BACTERIAL GENERA ASSOCIATED WITH THREE COTTON GROWING REGIONS OF THE COTTON BELT

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Abstract

A survey method is described for sampling and identifying bacterial genera associated with the seasonal population of bacteria on cotton grown in Florence and for different cotton growing regions. The method utilizes the MIDI Microbial Identification System (MIS) which uses whole cell fatty acid analysis by gas chromatography. A gram-index concept is introduced. Initial results support previous findings that California cottons generally contain low levels of endotoxin and gram-negative bacteria and that Mississippi cottons generally contain high levels of endotoxin and gram-negative bacteria. In California cottons, the bulk of gram-positive species consisted of *Bacillus* spp.; and in Mississippi and Texas region cottons, the bulk of gram-negative species was made up of *Pseudomonas* spp.

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. In addition when it became known that decreases in pulmonary function was highly correlated with endotoxin level (Castellan et. al., 1984 & 1987; Rylander et. al. 1985) and since endotoxin is a biological product of gram-negative bacteria, interest in the ecology of cotton bacteria has been intense.

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.

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Methods & Materials

Cotton

Three Region Study. Cottons used were all from the 1995 harvest year. The three cotton growing regions chosen were California, Texas, and Mississippi (Chun, 1989; Fischer & Jacobs, 1988; Olenchock et. al., 1984). 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 1-gm samples were used for testing. Dry weights of the composite samples were determined.

1995 Seasonal Cotton Study. Cotton was supplied by Dr. Oscar L. May III (Cotton Production Research, USDA, ARS, SAA, P.O. Box 3039, Florence, SC 29502-3039). 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 1-gm samples were removed for testing. Dry weight of the lint was determined.

Viable Microbial Count & Culturing Method for Bacterial Identification

Method for Bacterial Identification. Viable total bacterial populations were determined for each of the 1-gm samples from the Florence seasonal cotton and the cottons from the three growing regions as described in Chun & Perkins, Jr., 1991; except that instead of doing pour plates, spread plates were done 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 were used for the seasonal portion of the study. Version 3.9 of the Aerobic Method, and TSBA and CLIN aerobic package, which was released after the seasonal study was 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 regions: 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 were 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

Three Regions Study. Total bacterial population (Figure 1) was highest for cotton grown in Texas (3.4×10^6 cfu/gm, s.e. = 8.5×10^5 cfu/gm) but this difference in population was not significantly different from the bacterial population found in Mississippi (1.9×10^6 cfu/gm, s.e. = 2.1×10^5 cfu/gm). However, the populations found on California cottons (1.1×10^5 cfu/gm, s.e. = 4.9×10^4 cfu/gm) 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 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, 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 (Akinwunmi et. al., 1989; Borbon Reyes et. al., 1988; Fischer & Foarde, 1989; Simpson et. al., 1988). Of these 31 different genera categories, 14 genera made up less than 2% of the total species identified (Figure 2). The 'No Match' samples fell in this group. The number of species found in these genera were low; and chi-square testing did not suggest any unusual distribution of these genera to the growing regions.

