1 **Coaggregation between *Rhodococcus* and *Acinetobacter* strains isolated from**

2 **the food industry**

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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* MF3727and *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.

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

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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

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69 arrangements. Different species occupy spatially distinct regions of an oral biofilm and are

70 often associated with specific coaggregating partners. Spatial juxtaposition through

71 coaggregation is advantageous as this enhances metabolic and cell-cell signalling interactions

72 (Egland et al. 2004). Studies of the mechanisms involved in coaggregation have indicated that

73 the interactions are typically mediated by a polysaccharide-containing receptor and a

74 proteinaceous adhesin (Rickard et al. 2003). Although, protein adhesin-protein adhesin

75 interactions have been also indicated to mediate coaggregations (Daep et al. 2008). Beyond

76 studies of coaggregation between bacteria isolated from the human oral cavity, bacteria from

77 aquatic systems have received considerable attention. Not only are these environmentally

78 distinct studies of coaggregation but these investigations have highlighted certain potential

79 roles of coaggregation. In particular, Min and Rickard (2009) showed that coaggregation

80 between *Micrococcus* and *Sphingomonas* enhanced biofilm formation. Furthermore, work by

81 Simões et al. (2008) have shown that *Acinetobacter calcoaceticus* coaggregates with

82 numerous freshwater species and may act as a bridging organism between non-

83 coaggregating strains and facilitate their retention in freshwater biofilms. Furthermore, in

84 addition to altering biofilm development and biofilm community diversity, coaggregation has

85 been proposed to protect partner species against disinfectants (Gilbert et al. 2002). Thus,

86 studies of coaggregation between bacteria from a range of different environments have

87 indicated that coaggregation may contribute to the enhanced colonization, retention, and

88 protection of biofilm species. In context with the study described here, all these roles of

89 coaggregation will conceivably have important implications for biofilm development in

90 industrial food preparation environments.

91   The food industry is dependent on good hygienic practices to produce safe food of

92 high quality. Microorganisms present on surfaces and equipment in the production

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93 environments may contaminate food during processing. The majority of bacteria in the food

94 production environment are non-pathogenic (Bagge-Ravn et al. 2003; Møretrø et al. 2013;

95 Schirmer et al. 2013). These bacteria may be involved in reducing the quality of foods, but

96 importantly, may also facilitate colonization and survival of pathogenic bacteria. As an

97 example, *Acinetobacter calcoaceticus* has been shown to promote biofilm formation of the

98 pathogenic bacterium *E. coli* O157:H7 (Habimana et al. 2010). *Acinetobacter* spp. and

99 *Rhodococcus* spp. are frequently isolated from surfaces and equipment in the food industry

100 (Bore and Langsrud 2005; Møretrø et al. 2013; Schirmer et al. 2013). *Acinetobacter* sp. may

101 play a role in spoilage of foods (Barnes and Impey 1968; Hinton et al. 2004) and both

102 *Acinetobacter* sp. and *Rhodococcus* sp. may be opportunistic pathogens (Bell et al. 1998;

103 Towner 2009). Of particular interest also, *Acinetobacter calcoaceticus* strains isolated from

104 aquatic systems (Simões et al. 2008) and phenol degrading granules (Adav et al. 2008) have

105 previously been demonstrated to form coaggregates with bacteria from other genera.

106 In the present study we aimed to establish that coaggregation can occur between

107 *Acinetobacter* and *Rhodococcus* isolated from food processing surfaces and, furthermore, to

108 characterize the influence of environmental factors on coaggregation as well as the

109 mechanisms involved in coaggregation. Of significance, our findings indicate that species

110 belonging to these two genera have the potential to use coaggregation to recruit species into

111 biofilms, such as those found on industrial food preparation surfaces, and this may be

112 important for the integration and protection of pathogenic species.

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Materials and methods

Bacterial strains

Strains of *Rhodococcus* and *Acinetobacter* 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.

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Aggregation assays

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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.

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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

162   be tested was transferred into a cuvette with total volume of 1 ml and mixed by pipette for

163   10 s. To determine autoaggregation, 1 ml suspensions of individual strains were used. Then

164   the absorbance (650 nm) was read by spectrophotometer (Ultraspec 3000, Pharmacia

165   Biospec, Cambridge, UK) immediately and also after leaving the suspension completely still

166   for 1 and 2 h. The coaggregation percentage (after 2 h) was calculated as described

167   previously (Ekmekci et al. 2009).

