ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 1997, 4, 233–242

PROFILE OF BACTERIAL GENERA ASSOCIATED WITH COTTON FROM LOW ENDOTOXIN AND HIGH ENDOTOXIN GROWING REGIONS

David T.W. Chun, Henry H. Perkins, Jr.

Cotton Quality Research Station, USDA, ARS, Clemson, SC, USA

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

Abstract: 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. Approximately 1093 bacterial isolates were made and identified from cotton. The method uncovers a greater diversity of bacterial species from cotton than has hitherto been reported. In California, the bulk of bacterial species consisted of *Bacillus* spp.; and in Mississippi and Texas region cottons, the bulk of bacterial species consisted of *Pseudomonas* spp. No significant differences between populations were observed in the nonsticky, moderately sticky and sticky cottons. A Gram-index concept is introduced which relates the 'Gram-reaction character' of a cotton growing region.

Address for correspondence: David T.W. Chun, PhD, Cotton Quality Research Station, USDA, ARS, P.O. Box 792, Clemson, SC 29633, USA.
E-mail: dtwchun@clemson.campus.mci.net or usdaars@clemson.edu

Key words: endotoxin, cotton dust, Gram-positive bacteria, Gram-negative bacteria, cotton, methodology, identification, gas chromatography, fatty acid analysis, sticky cotton.

INTRODUCTION

Since the work of Prindle [23, 24, 25] and Clark *et al.* [13] on the association of bacteria and cotton lint, and the paper by Neal *et al.* [19] 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 [5, 6, 26] since endotoxin is a biological product of Gram-negative bacteria. Of more current interest to the industry was a report by Wyatt and Heintz [31] that associated capsule-producing coryneform bacteria with stickiness in cotton and the recent emphasis on relieving cotton stickiness by microbial decomposition of the insect honeydew which might increase the endotoxin potential of cotton [2, 17, 21]. This has created an intense interest in the ecology of cotton bacteria and the need for more precise identification of microorganisms on both normal and sticky cottons.

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 [3, 8, 9, 11, 30] or on determining levels of endotoxin [10, 15] which led investigators to find that different cotton growing regions may exhibit different bacterial populations and endotoxin levels [14, 15, 30]. 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

Received: 11 September 1996
Accepted: 14 April 1997

biochemical methods [3, 14, 29]. 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.

MATERIALS AND METHODS

Cotton. *Three Region Study*: Cottons used were all from the 1995 harvest year. The three cotton growing regions chosen were California, Texas, and Mississippi since these regions characteristically have low, variable, and high endotoxin cottons, respectively [7, 15, 20]. The cottons were originally sent to the Cotton Quality Research Station (CQRS; Clemson, SC) from the Agricultural Marketing Service (AMS) testing centers (of that region) for sugar analysis and were on their way to Cotton Inc. The cotton samples from a region were made into composite samples: Pinches of cotton were collected from the sampling bags from a region (to make a total of 15+ grams), and passed three times through a rotary cotton blender to produce the composite samples. From each composite regional sample, 12 one gram samples were used for testing. Dry weights of the composite samples were determined.

Seasonal Cotton Study: Cotton was supplied by Dr. O. L. May (USDA, ARS, SAA, Pee Dee Research and Education Center, 2200 Pocket Road, Florence, SC 29506-9706, USA) from the 1995 harvest year. Cotton collection began approximately at the time of boll crack and continued at approximately weekly intervals until the time of cotton harvest. Twelve bolls, each from a separate cotton plant, were collected at each harvest and packed in paper bags. The samples were mailed on the day of harvest using over-night delivery to CQRS. On receipt, each boll was hand ginned and from each boll one gram samples were removed for testing. Dry weight of the lint was determined.

Sticky Cotton Study: 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, USA) to CQRS 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 rated for stickiness on a thermodetector (TD) [4, 22]. Instead of using the four categories 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. These spots are countable sticky points/spots produced on an aluminum foil by the thermodetector test which indicates an area of stickiness on a web of cotton). The cotton samples were sorted by TD into the three categories (Tab. 3). Ten one gram samples were chosen from each stickiness category; and from these 30 one gram samples, ten samples were randomly chosen and processed at a time. Dry weights of the samples were determined.

Culturing Method for Bacterial Identification and Viable Microbial Count. Viable total bacterial populations were determined for each of the one gram samples [9, 12], except that instead of pour plates, spread plates were used and the plates were cultured for 24 hours at 28° ± 0.5°C before being counted. A 1:5 dilution series was used beginning with either a 1:75 or 1:150 initial dilution and ranged to a 1:18,750 dilution, depending on the sample. The initial dilution consisting of the one gram sample in either 74 or 149 ml of diluent [0.4 M NaCl, 1.0 mM $MgSO_4$, 0.01% gelatin, and 0.01% Tween-80 in 50 mM phosphate (K^+, Na^+) buffer, pH 7 ± 0.2; cooled in an ice bath] was shaken for 20 minutes on a Burrell Wrist-Action Laboratory Shaker (Model BT, Burrell Corporation, 2223 Fifth Avenue, Pittsburgh, PA 15219) at a setting of 10. Aliquots of 0.1 ml were removed from each dilution to make spread plate counts on TSBA plates (trypticase soy broth agar). Bacterial populations were based on colony forming units per gram cotton lint corrected for dry weight (cfu/g). Prior to counting the plates, bacterial cells from well isolated colonies were taken for identification and the number of species found in each genera was tabulated for comparison.

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 left to right, top to bottom. To eliminate bias, ten locations were chosen randomly (using a random number generator) for each sample and an individual, 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 one or more days on a fresh TSBA 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° ± 0.5°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 [27, 28]. 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 were used for the sticky cotton and seasonal cotton studies. Version 3.9 of the Aerobic Method, and TSBA and CLIN libraries, which was released after the studies had begun, was used for the regional cotton study). 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 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 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 logarithm (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 AND DISCUSSION

Three Regions Study. Total bacterial population (Fig. 1) was highest for cotton grown in Texas (3.4×10^6 cfu/g, S.E. = 8.5×10^5 cfu/g) but this difference in population was not significantly different from the bacterial population found in Mississippi (1.9×10^6 cfu/g, S.E. = 2.1×10^5 cfu/g). However, the populations found on California cottons (1.1×10^5 cfu/g, S.E. = 4.9×10^4 cfu/g) were distinctly and significantly lower than that of cottons grown in either Texas or Mississippi.

