

1. Akinwunmi, O. O., Templer, J. A., and Heintz, C. E. 1989. Viable counts and distribution of bacterial flora on cotton plant parts. Pages 19-23 in: Proc. Thirteenth Cotton Dust Res. Conf. R. R. Jacobs and P. J. Wakelyn, eds. National Cotton Council, Memphis, TN. 159 pp.

2. Balasubramanya, R. H., Bhatawdekar, S. P., and Paralikar, K. M. 1985. A new method for reducing the stickiness of cotton. Tex. Res. J. 55:227-232.

3. Borbon Reyes, J. C., Batson, Jr., W. E., Miller, D. P., and Greenough, D. 1988. Evaluation of populations of total and gram negative bacteria on cotton lint. Pages 36-37 in: 'Cotton Dust' Proc. Twelfth Cotton Dust Res. Conf., New Orleans, LA, January 6-7, 1988. R. R. Jacobs and P. J. Wakelyn, eds. National Cotton Council, Memphis, TN. 206 pp.

4. Brushwood, D. E. and Perkins, H. H., Jr. 1993. Cotton Sugar and stickiness test methods. Canadian Textile Journal 110(6):54-62.

5. Castellan, R. M., Olenchock, S. A., Kinsley, K. B., Hankinson, J. L. 1987. Inhaled endotoxin and decreased spirometric values: An exposure-response relation for cotton dust. N. Engl. J. Med. 317:605-610.

6. Chun, D. T. 1990. Airborne bacterial levels of card generated dust from 1988 cottons grown in different

locations. Pages 37-39 in: 'Cotton Dust' Proc. Fourteenth Cotton Dust Res. Conf., Las Vegas, NV, January 12-13, 1990. R. R. Jacobs, P. J. Wakelyn, and L. N. Domelsmith, eds. National Cotton Council, Memphis, TN. 153 pp.

7. Chun, D. T. and Perkins, Jr., H. H. 1991. Survey of bacterial population survival from cottons in storage. Pages 287-288, Proc. Fifteenth Cotton Dust Res. Conf. (Wakelyn, P. J., Domelsmith, L. N., and Jacobs, R. R., Editors), in: 1991 Proc. Beltwide Cotton Conferences, Vol. 1. D. J. Herber and D. A. Richter (eds.). National Cotton Council of America, Memphis, TN. 579 pp.
8. Chun, D. T. and Perkins, H. H., Jr. 1992. Survey of endotoxin and dust levels from cottons in storage. Pages 315-317, 16th Cotton Dust Res. Conf. (Wakelyn, P. J., Domelsmith, L. N., and Jacobs, R. R., Editors), in: 1992 Proc. Beltwide Cotton Conferences, Vol. 1 (Douglas J. Herber, Editorial Coordinator, and Deborah A. Richter, Asst. Editorial Coordinator). National Cotton Council of America, Memphis, TN. 528 pp.

9. Chun, D. T. and Perkins, Jr., H. H. 1993. Seasonal variations in bacterial population and dust generation potential of cotton. Pages 306-309, 17th Cotton Dust Res. Conf. (Domelsmith, L. N., Jacobs, R. R. and Wakelyn, P. J., Editors), in: 1993 Proc. Beltwide Cotton Conferences, Vol. 1, January 10-14, New Orleans, LA. (Douglas J. Herber, Editorial Coordinator, and Deborah A. Richter, Asst. Editorial Coordinator). National Cotton Council of America, Memphis, TN. 1-556 pp.

10. Chun, D. T. and Perkins, Jr., H. H. 1996. Bacterial genera associated with three cotton growing regions of the cotton belt. Pages ?-?, in: 1996 Proc. Beltwide Cotton Conferences, Vol. . D. A. Richter and J. Armour (eds.). National Cotton Council of America, Memphis, TN. ? pp. [**In Press**]

11. Clark, F. E., Hervey, R. J., and Blank, L. M. 1947. Occurrence of cotton fiber contaminated by Aerobacter cloacae. US Department of Agriculture Technical Bulletin No. 935 28 pp.

