BACTERIAL PROFILE OF THE COTTON DUST, FROM COTTON GROWN IN THE MISSISSIPPI DELTA REGION, COLLECTED FOR THE SECOND ENDOTOXIN ASSAY ROUND ROBIN STUDY

D. T. Chun and R. E. Harrison
USDA, ARS
Cotton Quality Research Station
Clemson, SC

Abstract

Cotton dust was collected in 1998 on PVC (polyvinyl chloride) and glass filters from the 1997-Harvest year cotton grown in the Mississippi Delta region. The dust was collected using vertical elutriators from strict low middling (grade 41) cotton, low middling light spotted (grade 53) cotton, and a 1:1 mixture of the two grades of cotton. The bacterial profile of the cotton dust approximately 1 year after collection will be presented: this will include the population density as well as the most common bacterial genera found on the dust from the three cotton sources and the two filter types used for collection. Filter samples used to develop the profile will be from the surplus set of filters not used in the second endotoxin assay round robin study.

Introduction

Last year, after results from the first round robin endotoxin assay study became known (Chun et al., 1999; Chun and the Endotoxin Assay Committee, 1999), it was decided to extend the endotoxin assay study to see if more comparable results could be obtained between laboratories when further standardization was adopted. In order to do this, additional dust samples were required. To this end, the Cotton Quality Research Station in Clemson, SC, generated cotton dust samples from three sources of cotton believed to contain high, medium and low concentrations of endotoxin to provide dust samples collected on glass and polyvinyl chloride filters (PVC) for further investigations (Chun et al., 1999a,b). The dust samples have not been fully characterized. Currently, only the weight and source of the dust samples are known so it was decided to further investigate the biological properties of the dust.

From earlier workers, a general idea of the makeup of the bacterial population was anticipated (Fisher et al., 1989; 1986, 1982; Chun and Perkins, 1997; Godby et al., 1995): that the dust bacterial population would probably be high and contain a high ratio of Gram-negative over Gram-positive bacteria within the viable population; and that the common genera found would be *Pseudomonas, Pantoe* (*Enterobacter agglomerans* has been transferred here), *Kluyera*, *Lebsiella*, *etc*. *spps*, characteristic of Mississippi Delta cotton. However, the early results indicated that the predicted biological stature of the dust would not reflect the expected characteristics of Mississippi grown cotton lint, but rather the characteristics of a bacterial population whose members were no longer viable so that what was observed and will be reported here, depicts the bacterial survivors on cotton dust.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:235-238 (2000)
National Cotton Council, Memphis TN

Methods and Materials

Cotton Dust Samples

The cotton dust sample collection was described by Chun et al., 1999a,b. Cotton dust was generated from Mississippi Delta region cotton from the 1997-harvest year. The dust samples were derived from three sources of cotton believed to contain a low, medium and high concentration of endotoxin. The cottons consisted of: strict low middling cotton (grade 41), Cotton Source 'A'; low middling light spotted cotton (grade 53), Cotton Source 'B'; and a 1:1 composite of Cottons 'A' and 'B', Cotton Source 'AB'. The cottons were carded in a model card room at the Cotton Quality Research Station at Clemson, SC and the generated dust was collected on glass or polyvinyl chloride (PVC) filters using vertical elutriators. Each dust sample was stored in a 50-ml screw-top polypropylene conical centrifuge tube in the dark at room temperature (~22˚±1˚C) until used. For this study, Ten samples from each filter and cotton source were used. The samples were chosen from the samples not used in the second endotoxin assay round robin study and taken from lots spanning the range of collection runs as much as possible. The samples had been collected in 1998 and had been stored approximately one year before they were used in this study.

Viable Microbial Count and Culturing Method for Bacterial Identification

Viable total bacterial populations were determined for each of the cotton dust filters as described in Chun & Perkins, 1991; except that the initial diluent was 20-ml and a 1:5 dilution series was used, that instead of doing pour plates, spread plates were done and that the plates were cultured for 24 hours at 28˚±0.5˚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. In this study, the bacterial populations were so low that often the plates contained less than the desired 50-250 colonies/plate. The methodology of profiling the bacterial genera associated with the samples was the same as described by Chun and Perkins, 1997, except that 196 0.5-cm^2 locations or squares was used instead of the 44 1-cm^2 locations or

squares. The circle contained the same area as the Petri plate bottoms used and was subdivided into 196 0.5-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 or longer for some isolates at 28˚±0.5˚C after which time the cells were harvested for fatty acid extraction.

Bacterial Identification

Identification was by 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 and version 4.0 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. For this study, samples lost during processing or not identified were lumped into the 'No matches' category and treated as a separate category so the 'No Match' category represents a more inclusive group than previously described.