For the California, Texas, and Mississippi growing regions, 120, 118 and 120 isolates were counted and identified, respectively. Even though only a few morphologically different colonial types appeared on the spread plates, when the different isolates were sorted to bacterial taxa, 68 species and 31 different genera, including the 'No Match' category, were obtained. 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 (Tab. 6) [1, 3, 14, 29]. Of these 31 different genera categories, 14 genera made up less than 2% of the total isolates identified (Fig. 2). The 'No Match' samples fell in this group. The number of isolates found in these genera were low; and chi-square testing did not suggest any unusual distribution of these genera to the growing regions. Genera containing 2% or more of the isolates identified did have a highly significant probability of being unevenly distributed between the three growing regions (Fig. 3). Most of the individual genera did not show significant distribution differences. However, some of the individual genera stood out as being more commonly found in one or more of the growing regions. *Salmonella* spp. were distributed significantly differently in the three regions ($p < 0.005$). No difference was observed between Texas and Mississippi; but, the 15 isolates of *Salmonella*

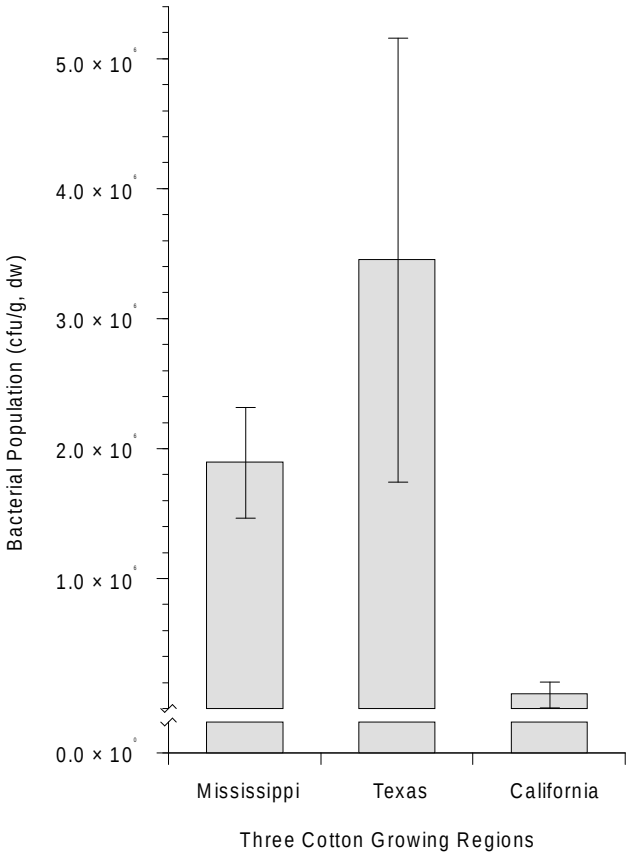

Figure 1. Average total bacterial populations found in three major cotton growing regions (cfu/g, corrected for dry weight; each half error bar represents 2 S.E.).

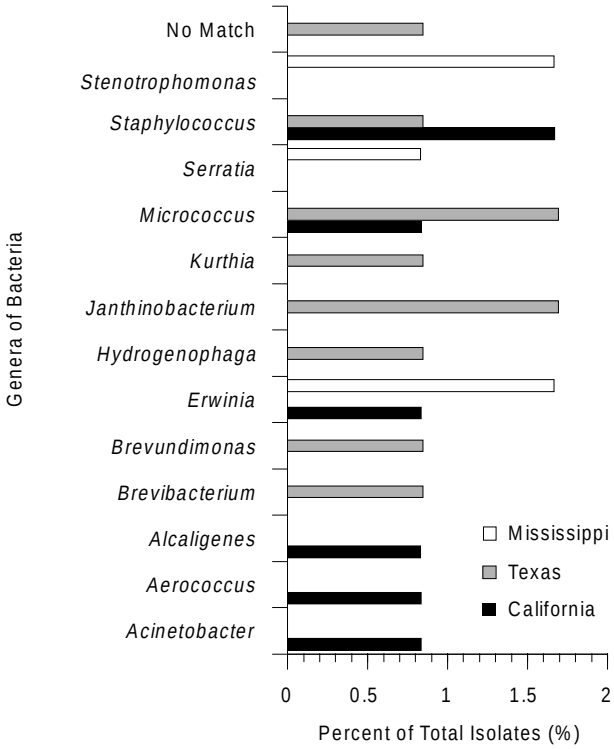

Figure 2. The genera of bacteria found in California, Texas and Mississippi growing regions making up less than 2% of the total number of isolates identified.

found in California was significantly higher than the total of 8 found in Texas and Mississippi ($p = 0.0009$). *Pantoea* spp., formerly grouped as *Erwinia* or *Enterobacter* species (both genera showing approximately the same distribution as *Pantoea* spp. - compare Figures 2 and 3, reflect changes due to the software library upgrade from version 3.8 and 3.9), also showed a significant distribution difference between the three regions ($p < 0.0001$). The distribution between California and Mississippi (12 and 19 isolates, respectively) was not significant; but the 3 found in Texas was significantly lower ($p = 0.0016$) than California and Mississippi. The opposite was found with *Listeria* spp. None were found in California and Mississippi, but 8 were found in Texas ($p = 0.000045$). *Kluyvera* spp. were also not uniformly distributed: 8, 1 and 17 isolates for California, Texas and Mississippi, respectively ($p = 0.003$). The chi-square test between California and Mississippi was not significant ($p = 0.06$); but the chi-square for *Kluyvera* spp. between Texas and both California and Mississippi was highly significant ($p = 0.001$). *Klebsiella* spp. also showed an uneven distribution between the three regions ($p = 0.05$). The distribution was not significant between California (3) and Mississippi (6); but was significant between Texas (0), California, and Mississippi ($p = 0.03$). *Flavimonas* spp. distribution was not different between California and Mississippi, 3 and 2 isolates, respectively; but, the 15 found in the Texas cottons was significant when contrasted to California and Mississippi ($p = 0.000038$). The occurrence of 4 *Escherichia* spp. isolates in the California lint vs. none in either the Texas or Mississippi lints was significant ($p = 0.005$). The distribution of 7 *Citrobacter* spp. isolates in the California region and none in Texas and Mississippi was highly significant ($p = 0.0002$). The occurrence of 16 *Cellulomonas* spp. isolates in Texas and the absence of any *Cellulomonas* spp. in California and Mississippi was highly significant ($p < 0.0001$).