On the other hand, using chi-square tests, genera containing 2% or more of the species identified did have a highly significant probability of being unevenly distributed between the three growing regions (Figure 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 3 regions ($P < 0.001$). When Texas and Mississippi were compared, the distribution was not significantly different. However, the 15 species of *Salmonella* 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 & 3], reflect changes due to the software library upgrade from version 3.8 and 3.9), also showed a significant distribution difference between the 3 regions ($P < 0.001$). The distribution between California and Mississippi (12 and 19, respectively) was not significant; but the 3 found in Texas was significantly lower than the distribution in California and Mississippi ($P = 0.0016$). The opposite was found with *Listeria* spp. Here, none were found in California and Mississippi; whereas 8 were found in Texas ($P = 0.000045$). *Kluyvera* spp. were also not uniformly distributed between the three regions: 8, 1 and 17 for California, Texas and Mississippi, respectively. The chi-square test between California and Mississippi was not significant ($P = 0.06$); but the chi-square for *Kluyvera* spp. between Texas and California and Mississippi was highly significant ($P = 0.001$). *Klebsiella* spp. also showed an uneven distribution between the three regions. The distribution was not significant between California (3) and Mississippi (6). However, the distribution was significant between Texas (0) and California and Mississippi ($P = 0.03$). *Flavimonas* spp. distribution was not significantly different between California and Mississippi, 3 and 2, respectively. However, the 15 found in the Texas cottons was significant when contrasted to California and Mississippi ($P = 0.000038$). The occurrence of 4 *Escherichia* spp. in the California lint vs. none being found in either the Texas or and Mississippi lints was significant ($P = 0.005$). The distribution of 7 *Citrobacter* spp. in the California region and none in Texas and Mississippi was highly significant ($P = 0.0002$). The occurrence of 17 *Cellulomonas* spp. in Texas and the absence of any *Cellulomonas* spp. in California and Mississippi was highly significant ($P < 0.001$).

Most of the above species 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 (Figure 3). *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). 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 significantly 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, respectively; $P < 0.002$). The difference in distribution of *Bacillus* spp. was also highly significant between Texas and California ($P < 0.001$). What I find interesting is that even though the percentage of *Bacillus* spp. in California is high, it is considerably lower than what was observed in a concurrent study involving 3 levels of sticky cottons from California (Chun & Perkins, Jr., 1996), where the percentage of *Bacillus* spp. averaged 67%. In this study 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 (non-sticky, moderately sticky and sticky cottons) was 0.9! In addition, no *Pseudomonas* spp. was observed on the cottons used in the sticky cotton study while in this study, *Pseudomonas* spp. made up 9.2% of the genera found in California. 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 (Table 1). 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 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 was 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. This thesis may focus future endeavor.

Seasonal Changes in Bacterial Genera Study. The spread plate method was used instead of the pour plate method for determining bacterial population resulting in greater variation as shown in Figures 1 & 4: September 18 (6.9×10^6 cfu/gm, s.e. = 3.6×10^6 cfu/gm), September 27 (3.3×10^7 cfu/gm, s.e. = 9.7×10^6 cfu/gm), October 9 (2.2×10^7 cfu/gm, s.e. = 8.2×10^6 cfu/gm) and October 23 (2.9×10^7 cfu/gm, s.e. = 1.2×10^7 cfu/gm). While this has obscured differences between the sampling dates, the general pattern of the seasonal bacterial population of Florence grown cotton (Figure 4) followed the same general pattern observed elsewhere (Borbon Reyes et. al., 1988; Fischer and Jacobs, 1988; Heintz et. al., 1988; Zuberer & Kenerley, 1993) 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/gm, s.e. = 3.6×10^6 cfu/gm), September 27 (3.3×10^7 cfu/gm, s.e. = 9.7×10^6 cfu/gm), October 9 (2.2×10^7 cfu/gm, s.e. = 8.2×10^6 cfu/gm) and October 23 (2.9×10^7 cfu/gm, s.e. = 1.2×10^7 cfu/gm), 1995, 117, 116, 120 and 120 species were identified, respectively. When sorted to genera, 26 different genera, including the 'No Match' category, were obtained. Of these,10 genera made up less than 2% of the total species identified (Figure 5). The 'No Match' category fell into the group containing more than 2% of the population. The number of species found in the genera

making up less than 2% of the total species group were too few to warrant chi-square testing.

The genera making up more than 2% of all the species identified are shown in Figure 6. No outstanding seasonal trends were observed. What stands out, however, was the high proportion of *Pseudomonas* spp. occurring throughout the growing season (Table 3). *Actinobacillus* spp. and *Flavimonas* spp. were the next most frequently occurring species and these two together paled in comparison to the occurrence 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 use in discriminate analysis to identify the origin of unknown cottons?

