Aggregation and biofilm formation of bacteria isolated from domestic drinking water
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ABSTRACT
The objective of this study was to investigate the autoaggregation, coaggregation and biofilm formation of four bacteria namely *Sphingobium*, *Xenophilus*, *Methylobacterium* and *Rhodococcus* isolated from drinking water. Auto and coaggregation studies were performed by both qualitative (DAPI staining) and semi-quantitative (visual coaggregation) methods and biofilms produced by either pure or dual-cultures were quantified by crystal violet method. Results from the semi-quantitative visual aggregation method did not show any immediate auto or coaggregation, which was confirmed by the 4',6 diamidino-2-phenylindole (DAPI) staining method. However, after 2 hours, *Methylobacterium* showed the highest autoaggregation of all four isolates. The *Methylobacterium* combinations showed highest coaggregation between dual species at extended period of times (72 hours). Biofilm formation by pure cultures was negligible in comparison to the quantity of biofilm produced by dual-species biofilms. The overall results show that coaggregation of bacteria isolated from drinking water was mediated by species-specific and time-dependent interactions with a synergistic type of biofilm formation. The results of this study are therefore a useful step in assisting the development of potential control strategies by identifying specific bacteria that promote aggregation or biofilm formation in drinking water distribution systems.

Key words | aggregation, bacteria, biofilms, coaggregation, DAPI, drinking water

INTRODUCTION
Biofilms formed on internal pipe surfaces within drinking water distribution systems (DWDS), can have a negative effect on the water quality (Lechevallier et al. 1993) by increasing microbial load due to sloughing off and transport (Jefferson 2004). Biofilms can also cause various water quality problems within DWDS such as obnoxious taste and odour, increased turbidity, reduced water pressure and flow, microbiologically influenced corrosion and release of pathogenic bacteria, which is a major public health concern (Berry et al. 2006). Biofilms can also act as a hiding place for potential pathogens such as *Helicobacter pylori*, *Escherichia coli* and *Legionella pneumophila*, which cause various illnesses and diseases to human beings (Szewzyk et al. 2000; Berry et al. 2006).

Biofilms form a complex structure which are composed of a community of microbes enclosed in self-produced extracellular polymeric matrix (Monds & O'Toole 2009). The different stages of biofilm formation are surface association, adhesion, and production of extracellular polymeric substances (EPS), multiplication and maturation (Stoodley et al. 2002). Biofilm formation and adhesion of bacteria on different surfaces and the EPS production by biofilm forming microbes have been well studied in general (Skillman et al. 1999; Tsuneda et al. 2003; Flemming et al. 2007; Simoes et al. 2007a; Furuhat et al. 2008). However, studies on biofilm formation and coaggregation by bacteria isolated from drinking water is very limited (Buswell et al. 1997; Simoes et al. 2007a, b; Simoes et al. 2008; Yu et al. 2010).

Aggregation is one of the essential steps towards biofilm formation and it depends on a range of interactions such as synergistic, antagonistic, mutualistic, competitive, and commensalism interactions (Simoes et al. 2007b). Coaggregation
is a highly specific interaction which was first reported in human oral bacterial communities (Gibbons & Nygaard 1970). Cisar et al. (1979) introduced a visual coaggregation scoring method to assess the coaggregation between two bacterial partners Actinomyces sp. and Streptococcus sp. isolated from dental plaque. The result of this study showed that coaggregation was mediated by specific cell surface interaction such as proteins and carbohydrates. Since then, various reports have shown that coaggregation occurs in different environments such as dental water systems, fresh water samples and activated sludge (Buswell et al. 1997; Moller et al. 1998; Stoodley et al. 2002; Rickard et al. 2003).

The aims of the present study were: (i) to investigate the auto and coaggregation of selected bacteria isolated from domestic drinking water by visual aggregation assay and 4',6 diamidino-2-phenylindole (DAPI) staining method in combination with epifluorescence microscopy; and (ii) to quantify the biofilm formed by the bacterial isolates by crystal violet method to find out their potential role in multispecies biofilm formation in DWDS. The long-term ambition of this work is to provide new knowledge to assist in the control of biofilm formation and therefore to help inform successful DWDS operation, maintenance and investment strategies.

MATERIALS AND METHODS

Isolation and identification of bacteria

Water samples were collected from domestic drinking water taps in Sheffield, UK. The water samples (2 L) were filtered on 0.22 μm white polycarbonate membrane filters (Millipore, UK), vortexed in sterile water, and an aliquot of 200 μL were spread on Petri plates containing R2A agar medium as suggested by Allen et al. (2004). The plates were incubated at 25 °C for 72 hours and the bacterial colonies were picked and purified based on colony morphology.