12. Fischer, J. J., and Foarde, K. 1989. The association of Enterobacter agglomerans and Pseudomonas syringae with cotton lint. Pages 13-18 in: 'Cotton Dust' Proc. Thirteenth Cotton Dust Res. Conf., Nashville, TN, January 5-6, 1989. R. R. Jacobs and P. J. Wakelyn, eds. National Cotton Council, Memphis, TN. 159 pp.

13. Fischer, J. J., Jacobs, R. R.. 1988. Endotoxin on cottons and bracts from four areas throughout the 1986 growing season Pages 40-42 in: 'Cotton Dust' Proc. Twelfth Cotton Dust Res. Conf., New Orleans, LA, January 6-7, 1988. R. R. Jacobs and P. J. Wakelyn, eds. National Cotton Council, Memphis, TN. 206 pp.

14. Heuer, B. and Plaut, Z. 1985. A new approach to reduce sugar content of cotton fibers and its consequence for fiber stickiness. Tex. Res. J. 55:263-266.

15. Morey, P. R., Fischer, J. J., and Sasser, P. E.. 1982. Endotoxin content and fiber properties of raw cotton. Textile Res. J. 52:524-528

16. Neal, P. A., Schneiter, R., and Caminita, B. H. 1942. Report on acute illness among rural mattress makers using low grade, stained cotton. J. Amer. Med. Assoc. 119(14):1074-1082

17. Olenchock, S. A., Castellan, R. M., and Hankinson, J. L. 1984. Endotoxin contamination of cotton: area of growth/varieties. Pages 64-66 in: 'Cotton Dust' Proc. Eighth Cotton Dust Res. Conf. P. J. Wakelyn and R. R. Jacobs, eds. National Cotton Council, Memphis, TN. 189 pp.

18. Perkins, Jr., H. H. 1993. Strategies for processing honeydew contaminated cotton. Pages 1461-1462, in: 1993 Proc. Beltwide Cotton Conferences, Vol. 3, January 10-14, New Orleans, LA. (Douglas J. Herber, Editorial Coordinator, and Deborah A. Richter, Asst. Editorial Coordinator). National Cotton Council of America, Memphis, TN. 1175-1660 pp.

19. Perkins, Jr., Henry H. and Brushwood, Donald E. 1995. Interlaboratory evaluation of the thermodetector cotton stickiness test method. Pages 1189-1191, in: 1995 Proc. Beltwide Cotton Conferences, Vol. 2, January 4-7, San Antonio, TX. (Deborah A. Richter, Editorial Coordinator, and Jim Armour, Asst. Editorial Coordinator). National Cotton Council of America, Memphis, TN. 723-1458 pp.

20. Prindle, B. 1934a. The microbiology of textile fibres. Textile Res. J. 4(9):413-428.

21. Prindle, B. 1934b. The microbiology of textile fibres. Textile Res. J. 4(10):463-478.

22. Prindle, B. 1934c. The microbiology of textile fibres. Textile Res. J. 5:11-31.

23. Rylander, R. , Haglind, P. , and Lundholm, M. 1985. Endotoxin in cotton dust and respiratory function decrement among cotton workers in an

experimental cardroom. Am. Rev. Respir. Dis. 131:209-213.

24. Sasser, M. 1990. Identification of bacteria through fatty acid analysis. Pps. 199-204. In: Methods in Phytobacteriology. Z. Klement, K. Rudolf, and D. Sands (eds.). Akademiai Kiado, Budapest. 568 pp.

25. Sasser, M. and Wichman, M. D. 1991. Identification of microorganisms through use or gas chromatography and high-performance liquid chromatography. Pages. 111-118, in Manual of Clinical Microbiology, fifth edition. A. Balows, W. J. Hausler, Jr., K. L. Herrman, H. D. Isenberg, and H. J. Shadomy (eds.). American Society for Microbiology, Washington, D. C. 1364 pp.