Gram Index

In the past, a gram-index number was calculated which was 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. However, in this study, no gram-negative genera were isolated so no Gram Index results are presented.

Statistical Analysis

Data manipulation and analysis were done with Microsoft EXCEL 97 SR-2 for Windows 95 (Microsoft Corporation, USA) and plotted using SigmaPlot for Windows Version 4 or Version 5 (SPSS, Inc., USA).

Results and Discussion

When the study was begun, the assumption was made that the cotton dust would in some small way or more reflect the properties of the cotton it was derived from. With this in mind, some loss of bacterial population was expected since in conventional cotton storage, no significant difference in bacterial population is anticipated after 1 year in storage; but after 2 years, the total bacterial count and to a similar but lesser degree, the Gram-negative bacterial population would begin to drop (Chun and Perkins, 1996). The early results from this study were of the bacterial population determinations and the results made it clear that the total bacterial population density was lower than expected from the normal fall off in cotton lint.

From earlier work on Mississippi cotton dust the average total bacterial population ranged from 72,550 to 109,648 cfu/mg cotton dust (Chun, 1989,1990) which is 20 to 30 times the average population density obtained in this study, 3,595 cfu/mg cotton dust. The specific population densities by filter type and cotton source is shown in Table 1. The PVC filters seem to collect or provide a support medium that favors a higher number of viable bacterial cells, both overall and regardless of cotton source, except for the cotton source 'B' where the population density for both filters is generally lower than from the other two sources and may inversely reflect the situation where cotton 'B' is expected to contain the highest population of Gram-negative bacteria – since Gram-negative bacterial counts tend to fall off at a greater rate during storage (Chun and Perkins, 1996). While the disparity between the results from the early Mississippi cotton dust and the dust filters from this study would be less if only the PVC filters were compared and the glass filters discounted, the older Mississippi results would still be about 14 to 20 times greater so some other consideration besides filter type must be explored. The early Mississippi cotton dust collection were made within a year or two years of the cotton harvest but population determinations were made shortly after the dust was collected. In this study, the dust collection was made about a year after harvest, but the population determinations were made after the dust collection filters were stored for about a year. So apparently, bacterial populations do not survive well on dust and especially poorly when the dust is collected and stored on glass filters. Fischer (1989) has suggested that cotton is an excellent

bacteriological medium for the organisms it contains and may provide something that prolongs its survival, at least as compared to dust. At this point, we have no good storage time reference as to the survival of bacteria on cotton dust so use of the MTM to generate dust samples for a storage/survival study is tentatively planned to further our understanding of bacteria on cotton.

As expected, the bacterial profile reflected this drop in population density and the especially greater drop of the Gram-negative bacterial component. The bacterial genera identified are not only the more resilient Gram-positive genera, but the range of genera (and species, not shown) identified is very narrow, more representative of survivors (see Chun and Perkins, 1996). All of the randomly selected isolates have been identified as belonging in Gram-positive genera (Table 2). While this type of profile is more characteristic of a low endotoxin region such as California (Chun and Perkins, 1997), the current incoming results from the second endotoxin assay study and in-house assay for endotoxin (not published) has already belied the fear that the dust contains little or no endotoxin. Also, the three sources of cotton do not have identical proportions of the bacterial genera (Figure 2). What is surprising is that not only is the number of viable bacteria different for the two filter types but that the two filters favor different genera of bacteria (Figures 1 and 3). This holds despite the cotton source (Figure 2) and is exaggerated going from the low endotoxin cotton source to the high endotoxin cotton source; for example, where the proportion of *Bacillus* and *Paenibacillus* isolates increases on the PVC filter (Figure 3). With no knowledge other than the population density and the bacterial profile, one could easily have believed that the cotton was from a low endotoxin cotton region and that it contained low levels of endotoxin -- endotoxin concentration is still the best parameter for byssinotic potential (Chun and Perkins, 1996). This underscores the need for studying the changing or non-changing profile of bacterial genera and population of cotton dust under storage conditions.

Summary

In summary, the bacterial density and bacterial profile of the cotton dust collected for the second round robin endotoxin assay test are consistent with a situation where the bacteria observed are believed to be the survivors and not truly representative of the former bacterial residents. While this needs to be confirmed by future study, the narrow range of bacterial genera and the low population density suggested that the survival rate on cotton dust is lower than on cotton lint. This phenomena could mislead an investigation where only the population density and genera of bacterial isolates are determined since the type of results here are more indicative of a low endotoxin growing region than would probably be shown when cotton dust had not been stored. This leaves again, endotoxin concentration as the best indicator of byssinotic potential.

