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Phylogenetic Relationships and Coaggregation Ability of Freshwater Biofilm Bacteria

Alex H. Rickard,[1] Stephen A. Leach,[2] Laurence S. Hall,[1] Clive M. Buswell,[2] Nicola J. High,[1] and Pauline S. Handley[1]*

School of Biological Sciences, University of Manchester, Manchester,[1] and Centre for Applied Microbiology and Research, Salisbury, Wiltshire, United Kingdom[2]

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Nineteen numerically dominant heterotrophic bacteria from a freshwater biofilm were identified by 16S ribosomal DNA gene sequencing, and their coaggregation partnerships were determined. Phylogenetic trees showed that both distantly related and closely related strains coaggregated at intergeneric, intrageneric, and intraspecies levels. One strain, *Blastomonas natatoria* 2.1, coaggregated with all 18 other strains and may function as a bridging organism in biofilm development.

Coaggregation between bacteria occurs when two or more genetically distinct strains interact by specific cell-cell recognition (12). The phenomenon was first recognized between different oral plaque-forming bacteria, where both intergeneric and intrageneric coaggregation occurs (11). Coaggregation also occurs between bacteria isolated from a freshwater biofilm (3, 19), and it has been suggested that coaggregation may also mediate in the sequential integration of species of bacteria into freshwater biofilms (8, 20). Recently, Rickard et al. (19) used partial 16S rRNA gene sequencing to identify four coaggregating strains of *Blastomonas natatoria* and one strain of *Micrococcus luteus* from an established freshwater biofilm community (3). Six coaggregation partnerships between these five strains were found and shown to be mediated by growth-phase-dependent lectin-saccharide interactions (19). These five coaggregating strains of *B. natatoria* and *M. luteus* were part of a larger community of 19 coaggregating strains that were all isolated simultaneously from a biofilm formed on glass in a chemostat (3). The identities of the other members of the consortium are unknown, and the extent of intergeneric and intrageneric and intraspecies coaggregation between all members of the community has not been analyzed previously. Since coaggregation may be an adhesion mechanism involved with integrating and establishing bacteria in the biofilm community, it is important to know the extent of this specific adhesion mechanism in the freshwater biofilm. It is also relevant to know how closely related coaggregating strains are, since this has implications for understanding the biodiversity of the biofilm community. Therefore, the work reported here had three main objectives: (i) identification by 16S rRNA gene sequencing of all strains in the freshwater biofilm community; (ii) construction of phylogenetic trees by the computation of evolutionary distance matrices and maximum-likelihood rooted dendrograms; and (iii) analysis of intergeneric and intrageneric and intraspecies (interstrain) coaggregation partnerships between members of the biofilm community deduced using the phylogenetic trees.

All strains used in this study were isolated from a 14-day-old biofilm on a glass coupon in a two-stage chemostat kept at 4°C, which was initially inoculated with water from a borehole water source (Porton borehole, Salisbury, United Kingdom) (3). The ionic composition of the water and temperature within the chemostat were very similar to the conditions found in the source borehole water (4). Strains from the biofilm were initially grown on R2A agar (17) and were inoculated separately into conical flasks (250 ml) containing 100 ml of R2A broth and shaken at 200 rpm at 25°C in a G20 orbital shaker (New Brunswick Scientific, New Brunswick, N.J.).

The strains were identified by the method of Rickard et al. (19). Approximately 650 bases of the 16S rRNA gene were sequenced. Amplification of 16S ribosomal DNA was performed by removing a single bacterial colony from R2A agar plates to provide template DNA. Partial 16S rRNA gene sequences corresponding to the *Escherichia coli* 16S ribosomal DNA nucleotide positions 8 to 806 were amplified and sequenced using the universal primers 8FPL (22) and 806R (23). Partial 16S rRNA gene sequences of each of the strains were initially compared to those in the databases by using the FASTA3 program, and unambiguous positions of representative sequences were then aligned by using CLUSTALX version 1.64b (21). Maximum-likelihood analysis was conducted using DNAML (6), and the trees were viewed using TREEVIEW (15).