26. Simpson, M. E., Choper, e. A., and Prickett, T. J. 1988. A report on the identification of gram-negative bacteria on cotton fiber from two U.S. field locations. Pages 6-9, in: 'Cotton Dust' Proc. Twelfth Cotton Dust Res. Conf. R. R. Jacobs and P. J. Wakelyn, eds. National Cotton Council, Memphis, TN. 206 pp.

27. Simpson, M. E., and Marsh, P. B.. 1985. Exceptionally low bacterial counts common on raw cotton fiber from the San Joaquin valley of California. Pages 69-71 in: 'Cotton Dust' Proc. Ninth Cotton Dust Res. Conf., New Orleans, LA, January 9-11, 1985. P. J. Wakelyn and R. R. Jacobs, eds. National Cotton Council, Memphis, TN. 181 pp.

28. Wyatt, B. G., and Heintz, C. E.. 1982. Capsule-producing coryneform bacteria associated with stickiness in cotton. Textile Res. J. 52:518-523

Table 1. Description of the categories — average percent sugar and number of thermal detector spots.

Cotton Category	Percent Sugar(%)[1]	TDspots
Nonsticky Cottons	0.49	3.9
Moderately Sticky Cottons	0.64	11.8
Sticky Cottons	1.70	89.3

[1]USDA potassium ferricyanide test (Brushwood and Perkins 1993)

Table 2. Average Number of Species Found in Each Genera in the Three Categories of Sticky Cottons.

Genera	Avg. No.[1]
Bacillus	60.33^{A}
NO MATCH	7.33^{B}
Staphylococcus	5.67^{CB}
Listeria	3.33^{CBD}
Arthrobacter	2.33^{CD}
Cellulomonas	1.33^{CD}
Rathayibacter	1.33^{CD}
Acinetobacter	1.00^{D}
Hydrogenophaga	0.67^{D}
Actinobacillus	0.67^{D}
Streptococcus	0.67^{D}
Chryseomonas	0.33^{D}
Enterobacter	0.33^{D}
Micrococcus	0.33^{D}
Microbacterium	0.33^{D}
Curtobacterium	0.33^{D}
Salmonella	0.33^{D}
Erwinia	0.33^{D}
Corynebacterium	0.33^{D1}

Mean separation within column by Duncan's multiple range test, 5% level. Means with the same letter are not significantly different.

Table 3. Gram-index of cottons from the three sticky cotton categories.

Stickiness	Raw Gram-Index[1]		Relative Gram-Index[2]	
Category	G(-)	G(+)	G(-)	G(+)
Nonsticky	0.0	1.0	0.1	4.4
Moderate	0.1	0.9	0.3	4.3
Sticky	0.1	0.9	0.3	4.6

[1][Total frequency of gram(-negative or -positive)] ÷ [∑(Total frequency of gram-negative + gram-positive)]
[2](Raw gram-index) x [$\log_{10}$(bacterial population)]

