NON-SPREADING CYTOPHAGAS IN ACTIVATED SLUDGE

H. Güde
Landesanstalt für Umweltschutz Baden-Württemberg
Institut für Seenforschung und Fischereiwesen
Untere Seestr. 81, D-7994 Langenargen
Federal Republic of Germany

Summary

Spreading and non-spreading colonies of yellow pigmented, flexirubin-positive bacteria were observed regularly on agar plates inoculated with samples from different activated sludge and trickling filter sources. One part of the non-spreading colonies formed spreading colonies after transfer to more suitable growth media. The other part was never observed to form spreading colonies on various growth media tested. However, when examined microscopically in wet mount preparations most of the non-spreading isolates showed the same type of gliding motility as the spreading isolates. In order to test the taxonomic homogeneity as well as possible relationships to "classical" cytophagas, 25 non-spreading isolates were examined for 41 morphological and physiological characteristics. Of the strains tested, 19 had more than 80 % of the characteristics in common. Besides their deviating colony growth, the isolates differed from "classical" cytophagas by their more complex growth requirements and by a less wide spectrum of degradation capacities. It is concluded that the non-spreading isolates represent a distinct cluster within a Cytophaga group comprising all strains with gliding motility, flexirubin pigmentation and a G + C base ratio in the range of 32 - 36 %.

Introduction

The widespread occurrence of bacteria belonging to the Cytophaga group in marine, freshwater, and soil habitats and the participation of these bacteria in the degradation of natural organic matter is now well documented /3/. Accordingly, one should expect that cytophagas could also represent a component of the microbial biocenoses respon-

sible for biological water purification in sewage plants. Indeed, during a long term study of activated sludge and trickling filter samples, yellow spreading colonies with "classical" Cytophaga characteristics could frequently be detected /5/. Furthermore, considerable numbers of non-spreading yellow pigmented colonies were regularly observed during this study. Of these yellow pigmented colonies, different percentages showed a positive reaction in the KOH test for flexirubin pigmentation, varying with season and sample source. Because of the restricted distribution of flexirubin pigments among eubacteria /17/, the question arose whether these non-spreading, flexirubin positive bacteria might also be related to the Cytophaga group. In the present study it was attempted to answer this question by examining selected isolates of these bacteria from different sewage plants for further morphological and physiological characteristics and by discussing the results in the light of current concepts of Cytophaga taxonomy.

Materials and Methods

Sampling, plating out, and isolating of the bacteria from the different activated sludge and trickling filter sources have been described in a previous study /5/. The strains further investigated in the present study are listed in Table 1 along with the respective sources they were isolated from.

Table 1: List of the strains investigated and of the respective isolation sources.

Strain	Source
V_2, V_3, WB_{29}, WB_{32}, WB_{34}	Sewage plant Wolfenbüttel, activated sludge
SB_2, SB_{16}, SB_{112}, SB_{117}	Sewage plant Salzgitter-Lebenstedt, activated sludge
WT_1, WT_8, WT_4, WT_{11}	Sewage plant Wolfenbüttel, trickling filter
ST_1, ST_2, ST_8, ST_3	Sewage plant Salzgitter-Lebenstedt, trickling filter
SG_8, SG_{10}	Model activated sludge reactor from "Schering" works, Wolfenbüttel
FN_1, FN_2, FN_3	Sewage plant Friedrichshafen, activated sludge
US_1, US_7, US_8	Sewage plant Unteres Schussental, activated sludge
C_R	Lake Mindel (near Lake Constance)

Tests for spreading growth and for gliding motility were performed on nutrient agar (NA, Merck), Casitone-yeast extract (Cy) agar, Vy/2 agar (yeast cells + vitamin B_{12}), and activated sludge extract (ASE) agar. The agar media were prepared as described before /5/. Microscopical examination of gliding motility was done with cells grown for 24 h on Vy/2 or ASE- agar. The bacteria were observed under the microscope after transfer of cell material from the edge of the colony together with a small piece of the underlying agar to the microscopical slide. Flagellation was tested with Leifson's stain /6/.

