Coaggregation between *Rhodococcus* and *Acinetobacter* strains isolated from the food industry

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Abstract

In this study coaggregation interactions between *Rhodococcus* and *Acinetobacter* strains isolated from food processing surfaces were characterized. *Rhodococcus* sp. MF3727 formed intra-generic coaggregates with *Rhodococcus* sp. MF3803 and inter-generic coaggregates with two strains of *Acinetobacter calcoaceticus* (MF3293, MF3627). Stronger coaggregation between *Acinetobacter calcoaceticus* MF3727and *Rhodococcus* sp. MF3293 was observed following growth in batch-culture at 30 °C as opposed to 20 °C, after growth in Tryptic Soy broth as compared to liquid R2A medium, and between cells in exponential and early stationary phase as compared to late stationary phase. The coaggregation ability of *Rhodococcus* sp. MF3727 was maintained even after heat and Proteinase K treatment, suggesting the ability to coaggregate was protein independent while the coaggregation determinants of the other strains involved proteinaceous cell-surface-associated polymers. Coaggregation was stable at pH 5-9. The mechanisms of coaggregation among *Acinetobacter* and *Rhodococcus* strains bare similarity to that displayed by coaggregating bacteria of oral and freshwater origin, with respect to binding between proteinaceous and non-proteinaceous determinants and the effect of environmental factors on coaggregation. Coaggregation may contribute to biofilm formation on industrial food surfaces, which can protect bacteria against cleaning and disinfection.

Key words: Biofilm; coaggregation; cell-cell adhesion; lectin

Introduction

In natural and man-made environments, microorganisms often form multispecies biofilms, where the constituent microorganisms interact with each other to create dynamic and responsive communities (Costerton et al. 1995; Stoodley et al. 2002). The interactivity of the cells contributes to significant phenotypic differences, in comparison to their planktonic counterparts (Hojo et al. 2009; Kolenbrander and Phucas 1984). For example, biofilm bacteria are more tolerant to environmental stress and antimicrobials than planktonic bacteria (Gilbert et al. 2002; Mah and O'Toole 2001). For biofilm development to occur, bacteria first must adhere to surfaces. These adherent bacteria then produce extracellular polymeric substances (EPS) that can enhance the integration of other bacteria into the developing biofilm. During this developmental process, different species of bacteria can also specifically recognize and adhere to each other and this is described as coaggregation (Rickard et al. 2003). Coaggregation, that was originally thought to occur only among human dental plaque bacteria, has now been described to occur between bacteria isolated from numerous environments. These environments include human oral biofilms (Kolenbrander et al. 2006), the female urogenital tract (Ekmekci et al. 2009; Reid et al. 1988) , gastrointestinal tract (Schachtsiek et al. 2004; Tareb et al. 2013), freshwater systems (Rickard et al. 2002), and most recently in biofilms growing on showerheads (Vornhagen et al. 2013).

When considering coaggregation interactions, the vast majority of research has been focused on coaggregating human dental bacteria. Furthermore, human dental plaque is arguably the most studied multi-species biofilm and from nearly every cultured oral genera, representative species have been shown to coaggregate atintra-generic and/or inter-generic levels (Kolenbrander et al. 2010; Kolenbrander et al. 2006). Oral biofilms can contain up to several hundred different types of bacteria that are organized in highly spatially structured

arrangements. Different species occupy spatially distinct regions of an oral biofilm and are often associated with specific coaggregating partners. Spatial juxtaposition through coaggregation is advantageous as this enhances metabolic and cell-cell signalling interactions (Egland et al. 2004). Studies of the mechanisms involved in coaggregation have indicated that the interactions are typically mediated by a polysaccharide-containing receptor and a proteinaceous adhesin (Rickard et al. 2003). Although, protein adhesin-protein adhesin interactions have been also indicated to mediate coaggregations (Daep et al. 2008). Beyond studies of coaggregation between bacteria isolated from the human oral cavity, bacteria from aquatic systems have received considerable attention. Not only are these environmentally distinct studies of coaggregation but these investigations have highlighted certain potential roles of coaggregation. In particular, Min and Rickard (2009) showed that coaggregation between *Micrococcus* and *Sphingomonas* enhanced biofilm formation. Furthermore, work by Simões et al. (2008) have shown that *Acinetobacter calcoaceticus* coaggregates with numerous freshwater species and may act as a bridging organism between non-coaggregating strains and facilitate their retention in freshwater biofilms. Furthermore, in addition to altering biofilm development and biofilm community diversity, coaggregation has been proposed to protect partner species against disinfectants (Gilbert et al. 2002). Thus, studies of coaggregation between bacteria from a range of different environments have indicated that coaggregation may contribute to the enhanced colonization, retention, and protection of biofilm species. In context with the study described here, all these roles of coaggregation will conceivably have important implications for biofilm development in industrial food preparation environments.