Four bacterial isolates were identified by polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene. The DNA was extracted using Ultra Clean Soil DNA Isolation Kit (Cambio Laboratories Inc., UK) following the manufacturers protocol. The 16S rRNA gene was PCR amplified by using primer sets, 27F and 1492R (Muyzer et al. 1995). The purified PCR products were sequenced by using primers 27F, 518F and 1492R and the sequences assembled using BioEdit sequence alignment editor program (http://www.mbio.ncsu.edu/bioedit/bioedit.html, Ibis Biosciences, Carlsbad, CA). The sequences were compared using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) queuing system to identify their closest relatives.

The four bacterial isolates used in the further studies were Sphingobium sp. (A), Xenophilus sp. (B), Methylobacterium sp. (C) and Rhodococcus sp. (D). The DNA sequences were submitted to GenBank under accession numbers JQ928370 to JQ928373. The bacterial isolates were selected based on their ubiquity (Sphingobium sp. and Methylobacterium sp.) in the water distribution systems on a worldwide basis and also for less common or unique presence (Rhodococcus sp. and Xenophilus sp.) in the chosen domestic drinking water.

Visual aggregation assay

Visual studies of the auto and coaggregation behaviour of the four selected bacterial isolates were undertaken following the procedure described previously (Cisar et al. 1979; Simoes et al. 2008). The individual bacterial cultures grown in R2A broth were harvested at stationary phase, centrifuged and the pellets were resuspended in sterile water and adjusted to optical density (OD) of 1.5 at a wavelength of 640 nm. For coaggregation, the individual isolates were mixed in six possible combinations (A + B, A + C, A + D, B + C, B + D, C + D) by strain elimination procedure and the degree of aggregation was scored at 0, 2, 24, 48 and 72 hours as described by Cisar et al. (1979). For autoaggregation, the degree of aggregation was scored at 0, 2, 24, 48 and 72 hours for the individual pure cultures.

Aggregation studied by DAPI staining method

A combination of DAPI staining and epifluorescence microscopy was used to qualitatively study the auto and coaggregation of bacterial isolates. The bacterial cultures previously grown in R2A broth were diluted to an OD of 0.1 at 595 nm wavelength and incubated at 25 °C with agitation at 150 rpm. The autoaggregation was studied by aggregation with pure cultures and coaggregation was...
studied by mixing the diluted pure cultures in equal volume followed by incubating them at 25 °C with agitation at 150 rpm. The cultures were harvested at 0, 24, 48 and 72 hours and the harvested cells were fixed in 50% ethanol and 100 μL of cultures were vacuum filtered on membrane filters, stained with DAPI and observed under an epifluorescence microscope equipped with CellB imaging system (Olympus UK Ltd, Watford) and the images were captured at a xy resolution of 1,360 × 1,024 pixels.

**Biofilm assay**

Single (A, B, C and D) and dual species biofilm formation by the four isolates were studied at different combinations (A + B, A + C, A + D, B + C, B + D, C + D) and R2A broth was used as a control. The biofilm assay was carried out by crystal violet staining method as described previously (Simoes et al. 2007b) after minor modifications. Approximately 200 μL of individual and mixed cultures previously grown in R2A broth were added into three 96 well plates and incubated for 24, 48 and 72 hours at 25 °C with agitation at 150 rpm. After the incubation period, the wells were rinsed with sterile water and then stained with 1% crystal violet solution. After a brief washing followed by drying for 30 minutes, 150 μL of 33% acetic acid was added into each well and shaken for 5 minutes at 500 rpm in an orbital shaker. The OD was measured at a wavelength of 595 nm using GENios 96 well microplate reader (TECAN, UK) in triplicates and the data were analysed by two-way analysis of variance (ANOVA) by using GraphPad Prism 5 (GraphPad software Inc., La Jolla, CA). All experiments were done in triplicate, at three different time points.

**RESULTS**

**Visual aggregation assay**

The results of visual aggregation assay of auto and coaggregation studies showed no visible flocs formed for the four pure cultures (Sphingobium (A), Xenophilus (B), Methylobacterium (C) and Rhodococcus (D)) and for the six possible paired combinations (A + B, A + C, A + D, B + C, B + D, C + D) after 30 seconds. Therefore, the autoaggregation and coaggregation was measured after 2, 24, 48 and 72 hours. The Methylobacterium aggregated at 2 hours with the visual score of 1 (small uniform aggregates in a turbid suspension) whereas the other three cultures (Sphingobium, Xenophilus, and Rhodococcus) did not autoaggregate at 2 hours. All cultures, both pure and paired, settled after 24 hours and remained so until 72 hours with the exception of the Xenophilus bacterium, which did not autoaggregate or settle even up to 72 hours.

