The composition of bacterial populations in soil fractions differing in their degree of adherence to barley roots

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Received 27 October 1998; received in revised form 9 February 1999; accepted 12 February 1999

Abstract

Soil fractions differing in their degree of adherence to barley roots were sampled, and their microbial populations were cultivated on a nutrient-poor bacterial medium at low temperature. The biomass of each population was harvested and analysed to determine its FAME pattern. The greater the degree of adherence of the soil sample to roots, the higher were the relative proportions of the fatty acids 15:0, 15:0 Iso, 15:0 Iso 3OH, 15:1 Iso, 17:0 Iso 3OH and 17:1 Iso. This trend was both pronounced and consistent. The opposite relationship was found between relative proportions of the fatty acids 10:0 3OH, 12:0, 12:0 2OH, 12:0 3OH, 16:0 and 18:1 and degree of root adherence. Differences in the FAME patterns can best be interpreted as differences in the relative proportions of the various types of groups constituting the bacterial populations. Specifically, as the degree of soil adherence to roots increased, there was a shift in favour of bacteria belonging to the Cytophaga–Flavobacterium group and a corresponding decrease in bacteria with FAME patterns similar to that of Pseudomonas. The distribution of the fatty acid 15:0 Anteiso indicated that the relative frequencies of certain Gram-positive bacterial strains with a high content of this acid were diminished in habitats close to roots. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Rhizoplane; Rhizosol; FAME; Cytophaga; Flavobacterium; Pseudomonas

1. Introduction

Plant roots, their surfaces and soil in their immediate vicinity constitute dynamic habitats densely colonised by soil-borne microbiota. The high microbial activity in these habitats is due to a flow of organic substances from the photosynthetic parts of the plants into the roots. This flow consists of low molecular weight organic substances (e.g. sugars, fatty acids and amino acids), as well as more complex substances (e.g. starch, cellulose and lipids). The chemical composition of this organic matter (the rhizodeposition) varies between plant species and growth stages and is affected by plant growth conditions (Curl and True-love, 1986).

This variation in the composition of rhizodeposition influences the composition of bacterial populations in the rhizosphere. There are, however, some consistent differences between the populations in the rhizosphere on the one hand and those in bulk soil on the other. Investigations have shown that Gram-negative, non-sporulating, rod-shaped bacteria dominate in the rhizosphere, with Pseudomonas spp being especially
frequent. The chemical diversity in the rhizosphere matches the metabolic diversity within this genus (Curl and Truelove, 1986; Vancura, 1988).

In studies aimed at characterising the rhizobacterial flora, one sound approach is to compare the compositions of populations in samples of soil (i.e. habitat) differing in their degree of adherence to roots. Only if the relative frequency of a given bacterial group is higher in the proximity of roots, would it make sense to categorise the group as a root colonising one, that is, rhizobacteria.

However, describing the composition of microbial populations can be tedious, especially when relying on approaches where the individual members are classified one by one. Thus it would be advantageous to find sensitive and reproducible methods for characterising whole populations as single units. To a certain degree, analyses of fatty acid composition may be useful for such investigations (Haack et al., 1994).

In an accompanying study on bacteria colonising barley roots, 1188 bacterial isolates were clustered with regard to their fatty acid content (Olsson et al., 1999). It was found that the rhizobacterial populations consisted mainly of two distinct groups. In one Pseudomonas was the dominating genus, and in the other there was a high frequency of bacteria belonging to the Cytophaga and Flavobacterium genera. Only a few isolates of Gram-positive bacteria were registered. These two main bacterial groups have very different fatty acid profiles: The lipid content among the Cytophaga/Flavobacterium group is dominated by acids with chains 15 or 17 carbons long (Bernardet et al., 1996), whereas the Pseudomonas spp. normally have even-numbered chains with 10, 12, 16 or 18 carbon atoms. In addition, the Gram-positives are mainly characterised by high contents of the fatty acid 15 : 0 Anteiso. Since these bacteria have such contrasting profiles, it should be possible to determine the relative proportions of these three groups in a given bacterial population by analysing the proportional distribution of fatty acids. Even small differences in composition should be detectable in this way.