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169   **Effect of culturing conditions on coaggregation**

170   To test the effect of culturing conditions on coaggregation, the strains *Rhodococcus* sp.

171   MF3727 and *Ac. calcoaceticus* MF3293 were cultivated under different conditions before

172   coaggregation was tested. The tested conditions were growth in R2A or TSB medium at 20 or

173   30 °C. Cultures were harvested at exponential phase (OD_{600nm} of 0.5 (reached after 2 h and

174   3.5 h incubation for *Acinetobacter calcoaceticus* MF3293 and *Rhodococcus* sp. MF3727,

175   respectively), and after 18 h and 42 h growth. The washing and re-suspension regime was as

176   described above. This experiment and all the aggregation experiments described below were

177   performed in triplicates. In all experiments coaggregation was determined both with the

178   visual- and the spectrophotometric assay.

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180   **Effect of washing and re-suspension solution on coaggregation**

181   Given that food processing biofilms may develop in different milieu, for example in water or

182   more ionic solutions (e.g food residues) the impact of the presence of salts/ions were tested.

183   To determine the effect of washing solution on coaggregation, suspensions of *Rhodococcus*

184   sp. MF3727 and *Ac. calcoaceticus* MF3293 were washed three times with either sterile dH_2O

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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.

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191   **Characterization of coaggregates**

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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 °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.

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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 1×10^8 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

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209   mol l^{-1} Tris (pH 7.5) + 0.1 mol l^{-1} NaCl, Dispersin B (40 mg l^{-1}, Kane Biotech Inc., Winnipeg,

210   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

211   volume of sterile de-ionized water was added to prepare control (untreated) suspensions.

212   The suspensions were incubated in a shaker-incubator for 60 min at 37 ^{o}C, washed and re-

213   suspended in coaggregation buffer. Coaggregation between different pairs of enzyme-treated

214   and control suspensions was determined.

215

216   **Effect of sugars**

217   Sugars may inhibit coaggregation by competitive inhibition. The capability of different sugars

218   (selected based on previous studies (Kolenbrander et al. 1993; Kolenbrander et al. 2006)) to

219   reverse or inhibit the coaggregation was studied. Durham tubes or cuvettes containing a

220   coaggregating pair was added each of the following sugars to a final concentration of 0.05

221   mol l^{-1}: Lactose monohydrate (Sigma), D (+) galactose (Sigma), α-L-fucose (Sigma), N-acetyl-

222   D-galactosamine (Sigma), D (+) glucose (Merck, Oslo, Norway) and D-mannose (Sigma).

223   Control was added sterile de-ionized water. The samples were vortexed for 10 s and

224   aggregation determined.

225

226   **Effect of chelating agents**

227   The capability of different chelating agents to disperse or inhibit coaggregation was studied

228   as described previously (Grimaudo et al. 1996; Malik et al. 2003). Durham tubes or cuvettes

229   containing a coaggregating pair was added each of the following: EDTA (Merck 0.05 mol l^{-1}),

230   EGTA (Merck, 0.05 mol l^{-1}) or citrate (Merck, 0.005 mol l^{-1}). A control sample was prepared by

231   adding the same volume of sterile de-ionized water. The samples were vortexed for 10

232   seconds and coaggreation were determined.

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Effect of pH

235 Fourteen tubes of cell suspensions were prepared separately for each pair of the

236 coaggregating strains. The pH of each suspension was set to be from 1 to 14 (in increments

237 of 1) by the addition of NaOH and HCl. Single-species cell suspensions were incubated for 20

238 min at room temperature. Subsequently, the different single-species suspensions of bacteria,

239 with same pH, were combined and coaggregation evaluated.