The food industry is dependent on good hygienic practices to produce safe food of high quality. Microorganisms present on surfaces and equipment in the production

environments may contaminate food during processing. The majority of bacteria in the food production environment are non-pathogenic (Bagge-Ravn et al. 2003; Møretrø et al. 2013; Schirmer et al. 2013). These bacteria may be involved in reducing the quality of foods, but importantly, may also facilitate colonization and survival of pathogenic bacteria. As an example, *Acinetobacter calcoaceticus* has been shown to promote biofilm formation of the pathogenic bacterium *E. coli* O157:H7 (Habimana et al. 2010). *Acinetobacter* spp. and *Rhodococcus* spp. are frequently isolated from surfaces and equipment in the food industry (Bore and Langsrud 2005; Møretrø et al. 2013; Schirmer et al. 2013). *Acinetobacter* sp. may play a role in spoilage of foods (Barnes and Impey 1968; Hinton et al. 2004) and both *Acinetobacter* sp. and *Rhodococcus* sp. may be opportunistic pathogens (Bell et al. 1998; Towner 2009). Of particular interest also, *Acinetobacter calcoaceticus* strains isolated from aquatic systems (Simões et al. 2008) and phenol degrading granules (Adav et al. 2008) have previously been demonstrated to form coaggregates with bacteria from other genera.

In the present study we aimed to establish that coaggregation can occur between *Acinetobacter* and *Rhodococcus* isolated from food processing surfaces and, furthermore, to characterize the influence of environmental factors on coaggregation as well as the mechanisms involved in coaggregation. Of significance, our findings indicate that species belonging to these two genera have the potential to use coaggregation to recruit species into biofilms, such as those found on industrial food preparation surfaces, and this may be important for the integration and protection of pathogenic species.

Materials and methods

Bacterial strains

Strains of *Rhodococcus* and *Acinetobacte*r that were used in this study are shown in Table 1. The strains *Ac. calcoaceticus* MF3293 and *Rhodococcus* sp. MF3727 were subject to an initial coaggregation study and shown to coaggregate with each other (A.H. Rickard, not published). All strains, including *Ac. calcoaceticus* MF3293 and *Rhodococcus* sp. MF3727, were isolated from surfaces of equipment/machines in the Norwegian food industry. Species designation was done by 16S rDNA sequencing of 800-1450 bp, followed by BLAST sequence comparison searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Culturing and preparation of cell suspensions for aggregation studies

Unless otherwise stated, bacterial suspensions for aggregation measurements were prepared as follows: A single colony from Tryptone Soy Agar (TSA, Oxoid, Basingstoke, UK) was transferred to a tube with 5 ml Tryptone Soy Broth (TSB, Oxoid). The tube was incubated overnight in a shaking incubator (200 rpm) at 30 °C, before 500 µl was transferred into an Erlenmeyer flask containing 50 ml tempered (30 °C) TSB, and incubated at 30 °C for 18 h at 200 rpm. 45 ml of culture was harvested by centrifugation at 2000 g for 20 min, and washed three times with 45 ml deionized water. Then cells were re-suspended in coaggregation buffer ($CaCl_2$ (10^{-4} mol l^{-1}), $MgCl_2$ (10^{-4} mol l^{-1}) and NaCl (0.15 mol l^{-1}) dissolved in 0.001 mol l^{-1} Tris (hydroxymethyl) aminomethane, adjusted to pH 8.0 (Kolenbrander and Phucas 1984)) to a OD_{650nm} of 1.5. The resulting suspensions were tested for coaggregation and autoaggregation.

Aggregation assays

Visual aggregation assay

The degree of aggregation was determined visually by observing flocculation and sedimentation of a bacterial suspension under a magnifying lamp, using the methodology and ranking system of Cisar et al. (1979), with slight modifications. To evaluate whether the observed aggregation was a result of coaggregation, all individual strains were also tested for autoaggregation by the same procedure. Prepared suspensions (200 µl) from each of the pair of strains to be tested were added into a Silica Durham tube (Borosilicate Glass 12x75 mm, Fisher brand, Waltham, MA, USA). The suspension was vortexed, rolled slowly for 10 s and then left for 30 s at room temperature. Then the suspensions were investigated visually under a magnifying lamp immediately, and after 1 and 2 h. If aggregation occurs, the bacterial cells stick together and result in a relatively transparent suspension with high sedimentation of flocs. An aggregation score rating scheme was used: Score “0” - no observable flocs/coaggregates formed. Score “1” - very small flocs formed. Score “2” -formation of flocs that are not sedimentary, leaving a turbid suspension. Score “3”- generation of flocs that are sedimentary but with a suspension with some degree of turbidity. Score “4”- generation of large flocs that sediment immediately and result in a suspension that is very transparent in its upper parts.