Thus, the aim of the present study was to compare the fatty acid profiles of microbial populations from soil habitats differing in their degree of adherence to roots. Special emphasis was placed on investigating whether differences in population profiles could be interpreted as differences in relative frequencies of the bacterial groups that constitute the populations.

2. Material and methods

The experimental work was carried out in five steps: (1) Soil samples were collected from various experimental fields; (2) the samples were used for cultivating a bait crop under standardised conditions; (3) roots of the bait crop and adhering soil were harvested and treated in order to separate the microbial habitats into fractions with regard to how closely they adhered to the roots; (4) these fractions of soil and/or roots were mixed with water and spread on agar plates with a diluted bacterial growth medium; (5) after incubation, the microbial populations on the plates were sampled, and their fatty acids were extracted and analysed in a gas chromatograph.

2.1. Fields, soil samples and bait crop

In autumn 1997, soil samples were collected from six experimental fields in the southern and middle parts of Sweden. Two of the fields, i.e. Ultuna (R4-007-1) and Säby (R4-1103-5), are situated near Uppsala in the middle part of Sweden. They are part of long-term crop rotation experiments run by the Department of Crop Production Science at the Swedish University of Agricultural Sciences. Three fields, situated in Skåne, are part of a long-term experiment focused on conventional and ecological cropping systems (L4-3410). These fields are named Ljungby (HL1162), Tomelilla (HL1163) and Önnestad (HL1164) and are managed by the Kristianstad Agricultural Society (Anita Gunnarson, Box 9084, 291 09 Kristianstad). In one experiment at Petersborg (L3-0014), the effects of sewage sludge applications on soil are being assessed. The latter experiment is being run by the Malmöhus County Agricultural Society (Borgeby, 237 91 Bjärred). Soil characteristics and pre-crops in the experimental fields are shown in Table 1.

Soil samples were taken with an auger from the upper 20 cm of the soil. Each sample consisted of 6–8 sub-samples that had been taken from a small square (20 × 20 cm²) in the field and then combined. The samples were stored dark and cold (2°C) in plastic
bags prior to use. The storage period ranged from 1 week to 5 months. Soil used for experiments was potted in bags made of thin polyethylene tubes (43 cm high and with a diameter of 3.2 cm), and 10 seeds of barley (cvs. Golf or Alexis) were sown as a bait crop in each tube. Small holes were made to allow air exchange in the lower parts of the tubes. The soil tubes with the barley bait crop were placed in a climate chamber with a 12 : 12 h light/dark cycle and a temperature of 9°C. This temperature was chosen because the average soil temperature 5 cm below the surface is approx. 9–10°C during the first weeks after sowing in the central part of Sweden (Rodskjer and Tuvesson, 1976). The bait crop was cultivated for 4–5 weeks before being harvested. At that time the plants had roots that were 30–40 cm deep.

2.2. Sampled habitats and incubation conditions

The soil and roots of the bait crop were harvested by cutting off the plastic tubes at a distance of 5 and 15 cm below the planted seeds. Within this interval the soil rhizosoil roots were separated into various fractions, chosen to reflect an ordered sequence of microbial habitats that differ in their degree of adherence to the roots. It was found that four such fractions could be experimentally handled in a sufficiently rigorous way. The fractions were separated as follows:

- **B-soil** is the bulk soil, which is assumed not to have been in contact with roots of the bait crop; it easily falls off when roots are sampled. Suspensions of B-soil were prepared by mixing 3 g soil (fresh weight) with 30 ml of water.

- **Rh-soil** is the soil that sticks to the roots at normal soil moisture contents (16–23% in these experiments) but which readily loosens when the roots are dipped into or gently shaken with water. The Rh-soil suspensions were prepared by dipping the barley roots into 20 ml of water. The dry weight of soil in these suspensions amounted to approx. 0.1 g/ml.

- **Rh-plane** is the soil/material still adhering to roots that have been dipped in water but which loosens when the roots are stored (9°C) in water for at least 1 h and then vigorously vibrated with a Vortex for 1 min. The Rh-plane suspensions were prepared in 20 ml of water, and amounts of dry soil were normally less than 0.01 g/ml.

- **In-root** is what is left in and on the roots once they have been treated to remove the Rh-plane fraction. These suspensions were prepared by macerating the seemingly soil-free barley roots in 10 ml of water.