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. In California, the bulk of gram-positive species consisted of *Bacillus* spp.; and in Mississippi and Texas region cottons, the bulk of g ram-negative species was made up of *Pseudomonas* spp. 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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Table 1. Gram-index of cottons from three cotton growing regions

Cotton Growing	Raw Gram-Index[1]		Relative Gram-Index[2]	
Region	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

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

Table 2. Seasonal gram-Index of Florence grown cotton

Harvest	Raw Gram-Index[1]		Relative Gram-Index[2]	
Date,1995	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

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

Table 3. Average Number of Species Found in Each Genera Over the Season

Genera	Avg.No.[1]			
Pseudomonas	64.25	A		
Actinobacillus	9.75		B	
Flavimonas	8.50		B	
Enterobacter	5.75	C	B	D
Xanthomonas	5.25	C	B	D
No Match	4.25	C	B	D
Yersinia	4.00	C		D
Salmonella	2.50	C		D
Flavobacterium	2.25			D
Sphingobacterium	2.00			D
Escherichia	1.50			D
Staphylococcus	1.50			D
Cedecea	1.25			D
Bacillus	1.00			D
Clavibacter	0.75			D
Weeksella	0.75			D
Streptococcus	0.50			D
Klebsiella	0.50			D
Hydrogen	0.25			D
Aureobacterium	0.25			D
Rhodobacter	0.25			D
Rathayibacter	0.25			D
Morganella	0.25			D
Microbacterium	0.25			D
Kurthia	0.25			D
Gluconobacter	0.25			D

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

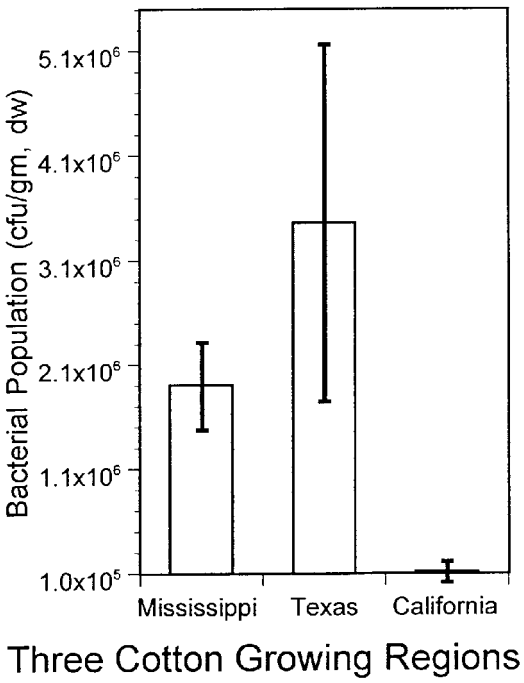

Figure 1. Average total bacterial population found in three major cotton growing regions (cfu/gm, corrected for dry weight; each half bar represents 2 s.e.).

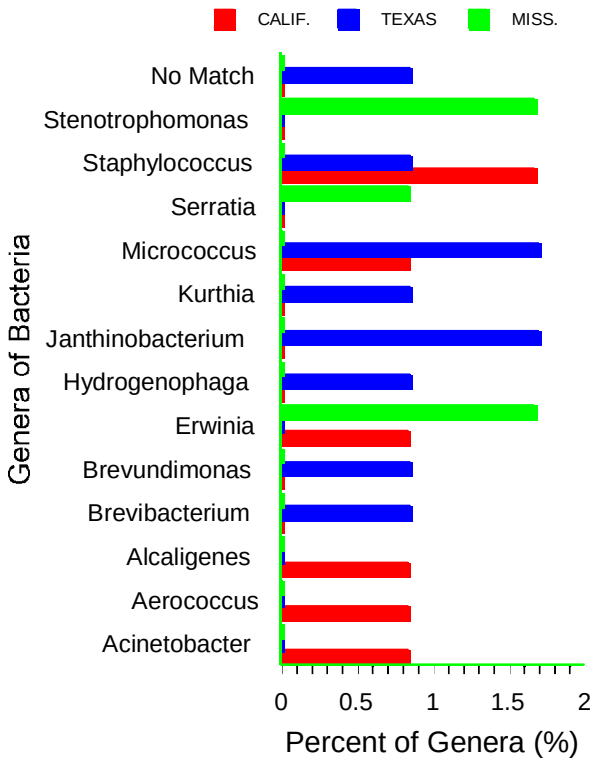

Figure 2. The genera of bacteria found in California, Texas and Mississippi growing regions making up less than 2% of the total number identified.