240

Scanning electron microscopy

242 In order to visualize coaggregates, scanning electron microscopy (SEM) was performed on

243 single-species and dual-species coaggregated suspensions. The initial fixation of bacteria was

244 performed by incubation of bacterial cells (pre-grown in TSB at 30 °C for 18 h, and prepared

245 for aggregation assays as described above) in 2.5% glutaraldehyde in PBS buffer (phosphate-

246 buffered saline). A cover slip pretreated with poly-L- lysine was placed in the cell suspension

247 for 30 min to allow the bacteria to attach. Loosely attached bacteria were removed by gentle

248 rinsing in 1 ml sterile de-ionized water for a couple of seconds. The cover slip was dried in

249 steps of 5 min in increasing concentrations of ethanol (70, 90, 96, 100 %). The step with

250 100% ethanol was repeated three times. The sample was dried by a critical point dryer (BAL-

251 TEC CPD 030, BAL-TEC AG, Blazers, Germany). The dry samples were sputter-coated with (5-7

252 nm) gold/ palladium (Sputter Coater, Polaron SC 7640, Quorum Technologies Ltd, East Sussex,

253 UK) before examination in the microscope (Zeiss EVO-50-EP, Carl Zeiss SMT Ltd, Cambridge,

254 UK).

Results

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

MF3727

259 Initially, coaggregation studies focused on the pair *Ac. calcoaceticus* MF3293 and

260 *Rhodococcus* sp. M3727 which had previously been observed to coaggregate. This pair was

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

262 strains of *Rhodococcus* sp. and *Acinetobacter* sp. that had been isolated from food processing

263 surfaces. Mixing of suspensions of *Ac. calcoaceticus* MF3293 and *Rhodococcus* sp. M3727

264 lead to formation of coaggregates/flocs followed by sedimentation (Fig. 1), which resulted in

265 a decrease in optical density (Fig. 2). According to the visual classification scheme, a score of

266 4 was observed for this coaggregation pair. No autoaggregation was observed in the visual

267 coaggregation assays (data not shown) while a small decrease in optical density was

268 observed for single suspensions (Fig. 2). In general there was good correlation between the

269 visual and spectrophotometric coaggregation tests, and all major observations in this work

270 were supported by data from both measurement methods. Consequently, for this work we

271 chose to focus and present mainly data based on the visual coaggregation test, as this is

272 most widely used in other publications. Scanning electron microscopy revealed that the

273 individual suspensions of *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 consisted

274 of single or pairwise cells, while the mixed suspension contained large coaggregates (Fig. 3).

275 *Acinetobacter calcoaceticus* MF3293 expressed clearly-visible surface-associated

276 appendages, and the coaggregates appeared to be inter-connected by these appendages.

277   The impact of cultivation conditions of *Rhodococcus* sp. MF3727 and *Ac.*

278 *calcoaceticus* MF3293 on coaggregation was tested. Coaggregation was lower after 42 h

279 growth, especially for *Rhodococcus* sp. MF3727, compared to exponential and 18 h growth

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280 (Fig. 4). Higher coaggregation was found for cells grown in TSB compared to R2A and cells

281 cultivated at higher temperature (30 vs 20 °C, data not shown). Figure 4 shows growth and

282 coaggregation of cells from different growth phases at the conditions resulting in the

283 strongest coaggregation (TSB at 30 °C). The same level of coaggregation was found for cells

284 washed with dH_2O and coaggregation buffer. Cells that after the washing-step were re-

285 suspended in coaggregation buffer or 0.85% NaCl had similar coaggregation (visual score 4),

286 while cells re-suspended in dH_2O did not coaggregate (score 0). Based on the results

287 presented above, we selected cultivation in TSB, at 30 °C for 18 h, washing cells with dH_2O,

288 final re-suspension in coaggregation buffer and scoring of coaggregation after incubation for

289 2 h as standard conditions that were used for coaggregation tests for the rest of the study,

290 unless otherwise stated.

291

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

293 Coaggregation was tested between three strains of *Rhodococcus* sp. (including MF3727) and

294 seven strains of *Acinetobacter* (including MF3293, Table 1), all isolated from the food

295 industry. Coaggregation was observed for three pairs, all involving *Rhodococcus* sp. MF3727:

296 MF3727 + *Ac. calcoaceticus* MF3293 (visual score 4), MF3727 + *Ac. calcoaceticus* MF3627

297 (score 3) and MF3727 + *Rhodococcus* sp. MF3803 (score 2). No coaggregation was observed

298 for the other 42 pairs tested (score 0) (data not shown). The strain pairs showing

299 coaggregation were subjected to further studies.