In three experiments, TYA medium was also used (2 g l⁻¹ Tryptone, 2.5 g l⁻¹ Yeast extract and 0.2 g l⁻¹ Sodium acetate). Some of the samples were picked out and diluted to estimate the CFU of the original suspensions.

Moreover, in some of the experiments, the effects of inoculum density were tested. The original suspensions were diluted 10-fold once or twice, and all samples representing all dilution levels were plated and harvested. In one experiment the suspensions were spread on replicate plates, which were harvested 5 and 6 days after plating.

To standardise the harvesting of the microbial populations, the agar plates were rotated in a bacterial spreader, whereupon their surfaces were swept with a plastic loop. In this way, approx. 150 μg, fresh weight, of microbial biomass was collected from each plate.

2.3. FAME analysis

The fatty acid methyl esters (FAMEs) of the sampled microbial populations were extracted as

<table>
<thead>
<tr>
<th>Field</th>
<th>Texture</th>
<th>pH</th>
<th>Pre-crops in sampled plots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Önnestad</td>
<td>Sandy loam</td>
<td>7.0</td>
<td>Winter rape, ley</td>
</tr>
<tr>
<td>Säby</td>
<td>Silt loam</td>
<td>6.1</td>
<td>Fallow, ley, winter turnip rape</td>
</tr>
<tr>
<td>Tomelilla</td>
<td>Silt loam</td>
<td>6.5</td>
<td>Winter rape, ley</td>
</tr>
<tr>
<td>Ultuna</td>
<td>Clay</td>
<td>6.2</td>
<td>Fallow</td>
</tr>
<tr>
<td>Petersborg</td>
<td>Silt loam</td>
<td>6.8</td>
<td>Winter wheat</td>
</tr>
<tr>
<td>Ljungby</td>
<td>Sandy loam</td>
<td>6.0</td>
<td>Winter rape, winter rye, potatoes</td>
</tr>
</tbody>
</table>
described by Sasser (1990). FAMEs were separated on a Hewlett Packard 5890 series II gas chromatograph, with a 25 m × 0.2 mm methyl silicone-fused silica capillary column, using hydrogen as carrier gas. Individual FAMEs were identified using the peak-naming table component of the Microbial Identification System (MIS, Microbial ID, US). As some new peaks appeared that could not be identified by MIS, the calculations to quantify the relative amounts were slightly modified. The new peaks were given provisional names, referring to their equivalent chain length (ECL), and these areas were also included when calculating the total named FAME peak area. The relative quantities of individual FAME peaks were expressed as percentages of the thus-defined total named FAME peak area.

2.4. Reference isolates

In a previous study, the FAME profiles of 1188 bacterial isolates had been analysed after being incubated for 24 h on TSA plates with 20 TSB per litre at 24°C (Olsson et al., 1999). To use these data for interpreting the FAME profiles of the microbial populations, representative isolates from that study were chosen to be incubated under the same conditions (e.g. on TSA plates with 5 TSB per litre and incubated for 6 days at 8°C) and analysed in the same way as the soil populations in the present study.

These isolates were chosen to represent the main clusters of FAME profiles and also included isolates that had been analysed after incubation according to the MIS standard procedure (30 g TSB and incubated at 28°C). They consisted of two Gram-positive bacteria, and eight isolates from each of the genera Cytophaga and Pseudomonas.

2.5. Experimental design and statistical analysis

The combination of fractions sampled in each field, numbers of soil samples collected, and other treatments are summarised in Table 2. In total, 325 microbial populations incubated on agar plates were harvested and analysed for their fatty acid composition.

The variables in the statistical analysis were the relative quantities of individual FAME peaks for each population expressed as percentages of the total named FAME peak areas. The analysis of the results was performed in six consecutive steps:

(a) the fatty acids were ranked according to their amount in the bacterial populations,
(b) the variation among populations in their fatty acid composition was summarised by a principle component analysis of the dominating fatty acids,
(c) the correlations between the principal components and the original variables were investigated and new variables defined,
(d) based on these new variables, the fatty acid profiles of hypothetical bacterial groups were defined,
(e) the fatty acid profiles of the hypothetical groups were compared with those of known bacterial groups,
(f) the relative proportions of bacteria from the hypothetical groups was estimated in the different soil habitats.