Most of the above isolates occurred as relatively small percentages of the genera observed. *Bacillus* spp. and *Pseudomonas* spp., however, hold greater interest because they were more frequently found and showed distinct distributions between the three regions (Fig. 3). *Pseudomonas* spp. was the most common genus found in the Mississippi cottons (51 isolates), followed by Texas cottons (29), and least often in the California cottons (11). The frequency in Mississippi was significantly greater than in the Texas and California cottons ($p < 0.0001$) and in Texas alone ($p < 0.0001$). The higher frequency of *Pseudomonas* spp. was highly significant for Texas compared to California ($p = 0.0015$). Almost the reverse was observed with the *Bacillus* spp. Mississippi had no *Bacillus* spp. and this frequency was significantly lower than observed for Texas and California (15 and 32

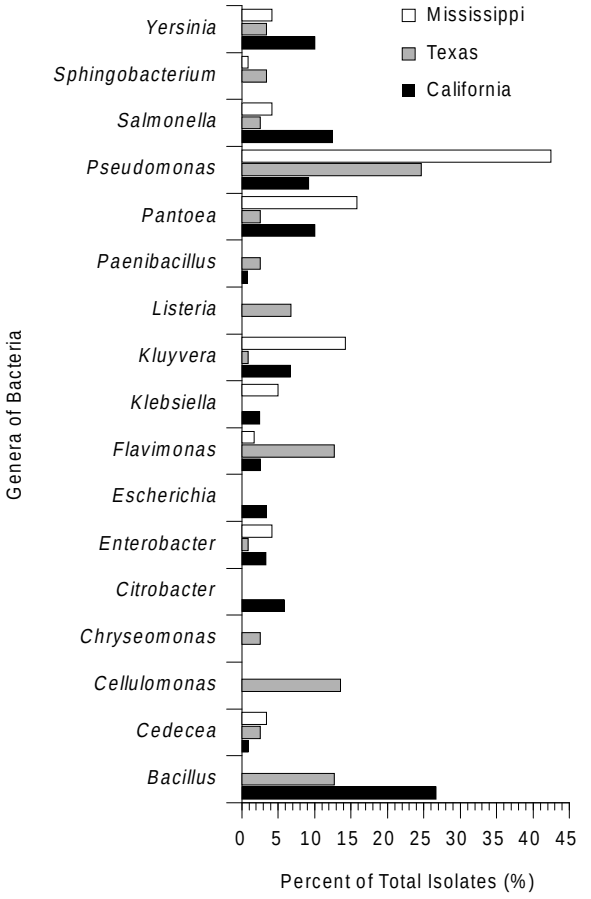

Figure 3. The genera of bacteria found in California, Texas and Mississippi growing regions making up 2% or more of the total number of isolates identified.

Table 1. Gram-index of cottons from three cotton growing regions.

Cotton Growing Region	Raw Gram-Index[a]		Relative Gram-Index[b]	
	G(-)	G(+)	G(-)	G(+)
California	0.7	0.3	3.5	1.6
Texas	0.6	0.4	3.8	2.7
Mississippi	1.0	0.0	6.3	0.0

[a][Total frequency of Gram(-negative or -positive)] ÷ [∑(Total frequency of Gram-negative + Gram-positive)]
[b](Raw Gram-index) × [$\log_{10}$(bacterial population)]

Table 2. Seasonal Gram-Index of Florence grown cotton.

Harvest Date,1995	Raw Gram-Index[a]		Relative Gram-Index[b]	
	G(-)	G(+)	G(-)	G(+)
Sept. 18	0.9	0.1	5.9	1.0
Sept. 27	1.0	0.0	7.4	0.1
Oct. 9	1.0	0.0	7.3	0.0
Oct. 23	1.0	0.0	7.3	0.1

[a][Total frequency of Gram(-negative or -positive)] ÷ [∑(Total frequency of Gram-negative + Gram-positive)]
[b](Raw Gram-index) × [$\log_{10}$(bacterial population)]

Table 3. Description of the categories — average percent sugar and number of thermal detector spots.

Cotton Category	Percent Sugar (%)[a]	TD spots
Nonsticky Cottons	0.49	3.9
Moderately Sticky Cottons	0.64	11.8
Sticky Cottons	1.70	89.3

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

isolates, respectively; $p < 0.0001$). The difference in distribution of *Bacillus* spp. was also highly significant between Texas and California ($p < 0.0001$). Even though the percentage of *Bacillus* spp. in California is high, it is considerably lower than what was observed in the 3 levels of western sticky cottons from California (Fig. 9), where the percentage of *Bacillus* spp. averaged 67%. Here the California percentage of *Bacillus* spp. was only 26.7%. This difference was more pronounced when the raw Gram-indexes were compared. In Table 1, the raw Gram-positive index for California was 0.3; whereas the average raw Gram-positive index for the sticky cottons was 0.9, Table 5. In addition, while no *Pseudomonas* spp. was observed on the sticky cottons, *Pseudomonas* spp. made up 9.2% of the genera found in California (Fig. 3). In the sticky cotton study, the average raw relative Gram-negative index was 0.06; whereas the raw Gram-negative index for California was 0.7 (Tables 1 and 5). The Gram-index follows trends of other studies which suggest that California is low in endotoxin levels and that Mississippi is high in endotoxin [14, 15, 30]. To verify the usefulness of this index would require additional studies to determine the correlation of, or lack of, an increased Gram-negative index with increased endotoxin levels. On speculation, a rational explanation might be that the cottons in this study were processed commercially before reaching CQRS and may have spent time stored in cotton modules before being ginned; whereas the cottons used in the sticky cotton study were sent direct to CQRS from researchers in the field. Conceivably, the time in storage may have contributed to the higher proportion of Gram-negative bacteria [12]. Morey *et al.* [18] have suggested that fiber yellowness was significantly and positively correlated with endotoxin content. Since endotoxin content is generally highly correlated with Gram-negative bacteria

Table 4. Average number of isolates found in each genera in the three categories of sticky cottons.

