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Aggregation and biofilm formation of bacteria isolated from domestic drinking water

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Abstract:

Auto and co-aggregation and biofilm formation of four bacteria (*Sphingobium, Xenophilus, Methylobacterium* and *Rhodococcus*) isolated from domestic drinking water was investigated in this study. Visual aggregation assay showed that both individual and combined isolates did not form any flocs immediately. DAPI imaging showed that except *Xenophilus*, the other three bacteria auto-aggregated at 24 h whereas, *Methylobacterium* combinations showed pronounced co-aggregation as compared to other combinations. Heat and protease treatment inhibited auto and co-aggregation of all bacterial combinations. However, sugar treatment showed varying results. Biofilm formation by pure culture bacteria was negligible as compared to multispecies biofilms. The overall results indicate that *Methylobacterium* showed more auto and co-aggregations and the aggregation was influenced by heat, protease and sugar treatments which may be mediated by lectin-polysaccharide interactions.

Key words: Aggregation; Biofilms; Drinking water

Introduction

Biofilms formed on inner surface of the pipe wall within drinking water distribution system have negative effect on the water quality by increasing microbial load due to sloughing off and transport (Lechevallier et al. 1993; Jefferson 2004). Aggregation is one of the essential steps towards biofilm formation and it depends on range of different interactions such as synergistic, antagonistic, mutualistic, competitive, and commensalism (Kolenbrander et al. 1985; Simoes et al. 2007). Auto-aggregation is defined as adherence of bacteria that belong to same strain, and co-aggregation is adherence as a result of two or more different bacterial species. Co-aggregation is a highly specific interaction, which was first reported in human oral bacterial communities (Gibbons and Nygaard 1970) and then in various environments such as dental water lines, freshwater samples and model drinking water biofilms (Rickard et al. 2003; Stoodley et al. 2002; Buswell et al. 1998; Simoes et al. 2007). In earlier studies, surface-associated molecules such as proteins and sugars were found to mediate the co-aggregation of bacteria, and such interactions contributed to the development of multispecies biofilm (Rickard e tal. 2003; Simoes et al. 2008). The aim of this study was to investigate the auto and co-aggregation and biofilm formation of bacteria isolated from domestic drinking water and to test the effect of surface associated molecules on the observed aggregation.

Materials and Methods

Water samples were collected from domestic drinking water in Sheffield, UK and four isolates, identified by 16S rRNA gene sequencing, were used for further studies

(*Sphingobium sp., Xenophilus sp., Methylobacterium sp. and Rhodococcus sp*.) based on their dominance and colony characteristics.

Table 1.1. The bacterial isolates used in this study and their identification by 16S rRNA gene sequencing.

Isolates	Sequence length (bp)	Closest relative in Genbank database (Accession number)	Similarity (%)	Phylogenetic affiliation
A	1408	*Sphingobium* sp. (DQ413165)	99	Alphaproteobacteria
B	1417	*Xenophilus* sp. (FJ605423)	99	Betaproteobacteria
C	1434	*Methylobacterium* sp. (AB252206)	94	Alphaproteobacteria
D	1460	*Rhodococcus* sp. (EF612291)	99	Actinobacteria

Visual aggregation assay: The visual auto and co-aggregation of the four isolates and in combinations were studied by scoring method as described by Cisar et al. (1979). The scoring criteria were 0=no aggregation; 1=small uniform aggregates in a turbid suspension, 3=clearly visible aggregates which settles leaving a clear supernatant, 4=large flocs of aggregates that settle instantaneously. The scoring was recorded after 30 seconds, 24, 48 and 72 hours.

Auto and co-aggregation study by DAPI staining method: A combination of DAPI staining and epifluorescence microscopy was used to study the auto- and co-aggregation of the four isolates and in combinations. Bacterial isolates were grown in R2A broth and the cells were harvested at 0, 24, 48 and 72 h. Cells were then filtered on 0.2 µm pore size membrane filters, stained with DAPI and analysed by epifluorescence microscopy.

Heat, protease and sugar treated cells: The surface associated molecules involved in aggregation were investigated by heat, protease and sugar treatment methods. The cells were treated at 80 ^{o}C for 30 min as described by Kolenbrander et al. (1985) and both treated and untreated cells were used for visual auto and co- aggregation assay. To understand the polymer mediated aggregation (Cookson et al 1995), Protease type XIV from *Streptomyces griseus* were used and the visual scoring of untreated and protease cells were determined. The reversal or inhibition of aggregation was determined by treating bacterial cultures with filter sterilised D(+) galactose, D(+) fucose and D(+) acetylglucosamine individually to a final concentration of 50mM and visual scores were determined (Simoes et al 2008).