300

301 **Characterization of coaggregates**

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303 **Effect of heat treatment and Proteinase K treatment**

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304   Given that coaggregation has been documented to involve heat and protease sensitive

305   adhesins, the effect of heat and enzymes on coaggregation were tested. Heat treatment had

306   no effect on the ability of *Rhodococcus* sp. MF3727 to form coaggregates with *Ac.*

307   *calcoaceticus* MF3293, *Ac. calcoaceticus* MF3627 or *Rhodococcus* sp. MF3803, however heat

308   treatment of the latter three strains completely inhibited (score 0) their ability to

309   coaggregate with *Rhodococcus* sp. MF3727 (Table 2). Using Proteinase K treatments, visual

310   coaggregation assays showed that Proteinase K significantly decreased the coaggregation

311   ability of *Ac. calcoaceticus* (MF3627 and MF3293) and *Rhodococcus* sp. MF3803 (Table 2). On

312   the other hand this enzyme had no effect on the ability of *Rhodococcus* sp. MF3727 to

313   coaggregate with the other strains. Dispersin B and DNase I had no effect on coaggregation

314   of any of the strains.

315

316   **Effect of sugars**

317   The potential for sugars to inhibit coaggregation, through competitively inhibiting adhesin-

318   receptor interactions, was evaluated. N-acetylgalactoseamine led to a reduction of

319   coaggregation between *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3627 from a visual

320   score of 3 to 2, but had only minor adverse effects on coaggregation of the two other

321   coaggregating pairs (data not shown). Lactose monohydrate inhibited coaggregation

322   between *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 and between *Rhodococcus*

323   sp. MF3727 and *Rhodococcus* sp. MF3803 by 17% and 15% according to the

324   spectrophotometric assay, respectively, while no effects were observed in the visual assay.

325   For D (+) galactose, α-L-fucose, D (+) glucose and D-mannose the registered inhibition was

326   less than 10% in the spectrophotometric assay, while no effects were observed with the

327   visual assay.

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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

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.

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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

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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

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392   exclude the possibility of a polysaccharide coaggregation polymer. This is because dispersin B

393   cleavage is specific for β 1,6 N-acetylglucosamine (Kaplan et al. 2004) and will not be active

394   against all polysaccharides. As heat treated *Rhodococcus* sp. MF3727 could form

395   coaggregates, this shows that bacteria may coaggregate even if they are inactivated. This is

396   because the cell wall with its cell-surface expressed polysaccharides may still remain

397   relatively unaffected by heat treatment. However, it is possible that bacteria may disintegrate

398   during other types of control measures, e.g. disinfection, so dead cells may not be able to

399   coaggregate on all occasions.

400   Coaggregation was relatively stable within a pH range relevant for food and food

401   production. The effects on coaggregation observed at extreme acidity/alkalinity can be

402   explained by alterations in the charge of proteins and carbohydrates that may affect

403   electrostatic interactions important for coaggregation (Min et al. 2010). The strains

404   *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 showed strongest coaggregation

405   when suspended in coaggregation buffer or 0.85% NaCl, while no coaggregation occurred in

406   dH_2O. This indicates that the presence of ions is important for the coaggregation binding.

407   However it is not clear whether anions or cations are most important since the chelators

408   citrate, EDTA and EGTA only had limited effect on coaggregation. Ionic strength of the

409   surroundings can affect electrostatic interactions between the surface-expressed

410   appendages of the coaggregation partners (Bos et al. 1999). Bacteria in the food industry are

411   likely to be exposed to ions from e.g. food residues, salts added to foods, tap water and

412   cleaning agents and disinfectants.

413   Coaggregation between *Rhodococcus* sp. MF3727 and *Ac. calcoaceticus* MF3293 was

414   stronger under conditions with high growth rate (TSB vs R2A, 30 vs 20 °C, exponential/early

415   stationary vs late stationary growth). It is possible that the cell-surface-associated polymers

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416   that mediate coaggregation are more highly expressed under conditions with high growth.