Genera	Avg. No[a]			
Bacillus	60.33			A
NO MATCH	7.33			B
Staphylococcus	5.67		C	B
Listeria	3.33	C	B	D
Arthrobacter	2.33		C	D
Cellulomonas	1.33		C	D
Rathayibacter	1.33		C	D
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			D

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

Table 5. Gram-index of cottons from the three sticky cotton categories.

Stickiness Category	Raw Gram-Index[a]		Relative Gram-Index[b]	
	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

[a][Total frequency of Gram(-negative or -positive)] ÷ [∑(Total frequency of Gram-negative + Gram-positive)]
[b](Raw Gram-index) × [$\log_{10}$(bacterial population)]

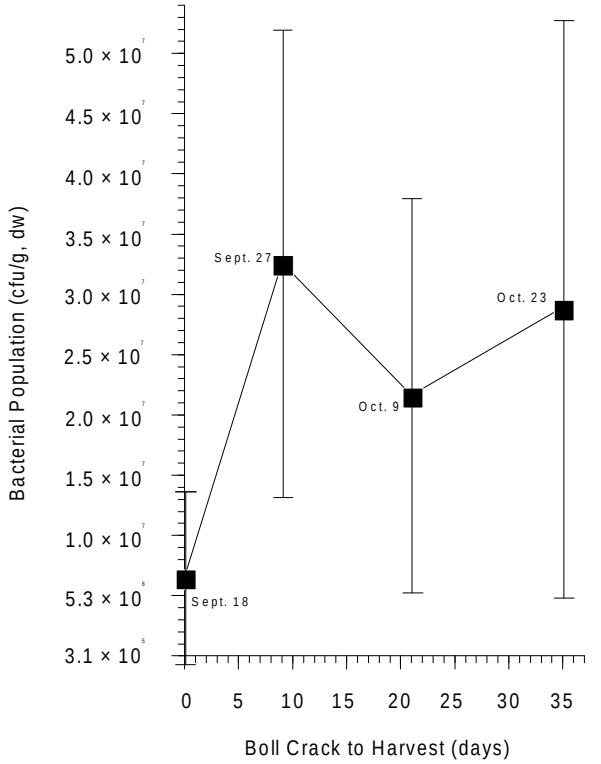

Figure 4. Average seasonal total bacterial population on Florence, South Carolina, grown cotton (cfu/g, corrected for dry weight; each half error bar represents 2 S.E.).

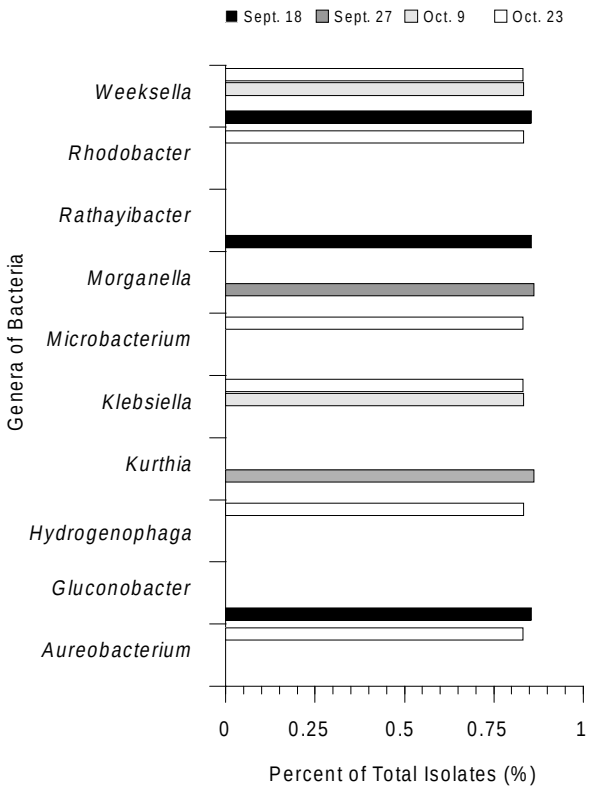

Figure 5. The seasonal genera of bacteria found on Florence, South Carolina, cotton from the time of boll crack to harvest. These genera make up less than 2% of the total number of isolates identified.

[15], the effect of module storage should be examined very closely as it relates to cotton color and quality. This thesis may focus future endeavor.

Seasonal changes in bacterial genera study. The spread plate method used here instead of the pour plate method resulted in greater variation as shown in Figures 1 and 4. While this has obscured differences between the sampling dates, the general pattern of the seasonal bacterial population of Florence grown cotton (Fig. 4) followed the same general pattern observed elsewhere [3, 15, 16, 32] for bacterial population from the time of boll crack (September 18) to harvest (October 23).

For the sampling dates, September 18 (6.9×10^6 cfu/g, S.E. = 3.6×10^6 cfu/g), September 27 (3.3×10^7 cfu/g, S.E. = 9.7×10^6 cfu/g), October 9 (2.2×10^7 cfu/g, S.E. = 8.2×10^6 cfu/g) and October 23 (2.9×10^7 cfu/g, S.E. = 1.2×10^7 cfu/g), 1995, - 117, 116, 120 and 120 isolates were identified, respectively. When sorted into bacterial taxa, 26 different genera comprising 46 species, including the 'No Match' category, were obtained. Of these, 10 genera made up less than 2% of the total isolates identified (Fig. 5). The 'No Match' category fell into the group containing more than 2% of the population. The number of isolates found in the genera making up less than 2% of the total isolates group were too few to warrant chi-square testing. No outstanding seasonal trends were observed in the genera making up more than 2% of all the isolates identified (Fig. 6). A high proportion of *Pseudomonas* spp. occurred throughout the growing season (Fig. 6). *Actinobacillus* spp. and *Flavimonas* spp. were the next most frequently occurring genera but even combined were fewer than the number of *Pseudomonas* spp. As expected, the Florence cotton appears most similar to the Mississippi region cotton (compare Figures 3 and 6) genera profile. Even the Gram-index suggests a close similarity to the Mississippi region (compare Tables 1 and 2). Through the entire season, the Florence cotton exhibited a high Gram-negative tendency. Taking into account the bacterial population, Florence cotton, like the cottons from the Mississippi region, exhibited a very high relative Gram-negative index. For now, based solely on the Florence genera profile example, there is no reason to believe major shifts occur within genera through the growing season for the California, Texas, or Mississippi region cotton. While outside the realm of this small study, one wonders if the genera profile of particular regions can act as a 'fingerprint' of that region which could later be used in discriminate analysis to identify the origin of unknown cottons?