Table 1 shows the percent nucleotide sequence identity of all 19 biofilm strains to the closest sequence in the EMBL database. The five strains identified by Rickard et al. (19) have been included for completeness. Eleven of the fourteen strains identified in this study could be assigned to a genus, but no species identification was possible, since the closest strains in the database were not assigned species (Table 1). The biochemical and morphological characteristics of each strain supported the assigned identities (data not presented). Only strain 2.17 could not be identified to the genus level, since it had a very low percent sequence identity (87.6%) with the closest matching 16S rRNA gene sequence in the database, which

* Corresponding author. Mailing address: University of Manchester, 1.800 Stopford Building, Oxford Rd., Manchester M13 9PT, United Kingdom. Phone: 44 (0)161 275 5265. Fax: 44 (0)161 275 5656. E-mail: p.s.handley@man.ac.uk.

TABLE 1. Identification of the aquatic biofilm strains by alignment with the sequences of organisms in the EMBL database

Strain no.	Database accession no.	Highest % identity to sequence in database	Proposed identity[a]
2.2	AJ299221	99.35	*Afipia* sp. 2.2
2.1	AJ250434	99.71	*B. natatoria* 2.1*
2.3	AJ250435	98.39	*B. natatoria* 2.3*
2.4	AJ299222	99.71	*B. natatoria* 2.4
2.6	AJ250436	99.85	*B. natatoria* 2.6*
2.8	AJ250437	99.85	*B. natatoria* 2.8*
2.7	AJ299223	98.68	*Methylobacterium* sp. 2.7
2.9	AJ299224	98.05	*Methylobacterium* sp. 2.9
2.13	AJ250438	98.88	*M. luteus* 2.13*
2.14	AJ299232	97.54	*Nocardioides* sp. 2.14
2.20	AJ299233	97.71	*Nocardioides* sp. 2.20
2.21	AJ299231	99.84	*P. marcusii* 2.21
2.19	AJ299230	99.07	*Pseudomonas* sp. 2.19
2.17	AJ299234	87.59	Unknown
2.10	AJ299225	99.51	*Sphingomonas* sp. 2.10
2.11	AJ299226	94.77	*Sphingomonas* sp. 2.11
2.12	AJ299227	96.76	*Sphingomonas* sp. 2.12
2.15	AJ299228	96.94	*Sphingomonas* sp. 2.15
2.18	AJ299229	98.39	*Sphingomonas* sp. 2.18

[a] Strains with closest sequence identity were named. *, strains previously sequenced and named by Rickard et al. (19).

belonged to *Salinicoccus roseus* (EMBL accession no. SR16SRRN1).

Maximum-likelihood phylogenetic trees inferred from the 16S rRNA sequences of all the 19 coaggregating biofilm strains showed that they are distributed over a wide range of bacterial taxonomic groups (Fig. 1 and 2). The 6 gram-negative genera are distributed between four groups of the alpha-*Proteobacteria* subclass of the *Proteobacteria*: the *Zymomonas*, *Methylobacterium*, *Bradyrhizobium*, and *Rhodobacter* groups, as well as a single group of the gamma-*Proteobacteria* subclass, the *Pseudomonas* group (Fig. 1). The two gram-positive genera, *Micrococcus* and *Nocardiodes,* were members of the high-G+C groups *Micrococcaceae* and *Propionibacterineae* (Fig. 2). Strain 2.17, which could not be identified, is not on the gram-positive phylogenetic tree but is a member of the low-G+C group occupied by *S. roseus.*

Most of the identified strains in this study were from the alpha-*Proteobacteria* (74%), and no beta-*Proteobacteria* were found. R2A agar has been shown to support the growth of alpha and gamma *Proteobacteria* rather than the less easily cultivable beta-*Proteobacteria* (9, 10). This could explain why the majority of these freshwater biofilm strains that were originally isolated by Buswell et al. (3) were from the alpha-*Proteobacteria*. However, the objective of this study was to establish the extent of coaggregation between cultivable heterotrophic bacteria, and enough strains have been isolated and identified to reveal that coaggregation is a common property of strains in this ecosystem. A considerable taxonomic diversity of heterotrophs in the biofilm was found, and all except strain 2.17 are very closely related to organisms that have previously been isolated from freshwater or soil environments.

The phylogenetic trees revealed that the biofilm contained clusters of very closely related strains or clones. These clonal groups included five *B. natatoria* strains, four *Sphingomonas* strains, two *Methylobacterium* strains (Fig. 1), and two *Nocardioides* strains (Fig. 2). Each clonal group contained strains with different coaggregation partners (see Fig. 5). It has been proposed that biofilms can act as reservoirs of clones of the same species and that this may enhance the survival of the species during environmental fluctuations (2, 16). It is not yet clear why closely related clones with such distinct coaggregation patterns can coexist within a freshwater biofilm.