Biofilm assay: Biofilm formation by individual and combined isolates was studied by colorimetric method with minor modifications as described by Simoes et al. (2008). Four bacterial isolates were grown individually in R2A broth for 24 h and diluted at OD_{595} to 0.01. The diluted cultures were mixed in required combinations and the OD of biofilm mass was measured at 24, 48 and 72 h at OD_{570} nm using a microplate reader.

Results and Conclusions:

Visual aggregation assay: Results of visual aggregation assay showed that after 30 seconds, the individual and combined isolates did not form any flocs. However, it was observed that the individual isolates, except for *Xenophilus* sp., settled down after 24 h.

Auto aggregation of *Xenophilus* sp. did not occur even up to 72 h. However when in combination with the other isolates *Xenophilus* sp. formed aggregates. This result indicates that the bacterium *Xenophilus* only formed aggregates in the presence of other bacteria, and that the overall aggregation process is time dependent.

Auto and co-aggregation by DAPI staining method. *Sphingobium sp*. and *Rhodococcus* sp. started to auto-aggregate after 24 h and aggregation was more pronounced at 48 and 72 h. *Methylobacterium sp*. showed aggregation starting from 24 hours by forming a rosette pattern which was not observed in other three bacteria used in this study. *Xenophilus sp.* did not show aggregation even after 72 h (Figure 1.1), which is in agreement with visual aggregation assay. The co-aggregation study showed that in the cases of *Methylobacterium* combinations, aggregation was more pronounced as compared to other combinations (Figure. 1.2).

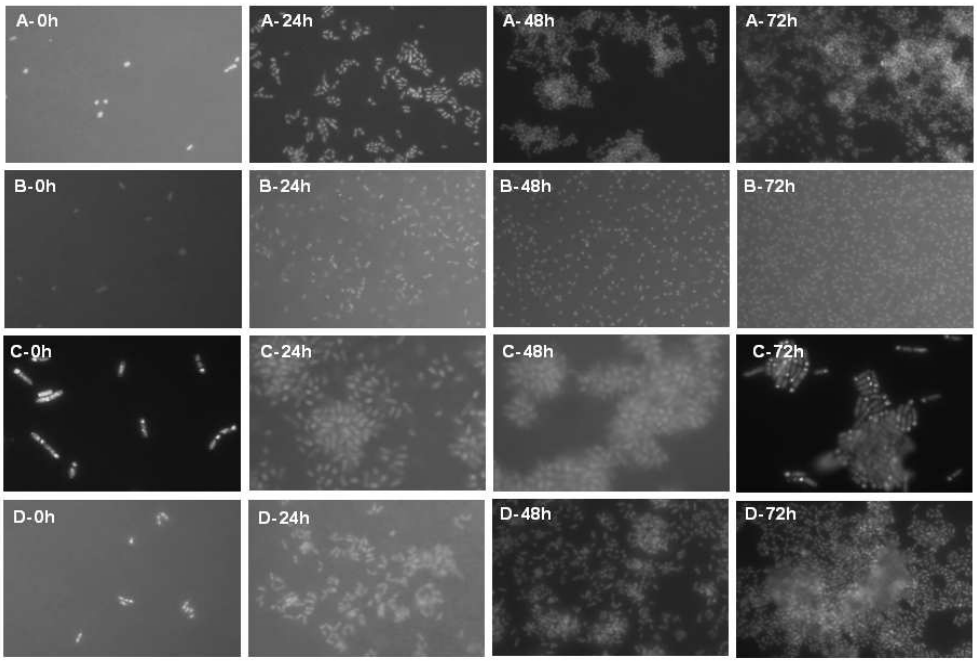

Figure 1.1. Auto-aggregation of the four isolates at various time intervals. (A=*Sphingobium* sp., B=*Xenophilius* sp., C=*Methylobacterium* sp., D=*Rhodococcus* sp.)

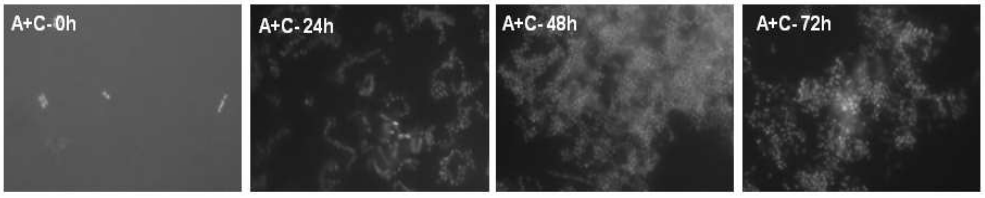

Figure 1. 2. Co-aggregation of *Shingobium* (A) and *Methylobacterium* (C) at various time intervals.