417   Changes in growth temperature may affect the expression of structures on cell surface that

418   mediate coaggregation or adhesion (Amano et al. 2001; Briandet et al. 1999). Also studies by

419   other researchers have indicated that stronger coaggregation interactions occur between

420   bacteria grown at higher temperatures (Jenkinson et al. 1990; Joe et al. 2009) and in more

421   nutritious growth media (Burdman et al. 1998; McIntire et al. 1978). Coaggregation was

422   observed under conditions relevant for the food industry. Food residues will be present on

423   many surfaces and will provide nutrients for bacterial growth. The temperature will vary

424   between types of production and within a processing plant but temperatures around 20-30

425   °C are not uncommon, e.g. in areas where food are heated and during cleaning and

426   disinfection.

427   The increased prevalence of antibiotic resistant *Acinetobacter* in foods is of concern

428   (Guerra et al. 2014). Although *Acinetobacter* is not frequently identified as the cause of

429   microbial spoilage, it has been associated with spoilage of meat, seafood and poultry stored

430   at cooling temperatures (Barnes and Impey 1968; Hinton et al. 2004). *Rhodococcus equi*

431   causes mainly lung associated infections in immunocompromised individuals, but also other

432   species of *Rhodococcus*, such as *R. erythropolis* have been reported to cause infections (Bell

433   et al. 1998). *Acinetobacter* and *Rhodococcus* species are commonly found in food processing

434   plants, and have the ability to form biofilms (Aaku et al. 2004; Bore and Langsrud 2005; Lewis

435   et al. 1989; Møretrø et al. 2013; Schirmer et al. 2013). *Acinetobacter calcoaceticus* has been

436   shown to promote biofilm formation by the pathogenic bacteria *Escherichia coli* O157:H7,

437   however the mechanisms involved were not investigated (Habimana et al. 2010). Also the

438   food borne pathogenic bacterium *Listeria monocytogenes* is a relatively poor biofilm former

439   (Møretrø et al. 2013), which adhesion and biofilm formation may be induced in multispecies

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440   biofilms (Bremer et al. 2001; Hassan et al. 2004). Given the available literature it is likely that

441   species of *Acinetobacter* and *Rhodococcus* may promote biofilm recruitment of each other

442   and other species including pathogenic bacteria and this could be in part due to

443   coaggregation interactions. For other environments coaggregation has been shown to occur

444   between bacteria co-isolated from the same biofilm, leading to the assumption that the

445   ability to coaggregate is advantageous for growth and survival in such environments

446   (Kolenbrander 1988; Rickard et al. 2003). Also it has been shown that coaggregation has a

447   positive effect on biofilm formation in multispecies biofilm (Min and Rickard 2009).

448   Bacteria adhere to each other, to equipment, and to food. The biofilms that form are

449   difficult to remove, act as a source for spreading of microorganisms to uncolonized surfaces

450   and act as a shelter against cleaning and disinfection (Bridier et al. 2015; Srey et al. 2013). A

451   better understanding of cooperation and interactions in biofilms, such as coaggregation, may

452   facilitate the development of improved and targeted strategies for control of biofilms.

453   Enzymatic treatment can degrade the biofilm matrix (Srey et al. 2013) and can also be a

454   strategy for breaking up coaggregates. In particular, while the information generated from

455   this early study is useful and will help direct future studies of coaggregation between

456   bacterial isolates from the food industry, more research is necessary to reveal how often and

457   under what conditions coaggregation occurs among other frequently isolated bacterial

458   species from the food industry. Ultimately, a key question, for which this work sets the

459   rationale to investigate, is whether bacterial coaggregation influences the growth, retention,

460   and survival of pathogenic species in food processing facilities.

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463   **Acknowledgements**

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References

Aaku, E.N., Collison, E.K., Gashe, B.A., and Mpuchane, S. 2004. Microbiological quality of milk from two processing plants in Gaborone Botswana. Food Control **15**(3): 181-186. doi: 10.1016/s0956-7135(03)00031-8.

Adav, S.S., Lee, D.-J., and Lai, J.-Y. 2008. Intergeneric coaggregation of strains isolated from phenol-degrading aerobic granules. Appl. Microbiol. Biotechnol. **79**(4): 657-661. doi: 10.1007/s00253-008-1456-8.

Amano, A., Premaraj, T., Kuboniwa, M., Nakagawa, I., Shizukuishi, S., Morisaki, I., and Hamada, S. 2001. Altered antigenicity in periodontitis patients and decreased adhesion of *Porphyromonas gingivalis* by environmental temperature stress. Oral Microbiol. Immunol. **16**(2): 124-128. doi: 10.1034/j.1399-302x.2001.016002124.x.