In order to detect all the coaggregation partnerships between strains, all 171 possible pairwise combinations of strains were tested using the visual coaggregation assay originally developed by Cisar et al. (5) and adapted by Rickard et al. (20). Because the ability to coaggregate has been found to be optimum for only a relatively short period in stationary phase and not to occur in exponential phase for *B. natatoria* 2.1 and *M. luteus* 2.13 (19), strains were harvested at three different times during stationary phase. The harvest times were the following: early stationary phase (36 h), mid-stationary phase (72 h), and later stationary phase (144 h) in batch culture (growth kinetics data not shown). In this way the optimum time in stationary phase at which coaggregation occurred could be determined. Cells were harvested and washed three times in sterile deionized water. Using a spectrophotometer (Cecil instruments, Cambridge, United Kingdom), the washed cells were then resuspended in deionized water to an optical density at 650 nm (OD_{650}) of 1.0 and concentrated to give a calculated final OD_{650} of 1.5. For coaggregation, pairs of strains were mixed at an OD_{650} of 1.5 in equal volumes (200 μl) at room temperature in 6- by 50-mm silica Durham tubes (Scientific Lab Supplies, Nottingham, United Kingdom). Mixtures were vortexed for 10 s, and the tubes were rolled gently for 30 s. The degree of coaggregation between each pair was scored as follows: 0, no coaggregates in suspension; 1+, small uniform aggregates in a turbid suspension; 2+, easily visible coaggregates in a turbid suspension; 3+, clearly visible coaggregates which settle, leaving a clear supernatant; 4+, large flocs of coaggregates that settle almost instantaneously, leaving a clear supernatant. Control tubes of each isolate on its own were also included to assess autoaggregation. Where present, the autoaggregation score was deducted from the coaggregation score.

Maximum visual coaggregation scores for different pairs ranged from 1+ to 4+ (Fig. 3), and 82 pairs out of the total of 171 pairs (48%) gave a positive score. Scores were always reproducible after growth in batch culture for a set period of time, and three batches of all cultures were tested separately to confirm the reproducibility of coaggregation. For all pairs, a microscopic examination of the coaggregating pairs showed flocs of coaggregating cells and the size of the coaggregates increased with increasing visual coaggregation scores (Fig. 4).

In order to represent the complexities of the many coaggregation partnerships, a matrix of all the pairings giving a score of 2+ or greater was constructed (Fig. 5). The growth time in batch culture (36, 72, or 144 h) at which optimum coaggregation scores occurred is indicated on the matrix. For 76 of the 82 coaggregating pairs (93%) the ability to coaggregate was maximum at only one of the three harvest times in stationary phase. After 36 h of growth in batch culture, 38 pairs (22%) coaggregated, after 72 h, 62 pairs (36.2%) coaggregated, and after 144 h of growth, 36 pairs (21%) coaggregated. The majority of the possible coaggregation partnerships occurred after