Table 2
The experimental design. SA indicates the combination sampled and analysed

<table>
<thead>
<tr>
<th>Field</th>
<th>No. of soil samples</th>
<th>Sampled populations</th>
<th>Additional treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B-soil</td>
<td>Rh-soil</td>
</tr>
<tr>
<td>Önnestad</td>
<td>16</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>Säby</td>
<td>16</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>Tomelilla</td>
<td>9</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>Ultna</td>
<td>10</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>Petersborg</td>
<td>10</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>Ljungby</td>
<td>5</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>Säby</td>
<td>3</td>
<td>SA</td>
<td>SA</td>
</tr>
</tbody>
</table>

*a These habitats represent an ordered sequence with regard to their degree of adherence to barley roots. The order is B-soil < Rh-soil < Rh-plane < In-root, where ‘B-soil’ stands for bulk soil and is the habitat furthest from the roots. (See also text for a more precise definition.)
3. Results

By the time that the populations were harvested, after 5–6 days of incubation, both bacteria and fungi had grown out on the plates. As a rule, fungal and bacterial colonies covered the whole surface of each plate, and bacteria strongly dominated the harvested biomass.

3.1. FAME profiles of the incubated microbial populations

In the analysis of the lipid contents of the microbial populations, more than 80 distinct fatty acids were detected. The 15 most dominant acids accounted for more than 90% of the fatty acid content of 75% of the populations; thus the first statistical analysis was restricted to these 15 acids.

A principal component analysis of these acids showed that the first four components represented 66, 14, 5 and 3% respectively, of the total variation among populations. The correlations among the 15 dominating fatty acids and the first two principal components are shown in Table 3. The table demonstrates that the main factor dividing the bacterial populations was the proportion of fatty acids with even or odd numbers of carbon atoms in the chains. A simple regression model revealed that the first component was highly correlated with the ordered sequence of habitats from which the microbial populations had been taken (Pearson correlation coefficient, 0.43; \( p \)-value < 10\(^{-13} \)). To facilitate the interpretation of this relationship in terms of bacterial genera the following two variables were therefore defined:

\[
F_{a_{\text{odd}}} = 15 : 0 + 15 : 0 \text{ Anteiso} + 15 : 0 \text{ Iso} \\
+ 15 : 1 \text{ Iso G} + 17 : 1 \text{ Iso} + 17 : 0 \text{ Iso 3OH} \\
+ 15 : 0 \text{ Iso 3OH}
\]

and

\[
F_{a_{\text{even}}} = 12 : 0 \text{ 3OH} + 10 : 0 \text{ 3OH} + 12 : 0 \text{ 2OH} \\
+ 18 : 1 + 16 : 0 + 12 : 0
\]

The acids 16 : 1 and 15 : 0 Iso 2OH could not be separated with our equipment and were therefore excluded from the analysis. The acid 17 : 0 CYCLO is also excluded as it deviated from the overall pattern.

As shown in Table 4, the higher the degree of adherence to roots of the soil samples analysed, the higher was the content of \( F_{a_{\text{odd}}} \) and the lower was the content of \( F_{a_{\text{even}}} \). This result was consistent for both types of bacterial substrates used for incubation and for all tested soils. When replicated plates from the same population were harvested after being incubated for 5 or 6 days, there was good agreement between the replicates in their content of \( F_{a_{\text{odd}}} \) and \( F_{a_{\text{even}}} \) respectively (Pearson correlation coefficient, 0.95; \( p \)-value < 10\(^{-14} \) for both variables).

A closer examination of the relationships between the acids making up \( F_{a_{\text{odd}}} \) indicates that 15 : 0 Anteiso showed a deviating pattern. More specifically, when the seven variables

\[
O_{15:0 \text{Anteiso}} = 15 : 0 \text{ Anteiso}/F_{a_{\text{odd}}}, \text{ etc.},
\]

were defined and analysed, it was found that \( O_{15:0 \text{ Anteiso}} \) was negatively correlated to five of the other variables (\( O_{15:0 \text{ Iso}} \), \( O_{15:1 \text{ Iso G}} \), \( O_{17:1 \text{ Iso}} \), \( O_{15:0 \text{ Iso 3OH}} \) and \( O_{17:0 \text{ Iso 3OH}} \); coefficients varying between –0.4 and –0.6; \( p \)-values < 10\(^{-6} \) for all individual correlations), while four of them were positively correlated with each other (\( O_{15:1 \text{ Iso G}} \), \( O_{17:1 \text{ Iso}} \), \( O_{17:0 \text{ Iso 3OH}} \) and \( O_{15:0 \text{ Iso 3OH}} \); coefficients between 0.3 and 0.9; \( p \)-values < 10\(^{-6} \) for all individual correlations).