The autoaggregation study indicated that the bacterium Methylobacterium might play a leading role in formation of multispecies aggregates, therefore the coaggregation studies were carried out with Methylobacterium combinations. The combinations of A + C, B + C and C + D showed coaggregation at 2 hours with the visual score of 2 (easily visible aggregates in a turbid solution). Since Methylobacterium (C) showed both auto and coaggregation more quickly (2 hours) as compared with other combinations, further coaggregation studies using the DAPI staining method were carried out in Methylobacterium combinations.

**Aggregation of bacteria studied by DAPI staining method**

**Autoaggregation of bacterial isolates**

As shown in Figure 1, and in agreement with the visual aggregation studies, all four cultures did not autoaggregate at 0 hours. The bacterium Sphingobium sp. started to autoaggregate after 24 hours and the aggregation became more pronounced at 48 and 72 hours. The bacterium, Xenophilus sp. did not show autoaggregation even after 72 hours while Methylobacterium sp. showed autoaggregation starting from 24 hours by forming a rosette pattern of aggregation which was not observed in the other three bacteria investigated in this study (Figure 1). Autoaggregation of Methylobacterium sp. was very well pronounced at 72 hours and Rhodococcus sp. started to autoaggregate at 24 hours and the aggregation increased with time (Figure 1).

**Coaggregation of bacterial isolates**

Figure 2 shows the combination of Sphingobium + Methylobacterium (A + C), which did not result in any
coaggregation at 0 hours. Coaggregation between these two pairs was seen at 48 and 72 hours (Figure 2). The second combination of \textit{Xenophilus} + \textit{Methylobacterium} (B + C) also did not show coaggregation at 0 hours. However, the isolates started to coaggregate at 24 hours and the coaggregation was more pronounced at 48 and 72 hours. The third combination of \textit{Rhodococcus} + \textit{Methylobacterium} (D + C) only started to coaggregate at 48 hours.
hours and coaggregation was more pronounced at 72 hours (Figure 2).

**Biofilm assay**

*Figure 3* shows that the amount of biofilm formed by the pure cultures was negligible after 72 hours, whereas the biofilm formed between dual species varied depending upon the strains present. Between dual species biofilm formation, combinations of *Sphingobium* + *Methylobacterium* and *Methylobacterium* + *Rhodococcus* \( (P < 0.0001) \) formed more biofilms over time than the other three combinations.

Biofilms formed by *Methylobacterium* combinations were more than biofilms formed with single cultures (two-way ANOVA; \( P < 0.01 \) to \( P < 0.0001 \)). These results show that biofilm formation between dual species were influenced by the presence of *Methylobacterium* suggesting that this bacterium might act as a bridging bacterium towards multispecies biofilm formation within the water distribution system.

**DISCUSSION**

Drinking water harbours a diverse and dynamic microbial community (*Szewzyk et al. 2000; Berry et al. 2006*). In this study, the chosen bacteria include both Gram-negative (*Sphingobium*, *Xenophilus* and *Methylobacterium*) and Gram-positive bacteria (*Rhodococcus*). The bacterial isolates *Sphingobium* sp. and *Methylobacterium* sp. have been commonly found in drinking water (*Simoes et al. 2007a, b, 2008; Kormas et al. 2009*). The bacterium *Rhodococcus* is not commonly found in drinking water, however the presence of this bacterium in chlorinated drinking water samples has been previously reported (*Norton & LeChevallier 2000*). The bacterium *Xenophilus*, which was found in the present study, has been rarely reported in drinking water and source waters (*Hoefel et al. 2005*). This study therefore provided an opportunity to explore biofilm and aggregate forming abilities of both common drinking water bacteria and bacteria that are unique to domestic drinking water.