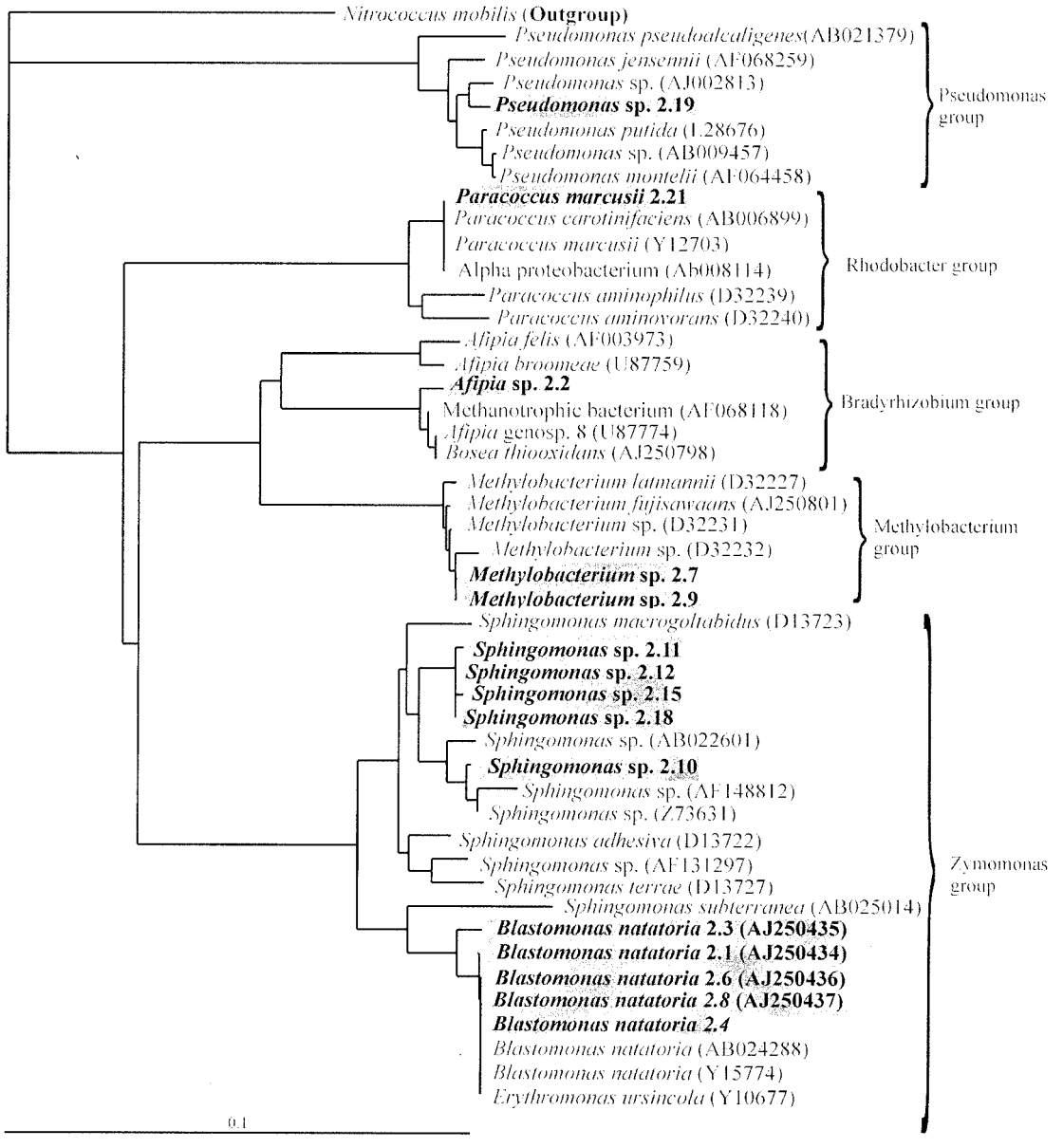

FIG. 1. Maximum-likelihood phylogenetic tree constructed using partial 16S rRNA gene sequences of closely related speciated and nonspeciated strains belonging to the alpha- and gamma-*Proteobacteria*. The tree is rooted using *Nitrococcus mobilis* (L35510) as the outgroup. Nucleotide sequence database accession codes are shown in the brackets. The length of each branch is proportional to the estimated number of substitutions per position. Sequenced strains are highlighted in boxes; most-likely identities were assigned.

incubation in batch culture for 72 h, although 7% of the possible 171 coaggregation partnerships showed a consistent score which was not influenced by the harvest time (Fig. 5). In freshwater the bacteria in biofilms are probably in stationary phase for the majority of the time (7) and may therefore have evolved to express the ability to coaggregate while in this starved physiological state.

As shown in the coaggregation matrix (Fig. 5), *B. natatoria* 2.1 was able to coaggregate with all other strains tested. Other highly interactive strains included *Sphingomonas* sp. strain 2.15, with 15 partner strains, and *Afipia* sp. strain 2.2, with 13 partner strains. In contrast, *Pseudomonas* sp. strain 2.19 and *B. natatoria* 2.4 had the lowest number of partnerships, each coaggregating with two and four partners, respectively. Each strain possessed different coaggregation abilities, with respect to numbers and identity of partners as well as the size of the flocs formed.

