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

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24 Abstract

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

42

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

44

45

46 **Introduction**

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

64 When considering coaggregation interactions, the vast majority of research has been
65 focused on coaggregating human dental bacteria. Furthermore, human dental plaque is
66 arguably the most studied multi-species biofilm and from nearly every cultured oral genera,
67 representative species have been shown to coaggregate at intra-generic and/or inter-generic
68 levels (Kolenbrander et al. 2010; Kolenbrander et al. 2006). Oral biofilms can contain up to
69 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

94 environments may contaminate food during processing. The majority of bacteria in the food
95 production environment are non-pathogenic (Bagge-Ravn et al. 2003; Mørretrø et al. 2013;
96 Schirmer et al. 2013). These bacteria may be involved in reducing the quality of foods, but
97 importantly, may also facilitate colonization and survival of pathogenic bacteria. As an
98 example, *Acinetobacter calcoaceticus* has been shown to promote biofilm formation of the
99 pathogenic bacterium *E. coli* O157:H7 (Habimana et al. 2010). *Acinetobacter* spp. and
100 *Rhodococcus* spp. are frequently isolated from surfaces and equipment in the food industry
101 (Bore and Langsrud 2005; Mørretrø et al. 2013; Schirmer et al. 2013). *Acinetobacter* sp. may
102 play a role in spoilage of foods (Barnes and Impey 1968; Hinton et al. 2004) and both
103 *Acinetobacter* sp. and *Rhodococcus* sp. may be opportunistic pathogens (Bell et al. 1998;
104 Towner 2009). Of particular interest also, *Acinetobacter calcoaceticus* strains isolated from
105 aquatic systems (Simões et al. 2008) and phenol degrading granules (Adav et al. 2008) have
106 previously been demonstrated to form coaggregates with bacteria from other genera.
107 In the present study we aimed to establish that coaggregation can occur between
108 *Acinetobacter* and *Rhodococcus* isolated from food processing surfaces and, furthermore, to
109 characterize the influence of environmental factors on coaggregation as well as the
110 mechanisms involved in coaggregation. Of significance, our findings indicate that species
111 belonging to these two genera have the potential to use coaggregation to recruit species
112 into biofilms, such as those found on industrial food preparation surfaces, and this may be
113 important for the integration and protection of pathogenic species.

114

115

116 **Materials and methods**

117

118 **Bacterial strains**

119 Strains of *Rhodococcus* and *Acinetobacter* that were used in this study are shown in Table 1.

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

126

127 **Culturing and preparation of cell suspensions for aggregation studies**

128 Unless otherwise stated, bacterial suspensions for aggregation measurements were
129 prepared as follows: A single colony from Tryptone Soy Agar (TSA, Oxoid, Basingstoke, UK)
130 was transferred to a tube with 5 ml Tryptone Soy Broth (TSB, Oxoid). The tube was
131 incubated overnight in a shaking incubator (200 rpm) at 30 °C, before 500 µl was transferred
132 into an Erlenmeyer flask containing 50 ml tempered (30 °C) TSB, and incubated at 30 °C for
133 18 h at 200 rpm. 45 ml of culture was harvested by centrifugation at 2000 g for 20 min, and
134 washed three times with 45 ml deionized water. Then cells were re-suspended in
135 coaggregation buffer (CaCl_2 (10^{-4} mol l $^{-1}$), MgCl_2 (10^{-4} mol l $^{-1}$) and NaCl (0.15 mol l $^{-1}$) dissolved
136 in 0.001 mol l $^{-1}$ Tris (hydroxymethyl) aminomethane, adjusted to pH 8.0 (Kolenbrander and
137 Phucas 1984)) to a OD_{650nm} of 1.5. The resulting suspensions were tested for coaggregation
138 and autoaggregation.

139

140 **Aggregation assays**

141

142 **Visual aggregation assay**

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

159

160 **Spectrophotometric aggregation assay**

161 In addition to a visual assay, a quantitative optical density method was used to measure
162 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 dH₂O

186 or coaggregation buffer before re-suspension in coaggregation buffer and measurement of
187 coaggregation. To study the effect of the presence of salts during the coaggregation test,
188 coaggregation of *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 was determined
189 for bacterial suspensions washed with dH₂O and re-suspended in coaggregation buffer,
190 0.85% NaCl or dH₂O, respectively.

191

192 **Characterization of coaggregates**

193

194 **Effect of heat treatment**

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

201

202 **Effect of enzymatic treatment**

203 Enzymatic treatment may destabilize cell-surface associated polymers, and can indicate the
204 nature of the coaggregation determinants, e.g. inhibition of coaggregation by Proteinase K,
205 indicate that the coaggregation determinant is proteinaceous. The effect of different
206 enzymes on aggregation was tested according to previous research (Chaignon et al. 2007;
207 Schachtsiek et al. 2004), with modifications. Cells washed three times in dH₂O were re-
208 suspended to 1 x 10⁸ cells ml⁻¹ in buffer added enzymes. The following combinations of
209 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 CaCl₂. 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.