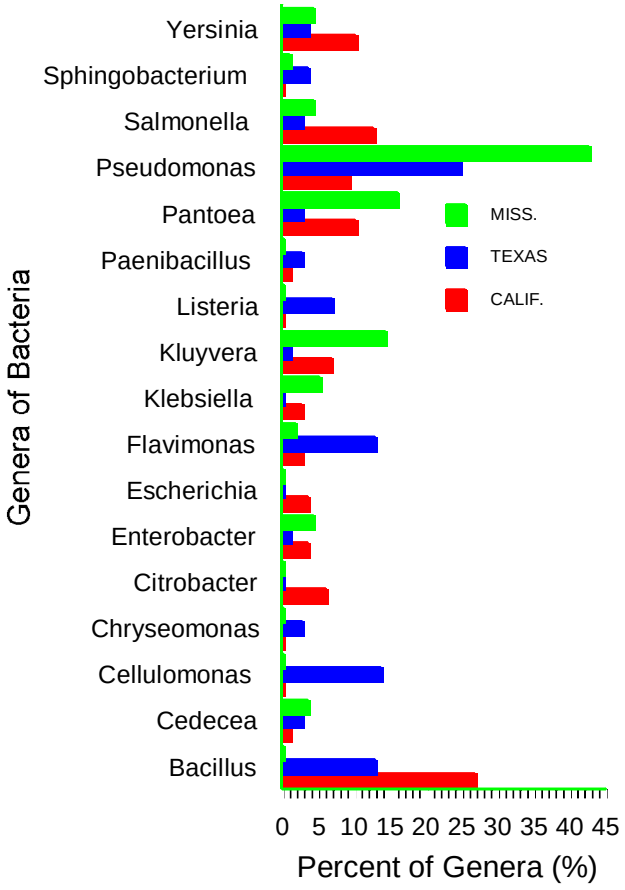

Figure 3. The genera of bacteria found in California, Texas and Mississippi growing regions making up 2% or more of the total number of species identified.

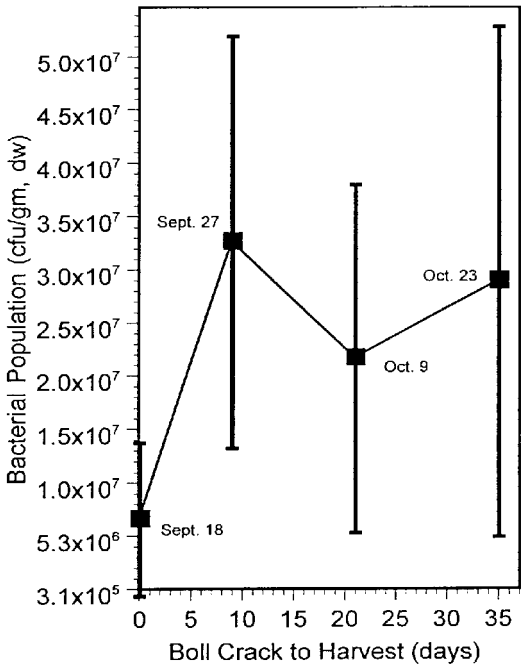

Figure 4. Average seasonal total bacterial population on Florence (South Carolina) grown cotton (cfu/gm, corrected for dry weight; each half bar represents 2 s.e.).