Spectrophotometric aggregation assay

In addition to a visual assay, a quantitative optical density method was used to measure aggregation (Ekmekci et al. 2009). After preparing the suspensions, 0.5 ml of each strain to

be tested was transferred into a cuvette with total volume of 1 ml and mixed by pipette for 10 s. To determine autoaggregation, 1 ml suspensions of individual strains were used. Then the absorbance (650 nm) was read by spectrophotometer (Ultraspec 3000, Pharmacia Biospec, Cambridge, UK) immediately and also after leaving the suspension completely still for 1 and 2 h. The coaggregation percentage (after 2 h) was calculated as described previously (Ekmekci et al. 2009).

Effect of culturing conditions on coaggregation

To test the effect of culturing conditions on coaggregation, the strains *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 were cultivated under different conditions before coaggregation was tested. The tested conditions were growth in R2A or TSB medium at 20 or 30 °C. Cultures were harvested at exponential phase (OD_{600nm} of 0.5 (reached after 2 h and 3.5 h incubation for *Acinetobacter calcoaceticus* MF3293 and *Rhodococcus* sp. MF3727, respectively), and after 18 h and 42 h growth. The washing and re-suspension regime was as described above. This experiment and all the aggregation experiments described below were performed in triplicates. In all experiments coaggregation was determined both with the visual- and the spectrophotometric assay.

Effect of washing and re-suspension solution on coaggregation

Given that food processing biofilms may develop in different milieu, for example in water or more ionic solutions (e.g food residues) the impact of the presence of salts/ions were tested. To determine the effect of washing solution on coaggregation, suspensions of *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 were washed three times with either sterile dH_2O

185 or coaggregation buffer before re-suspension in coaggregation buffer and measurement of
186 coaggregation. To study the effect of the presence of salts during the coaggregation test,
187 coaggregation of *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 was determined for
188 bacterial suspensions washed with dH_2O and re-suspended in coaggregation buffer, 0.85%
189 NaCl or dH_2O, respectively.
190

191 Characterization of coaggregates

192

193 **Effect of heat treatment**

194 Proteinaceous coaggregation determinants will be inactivated by heat treatment. The effect
195 of heat treatment on coaggregation was studied. Cell suspensions (1.5 ml) were heat-treated
196 at 85 ^{o}C for 30 min (Eppendorf, Thermomixer 5436, Hamburg, Germany). The suspensions
197 were cooled in a water bath at 20 °C for 20 min and coaggregation of different pairs of heat-
198 treated and control suspensions (no heat treatment, but otherwise same treatment as heat
199 treated suspension) were determined.
200

201 **Effect of enzymatic treatment**

202 Enzymatic treatment may destabilize cell-surface associated polymers, and can indicate the
203 nature of the coaggregation determinants, e.g. inhibition of coaggregation by Proteinase K,
204 indicate that the coaggregation determinant is proteinaceous. The effect of different
205 enzymes on aggregation was tested according to previous research (Chaignon et al. 2007;
206 Schachtsiek et al. 2004), with modifications. Cells washed three times in dH_2O were re-
207 suspended to 1 x 10^8 cells ml^{-1} in buffer added enzymes. The following combinations of
208 buffers and enzymes were used: Proteinase K (1 g l^{-1}, Sigma Aldrich, Oslo, Norway) in 0.02

mol l^{-1} Tris (pH 7.5) + 0.1 mol l^{-1} NaCl, Dispersin B (40 mg l^{-1}, Kane Biotech Inc., Winnipeg, Canada) in PBS, DNAse I (0.1 g l^{-1}, Sigma) in 0.15 mol l^{-1} NaCl + 0.001 mol l^{-1} $CaCl_2$. The same volume of sterile de-ionized water was added to prepare control (untreated) suspensions. The suspensions were incubated in a shaker-incubator for 60 min at 37 °C, washed and re-suspended in coaggregation buffer. Coaggregation between different pairs of enzyme-treated and control suspensions was determined.