216

217 **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.

226

227 **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 coaggregation were determined.

234

235 Effect of pH

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

241

242 Scanning electron microscopy

243 In order to visualize coaggregates, scanning electron microscopy (SEM) was performed on
244 single-species and dual-species coaggregated suspensions. The initial fixation of bacteria was
245 performed by incubation of bacterial cells (pre-grown in TSB at 30 °C for 18 h, and prepared
246 for aggregation assays as described above) in 2.5% glutaraldehyde in PBS buffer (phosphate-
247 buffered saline). A cover slip pretreated with poly-L- lysine was placed in the cell suspension
248 for 30 min to allow the bacteria to attach. Loosely attached bacteria were removed by gentle
249 rinsing in 1 ml sterile de-ionized water for a couple of seconds. The cover slip was dried in
250 steps of 5 min in increasing concentrations of ethanol (70, 90, 96, 100 %). The step with
251 100% ethanol was repeated three times. The sample was dried by a critical point dryer (BAL-
252 TEC CPD 030, BAL-TEC AG, Blazers, Germany). The dry samples were sputter-coated with (5-
253 7 nm) gold/ palladium (Sputter Coater, Polaron SC 7640, Quorum Technologies Ltd, East
254 Sussex, UK) before examination in the microscope (Zeiss EVO-50-EP, Carl Zeiss SMT Ltd,
255 Cambridge, UK).

256 Results

257

258 **Coaggregation of *Acinetobacter calcoaceticus* MF3293 and *Rhodococcus* sp.**259 **MF3727**260 Initially, coaggregation studies focused on the pair *Ac. calcoaceticus* MF3293 and261 *Rhodococcus* sp. M3727 which had previously been observed to coaggregate. This pair was

262 studied in depth to create a standardized protocol for study of coaggregation between

263 strains of *Rhodococcus* sp. and *Acinetobacter* sp. that had been isolated from food264 processing surfaces. Mixing of suspensions of *Ac. calcoaceticus* MF3293 and *Rhodococcus* sp.

265 M3727 lead to formation of coaggregates/flocs followed by sedimentation (Fig. 1), which

266 resulted in a decrease in optical density (Fig. 2). According to the visual classification

267 scheme, a score of 4 was observed for this coaggregation pair. No autoaggregation was

268 observed in the visual coaggregation assays (data not shown) while a small decrease in

269 optical density was observed for single suspensions (Fig. 2). In general there was good

270 correlation between the visual and spectrophotometric coaggregation tests, and all major

271 observations in this work were supported by data from both measurement methods.

272 Consequently, for this work we chose to focus and present mainly data based on the visual

273 coaggregation test, as this is most widely used in other publications. Scanning electron

274 microscopy revealed that the individual suspensions of *Rhodococcus* sp. MF3727 and *Ac.*275 *calcoaceticus* MF3293 consisted of single or pairwise cells, while the mixed suspension276 contained large coaggregates (Fig. 3). *Acinetobacter calcoaceticus* MF3293 expressed clearly-

277 visible surface-associated appendages, and the coaggregates appeared to be inter-

278 connected by these appendages.

279 The impact of cultivation conditions of *Rhodococcus* sp. MF3727 and *Ac.*280 *calcoaceticus* MF3293 on coaggregation was tested. Coaggregation was lower after 42 h

281 growth, especially for *Rhodococcus* sp. MF3727, compared to exponential and 18 h growth
282 (Fig. 4). Higher coaggregation was found for cells grown in TSB compared to R2A and cells
283 cultivated at higher temperature (30 vs 20 °C, data not shown). Figure 4 shows growth and
284 coaggregation of cells from different growth phases at the conditions resulting in the
285 strongest coaggregation (TSB at 30 °C). The same level of coaggregation was found for cells
286 washed with dH₂O and coaggregation buffer. Cells that after the washing-step were re-
287 suspended in coaggregation buffer or 0.85% NaCl had similar coaggregation (visual score 4),
288 while cells re-suspended in dH₂O did not coaggregate (score 0). Based on the results
289 presented above, we selected cultivation in TSB, at 30 °C for 18 h, washing cells with dH₂O,
290 final re-suspension in coaggregation buffer and scoring of coaggregation after incubation for
291 2 h as standard conditions that were used for coaggregation tests for the rest of the study,
292 unless otherwise stated.

293

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

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

302

303 **Characterization of coaggregates**

304

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.