Sticky Cotton Study. The total bacterial population tended to increase with stickiness (Fig. 7); however, total population was not significantly different for the three categories of sticky cotton (nonsticky = 2.9×10^4 cfu/g, S.E. = 6.6×10^3 cfu/g; moderately sticky = 3.6×10^4 cfu/g, S.E. = 1.7×10^4 cfu/g; and sticky = 7.6×10^4 cfu/g, S.E. = 3.2×10^4 cfu/g). The low bacterial population was consistent with California/Western cottons [7, 30].

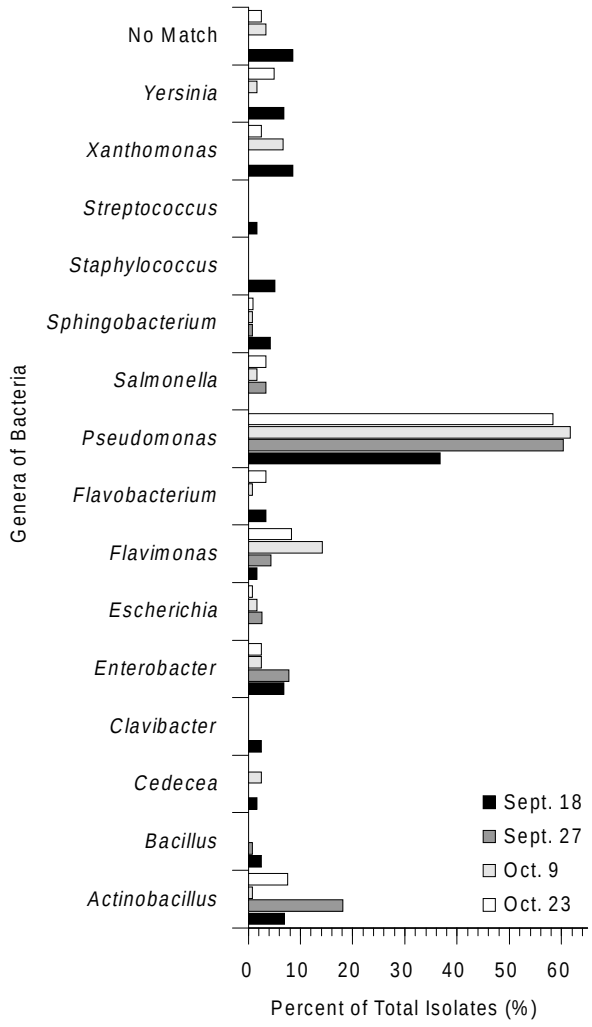

Figure 6. The seasonal genera of bacteria found on Florence, South Carolina, cotton from the time of boll crack to harvest. These genera make up more than 2% of the total number of isolates identified.

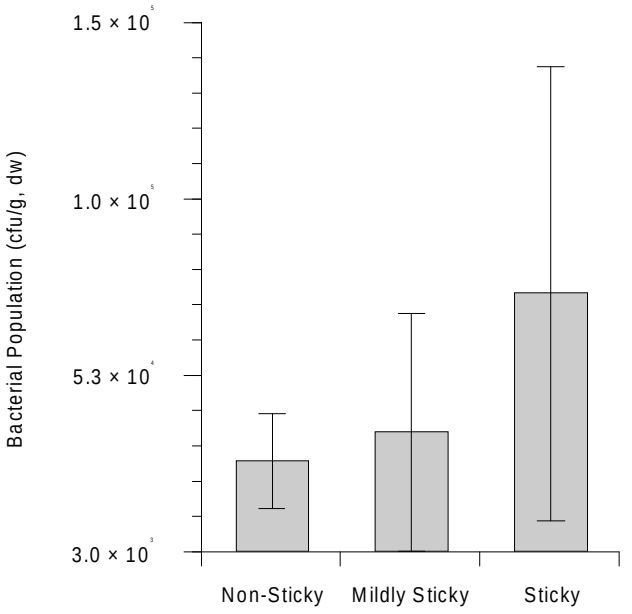

Figure 7. Average total bacterial populations found in nonsticky, moderately sticky and sticky cottons (cfu/g, corrected for dry weight; each half error bar represents 2 S.E.).

For the nonsticky, moderately sticky and sticky cottons, 90, 85 and 87 isolates 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 isolates were sorted to bacterial taxa, 19 different genera comprising 52 species, including the 'No Match' category, were obtained. 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 (Tab. 6) [1, 3, 14, 29]. Of these 19 different genera categories, 10 genera made up less than 2% of the total isolates identified (Fig. 8). The number of isolates 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 isolates identified did have a highly significant probability of being unevenly distributed between the three categories (Fig. 9). The 'No Match' samples fell in this group. Most of the individual