Effect of sugars

Sugars may inhibit coaggregation by competitive inhibition. The capability of different sugars (selected based on previous studies (Kolenbrander et al. 1993; Kolenbrander et al. 2006)) to reverse or inhibit the coaggregation was studied. Durham tubes or cuvettes containing a coaggregating pair was added each of the following sugars to a final concentration of 0.05 mol l^{-1}: Lactose monohydrate (Sigma), D (+) galactose (Sigma), α-L-fucose (Sigma), N-acetyl-D-galactosamine (Sigma), D (+) glucose (Merck, Oslo, Norway) and D-mannose (Sigma). Control was added sterile de-ionized water. The samples were vortexed for 10 s and aggregation determined.

Effect of chelating agents

The capability of different chelating agents to disperse or inhibit coaggregation was studied as described previously (Grimaudo et al. 1996; Malik et al. 2003). Durham tubes or cuvettes containing a coaggregating pair was added each of the following: EDTA (Merck 0.05 mol l^{-1}), EGTA (Merck, 0.05 mol l^{-1}) or citrate (Merck, 0.005 mol l^{-1}). A control sample was prepared by adding the same volume of sterile de-ionized water. The samples were vortexed for 10 seconds and coaggreation were determined.

Effect of pH

Fourteen tubes of cell suspensions were prepared separately for each pair of the coaggregating strains. The pH of each suspension was set to be from 1 to 14 (in increments of 1) by the addition of NaOH and HCl. Single-species cell suspensions were incubated for 20 min at room temperature. Subsequently, the different single-species suspensions of bacteria, with same pH, were combined and coaggregation evaluated.

Scanning electron microscopy

In order to visualize coaggregates, scanning electron microscopy (SEM) was performed on single-species and dual-species coaggregated suspensions. The initial fixation of bacteria was performed by incubation of bacterial cells (pre-grown in TSB at 30 °C for 18 h, and prepared for aggregation assays as described above) in 2.5% glutaraldehyde in PBS buffer (phosphate-buffered saline). A cover slip pretreated with poly-L- lysine was placed in the cell suspension for 30 min to allow the bacteria to attach. Loosely attached bacteria were removed by gentle rinsing in 1 ml sterile de-ionized water for a couple of seconds. The cover slip was dried in steps of 5 min in increasing concentrations of ethanol (70, 90, 96, 100 %). The step with 100% ethanol was repeated three times. The sample was dried by a critical point dryer (BAL-TEC CPD 030, BAL-TEC AG, Blazers, Germany). The dry samples were sputter-coated with (5-7 nm) gold/ palladium (Sputter Coater, Polaron SC 7640, Quorum Technologies Ltd, East Sussex, UK) before examination in the microscope (Zeiss EVO-50-EP, Carl Zeiss SMT Ltd, Cambridge, UK).

Results

Coaggregation of *Acinetobacter calcoaceticus* MF3293 and *Rhodococcus* sp. MF3727

Initially, coaggregation studies focused on the pair *Ac. calcoaceticus* MF3293 and *Rhodococcus* sp. M3727 which had previously been observed to coaggregate. This pair was studied in depth to create a standardized protocol for study of coaggregation between strains of *Rhodococcus* sp. and *Acinetobacter* sp. that had been isolated from food processing surfaces. Mixing of suspensions of *Ac. calcoaceticus* MF3293 and *Rhodococcus* sp. M3727 lead to formation of coaggregates/flocs followed by sedimentation (Fig. 1), which resulted in a decrease in optical density (Fig. 2). According to the visual classification scheme, a score of 4 was observed for this coaggregation pair. No autoaggregation was observed in the visual coaggregation assays (data not shown) while a small decrease in optical density was observed for single suspensions (Fig. 2). In general there was good correlation between the visual and spectrophotometric coaggregation tests, and all major observations in this work were supported by data from both measurement methods. Consequently, for this work we chose to focus and present mainly data based on the visual coaggregation test, as this is most widely used in other publications. Scanning electron microscopy revealed that the individual suspensions of *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 consisted of single or pairwise cells, while the mixed suspension contained large coaggregates (Fig. 3). *Acinetobacter calcoaceticus* MF3293 expressed clearly-visible surface-associated appendages, and the coaggregates appeared to be inter-connected by these appendages.