Bagge-Ravn, D., Ng, Y., Hjelm, M., Christiansen, J.N., Johansen, C., and Gram, L. 2003. The microbial ecology of processing equipment in different fish industries - analysis of the microflora during processing and following cleaning and disinfection. Int. J. Food Microbiol. **87**(3): 239-250.

Barnes, E.M., and Impey, C.S. 1968. Psychrophilic spoilage bacteria of poultry. J. Appl. Bacteriol. **31**(1): 97-&. doi: 10.1111/j.1365-2672.1968.tb00345.x.

Bell, K.S., Philp, J.C., Aw, D.W.J., and Christofi, N. 1998. A review - The genus *Rhodococcus*. J. Appl. Microbiol. **85**(2): 195-210.

Bore, E., and Langsrud, S. 2005. Characterization of micro-organisms isolated from dairy industry after cleaning and fogging disinfection with alkyl amine and peracetic acid. J. Appl. Microbiol. **98**(1): 96-105. doi: 10.1111/j.1365-2672.2004.02436.x.

Bos, R., van der Mei, H.C., and Busscher, H.J. 1999. Physico-chemistry of initial microbial adhesive interactions - its mechanisms and methods for study. FEMS Microbiol. Rev. **23**(2): 179-230. doi: 10.1016/s0168-6445(99)00004-2.

Bremer, P.J., Monk, I., and Osborne, C.M. 2001. Survival of *Listeria monocytogenes* attached to stainless steel surfaces in the presence or absence of *Flavobacterium* spp.. J. Food Prot. **64(**9): 1369-1376.

Briandet, R., Meylheuc, T., Maher, C., and Bellon-Fontaine, M.N. 1999. *Listeria monocytogenes* Scott A: Cell surface charge, hydrophobicity, and electron donor and acceptor characteristics under different environmental growth conditions. Appl. Environ. Microbiol. **65**(12): 5328-5333.

Bridier, A., Sanchez-Vizuete, P., Guilbaud, M., Piard, J.C., Naitali, M., and Briandet, R. 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiol. **45**: 167-178. doi: 10.1016/j.fm.2014.04.015.

Burdman, S., Jurkevitch, E., Schwartsburd, B., Hampel, M., and Okon, Y. 1998. Aggregation in *Azospirillum brasilense*: effects of chemical and physical factors and involvement of extracellular components. Microbiology-Uk **144**: 1989-1999.

Chaignon, P., Sadovskaya, I., Ragunah, C., Ramasubbu, N., Kaplan, J.B., and Jabbouri, S. 2007. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. Appl. Microbiol. Biotechnol. **75**(1): 125-132. doi: 10.1007/s00253-006-0790-y.

Cisar, J.O., Kolenbrander, P.E., and McIntire, F.C. 1979. Specificity of coaggregation reaction between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii* Infect. Immun. **24**(3): 742-752.