Disclaimer

Mention of a trademark, warranty, proprietary product or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

References

Chun, D.T. 1989. Airborne bacterial levels of card generated dust from cottons grown in different locations and seasons. Pages 34-36 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.

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.

Chun, D. T., Chew, V. Bartlett, K., Gordon, T., Jacobs, R. R., Larsson, B., Larsson, L., Lewis, D. M., Liesivuori, J., Michel, O., Milton, D. K., Rylander, R., Thorne, P. S., and White, E. M. and Brown, M. E. 1999. Preliminary report on the results of the second phase of a round robin endotoxin assay study using cotton dust. *Applied Occupational & Environmental Hygiene.* [in press]

Chun, D.T. and the Endotoxin Assay Committee. 1999. A quick summary of the highlights of parts 1 and 2 of the first round Robin endotoxin assay study and a brief preview of the next round. Pages 132-138, in: 1999 Proc. Beltwide Cotton Conferences, Vol. 1. D. J. Herber and D. A. Richter (eds.). National Cotton Council of America, Memphis, TN. 754 pp.

Chun, D.T., Harrison, R.E., and Chew, V. 1999a. Second collection of uniform, card generated, vertically elutriated dust for interlaboratory comparison of dust endotoxin assays. Pages 129-132, in: 1999 Proc. Beltwide Cotton Conferences, Vol. 1. D. J. Herber and D. A. Richter (eds.). National Cotton Council of America, Memphis, TN. 754 pp.

Chun, D.T., Harrison, R.E., and Chew, V. 1999b. Second collection of card-generated, vertically elutriated dust for comparison endotoxin assays. The *Journal of Cotton Science* 3(4):xxx-xxx [In Press].

Chun, D.T. and Perkins, H.H., Jr. 1996. Effects of conventional cotton storage on dust generation potential, bacterial survival, and endotoxin content of lint and dust. *Ann. Agric. Environ. Med.* 3:19-25.

Chun, D.T. and Perkins, H.H., Jr. 1997. Profile of bacterial genera associated with cotton from low and high endotoxin growing regions. *Ann. Agric. Environ. Med.* 4:233-242.

Fischer, J. J., Foarde, K., and Jacobs, R. R. 1989. Endotoxin on cottons and bracts from four areas throughout the 1987 growing season. Pages 31-33 **In**: Proc. Thirteenth Cotton Dust Res. Conf. R. R. Jacobs and P. J. Wakelyn, eds. National Cotton Council, Memphis, TN. 159 pp.

Fischer, J. J., Morey, P. R., and Foarde, K. K.. 1986. The distribution of gram negative bacteria and endotoxin on raw cotton components. *Am. Ind. Hyg. Assoc. J.* 47(7):421-426.

Fischer, J. J., Morey, P. R., Sasser, P. E., and Foarde, K. K. 1982. Microbial and endotoxin content of botanical trash in raw cotton. *Am. Ind. Hyg. Assoc. J.* 43:492- 494.

Godby, M. W., Odencrantz, J. R., Whitmer, M. P., Harrison, R. E., and Perkins, H. H., Jr. 1995. USDA cotton classing correlated to endotoxin content of cardroom dust. Pages 280-283, 19th Cotton and Other Organic Dust Res. Conf. (Wakelyn, P. J., Jacobs, R. R. and Rylander, R., Editors), in: 1995 Proc. Beltwide Cotton Conferences, Vol. 1, January 4-7, San Antonio, TX (Deborah A. Richter, Editorial Coordinator, and Jim Armour, Asst. Editorial Coordinators). National Cotton Council of America, Memphis, TN. 722 pp.

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

Sasser, M., and Wichman, M.D. 1991. Identification of microorganisms through use of gas chromatography and high-performance liquid chromatography. **In:** Balows A., Hausler, W.J., Herrman, K.L., Isenberg, H.D., and Shadomy, H.J. (Eds); Manual of Clinical Microbiology, 5th Edition, 111-118. American Society for Microbiology, Washington, D.C.

Table 1. Bacterial population density of cotton dust collected on Glass and PVC filters from cottons containing low, medium and high endotoxin concentration, CFU/mg cotton dust.

Cotton Source[1]	Glass Filter	PVC Filter
A, AB, B[2]**	1,986	5,204
A*	4,485	8,588
AB**	317	5,927
B	1,155	1,096

[1] Cotton A = strict low middling; Cotton B = low middling light spotted; Cotton AB = 1:1 blend of strict low middling to low middling light spotted.

[2] t-test, average dust weight difference between PVC and Glass filters is equal to zero: *, $P < 0.05$; and **, $P < 0.01$.

Table 2. Total number and frequency of bacterial genera identified from cotton dust.