The impact of cultivation conditions of *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 on coaggregation was tested. Coaggregation was lower after 42 h growth, especially for *Rhodococcus* sp. MF3727, compared to exponential and 18 h growth

(Fig. 4). Higher coaggregation was found for cells grown in TSB compared to R2A and cells cultivated at higher temperature (30 vs 20 °C, data not shown). Figure 4 shows growth and coaggregation of cells from different growth phases at the conditions resulting in the strongest coaggregation (TSB at 30 °C). The same level of coaggregation was found for cells washed with dH_2O and coaggregation buffer. Cells that after the washing-step were re-suspended in coaggregation buffer or 0.85% NaCl had similar coaggregation (visual score 4), while cells re-suspended in dH_2O did not coaggregate (score 0). Based on the results presented above, we selected cultivation in TSB, at 30 °C for 18 h, washing cells with dH_2O, final re-suspension in coaggregation buffer and scoring of coaggregation after incubation for 2 h as standard conditions that were used for coaggregation tests for the rest of the study, unless otherwise stated.

Coaggregation among *Acinetobacter* and *Rhodococcus* from the food industry

Coaggregation was tested between three strains of *Rhodococcus* sp. (including MF3727) and seven strains of *Acinetobacter* (including MF3293, Table 1), all isolated from the food industry. Coaggregation was observed for three pairs, all involving *Rhodococcus* sp. MF3727: MF3727 + *Ac. calcoaceticus* MF3293 (visual score 4), MF3727 + *Ac. calcoaceticus* MF3627 (score 3) and MF3727 + *Rhodococcus* sp. MF3803 (score 2). No coaggregation was observed for the other 42 pairs tested (score 0) (data not shown). The strain pairs showing coaggregation were subjected to further studies.

Characterization of coaggregates

Effect of heat treatment and Proteinase K treatment

Given that coaggregation has been documented to involve heat and protease sensitive adhesins, the effect of heat and enzymes on coaggregation were tested. Heat treatment had no effect on the ability of *Rhodococcus* sp. MF3727 to form coaggregates with *Ac. calcoaceticus* MF3293, *Ac. calcoaceticus* MF3627 or *Rhodococcus* sp. MF3803, however heat treatment of the latter three strains completely inhibited (score 0) their ability to coaggregate with *Rhodococcus* sp. MF3727 (Table 2). Using Proteinase K treatments, visual coaggregation assays showed that Proteinase K significantly decreased the coaggregation ability of *Ac. calcoaceticus* (MF3627 and MF3293) and *Rhodococcus* sp. MF3803 (Table 2). On the other hand this enzyme had no effect on the ability of *Rhodococcus* sp. MF3727 to coaggregate with the other strains. Dispersin B and DNase I had no effect on coaggregation of any of the strains.

Effect of sugars

The potential for sugars to inhibit coaggregation, through competitively inhibiting adhesin-receptor interactions, was evaluated. N-acetylgalactoseamine led to a reduction of coaggregation between *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3627 from a visual score of 3 to 2, but had only minor adverse effects on coaggregation of the two other coaggregating pairs (data not shown). Lactose monohydrate inhibited coaggregation between *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 and between *Rhodococcus* sp. MF3727 and *Rhodococcus* sp. MF3803 by 17% and 15% according to the spectrophotometric assay, respectively, while no effects were observed in the visual assay. For D (+) galactose, α-L-fucose, D (+) glucose and D-mannose the registered inhibition was less than 10% in the spectrophotometric assay, while no effects were observed with the visual assay.

328

329 **Effect of chelating agents**

330 The dependency of cations on coaggregation was investigated by adding chelating agents to
331 coaggregating suspensions. It was observed that citrate slightly limited the coaggregation
332 ability (reduction from 4 to 3) of the coaggregating pair *Rhodococcus* sp. MF3727 + *Ac.*
333 *calcoaceticus* MF3293. No effects on coaggregation were observed with the visual assay for
334 EDTA and EGTA, while a 4-18% inhibition of coaggregation was observed in the
335 spectrophotometric test.

336

337 **Effect of pH**

338 The effect of pH on coaggregation was evaluated by combining suspensions of coaggregating
339 cells with similar pH for the range pH 1-14. The results showed that coaggregation was stable
340 at pH 5-9 for all three pairs of bacteria tested. For two of the pairs, coaggregation decreased
341 at higher pH, while for the pair *Rhodococcus* sp. MF3727 + *Rhodococcus* sp. MF3803,
342 coaggregation increased at low pH (Fig. 5). It should be noted that cells tested outside a pH-
343 range of 3-11 were observed to undergo lysis and no coaggregation occurred.