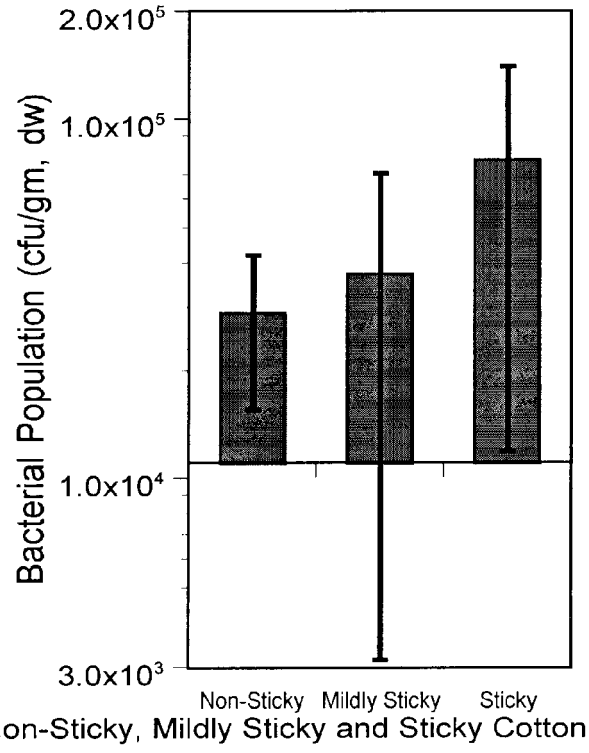

Figure 1. Average total bacterial population found in nonsticky, moderately sticky and sticky cottons, (cfu/gm, corrected for dry weight; each half bar represents 2 s.e.).

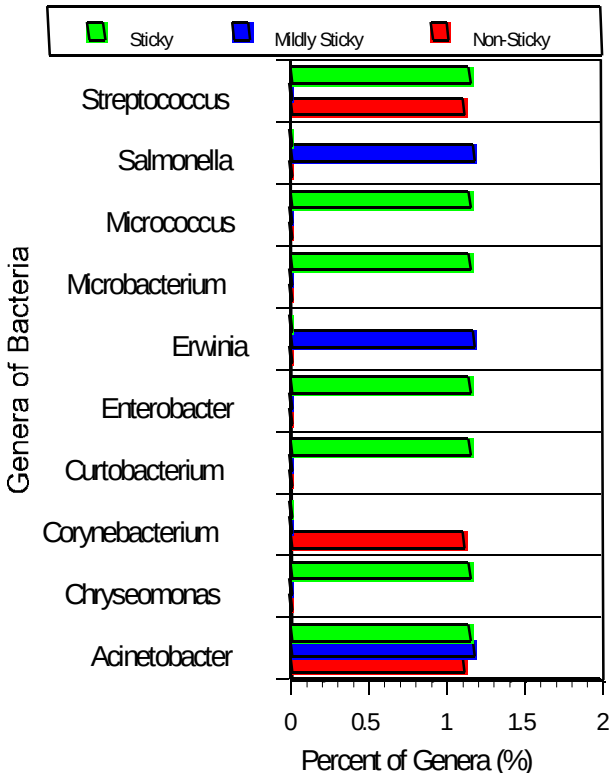

Figure 2. The genera of bacteria found in nonsticky, moderately sticky and sticky cottons making up less than 2% of the total number identified.

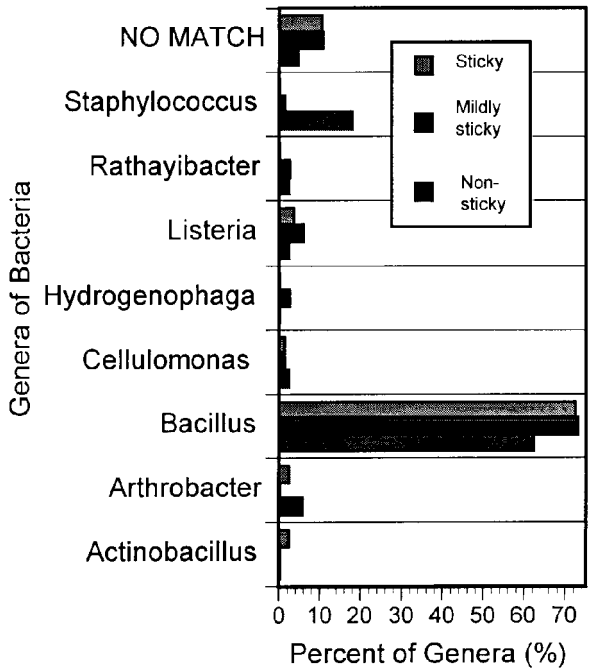

Figure 3. The genera of bacteria found in nonsticky, moderately sticky and sticky cottons making up 2% or more of the total number of species identified.