When considering the phylogenetic relationships and coaggregation partnerships, coaggregation at an intergeneric level was extremely common and every strain tested coaggregated intergenerically with at least one other strain from a different genus at a level of 2+ or greater (Fig. 5). For example, strain *B. natatoria* 2.1 coaggregated with strains from seven other genera—*Sphingomonas*, *Nocardioides*,

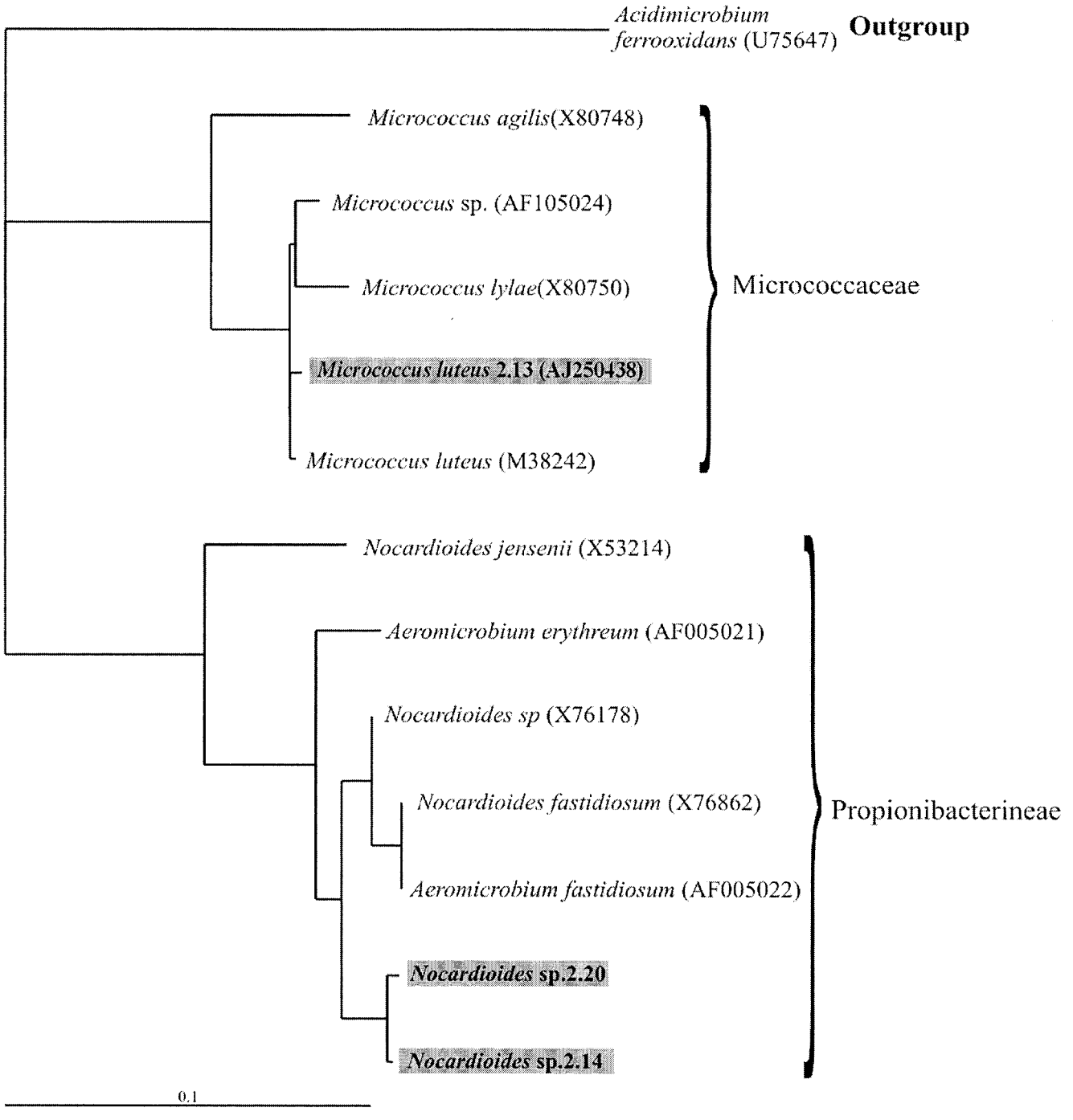

FIG. 2. . Maximum-likelihood phylogenetic tree constructed using partial 16S rRNA gene sequences of closely related speciated and nonspeciated strains belonging to the high-G+C gram-positive bacteria. The tree is rooted using *Acidimicrobium ferrooxidans* (U75647) as the outgroup. Nucleotide sequence database accession codes are shown in the brackets. The length of each branch is proportional to the estimated number of substitutions per position. Sequenced strains are highlighted in boxes; most-likely identities were assigned.