344

Discussion

In this work, strain-specific coaggregation was observed *in vitro* between *Rhodococcus* and *Acinetobacter* isolates from food production environments. In order to identify coaggregation interactions between strains from these genera we optimized the sensitivity for testing coaggregation using a model pair of strains isolated from food processing surfaces and explored the parameters that influenced the strength of expression of coaggregation. This yielded insight, concerning conditions that may allow food processing bacteria to coaggregate, which allowed additional strains to be tested. Two of these additional strains, that were isolated from different food processing surfaces, were also shown to coaggregate suggesting that coaggregation may be common to biofilms containing *Rhodococcus* and *Acinetobacter*. For *Acinetobacter,* coaggregation seemed to differ between species as both strains of *Ac. calcoaceticus* coaggregated*,* while coaggregation was not observed for strains from other species of *Acinetobacter*. Although there have been many studies of multispecies biofilms involving bacteria from the food industry (for a review, see Srey et al. 2013), to our knowledge this is the first time coaggregation among bacteria from food industry has been reported. Also, we are not aware of reports describing coaggregation between *Acinetobacter* sp. and *Rhodococcus* sp. from other sources, but the ability of *Acinetobacter* to coaggregate with *Oligotropha carboxidovorans*, *Methylobacterium* sp. and *Staphylococcus* sp. has previously been reported (Malik et al. 2003; Simões et al. 2008).

Our results from heat and Proteinase K treatments of bacterial suspensions indicated that the coaggregation polymers expressed by *Rhodococcus* sp. MF3803 and *Ac. calcoaceticus* MF3293 and MF3627 were proteinaceous, while the coaggregation polymers

368 of *Rhodococcus* sp. MF3727 were non-proteinaceous. The protein structures involved in
369 coaggregation of *Ac. calcoaceticus* MF3293 might be part of the appendages or fimbriae
370 observed by scanning electron microscopy (Fig. 3). It has previously been indicated that
371 *Acinetobacter* has proteinaceous cell-surface expressed coaggregation polymers (Malik et al.
372 2003; Simões et al. 2008). Reports indicate that coaggregation between bacteria isolated
373 from the human oral cavity and between bacteria isolated from drinking water is mediated
374 by cell-surface expressed adhesins (proteinacious lectin-like polymers) and cell-surface
375 expressed receptors (polysaccharides containing polymers)(Kolenbrander 1988; Rickard et al.
376 2004). Based on this it is likely that the coaggregation determinant of *Rhodococcus* sp.
377 MF3727 is a polysaccharide. A schematic overview of the proposed adhesin-receptor based
378 coaggregation between *Rhodococcus* and *Acinetobacter* is shown in Figure 6. Previous
379 studies of coaggregation between oral and between freshwater bacteria, as well as between
380 bacteria from a other environments, has shown that certain simple sugars are able to
381 competitively inhibit adhesin-receptor coaggregation interactions (Kolenbrander 1988;
382 Kolenbrander et al. 1993). In this study, however only limited inhibition was observed by
383 adding selected sugars to the coaggregating suspensions. Even if N-acetyl-D-galactosamine
384 and lactose exerted a slightly inhibitory effect on some coaggregation pairs involving
385 *Rhodococcus* sp. MF3727, other coaggregation pairs involving *Rhodococcus* sp. MF3727 were
386 unaffected. Thus the sugar involved in the coaggregation binding was not identified.
387 However, it cannot be ruled out that the coaggregation associated polymer was a
388 polysaccharide, because sugars other than those tested in the present study may inhibit the
389 interaction. Other sugars could be tested in future studies, but we did test the activity of
390 dispersin B. While dispersin B did not have any inhibitory effect either, the absence of a
391 discernable inhibitory effect of adding dispersin B to coaggregates, does not necessarily

exclude the possibility of a polysaccharide coaggregation polymer. This is because dispersin B cleavage is specific for β 1,6 N-acetylglucosamine (Kaplan et al. 2004) and will not be active against all polysaccharides. As heat treated *Rhodococcus* sp. MF3727 could form coaggregates, this shows that bacteria may coaggregate even if they are inactivated. This is because the cell wall with its cell-surface expressed polysaccharides may still remain relatively unaffected by heat treatment. However, it is possible that bacteria may disintegrate during other types of control measures, e.g. disinfection, so dead cells may not be able to coaggregate on all occasions.

Coaggregation was relatively stable within a pH range relevant for food and food production. The effects on coaggregation observed at extreme acidity/alkalinity can be explained by alterations in the charge of proteins and carbohydrates that may affect electrostatic interactions important for coaggregation (Min et al. 2010). The strains *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 showed strongest coaggregation when suspended in coaggregation buffer or 0.85% NaCl, while no coaggregation occurred in dH_2O. This indicates that the presence of ions is important for the coaggregation binding. However it is not clear whether anions or cations are most important since the chelators citrate, EDTA and EGTA only had limited effect on coaggregation. Ionic strength of the surroundings can affect electrostatic interactions between the surface-expressed appendages of the coaggregation partners (Bos et al. 1999). Bacteria in the food industry are likely to be exposed to ions from e.g. food residues, salts added to foods, tap water and cleaning agents and disinfectants.