Anaerobic growth was tested on nutrient agar in anaerobic jars incubated for 2 weeks at 25°C. Growth temperature studies were performed with bacteria growing in shaking flasks on nutrient broth (NB, Merck) at the respective temperatures. Growth requirements of the bacteria were studied in liquid media containing a mineral salts medium and either glucose (5 g/l), or glucose + Bacto casamino acids (5 g/l, Difco), or glucose + casamino acids + a vitamin solution.

All biochemical tests were carried out according to the prescriptions given in the handbook of Harrigan and McCance /6/.

Results

Because the formation of spreading colonies can depend on the composition of the agar media used /3/, it was suggested that the flexirubin positive bacteria not spreading on the original enumeration and isolation agar (Cy agar) would perhaps demonstrate spreading growth on other agar media. Indeed, 22 out of 59 strains not spreading on the nutrient rich NA and Cy media were shown to form spreading colonies on ASE or Vy/2 agar which are comparatively poor in easily available substrates (Table 2). Consequently, these spreading strains could be regarded as fitting into the "classical" Cytophaga concept and were not further investigated here. Therefore, in the following the investigation has been concentrated on those strains which could never be shown to form spreading colonies in spite of the fact that gliding motility was observed with the majority of them (Table 2).

Table 2: Comparison of spreading growth and gliding motility on different agar media among 59 flexirubin-positive isolates from originally non-spreading colonies.

Agar media	NA	Cy	ASE	Vy/2
Spreading growth	0	0	22	23
Gliding motility	9	15	51	51

Among 25 of these non-spreading strains, 19 were microscopically observed to glide (Table 3). These were put together in group A. Group B consisted of the remaining 6 strains (strains WT_1, WT_{11}, ST_3, SG_8, SG_{10}, and US_8). Both strains were compared with strain C_R which could be considered as a typical Cytophaga sp.

Table 3: Comparison of morphological and growth characteristics of flexirubin-positive, non-spreading strains with those of the Cytophaga strain C_R. Group A consisted of 19 strains, group B of 6 strains. Numbers represent percentage frequencies of positive characters among the respective strains.

Characteristics	Group A	Group B	Strain C_R
Orange-yellow pigmentation on NA	100	66	+
Spreading on Vy/2 agar	0	0	+
Gliding motility	100	0	+
Flagellation	0	0	-
Short (3 /um) rods on ASE agar	100	100	+
Elongated rods (8 /um) on Cy agar	89	33	+
Anaerobic growth	11	50	±
Growth in NB at			
5°C	74	0	+
10°C	95	33	+
20°C	100	100	+
30°C	79	100	-
37°C	5	17	-
Growth in mineral media + glucose	0	17	+
Growth in mineral media + glucose + amino acids	68	66	+
Growth in mineral media + glucose + amino acids + vitamins	95	100	+

Morphologically, it was common to group A and strain C_R that the cells grew as elongated rods in nutrient rich media, whereas only small rods were observed in nutrient poor media (Table 3). In contrast the majority of group B grew as small rods in both media.

Table 4: Comparison of biochemical characteristics of group A, group B and strain C_R (further explications: see Table 3)

Characteristics	Group A	Group B	Strain C_R
Utilization of:			
Glucose	83	66	+
Galactose	68	50	+
Mannose	63	50	+
Lactose	58	50	+
Maltose	63	50	+
Ribose	42	33	+
Arabinose	74	66	+
Xylose	79	66	+
Citrate	5	33	-
Lysis of:			
Casein	100	83	+
Blood	83	66	-
Gelatin	89	66	+
Chitin	0	0	+
Dextran	0	0	+
Pectin	0	0	+
Starch	68	33	+
Xylan	0	0	+
Yeast cells	68	50	+
Catalase	95	33	+
Oxidase	89	33	+
Phosphatase	100	66	+
Lecithinase	95	33	-
Urease	0	50	-
Nitrate reduction	63	50	+
H_2S formation	0	33	-
Indole formation	11	17	-

With respect to growth characteristics, there were remarkable differences between group A and strain C_R (Table 3). The latter showed better growth at lower temperatures and did not grow at temperatures of 30°C and above. Furthermore, strain C_R had many fewer growth requirements than the majority of group A strains. Group B strains also grew better at higher temperatures and had more complex growth requirements. Additionally, 50 % of them could grow well anaerobically, a characteristic only seldom observed in group A

strains, and only weakly expressed in strain C_R.