Paracoccus, *Methylobacterium*, *Micrococcus*, *Pseudomonas*, and strain 2.17. Coaggregation at the interstrain (intraspecies) level was common, occurring between closely related strains of *Sphingomonas* and *Blastomonas* clustering in the same phylogenetic groups. Coaggregation between *B. natatoria* 2.1 and three other strains of *B. natatoria* (2.3, 2.8, and 2.6) has been reported previously (19); however, this study shows that *B. natatoria* 2.4 is a very poor coaggregator and it cannot coaggregate at the interstrain level with the other *Blastomonas* strains. Intergeneric coaggregation is common between oral bacteria (13), but intraspecies (interstrain) coaggregation has not yet been reported between plaque

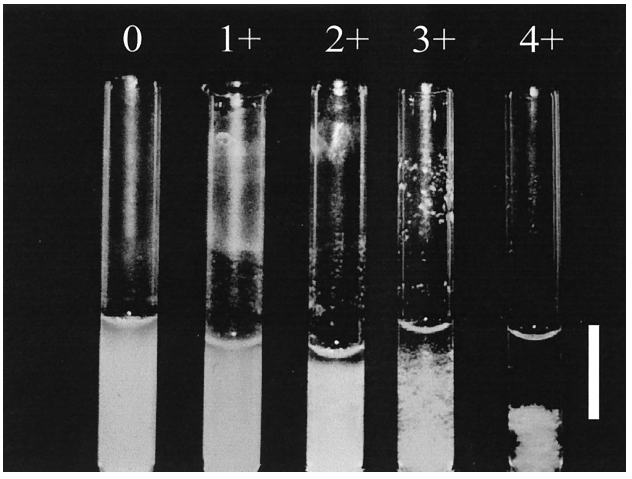

FIG. 3. Visual coaggregation scores for different pairs of strains. Cells were grown to stationary phase and harvested after 36, 71, or 144 h. *Sphingomonas* sp. strain 2.10 and *Afipia* sp. strain 2.2 (36 h) showed a score of 0 (no coaggregation), *P. marcusii* 2.21 and *B. natatoria* 2.3 (72 h) showed a score of 1+, *Sphingomonas* sp. strain 2.18 and *Afipia* sp. strain 2.2 (144 h) showed a score of 2+, *B. natatoria* 2.1 and *Nocardioides* sp. strain 2.20 (144 h) showed a score of 3+, and *B. natatoria* 2.1 with *M. luteus* 2.13 (72 h) showed a score of 4+. Bar, 1 cm.

bacteria. Thus, intraspecies coaggregation may well be a characteristic that is unique to freshwater biofilm bacteria.

While intergeneric and interstrain coaggregations are common between these aquatic biofilm community members, there is only one example of intrageneric coaggregation. *Sphingomonas* 2.10 and *Sphingomonas* 2.15 coaggregate with each other after 144 h (Fig. 5) with a score of 4+, and the evidence indicates that they are likely to be from different, as yet unnamed species. First, the phylogenetic tree (Fig. 1) shows a cluster of very closely related *Sphingomonas* strains (2.11, 2.12, 2.15, and 2.18) with strain 2.10 in a separate subgroup. The group of four strains all have >99.1% sequence homology to each other and none were 100% identical. However, strain 2.10 had only from 97.7 to 98.4% sequence homology with the four closely related strains. Secondly, typing of the *Sphingomonas* strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of whole-cell proteins shows 20 visible protein bands for strain 2.10, while for the strains in the group of four, only five identical bands were visible (18). In addition, all the *Sphingomonas* strains possessed distinct colony and cell morphologies. Taken together, the evidence indicates that 2.10 is likely to be a different species from the other four *Sphingomonas* strains. Therefore, we propose that intrageneric coaggregation can occur in both aquatic and oral biofilms.

In dental plaque, *Fusobacterium nucleatum* can coaggregate with all other oral bacteria so far tested (1, 14) and has therefore been described as a "promiscuous" coaggregator which is proposed to have a very significant role as a bridging organism linking primary and secondary colonizers that cannot coaggregate with each other (11). Since *B. natatoria* 2.1 coaggregated with all 18 other biofilm strains, it may also be described as promiscuous. *B. natatoria* 2.1 may have a role equivalent to that of *F. nucleatum* in the development of this freshwater biofilm and may have the same role in other biofilms. It is not yet known whether colonization during freshwater biofilm formation involves a succession of primary and secondary colonizers, as occurs in the development of dental plaque. However, it is possible that highly coaggregating genera such as *Blastomonas, Afipia*, and *Sphingomonas* spp. could be quantitatively important members of freshwater biofilm communities and that their ability to adhere to other community members in a biofilm would give them a colonization advantage.