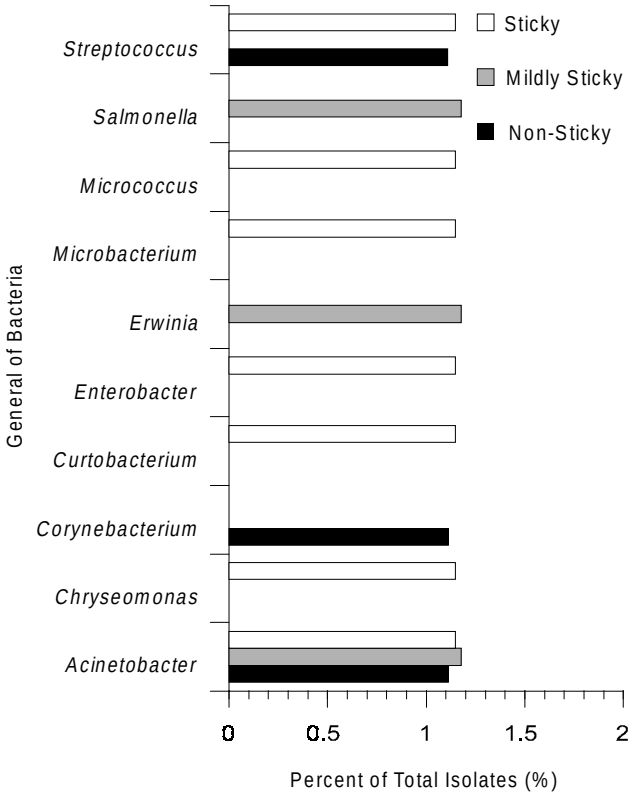

Figure 8. The genera of bacteria found in nonsticky, moderately sticky and sticky cottons making up less than 2% of the total number of isolates identified.

Table 6. Full list of the number of bacterial species isolated and identified from cotton in the three region study, the seasonal cotton study and the sticky cotton study.

Bacterial Species	A[ab]	B[ab]	C[ab]
Acinetobacter calcoaceticus	1	0	0
A. johnsonii	0	0	1
A. genospecies	0	0	1
Actinobacillus lignieresii	0	39	3
Aerococcus viridans	1	0	0
Alcaligenes piechaudii	1	0	0
Arthrobacter oxydans	0	0	1
A. pascens	0	0	1
A. protophormiae/ramosus	0	0	2
A. viscosus	0	0	3
Aureobacterium testaceum	0	1	0
Bacillus alcalophilus	2	0	0
B. alvei	0	0	4
B. aminovorans	1	1	1
B. amyloliquefaciens	1	0	0
B. atrophaeus	5	0	15
B. brevis	0	0	10
B. cereus	0	0	2
B. chitinosporus	0	0	1
B. circulans	4	0	11
B. filicolonicus	0	0	7
B. firmus	0	0	3
B. freudenreichii	0	0	1
B. gordonae	0	0	2
B. laterosporus	0	0	10
B. lentus	0	0	4
B. licheniformis	0	0	1
B. macerans	0	2	4
B. megaterium	2	0	12
B. pabuli	0	0	1
B. pantothenticus	0	0	1
B. polymyxa	0	0	1
B. pumilus	26	1	67
B. sphaericus	1	0	0
B. subtilis	5	0	22
B. thuringiensis	0	0	1
Brevibacterium acetylicum	1	0	0
Brevundimonas vesicularis	1	0	0
Cedecea davisae	1	0	0
C. neteri	7	5	0
Cellulomonas biazotea	7	0	4
C. cartae	1	0	0
C. flavigena	4	0	0
C. gelda	1	0	0
C. turbata	3	0	0
Chryseomonas luteola	3	0	1
Citrobacter amalonaticus	6	0	0
C. freundii	1	0	0
Clavibacter michiganense	0	3	0
Corynebacterium bovis	0	0	1
Curtobacterium flaccumfaciens	0	0	1
Enterobacter agglomerans	0	19	1
E. asburiate	1	0	0
E. cancerogenus	0	4	0
E. cloacae	3	0	0
E. taylorae	6	0	0
Erwinia carotovora	3	0	1
Escherichia coli	0	6	0
E. vulneris	4	0	0
Flavimonas oryzihabitans	20	34	0
Flavobacterium indologenes	0	8	0
F. meningosepticum	0	1	0
Gluconobacter asaii	0	1	0
Hydrogenophaga palleronii	0	1	0
H. pseudoflava	1	0	2
Janthinobacterium lividum	2	0	0
Klebsiella pneumoniae	7	0	0
K. trevisanii	2	2	0
Kluyvera ascorbata	12	0	0
K. cryocrescens	14	0	0
Kurthia gibsonii	1	1	0
Listeria grayi	0	0	1
L. innocua	0	0	1
L. ivanovii	7	0	5
L. monocytogenes	0	0	2
L. seeligeri	1	0	1
Microbacterium imperiale	0	1	1
Micrococcus halobius	2	0	0
M. luteus	0	0	1
M. varians	1	0	0
Morganella morganii	0	1	0
NO MATCH	1	17	22
Paenibacillus alvei	1	0	0
P. macerans	2	0	0
P. pabuli	1	0	0
Pantoea agglomerans	28	0	0
P. ananas	6	0	0
Pseudomonas aeruginosa	7	36	0
P. alcaligenes	0	2	0
P. chlororaphis	0	4	0
P. corrugata	1	0	0
P. fluorescens	3	0	0
P. mendocina	9	65	0
P. pseudoalcaligenes	0	33	0
P. pumilus	0	1	0
P. putida	69	110	0
P. saccharophila	0	2	0
P. savastanoi pv. oleae	0	1	0
P. syringae	1	3	0
P. savastanoi pv. fraxinus	1	0	0
Rathayibacter rathayi	0	0	4
R. tritici	0	1	0
Rhodobacter capsulatus	0	1	0
Salmonella choleraesuis	17	9	1
S. enteritidis	1	1	0
S. typhimurium	5	0	0
Serratia proteamaculans	1	0	0
Sphingobacterium multivorum	1	3	0
S. paucimobilis	1	0	0
S. spiritivorum	1	5	0
S. thalpophilum	2	0	0
Staphylococcus aureus	1	3	1
S. chromogenes	1	0	0
S. epidermidis	1	0	4
S. hominis	0	0	1
S. lugdunensis	0	1	0
S. warneri	0	2	11
Stenotrophomonas maltophilia	2	0	0
Streptococcus aureus	0	0	1
S. mutans	0	2	0
S. pyogenes	0	0	1
Weeksella zoohelcum	0	3	0
Xanthomonas campestris	0	6	0
X. fragariae	0	1	0
X. maltophilia	0	14	0
Yersinia enterocolitica	1	8	0
Y. frederiksenii	20	8	0

[a] A = Three Region Study; B = Seasonal Changes Study; C = Sticky Cotton Study. For each species, the number of isolations is shown; [b] Sherlock system software, version 1.06: Version 3.8 of the Aerobic Method, and TSBA and CLIN libraries were used for the sticky cotton and seasonal cotton studies. Version 3.9 of the Aerobic Method, and TSBA and CLIN libraries, which was released after the studies had begun, was used for the regional cotton study. 'No matches' were few and treated as a separate category.