In view of these findings, the proportions of odd-numbered fatty acids in different populations were
further analysed. Results from this analysis, including eight fatty acids, are presented in Fig. 1. It is obvious from the figure that 15:0 Anteiso constitutes a higher proportion of the odd-numbered acids in populations originating from habitats more distant from roots. A regression model revealed that the variable \( O_{15:0} \) Anteiso was correlated with the ordered sequence of habitats (Pearson correlation coefficient, 0.59; \( p \)-value < 10\(^{-14} \)). The even-numbered fatty acids used to calculate the sum \( F_{\text{even}} \) were analysed in the same way as the odd-numbered ones, but no consistent patterns were found with regard to degree of adherence.

### 3.2. Effect of initial inoculum density of the plated populations on FAME profiles

In most of the tests, the initial inoculum density of the plated populations varied between 10\(^5\) and 10\(^6\) CFU per plate. Some experiments were performed in order to elucidate the influence of inoculum density. These experiments did not indicate any consistent effect of this factor on the variables \( F_{\text{odd}} \) and \( F_{\text{even}} \). When the initial inoculum was diluted hundred-fold from approximately 10\(^5\) to 10\(^3\) CFU per plate, there was a tendency for the content of \( F_{\text{odd}} \) to decrease in populations from B-soil habitat. The same dilution of inoculum from the Rh-plane habitats, on the other hand, tended to cause an increase in the \( F_{\text{odd}} \) content of these populations. Neither of these changes were statistically significant and they did not interfere with the main pattern.

### 3.3. Composition of the microbial populations

The incubation conditions did not substantially affect the proportions of odd- and even-numbered fatty acids in the reference bacterial isolates (Table 5). Moreover, a striking similarity was found between the profiles of Cytophaga sp. cultivated at 8°C (Fig. 2) and those of the microbial populations originating from root-attached habitats (Fig. 1).

**Table 4**

<table>
<thead>
<tr>
<th>Comparison between populations from</th>
<th>( F_{\text{odd}}^a )</th>
<th>( p)-value(^d)</th>
<th>( F_{\text{even}}^b )</th>
<th>( p)-value(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-soil and Rh-soil</td>
<td>4 and 7</td>
<td>0.048</td>
<td>49 and 47</td>
<td>0.24</td>
</tr>
<tr>
<td>B-soil and Rh-plane</td>
<td>3 and 12</td>
<td>0.035</td>
<td>50 and 43</td>
<td>0.004</td>
</tr>
<tr>
<td>Rh-soil and Rh-plane</td>
<td>11 and 18</td>
<td>&lt;0.0001</td>
<td>43 and 37</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Rh-soil and In-root</td>
<td>6 and 15</td>
<td>0.0002</td>
<td>48 and 40</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\( a F_{\text{odd}} = 15:0 + 15:0 \) Anteiso + 15:0 Iso + 15:1 Iso G + 17:1 Iso + 17:0 Iso 3OH + 15:0 Iso 3OH.

\( b F_{\text{even}} = 12:0 \) 3OH + 10:0 3OH + 12:0 2OH + 18:1 + 16:0 + 12:0.

\( c \) Samples represent soil habitats differing with regard to their degree of adherence to barley roots, in the following order: B-soil < Rh-soil < Rh-plane < In-root, where B-soil is the habitat most detached from roots. (See text for a more precise definition.)

\( d \) \( p\)-values calculated using a sign test for paired samples.