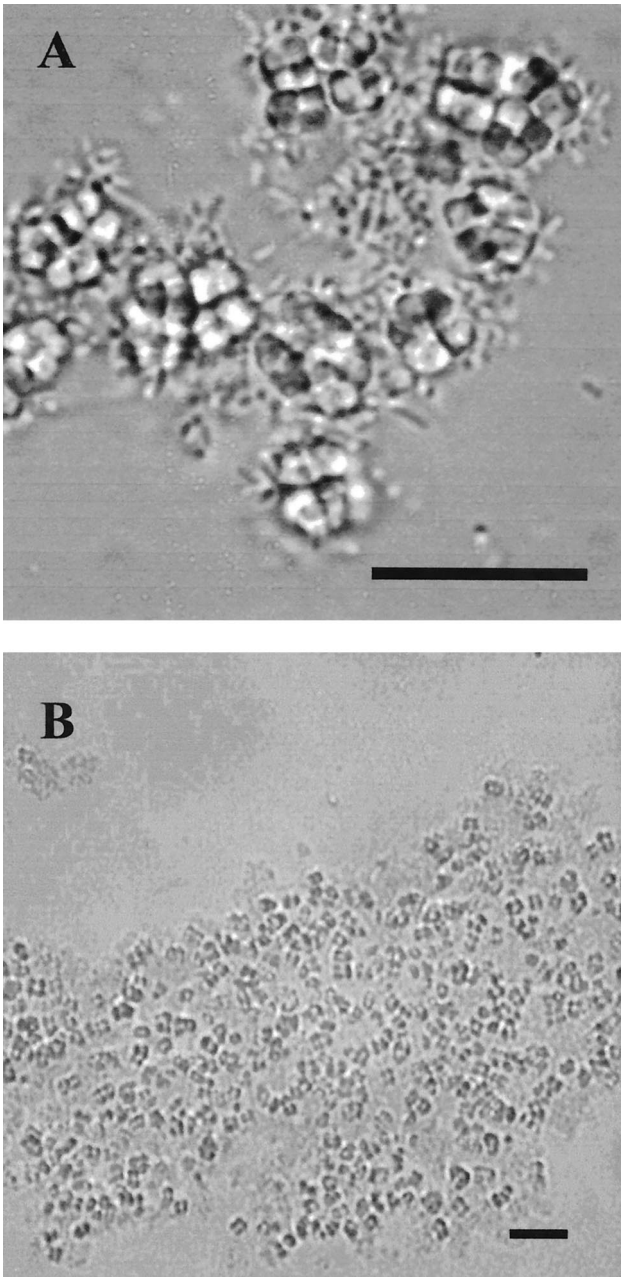

FIG. 4. Light micrographs showing rod-shaped cells of *B. natatoria* 2.1 coaggregating to tetrads of coccus-shaped cells of *M. luteus* 2.13. Bar, 10 μm.

In conclusion, this study has revealed that intergeneric and intraspecies coaggregation between freshwater bacteria are common phenomena that occur between strains from a laboratory model aquatic biofilm. In addition, expression of coag-