Heat, protease and sugar treated cells: No-aggregation was seen in all four isolates and their combinations using the visual aggregation method after heat and protease treatment from 30 seconds up to 72 h. As mentioned earlier aggregation was seen however with the untreated isolates after 24 h and up to 72 h (except for *Xenophilus* sp).. This suggests that the presence of heat sensitive and protein-like molecules are

involved in aggregation of the isolates *Sphingobium* sp., *Methylobacterium* sp., and *Rhodococcus* sp. specifically.

Using the visual aggregation assay, after 30 seconds, *Sphingobium* and *Xenophilus* auto-aggregated in the presence of N-acetyl glucosamine and *Xenophilus* auto-aggregated with galactose. The *Methylobacterium* sp. aggregated only in presence of fucose where as *Rhodococcus* did not auto aggregate with any of the chosen sugars (Table 1.2). *Xenophilus* sp. showed good co-aggregation with the other three bacteria in the presence of N-acetyl glucosamine and galactose where as *Methylobacterium* and its combinations showed aggregation with fucose (Table 1.3). These results indicate that the presence of sugars can promote aggregation that was not observed before e.g. auto aggregation of *Xenophilus* sp. However the overall influence of sugars on aggregation is dependent not only on the type of sugar but also the specific bacterial isolate.

Table 1.2. Auto-aggregation of bacteria with sugars studied by visual scoring method (+ = aggregation observed; - = no aggregation; +/- = not very clear aggregation).

Bacteria	D(+) Galactose	D (+) Fucose	D (+) N-Acetyl glucosamine
Sphingobium	-	-	+
Xenophilus	+	-	+
Methylobacterium	-	+	-
Rhodococcus	-	-	-

Table 1.3. Co-aggregation of bacteria with sugars studied by visual scoring method (+ = aggregation observed; - = no aggregation; +/- = not very clear aggregation). (A=*Sphingobium* sp., B=*Xenophilius* sp., C=*Methylobacterium* sp., D=*Rhodococcus* sp.)

Bacteria	D(+) Galactose	D (+) Fucose	D (+) N-Acetyl glucosamine
A+B	+	-	+
A+C	-	+	-/+
A+D	-	-	-
B+C	+	+	+
B+D	+	+	+
C+D	-	+	-

Biofilm assay: The amount of biofilm formed by individual cultures was negligible up to 72 h, whereas biofilm formed between dual species varied depending upon the species present. Combinations of *Sphingobium*+*Methylobacterium* and *Methylobacterium*+*Rhodococcus* formed more biofilms over time than other combinations. Interestingly, biofilm formation of the combined isolates was reduced when formed in combinations without *Methylobacterium* sp. indicating that this bacterium might act as bridging bacterium for multispecies biofilm formation (Figure 1.3.). However, further investigation is required to confirm this result.

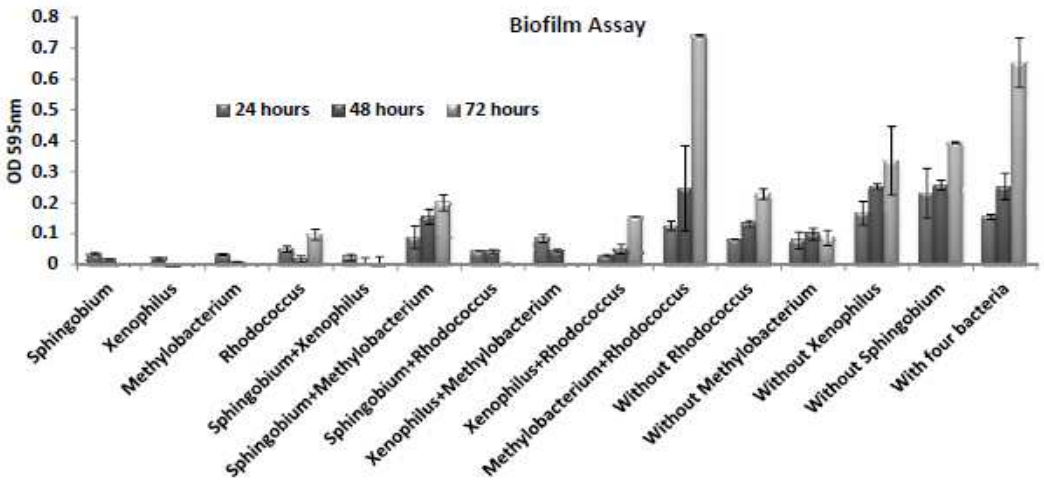

Figure 1.3. Biofilm formation by drinking water isolates at various time points.

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