Fig. 1. Proportions of 8 fatty acids in microbial populations from soil habitats differing in their degree of adherence to barley roots. The ordering of habitats, form strongest to weakest adherence, is as follows: In-root > Rh-plane > Rh-soil > B-soil where 'B-soil' stands for 'bulk soil'. (cf. Fig. 2).
Since the FAME profiles were largely unaffected with regard to \( Fa_{odd} \) and \( Fa_{even} \) by the incubation conditions, more information could be gleaned from a database including profiles of 1188 individual bacterial strains obtained in a former study (Olsson et al., 1999). This database was searched for strains fulfilling the following criteria:

(X) \( (Fa_{odd} < Fa_{even}) \) and \( (Fa_{even} > 40\% ) \). Based on the present findings (Table 4), it was predicted that isolates fulfilling these conditions should dominate in most populations but tend to be less common in habitats with intimate contact with roots.

(Y) \( (Fa_{odd} > Fa_{even}) \) and \( [(17:1 \text{ Iso} + 15:1 \text{ Iso G} + 17:0 \text{ Iso} + 17:0 \text{ Iso }3\text{OH} + 15:0 \text{ Iso }3\text{OH})] > 15:0 \text{ Anteiso]. Proportions of such isolates should increase with the level of root contact (Table 4 and Fig. 1).

(Z) \( (Fa_{odd} > Fa_{even}) \) and \( [(17:1 \text{ Iso} + 15:1 \text{ Iso G} + 17:0 \text{ Iso} + 17:0 \text{ Iso }3\text{OH} + 17:1 \text{ w6c} + 15:1 \text{ w6c}) > 15:0 \text{ Anteiso]. This third set of conditions should catch a marginal group of isolates that get even rarer as the root surface is approached (Fig. 1).

The bacterial groups defined in Olsson et al. (1999) (Groups A, B and C), almost completely correspond with the groups defined by the criteria X, Y and Z (Table 6). In Group A (or X) there were 400 identified isolates, 366 of which were *Pseudomonas*. In Group B

### Table 5

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>( Fa_{odd} )^a</th>
<th>( Fa_{even} )^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+8°C^c</td>
<td>+24°C^d</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Cytophaga</em> spp.</td>
<td>59.0</td>
<td>68.8</td>
</tr>
<tr>
<td>Gram-positives</td>
<td>63.8</td>
<td>76.9</td>
</tr>
</tbody>
</table>

^a\( Fa_{odd} = 15:0 + 15:0 \text{ Anteiso} + 15:1 \text{ Iso} + 15:1 \text{ Iso G} + 17:1 \text{ Iso} + 17:0 \text{ Iso }3\text{OH} + 15:0 \text{ Iso }3\text{OH}.\)

^b\( Fa_{even} = 12:0 \text{ 3OH} + 10:0 \text{ 3OH} + 12:0 \text{ 2OH} + 18:1 + 16:0 + 12:0.\)

^c Incubated at +8°C for 6 days on agar medium with 5 g TSB per litre.

^d Incubated at +24°C for 1 day on agar medium with 20 g TSB per litre.

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![Fig. 2](image-url) **Fig. 2.** Relative contents of 8 fatty acids in 8 *Cytophaga* and 2 Gram-positive isolates incubated under different conditions. At 8°C bacteria were incubated for 6 days on agar medium with 5 g TSB per litre; at 24°C for 1 day on agar medium with 20 g TSB per litre (cf. Fig. 1).
(or Y) the genus *Cytophaga* is dominant (213 out of 234 isolates), and Gram-positive bacteria are found in Group C (or Z).

### 3.4. Estimating the relative proportions

The aim of the following calculation is to roughly predict the compositions of soil-borne microbial populations from habitats differing in their degree of contact with roots.

First, let us assume that the collected and analysed microbial biomass consisted mainly of a mixture of bacterial isolates belonging to Groups X or Y, as defined above; and let us, for a moment, ignore the possibility that there could be differences among bacterial strains with regard to the amount of extracellular fatty acid per gram fresh weight (Haack et al., 1994). Average contents of $F_{\text{a even}}$ and $F_{\text{a odd}}$ in bacterial strains in Group X were found to be 60 and 0%, respectively, while the corresponding values for Groups Y were 2 and 69%. These figures are from the database compiled on the basis of bacteria incubated at 24°C for 24 h. Assuming that, on average, the performance of these isolates under the cooler incubation conditions (8°C for 6 days) used in the present experiment would be similar to that of tested isolates, it can be estimated that their contents should be about 56 and 0% and 1 and 59%, respectively, under the latter conditions (Table 5). Thus, the sum of $F_{\text{a even}}$ and $F_{\text{a odd}}$ should range between 56 and 59% for populations consisting exclusively of bacteria belonging to these two groups. Thus, the assumption that the major part of the microbial biomass of the incubated populations consists of bacteria belonging to Groups X and Y seems to be valid.