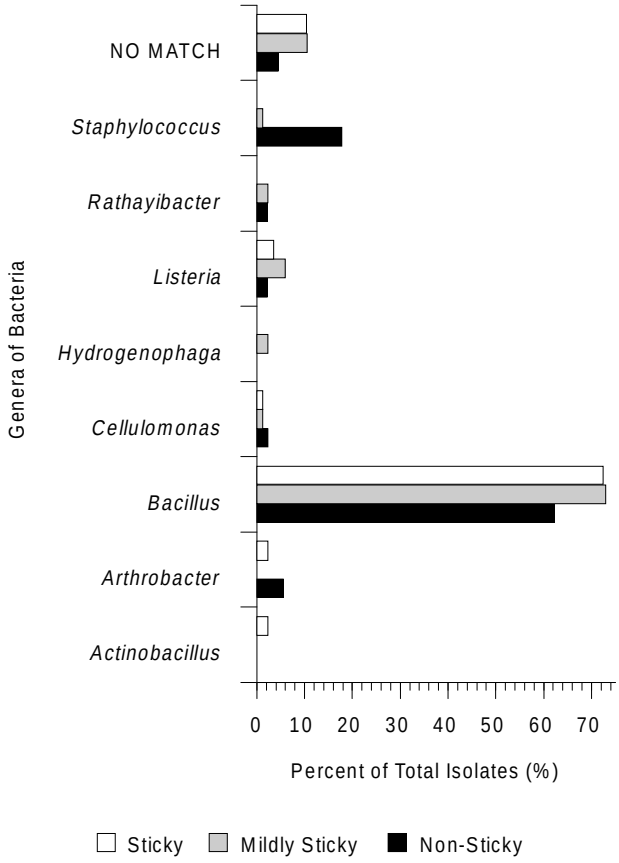

Figure 9. The genera of bacteria found in nonsticky, moderately sticky and sticky cottons making up 2% or more of the total number of isolates identified.

genera did not show significant distribution differences. However, the 16 isolates 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 combined ($p < 0.001$). In a concurrent study looking at regional differences, *Pseudomonas* spp. was the most common genus found in the Mississippi cottons (51 isolates), followed by Texas cottons (29), and least often in the California cottons (11). In this study, *Pseudomonas* spp. were not found. Also, only a single *Corynebacterium* sp. was found and it was isolated from the nonsticky cottons which argues against bacteria being associated with stickiness [31]. The genus with the largest number of isolates identified was *Bacillus* spp. When the chi-square test was applied, no significant difference could be found between the three categories of stickiness. However, the occurrence of *Bacillus* spp. was significantly more common than the other genera (Tab. 4). This difference was more pronounced when the raw Gram-indexes were compared. The raw relative Gram-negative index values ranged from 0.0 to 0.1 for the nonsticky, mildly sticky and sticky cottons (Tab. 5); whereas the raw Gram-negative index for California cottons from the regional study was 0.7.

CONCLUSIONS

We have presented a survey method 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 is capable of identifying a large sampling of the bacterial population and it has uncovered a greater diversity of bacterial species than has been reported on cotton. In addition, the frequency of specific genera and species can be determined which may permit characterization or profiling of specific cotton. In regard to this, a Gram-index concept was introduced which relates the 'Gram-reaction character' of a cotton growing region and is consistent with the general level of endotoxin attributed with cotton grown in California, Texas and Mississippi.

Acknowledgment

Appreciation and thanks are due to Dr. O. L. May at the USDA-ARS, SAA, Pee Dee Research and Education Center for harvesting and mailing cotton samples used in the seasonal study; and to Victor Chew at USDA, ARS, SAA (stationed at University of Florida, Gainesville, FL) for statistical support.

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

1. Akinwunmi OO, Templer JA, Heintz CE: Viable counts and distribution of bacterial flora on cotton plant parts. **In:** Jacobs RR, Wakelyn PJ (Eds): Cotton Dust. *Proceedings of the Thirteenth Cotton Dust Research Conference, Beltwide Cotton Research Conferences, Nashville, TN, January 5-6, 1989,* 19-23. National Cotton Council of America, Memphis, TN 1989.

2. Balasubramanya RH, Bhatawdekar SP, Paralikar KM: A new method for reducing the stickiness of cotton. *Textile Res J* 1985, **55**, 227-232.

3. Borbon Reyes JC, Batson WE Jr, Miller DP, Greenough D: Evaluation of populations of total and gram negative bacteria on cotton lint. **In:** Jacobs RR, Wakelyn PJ (Eds): Cotton Dust. *Proceedings of the Twelfth Cotton Dust Research Conference, Beltwide Cotton Research Conferences, New Orleans, LA, January 6-7, 1988*, 36-37. National Cotton Council of America, Memphis, TN 1988.

4. Brushwood DE, Perkins HH Jr: Cotton sugar and stickiness test methods. *Canadian Textile J* 1993, **110(6),** 54-62.

5. Castellan RM, Olenchock SA, Hankinson JL, Millner PD, Cocke JB, Bragg CK, Perkins HH Jr, Jacobs RR: Acute bronchoconstriction induced by cotton dust: dose-related responses to endotoxin and other dust factors. *Ann Int Med* 1984, **101(2),** 157-163.

6. Castellan RM, Olenchock SA, Kinsley KB, Hankinson JH: Inhaled endotoxin and decreased spirometric values. *N Engl J Med* 1987, **317**, 605-610.

7. Chun DT: Airborne bacterial levels of card generated dust from cottons grown in different locations and seasons. **In:** Jacobs RR, Wakelyn PJ (Eds): Cotton Dust. *Proceedings of the Thirteenth Cotton Dust Research Conference, Beltwide Cotton Research Conferences,*

Nashville, TN, January 5-6, 1989, 34-36. National Cotton Council, Memphis, TN 1989.