Also with regard to the biochemical characteristics there were considerable differences between group A and strain C_R (Table 4). No member of group A strains was able to attack any of the polysaccharides except starch. Additionally, the utilization of sugars was more restricted among the group A strains, whereas haemolysis and lecithinase was not observed with strain C_R. However, it must not be overlooked that there were also several characteristics in common such as the presence of catalase, oxidase, and phosphatase, and the absence of urease, H_2S and indole formation, and the lack of citrate utilization. Also in this case, significant differences between group A and group B could be observed. Furthermore, group A with a mean similarity of 87 % (calculated on the base of negative and positive matches) proved to be much more homogenous than group B, the corresponding similarity value of which amounted to only 67 %.

Discussion

The non-spreading activated sludge isolates investigated in this study demonstrated a lot of similarities, but also several important differences to typical cytophagas. Therefore, the question as to the relationship of these bacteria to the Cytophaga group can not be answered without entering deeper into the problems concerning the taxonomic differentiation of this group.

Because of the lacking demonstration of spreading growth in all strains and because of the failing of gliding motility in group B strains, it becomes obvious that also in this case the long discussed problem of the differentiation between cytophagas and flavobacteria proves to be of central importance /3, 7, 9, 10, 12, 13, 17, 19, 20/. At present, the most accepted strategy is to allocate the non-motile strains with a low G + C content to the Flavobacterium group, whereas the Cytophaga group would comprise the low G + C base content bacteria showing unequivocal gliding motility. Accordingly, at least group A strains could be regarded as cytophagas due to a low G + C base content (determined for two strains by Behrens /1/) and due to gliding motility. However, it must be mentioned that the type of motion observed with group A strains resembled to a high degree that described by Perry /15/ inasmuch as the bacteria glided only over comparatively short distances (maximum three cell lengths) with frequent changes of direction. It has been argued /7,12/ that Perry's observations

would perhaps be better described as what has been defined by Henrichsen /8/ as "twitching" motion. However, Acinetobacter calcoaceticus, a typical representative of twitching strains showed jerky and irregular motions, whereas the movements of group A strains regularly followed the long axis of the cell. Additionally, typical cytophagas also moved in the same way when observed under the described conditions.

In any case, it becomes apparent by this example that a taxonomic differentiation based on gliding motility alone, must be regarded as unsatisfactory. Thus, the relationships between Cytophaga and Flexibacter were never doubted, in spite of the fact the G + C base contents of this group showed remarkable differences /1/. In contrast, relationships between cytophagas and flavobacteria would implicitly be excluded by the application of this criterium, although they could be much more frequent because of similar G + C content values /1/.

These considerations make it evident that final decisions as to these relationships can only be made on the base of genetic homology studies with these strains. A first step in this direction was done by Behrens /1/ who investigated the DNA homology of strains having a G + C base content in the range of 32 - 36 %, including two strains of group A (V_3 and SB_2). Both strains were shown to be closely related to each other, whereas homologies to other cytophagas were much lower. However, it must be noted that also many other cytophagas which phenotypically would clearly have been designated as typical, were demonstrated to have low DNA homology values.

After all, it can be stated that the group A strains at least can be considered as being related to the Cytophaga group, based on DNA characteristics, pigmentation patterns (studied with two isolates by Fautz /4/), and on the morphological and physiological characteristics (investigated in this study and by Hirsch /9/). Thus, these bacteria would represent a distinct cluster within a Cytophaga group comprising several clusters with the common characteristics of gliding motility, flexirubin pigmentation, and a G + C base content in the range of 32 - 34 %.

Here, it can not be decided whether the occurrence of these bacteria is restricted to sewage plant habitats, because so far no strains from other sources were examined. However, the fact that these bacteria could easily be found in several different sewage plants indicate that they may represent regular inhabitants of activated

sludge and trickling filter biocenoses. Consequently, it can be assumed that at least a part of those bacteria designated as flavobacteria may belong to this Cytophaga-cluster /1, 11, 16/.

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