To estimate the proportions of the total biomass accounted for by Groups X and Y, we used the following expression:

$$\text{Group X}/\text{Group Y} = (F_{\text{a even}}/0.55)/(F_{\text{a odd}}/0.6).$$

Distributions of ratio values for the separate habitats are plotted in Fig. 3. These ratios were assumed to reflect the ratio of Group X (mainly *Pseudomonas* spp.) to Group Y (mainly *Cytophaga* spp.) bacteria in an incubated bacterial mixture consisting exclusively of these two groups. It is obvious that the proportion of *Cytophaga* spp. increases with the level of barley root contact of the sampled population.

### 4. Discussion

We analysed the fatty acid composition of the biomass of incubated microbial populations originating from soil samples with various degrees of adherence to barley roots. As the degree of contact/adherence increased, there was a clear and consistent increase in the relative proportions of the acids 15 : 0, 15 : 0 Iso, 15 : 1 Iso G, 17 : 1 Iso, 15 : 0 Iso 3OH and 17 : 0 Iso 3OH and a corresponding decrease in the proportions of 10 : 0 3OH, 12 : 0, 12 : 0 2OH, 12 : 0 3OH, 16 : 0 and 18 : 1. These trends were highly significant even when tested by conservative statistical models. They were consistent for all types of soil sampled. The differences among populations in their relative contents of these odd-numbered and even-numbered fatty acids can best be interpreted as differences in their composition with regard to certain bacterial groups. Specifically, a high relative content

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Table 6

<table>
<thead>
<tr>
<th>Grouping criteria based on individual bacterial isolates</th>
<th>Grouping criteria based on bacterial populations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Criteria X</td>
<td>Criteria Y</td>
</tr>
<tr>
<td>Group A</td>
<td>642</td>
</tr>
<tr>
<td>Group B</td>
<td>0</td>
</tr>
<tr>
<td>Group C</td>
<td>0</td>
</tr>
</tbody>
</table>

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of odd-numbered fatty acids indicates that a high proportion of the microbial biomass is made up of bacteria belonging to the genus *Cytophaga*. A high relative content of even-numbered acids, on the other hand, is indicative of a high proportion of *Pseudomonas* spp. A rough estimate of the plated populations showed that the ratio of *Pseudomonas* biomass to *Cytophaga* biomass averages 10 : 1 in the rhizosoil habitats (Rh-soil) and 5 : 1 in the rhizoplane (Rh-plane) habitats.

To avoid controversies concerning bacterial systematic in the following discussion it is better to use the broader concept ‘*Cytophaga*–*Flavobacterium* group’ instead of *Cytophaga*. There seems to be a consensus among taxonomists that the genera *Cytophaga*, *Flavobacterium* and *Flexibacter* form a distinct phylogenetic group, separated from Purple bacteria (Prescott et al., 1996; Marguils and Schwartz, 1998). Moreover, it has been demonstrated that lipids within the *Cytophaga*–*Flavobacterium* group are mainly composed of odd-numbered fatty acids (Bernardet et al., 1996). The $F_{\text{odd}}$ contents in the *Cytophaga*, *Flavobacterium* and *Flexibacter* isolates in their study varied between 41 and 89%, with a mean of 63%. When 15 : 0 Anteiso was excluded from $F_{\text{odd}}$ the average content was still 60% (for the incubation conditions in their study: 48 h on modified Shieh agar at +25°C). It has, however, been found that the main features of fatty acid profiles characterising the genus are not affected by growth conditions (Haack et al., 1994), and our results confirm that conclusion. Thus it can be safely stated that the high contents of the above-mentioned odd-numbered fatty acids are indicative of high proportions of bacteria belonging to the *Cytophaga*–*Flavobacterium* group.