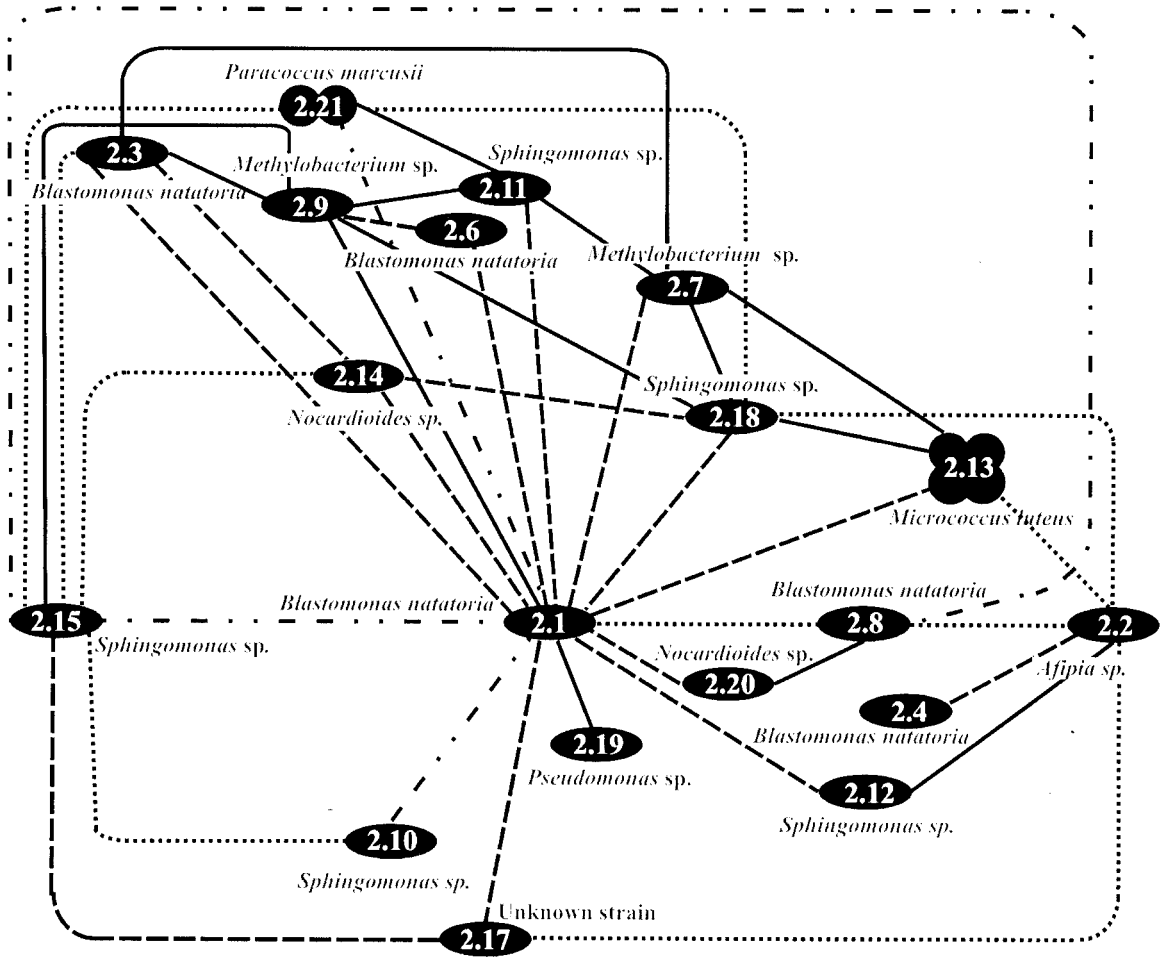

FIG. 5. A diagrammatic representation of the specificity of coaggregation of all 19 identified strains when grown separately in batch culture and harvested after 36, 72, and 144 h. Optimal expression of coaggregation at either 36 (—), 72 (- - -), or 144 h is shown for each pair. Expression of coaggregation that was constant between pairs at 36, 72, and 144 h is also shown (-·-·-). Visual coaggregation scores below a score of 2 are not included.

gregation is dependent on cells being in the optimum physiological state for coaggregation, which usually occurs in stationary phase. It is therefore possible that since cells grow very slowly in nutrient-limited biofilms, a natural freshwater biofilm would provide suitable conditions for expression of coaggregation.

Nucleotide sequence accession numbers. Partial 16SrRNA sequences for *M. luteus* 2.13 and the four *B. natatoria* strains (2.1, 2.3, 2.6, and 2.8) have been assigned accession numbers previously (19). The sequences of all the remaining identified strains were also deposited in the EMBL database. The EBML accession numbers of the sequences were the following: for *Afipia* sp. strain 2.2, accession no. AJ299221; for *B. natatoria* 2.1, 2.3, 2.4, 2.6, and 2.8, accession numbers AJ250434, AJ250435, AJ299222, AJ250436, and AJ250437, respectively; for *Methylobacterium* sp. strains 2.7 and 2.9, accession numbers AJ299223 and AJ299224, respectively; for *M. luteus* 2.13, accession number AJ250438; for *Nocardioides* sp. strains 2.14 and 2.17, accession numbers AJ299232 and AJ299233, respectively; for *Paracoccus marcusii* 2.21, accession no. AJ299231; for *Pseudomonas* sp. strain 2.19, accession no. AJ299230; for *Sphingomonas* sp. strains 2.10, 2.11, 2.12, 2.15, and 2.18, accession numbers AJ299225, AJ299226, AJ299227, AJ299228, and AJ299229, respectively; for unknown bacterial heterotroph 2.17, accession no. AJ299234.

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