Coaggregation between *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 was stronger under conditions with high growth rate (TSB vs R2A, 30 vs 20 °C, exponentially/early stationary vs late stationary growth). It is possible that the cell-surface-associated polymers

that mediate coaggregation are more highly expressed under conditions with high growth. Changes in growth temperature may affect the expression of structures on cell surface that mediate coaggregation or adhesion (Amano et al. 2001; Briandet et al. 1999). Also studies by other researchers have indicated that stronger coaggregation interactions occur between bacteria grown at higher temperatures (Jenkinson et al. 1990; Joe et al. 2009) and in more nutritious growth media (Burdman et al. 1998; McIntire et al. 1978). Coaggregation was observed under conditions relevant for the food industry. Food residues will be present on many surfaces and will provide nutrients for bacterial growth. The temperature will vary between types of production and within a processing plant but temperatures around 20-30 °C are not uncommon, e.g. in areas where food are heated and during cleaning and disinfection.

The increased prevalence of antibiotic resistant *Acinetobacter* in foods is of concern (Guerra et al. 2014). Although *Acinetobacter* is not frequently identified as the cause of microbial spoilage, it has been associated with spoilage of meat, seafood and poultry stored at cooling temperatures (Barnes and Impey 1968; Hinton et al. 2004). *Rhodococcus equi* causes mainly lung associated infections in immunocompromised individuals, but also other species of *Rhodococcus*, such as *R. erythropolis* have been reported to cause infections (Bell et al. 1998). *Acinetobacter* and *Rhodococcus* species are commonly found in food processing plants, and have the ability to form biofilms (Aaku et al. 2004; Bore and Langsrud 2005; Lewis et al. 1989; Møretrø et al. 2013; Schirmer et al. 2013). *Acinetobacter calcoaceticus* has been shown to promote biofilm formation by the pathogenic bacteria *Escherichia coli* O157:H7, however the mechanisms involved were not investigated (Habimana et al. 2010). Also the food borne pathogenic bacterium *Listeria monocytogenes* is a relatively poor biofilm former (Møretrø et al. 2013), which adhesion and biofilm formation may be induced in multispecies

biofilms (Bremer et al. 2001; Hassan et al. 2004). Given the available literature it is likely that species of *Acinetobacter* and *Rhodococcus* may promote biofilm recruitment of each other and other species including pathogenic bacteria and this could be in part due to coaggregation interactions. For other environments coaggregation has been shown to occur between bacteria co-isolated from the same biofilm, leading to the assumption that the ability to coaggregate is advantageous for growth and survival in such environments (Kolenbrander 1988; Rickard et al. 2003). Also it has been shown that coaggregation has a positive effect on biofilm formation in multispecies biofilm (Min and Rickard 2009).

Bacteria adhere to each other, to equipment, and to food. The biofilms that form are difficult to remove, act as a source for spreading of microorganisms to uncolonized surfaces and act as a shelter against cleaning and disinfection (Bridier et al. 2015; Srey et al. 2013). A better understanding of cooperation and interactions in biofilms, such as coaggregation, may facilitate the development of improved and targeted strategies for control of biofilms. Enzymatic treatment can degrade the biofilm matrix (Srey et al. 2013) and can also be a strategy for breaking up coaggregates. In particular, while the information generated from this early study is useful and will help direct future studies of coaggregation between bacterial isolates from the food industry, more research is necessary to reveal how often and under what conditions coaggregation occurs among other frequently isolated bacterial species from the food industry. Ultimately, a key question, for which this work sets the rationale to investigate, is whether bacterial coaggregation influences the growth, retention, and survival of pathogenic species in food processing facilities.

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636

637 **Table 1. Bacterial strains of *Acinetobacter* and *Rhodococcus* used in this study**