Genera of Bacterial Isolates	Number of Isolates	Percent of total Isolates (%)
Aerococcus	1	0.3
Bacillus	204	51.1
Cellulomonas	7	1.8
No Match	29	7.3
Paenibacillus	79	19.8
Staphylococus	16	4.0
Streptococcus	63	15.8
Total	399	100.0

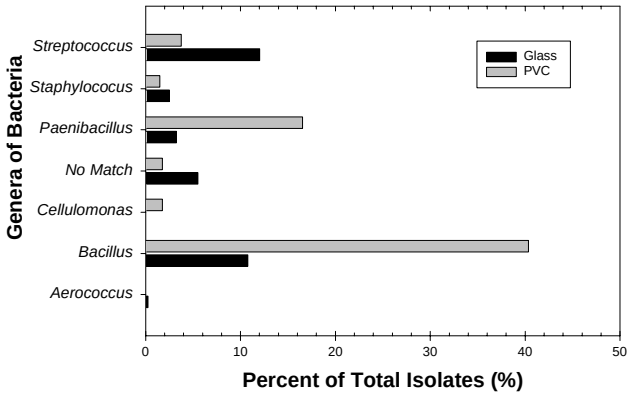

Figure 1. Percent of total isolates of genera of bacteria found on cotton dust on glass and polyvinyl chloride filters.

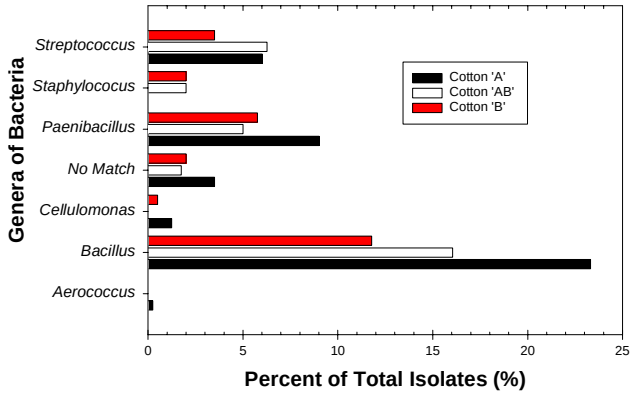

Figure 2. The percent of total isolates of each genera of bacteria from Cotton ‘A’, ‘AB’ and ‘B’.

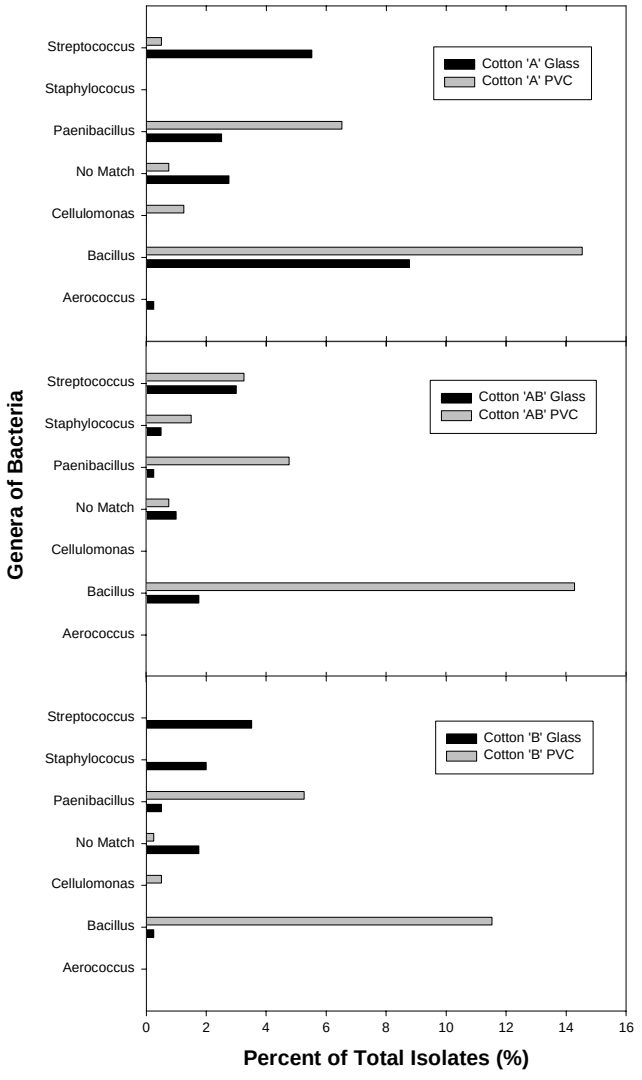

Figure 3. The percent of total isolates of each genera of bacteria from Cotton ‘A’, ‘AB’ and ‘B’ on glass and polyvinyl chloride filters.