The auto and coaggregation of bacterial isolates were studied to identify the existence of any potential species specific interactions. The result of the visual aggregation assay showed that the isolates did not form any flocs after mixing for up to 30 seconds. It has been reported previously that some bacteria need the presence of other bacteria to form flocs via a process of coaggregation (*Simoes et al. 2007b*). This may be due to the production of secondary metabolites by other organisms to form coaggregates (*Wimpenny & Colasanti 2004*). In our case however, no visible flocs were formed after mixing for 50 seconds when the isolates were combined to study coaggregation.
No visual autoaggregation or sedimentation by the bacterium *Xenophilus* was observed which agrees with the DAPI stained images in which no autoaggregation was observed even after 72 hours. However, according to Buswell et al. (1997) low visual aggregation scores may not necessarily indicate a lack of interaction. In this study, settlement was found after 24 hours for all autoaggregated cultures except *Xenophilus*. This suggests that there is some level of interaction which is not qualified using the scoring system described by Cisar et al. (1979). This is further evidenced by the qualitative measure of autoaggregation using the DAPI staining and microscopy (Figure 1). In this case, autoaggregation of the three bacteria (*Sphingobium, Methylobacterium* and *Rhodococcus*) was observed after 24 hours, which would promote the observed settlement. This highlights that the scores from visual aggregation assay need to be used with caution as they are not an accurate measurement of relative interaction strength between the cells (Buswell et al. 1997). This approach of combining DAPI staining with the visual aggregation assay has also been used by Simoes et al. (2008), who again found a higher degree of aggregation using the DAPI staining method than what was observed using the visual aggregation assay.

The autoaggregation and the preliminary study of coaggregation indicated that *Methylobacterium* might play a vital role in the formation of coaggregates and multispecies biofilm. Therefore, the other three isolates were combined with *Methylobacterium* for further coaggregation and biofilm studies. In this case, coaggregation was observed at longer time periods (after 24 hours) (Figure 2). The non-aggregating bacterium *Xenophilus* was also found to aggregate but only in the presence of *Methylobacterium*, which indicates the role of the later bacterium in cross species aggregate formation.

In order to understand the multispecies biofilm formation, both single and dual cultures biofilm assay was performed (Figure 3). All four bacteria, when grown individually to form biofilms in a 96 well microtitre plate, did not produce biofilms even after 72 hours, even though settlement was observed for the pure cultures after 24 hours. The contrast between aggregation (i.e. attachment in the liquid phase without a surface) and biofilm formation (attachment to a surface) is not currently known but is expected to be related to the surface characteristics of the microtitre plates. These plates were used however to provide a comparison with other studies in the literature via a standardised crystal violet biofilm assay. However despite the lack of biofilm formation for pure cultures, significant biofilm formation was found for dual species biofilms with *Methylobacterium* and *Rhodococcus* pairs forming the most biofilm.

The concept of enhanced aggregation and biofilm formation due to coculture, as shown in Figure 2 and Figure 3, has also been studied by Min & Rickard (2009) for *Sphingomonas natatoria* strains with *Micrococcus luteus*. In this case, Min & Rickard (2009) showed that naturally aggregating strain *Sphingomonas natatoria* 2.1gfp formed significant biofilms in the presence of *M. luteus*, whereas the non-coaggregating variant *Sphingomonas natatoria* 2.1COGgfp did not coaggregate in the presence of *M. luteus*, with only a few cells present in the dual biofilms formed. This species specific interaction result is in agreement with the present study in which *Sphingobium* sp. produced negligible amount of biofilm as a single species, whereas when grown with other partners more biofilm was produced. However, this enhanced biofilm formation was species specific with the greatest amount of biofilm formed in combination with *Methylobacterium* sp.

Interestingly, *Methylobacterium* produced more biofilm in the presence of all other combinations than as a pure culture. This suggests that *Methylobacterium* may play a role as a bridging bacterium. The concept of a bridging bacterium was also shown by Min & Rickard (2009) who found that *Sphingomonas natatoria* acted as bridging bacteria towards multispecies biofilm formation. In a related study, Simoes et al. (2008) reported that *Acinetobacter calcoaceticus* acted as a bridging bacteria in multispecies biofilm formation. The identification of different bacteria isolated from drinking water acting as a potential bridging bacterium indicates that biofilm formation in DWDS is influenced by various bacterium which acts as a bridging organism to influence the multispecies biofilm formation.

**CONCLUSIONS**

The formation of biofilm and aggregates in DWDS can have a negative impact on the microbiological quality of the
drinking water. In this study a visual aggregation assay and a DAPI staining method in combination with microscopy were used to study the ability of the common and unique bacterial isolate from domestic drinking water to form biofilms and aggregates as a pure culture and in combinations. The overall study reveals that the bacterium Methylobacterium sp. form autoaggregates and influenced the formation of coaggregates and multispecies biofilms by interacting with other bacteria including the non-aggregate forming (or non-flocculating) bacterium Xenophilus. This interesting finding therefore highlights the role of specific bridging bacteria within water distribution systems and new knowledge that may assist in developing strategies for biofilm control by focussing on controlling the bridging bacteria.

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