Bacterial genera	Species designation (% identity)[†]	Strain number*	Origin	Reference
Rhodococcus	*erythropolis/ qingshengii* (100)	3727	Drain, small scale cheese producer	(Schirmer et al. 2013)
Rhodococcus	*erythropolis/ qingshengii* (100)	3803	Floor, small scale cheese producer	(Schirmer et al. 2013)
Rhodococcus	*erythropolis/ qingshengii* (100)	4633	Slicing machine, meat processing plant	T. Møretrø, not published
Acinetobacter	*calcoaceticus* (100)	3293	Disinfecting footbath with hypochlorite, dairy	(Langsrud et al. 2006)
Acinetobacter	*calcoaceticus* (99)	3627	Platform evisceration, meat slaughterhouse	(Møretrø et al. 2013)
Acinetobacter	*johnsonii* (99)	4091	Conveyor belt, salmon processing plant	E. Heir, not published
Acinetobacter	*johnsonii* (99)	4112	Filet machine, salmon processing plant	E. Heir, not published
Acinetobacter	*guilloniae* (99)	4117	Conveyor belt, salmon processing plant	E. Heir, not published
Acinetobacter	*johnsonii* (99)	4130	Conveyor belt, salmon processing plant	E. Heir, not published
Acinetobacter	*bouvetii/johnsonii* (98)	4206	Conveyor belt, salmon slaughterhouse	E. Heir, not published

638 *Numbers refer to MF number in Nofima strain collection.

† Species with highest 16S rDNA sequence similarities to known sequences using BLAST, National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast) are shown. Comparisons and reported percent identity (in parenthesis) are based on partial 16S rDNA sequences of 800 to 1450 bp. Where more than one species are reported, identical 16S rDNA matches to the reported species were obtained.

Table 2. Coaggregation (visual score) of pairs of *Rhodococcus* sp. MF3727 and *Acinetobacter calcoaceticus* MF3293, *Ac. calcoaceticus* MF3627 or *Rhodococcus* sp. MF3803 pretreated with heat or Proteinase K or untreated (control)

		MF3293
		MF3627
		MF3803
MF3727		
		Control
		Heat*
		ProtK†
		Control
		Heat
		ProtK
		Control
		Heat
		ProtK
Control		
		4‡
		0
		1
		3
		0
		1
		2
		0
		0

Heat		
		4
		0
		NT[§]
		3
		0
		NT
		2
		0
		NT
ProtK		
		4
		NT
		1
		3
		NT
		1
		2
		NT
		0

*Heat treated (85° C, 30 min)

[†]Proteinase K treated

[‡]Data shown are visual coaggregation scores after 2h, the same scores were obtained for three independent experiments

[§]Not tested

Figure captions:

Figure 1. Coaggregation between *Rhodococcus* sp. MF3727 and *Acinetobacter calcoaceticus* MF3293. The pictures show tubes with coaggregate formation and sedimentation A: immediately after mixing the strains. B: after 10 min C: after 30 min. D: after 2 h.

Figure 2. Decreased optical density after incubation of individual and mixed suspensions of *Rhodococcus* sp. MF3727 and *Acinetobacter calcoaceticus* MF3293. Data represent means and standard deviations from three independent replicates.

Figure 3. Scanning electron microscopy of A: individual suspension of *Rhodococcus* sp. MF3727; B: individual suspension of *Acinetobacter calcoaceticus* MF3293; C-F. Mixed suspension of MF3727 and MF3293. The arrows refer to appendages on the cell surfaces. A-C 10,000 X magnification, D 20,000 X, E 60,000 X, E 40,000 X.

Figure 4. Growth (**a**) and coaggregation (**b**) of *Rhodococcus* sp. MF3727 and *Acinetobacter calcoaceticus* MF3293 in TSB medium at 30° C. Bacteria were harvested at three time points: A; Exponential growth OD_{600nm}=0.5 (reached after 2h and 3.5 h incubation for MF3293 and MF3727, respectively), B: 18h, C: 42 h. Combinations of cells from various growth phases (A, B, C) were tested for coaggregation. Data presented are based on the visual coaggregation score. The same scores were obtained in three independent experiments.

Figure 5. Coaggregation at different pH of of *Rhodococcus* sp. MF3727 combined in pairs with *Acinetobacter calcoaceticus* MF3293 (■), *Acinetobacter calcoaceticus* MF3627 (□) or

720 *Rhodococcus sp.* MF3803 (嬭), respectively. Data presented are visual coaggregation scores.
721 The same scores were obtained in three independent experiments.
722
723 **Figure 6.** Schematic presentation of the proposed receptor-adhesin mediated coaggregation
724 between *Rhodococcus* spp. and *Acinetobacter* spp. Three of the strains have proteinaceous
725 adhesins (cup-shaped structures) that interact with the receptors (squares) of *Rhodococcus*
726 sp. MF 3727, leading to the formation of coaggregates, while the other three strains are
727 examples of strains that do not have adhesins (cup-shaped structures) interacting with the
728 receptors of *Rhodococcus* sp. MF3727, thus no coaggregation can occur. *Rhodococcus* strains
729 are shown in red and *Acinetobacter* strains are blue. Cell sizes and cell shapes are not to scale
730 and approximate.
731