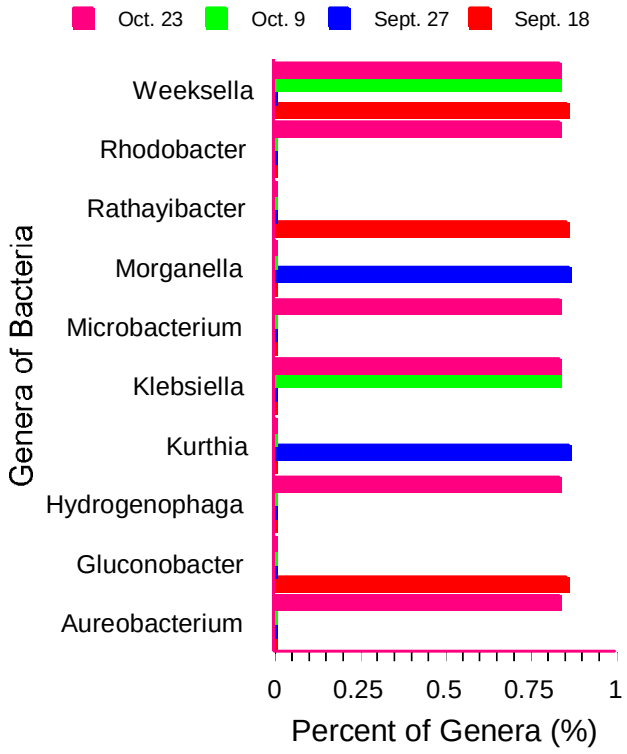

Figure 5. The seasonal genera of bacteria found on Florence, South Carolina, cotton from the time of boll crack to harvest. These genera make up less than 2% of the total number identified.

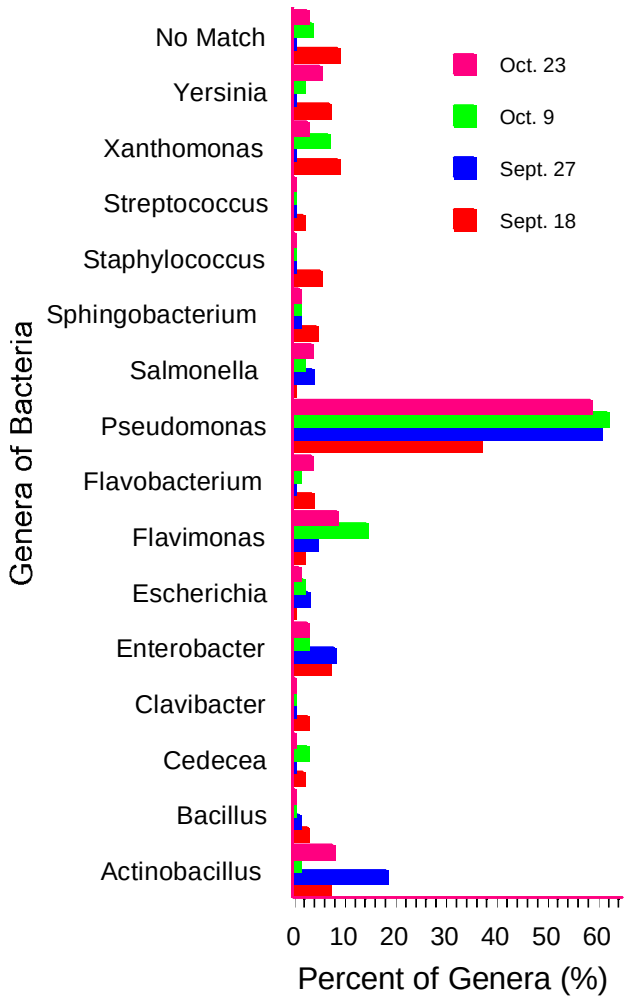

Figure 6. The seasonal genera of bacteria found on Florence, South Carolina, cotton from the time of boll crack to harvest. These genera make up more than 2% of the total number identified.