This estimate is based on extractable fatty acids of the biomass of incubated populations. Although we would have liked to have been able to calculate the proportions of viable units in the original sampled populations directly on the basis of these data, this was not possible. As already mentioned, the yield of extractable fatty acids per amount of biomass differs among bacterial strains (Haack et al., 1994). Moreover, the relative amount of biomass harvested from a certain bacterial strain is a function both of the relative numbers of viable units and the various growth rates of the cells present in that inoculum. In addition, growth rates differ depending on the stage of incubation, the relative duration of which depends on the amount of initial inoculum. Lower concentrations will result in

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Fig. 3. Composition, estimated as the ratio of Group X to Group Y bacteria, of microbial biomass of populations form the B-soil (furthest away from roots), Rh-soil, Rh-plane and In-root (greatest adherence to roots) fractions. Groups X and Y are mainly dominated by the genera *Pseudomonas* and *Cytophaga*, respectively. A precise definition of the different fractions and the methods used to calculate the Group X/Group Y ratio is presented in text.

longer periods during which individual inoculum units can grow in the absence of competition from other units; thus dilution may—or may not—change the final composition of the biomass. It was too complicated to take all of these effects into account in calculations/calibrations; therefore no effort was made to estimate the composition of viable units in the initial inoculum.

Nevertheless, with this approach, valid and interpretable results can be obtained when comparing populations inoculated with approximately the same amount of cultivable units. The general conclusion, that is, that the proportion of bacteria belonging to the Cytophaga–Flavobacterium group in a soil sample increases with the degree of contact with barley roots, is still valid even if it is not possible to quantify these proportions in terms of viable units.

The spatial distribution of the acid 15 : 0 Anteiso indicated that the frequency of Gram-positive bacterial strains, which generally have a high proportion of this acid, diminished in habitats close to roots. In earlier studies it has been shown that Gram-positives, in general, do not frequently colonise roots (Curl and Truelove, 1986), thus helping to confirm the validity of using fatty acid data for comparing the composition of microbial populations.

Four different microbial habitats were investigated in this study. They can be combined into two comparisons of biological importance. First, one can compare the bulk soil with the root-associated habitats, which differ distinctly in terms of their distance from roots. The fact that the proportion of Cytophaga–Flavobacterium was higher close to roots than in bulk soil indicates that these bacteria are well-adapted for colonising roots. This finding is in accordance with the results of Mawdsley and Burns (1994a). Since these bacteria feed on decomposing macromolecules (Christensen, 1977), they can meet their nutritional requirements in the vicinity of roots without having to compete with Pseudomonas spp., which mainly utilise small molecular substances.

The other comparison of importance is that between the rhizosol and rhizoplane. In this case the distinction between the habitats is more fluent. It is not possible to exactly determine where the rhizosol ends and the rhizoplane begins. Nevertheless, our results show very clearly that the proportion of bacteria belonging to the Cytophaga–Flavobacterium group was higher in the rhizoplane than in the rhizosol. This difference can probably be attributed to the fact that most strains in this group produce extracellular slime (Christensen, 1977; Prescott et al., 1996) which has been shown to have a strong binding capacity (Watt et al., 1993). This slime could thus allow Cytophaga–Flavobacterium sp. to bind more strongly to the root surface compared with Pseudomonas sp.

Based on the results of these comparisons, we suggest that bacteria in the Cytophaga–Flavobacterium group are a more important component of the rhizosphere than previously assumed. It has been shown that inoculation with Flavobacterium sp. alters enzyme activities in the rhizosphere (Mawdsley and Burns, 1994c). It is realistic to assume that the mucilage produced could facilitate contact between roots and soil and thus enhance water uptake by plants. Since the mechanisms controlling water relations in terrestrial plants are ancient in evolutionary terms, one of these mechanisms could be controlling the rhizobacterial flora. Mawdsley and Burns (1994b) found that the survival of a Flavobacterium strain on wheat roots was better under dry soil conditions. Was this effect to some degree caused by the wheat plants?

With the method described in this study, it is rather easy to detect differences between populations with regard to their relative content of Cytophaga–Flavobacterium. Thus, it should be possible to clarify the ecological importance of this group in the root zone.

Acknowledgements

Financial support was provided by the Swedish Farmers Foundation for Agricultural Research. We thank Dr J. Yuen for valuable comments about the statistical analysis and Dr S. Ahlström for critical reading of the manuscript.

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