317

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

328 less than 10% in the spectrophotometric assay, while no effects were observed with the
329 visual assay.

330

331 **Effect of chelating agents**

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

338

339 **Effect of pH**

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

346

347

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

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

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

403 Coaggregation was relatively stable within a pH range relevant for food and food
404 production. The effects on coaggregation observed at extreme acidity/alkalinity can be
405 explained by alterations in the charge of proteins and carbohydrates that may affect
406 electrostatic interactions important for coaggregation (Min et al. 2010). The strains
407 *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 showed strongest coaggregation
408 when suspended in coaggregation buffer or 0.85% NaCl, while no coaggregation occurred in
409 dH₂O. This indicates that the presence of ions is important for the coaggregation binding.
410 However it is not clear whether anions or cations are most important since the chelators
411 citrate, EDTA and EGTA only had limited effect on coaggregation. Ionic strength of the
412 surroundings can affect electrostatic interactions between the surface-expressed
413 appendages of the coaggregation partners (Bos et al. 1999). Bacteria in the food industry are
414 likely to be exposed to ions from e.g. food residues, salts added to foods, tap water and
415 cleaning agents and disinfectants.

416 Coaggregation between *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 was
417 stronger under conditions with high growth rate (TSB vs R2A, 30 vs 20 °C, exponentially/early
418 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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640 **Table 1. Bacterial strains of *Acinetobacter* and *Rhodococcus* used in this study**

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

			plant	published
<i>Acinetobacter</i>	<i>bouvetii/johnsonii</i> (98)	4206	Conveyor belt, salmon	E. Heir, not
			slaughterhouse	published

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

642 [†] Species with highest 16S rDNA sequence similarities to known sequences using BLAST, National Center for Biotechnology Information

643 (<http://blast.ncbi.nlm.nih.gov/Blast>) are shown. Comparisons and reported percent identity (in parenthesis) are based on partial 16S rDNA

644 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

657 **Figure captions:**

658
659 **Figure 1.** Coaggregation between *Rhodococcus* sp. MF3727 and *Acinetobacter calcoaceticus*
660 MF3293. The pictures show tubes with coaggregate formation and sedimentation A:
661 immediately after mixing the strains. B: after 10 min C: after 30 min. D: after 2 h.

662
663 **Figure 2.** Decreased optical density after incubation of individual and mixed suspensions of
664 *Rhodococcus* sp. MF3727 and *Acinetobacter calcoaceticus* MF3293. Data represent means
665 and standard deviations from three independent replicates.

666
667 **Figure 3.** Scanning electron microscopy of A: individual suspension of *Rhodococcus* sp.
668 MF3727; B: individual suspension of *Acinetobacter calcoaceticus* MF3293; C-F. Mixed
669 suspension of MF3727 and MF3293. The arrows refer to appendages on the cell surfaces. A-
670 C 10,000 X magnification, D 20,000 X, E 60,000 X, E 40,000 X.

671
672 **Figure 4.** Growth (a) and coaggregation (b) of *Rhodococcus* sp. MF3727 and *Acinetobacter*
673 *calcoaceticus* MF3293 in TSB medium at 30° C. Bacteria were harvested at three time points:
674 A; Exponential growth OD_{600nm}=0.5 (reached after 2h and 3.5 h incubation for MF3293 and
675 MF3727, respectively), B: 18h, C: 42 h. Combinations of cells from various growth phases (A,
676 B, C) were tested for coaggregation. Data presented are based on the visual coaggregation
677 score. The same scores were obtained in three independent experiments.

678
679 **Figure 5.** Coaggregation at different pH of of *Rhodococcus* sp. MF3727 combined in pairs
680 with *Acinetobacter calcoaceticus* MF3293 (■), *Acinetobacter calcoaceticus* MF3627 (□) or

681 *Rhodococcus* sp. MF3803 (▲), respectively. Data presented are visual coaggregation scores.
682 The same scores were obtained in three independent experiments.
683
684 **Figure 6.** Schematic presentation of the proposed receptor-adhesin mediated coaggregation
685 between *Rhodococcus* spp. and *Acinetobacter* spp. Three of the strains have proteinaceous
686 adhesins (cup-shaped structures) that interact with the receptors (squares) of *Rhodococcus*
687 sp. MF 3727, leading to the formation of coaggregates, while the other three strains are
688 examples of strains that do not have adhesins (cup-shaped structures) interacting with the
689 receptors of *Rhodococcus* sp. MF3727, thus no coaggregation can occur. *Rhodococcus* strains
690 are shown in red and *Acinetobacter* strains are blue. Cell sizes and cell shapes are not to
691 scale and approximate.

Fig.1.

Fig 4a

Fig 4b

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