8. Chun DT: Airborne bacterial levels of card generated dust from 1988 cottons grown in different locations. **In:** Jacobs RR, Wakelyn PJ, Domelsmith LN (Eds): Cotton Dust. *Proceedings of the Fourteenth Cotton Dust Research Conference, Beltwide Research Conferences, Las Vegas, NV, January 12-13, 1990*, 37-39. National Cotton Council of America, Memphis, TN 1990.

9. Chun DT, Perkins HH Jr: Survey of bacterial population survival from cottons in storage. **In:** Herber DJ, Richter DA (Eds): *1991 Proceedings Beltwide Cotton Conferences*, Vol. 1, 287-288. National Cotton Council of America, Memphis, TN 1991.

10. Chun DT, Perkins HH Jr: Survey of endotoxin and dust levels from cottons in storage. **In:** Herber DJ, Richter DA (Eds): *1992 Proceedings Beltwide Cotton Conferences*, Vol. 1, 315-317. National Cotton Council of America, Memphis, TN 1992.

11. Chun DT, Perkins HH Jr: Seasonal variations in bacterial population and dust generation potential of cotton. **In:** Herber DJ, Richter DA (Eds): *1993 Proceedings Beltwide Cotton Conferences, New Orleans, LA, January 10-14, 1993*, Vol. 1, 306-309. National Cotton Council of America, Memphis, TN 1993.

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

13. Clark FE, Hervey RJ, Blank LM: *Occurrence of Cotton Fiber Contaminated by Aerobacter Cloacae*. United States Department of Agriculture, USDA Technical Bulletin No. 935, Washington, D.C. 1947.

14. Fischer JJ, Foarde K: The association of *Enterobacter agglomerans* and *Pseudomonas syringae* with cotton lint. **In:** Jacobs RR, Wakelyn PJ (Eds): Cotton Dust. *Proceedings of the Thirteenth Cotton Dust Research Conference, Beltwide Cotton Research Conferences, Nashville, TN, January 5-6, 1989*, 13-18. National Cotton Council of America, Memphis, TN 1989.

15. Fischer JJ, Jacobs RR: Endotoxin on cottons and bracts from four areas throughout the 1986 growing season. **In:** Jacobs RR, Wakelyn PJ (Eds): Cotton Dust. *Proceedings of the Twelfth Cotton Dust Research Conference, Beltwide Cotton Research Conferences, New Orleans, LA, January 6-7, 1988*, 40-42. National Cotton Council of America, Memphis, TN 1988.

16. Heintz CE, Fisher [Fischer] JJ, Brashears AD: Viable counts of bacteria on cotton grown in Lubbock, Texas in 1986. **In:** Wakelyn PJ, Jacobs RR (Eds): Cotton Dust. *Proceedings of the Twelfth Cotton Dust Research Conference, Beltwide Cotton Research Conferences, Nashville, TN, January 6-7*, 1988, 38-39. National Cotton Council, Memphis, TN 1988.

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

18. Morey PR, Fischer JJ, Sasser PE: Endotoxin content and fiber properties of raw cotton. *Textile Res J* 1982, **52**, 524-528.

19. Neal PA, Schneiter R, Caminita BH: Report on acute illness among rural mattress makers using low grade cotton. *J Am Med Assoc* 1942, **119**, 1074-1082.

20. Olenchock SA, Castellan RM, Hankinson JL: Endotoxin contamination of cotton: area of growth/varieties. **In:** Wakelyn PJ, Jacobs RR (Eds): Cotton Dust. *Proceedings of the Eighth Cotton Dust Research Conference, Beltwide Cotton Production Research Conferences, Atlanta, GA, January 9-10, 1984*, 64-66. National Cotton Council of America, Memphis, TN 1984.

21. Perkins HH Jr: 1993. Strategies for processing honeydew contaminated cotton. **In:** Herber DJ, Richter DA (Eds): *1993 Proceedings Beltwide Cotton Conferences, New Orleans, LA, January 10-14, 1993*, Vol. 1, 1461-1462. National Cotton Council of America, Memphis, TN 1993.

22. Perkins HH Jr, Brushwood DE: Interlaboratory evaluation of the thermodetector cotton stickiness test method. **In**: Richter DA, Armour J. (Eds): *1995 Proceedings Beltwide Cotton Conferences, San Antonio, TX, January 4-7, 1995*, Vol. 2, 1189-1191. National Cotton Council of America, Memphis, TN 1995.

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

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

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

26. Rylander R, Haglind P, Lundholm M: Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. *Am Rev Respir Dis* 1985, **131,** 209-213.

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

28. Sasser M, Wichman MD: Identification of microorganisms through use of gas chromatography and high-performance liquid chromatography. **In:** Balows A, Hausler WJ Jr, Herrman KL, Isenberg HD, Shadomy HJ (Eds): *Manual of Clinical Microbiology, 5th Edition*, 111-118. American Society for Microbiology, Washington, D.C. 1991.

29. Simpson ME, Choper EA, Prickett TJ: A report on the identification of gram-negative bacteria on cotton fiber from two U.S. field locations. **In:** Wakelyn PJ, Jacobs RR (Eds): Cotton Dust. *Proceedings of the Twelfth Cotton Dust Research Conference, Beltwide Cotton Research Conferences, Nashville, TN, January 6-7, 1988*, 6-9. National Cotton Council, Memphis, TN 1988.

30. Simpson ME, Marsh PE: Exceptionally low bacterial counts common on raw cotton fiber from the San Joaquin Valley of California. **In:** Wakelyn PJ, Jacobs RR (Eds): Cotton Dust. *Proceedings of the Ninth Cotton Dust Research Conference, Beltwide Cotton Research Conferences, Nashville, TN, January 9-11, 1985*, 69-71. National Cotton Council, Memphis, TN 1985.

31. Wyatt BG, Heintz CE: Capsule-producing coryneform bacteria associated with stickiness in cotton. *Textile Res J* 1982, **52,** 518-523.

32. Zuberer DA, Kenerley CM: Seasonal dynamics of bacterial colonization of cotton fiber and effects of moisture on growth of bacteria within the cotton boll. *Appl Environ Microbiol* 1993, **59(4)**, 974-980.