22

516 Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. 1995.
517 Microbial biofilms. Annu. Rev. Microbiol. **49**: 711-742.
518 Daep, C.A., Lamont, R.J., and Demuth, D.R. 2008. Interaction of *Porphyromonas gingivalis*
519 with oral streptococci requires a motif that resembles the eukaryotic nuclear receptor box
520 protein-protein interaction domain. Infect. Immun. **76**(7): 3273-3280. doi: 10.1128/iai.00366-
521 08.
522 Egland, P.G., Palmer, R.J., and Kolenbrander, P.E. 2004. Interspecies communication in
523 *Streptococcus gordonii-Veillonella atypica* biofilms: Signaling in flow conditions requires
524 juxtaposition. Proc. Natl. Acad. Sci. USA **101**(48): 16917-16922. doi:
525 10.1073/pnas.0407457101.
526 Ekmekci, H., Aslim, B., and Ozturk, S. 2009. Characterization of vaginal lactobacilli
527 coaggregation ability with *Escherichia coli*. Microbiol. Immunol. **53**(2): 59-65. doi:
528 10.1111/j.1348-0421.2009.00115.x.
529 Gilbert, P., Maira-Litran, T., McBain, A.J., Rickard, A.H., and Whyte, F.W. 2002. The physiology
530 and collective recalcitrance of microbial biofilm communities. Adv. Microb. Physiol. **46**: 202-
531 256.
532 Grimaudo, N.J., Nesbitt, W.E., and Clark, W.B. 1996. Coaggregation of *Candida albicans* with
533 oral *Actinomyces* species. Oral Microbiol. Immunol. **11**(1): 59-61. doi: 10.1111/j.1399-
534 302X.1996.tb00337.x.
535 Guerra, B., Fischer, J., and Helmuth, R. 2014. An emerging public health problem: Acquired
536 carbapenemase-producing microorganisms are present in food-producing animals, their
537 environment, companion animals and wild birds. Vet. Microbiol. **171**(3-4): 290-297. doi:
538 10.1016/j.vetmic.2014.02.001.
539 Habimana, O., Heir, E., Langsrud, S., Asli, A.W., and Møretrø, T. 2010. Enhanced surface
540 colonization by *Escherichia coli* O157:H7 in biofilms formed by an *Acinetobacter*
541 *calcoaceticus* Isolate from meat-processing environments. Appl. Environ. Microbiol. **76**(13):
542 4557-4559. doi: 10.1128/aem.02707-09.
543 Hassan, A.N., Birt, D.M., and Frank, J F. 2004. Behavior of *Listeria monocytogenes* in a
544 *Pseudomonas putida* biofilm on a condensate-forming surface. J. Food Prot. **67**(2): 322-327.
545 Hinton, A., Cason, J.A., and Ingram, K.D. 2004. Tracking spoilage bacteria in commercial
546 poultry processing and refrigerated storage of poultry carcasses. Int. J. Food Microbiol. **91**(2):
547 155-165. doi: 10.1016/s0168-1605(03)00377-5.
548 Hojo, K., Nagaoka, S., Ohshima, T., and Maeda, N. 2009. Bacterial interactions in dental
549 biofilm development. J. Dent. Res. **88**(11): 982-990. doi: 10.1177/0022034509346811.
550 Jenkinson, H.F., Lala, H.C., and Shepherd, M.G. 1990. Coaggregation of *Streptococcus sanguis*
551 and other streptococci with *Candida albicans*. Infect. Immun. **58**(5): 1429-1436.
552 Joe, M.M., Jaleel, C.A., Sivakumar, P.K., Zhao, C.-X., and Karthikeyan, B. 2009. Co-aggregation
553 in *Azospirillum brasilensense* MTCC-125 with other PGPR strains: Effect of physical and
554 chemical factors and stress endurance ability. J. Taiwan Inst. Chem. Eng. **40**(5): 491-499. doi:
555 10.1016/j.jtice.2009.02.006.
556 Kaplan, J.B., Velliyagounder, K., Ragunath, C., Rohde, H., Mack, D., Knobloch, J.K.M., and
557 Ramasubbu, N. 2004. Genes involved in the synthesis and degradation of matrix
558 polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus*
559 *pleuropneumoniae* biofilms. J. Bacteriol. **186**(24): 8213-8220. doi: 10.1128/jb.186.24.8213-
560 8220.2004.
561 Kolenbrander, P.E. 1988. Intergeneric coaggregation among human oral bacteria and ecology
562 of dental plaque. Annu. Rev. Microbiol. **42**: 627-656.

23

563 Kolenbrander, P.E., Ganeshkumar, N., Cassels, F.J., and Hughes, C.V. 1993. Coaggregation -
564 specific adherence among human oral plaque bacteria. FASEB J. **7**(5): 406-413.
565 Kolenbrander, P.E., Palmer, R.J., Jr., Periasamy, S., and Jakubovics, N.S. 2010. Oral multispecies
566 biofilm development and the key role of cell-cell distance. Nat. Rev. Microbiol. **8**(7): 471-480.
567 doi: 10.1038/nrmicro2381.
568 Kolenbrander, P.E., Palmer, R.J., Rickard, A.H., Jakubovics, N.S., Chalmers, N.I., and Diaz, P.I.
569 2006. Bacterial interactions and successions during plaque development. Periodontol. 2000
570 **42**: 47-79. doi: 10.1111/j.1600-0757.2006.00187.x.
571 Kolenbrander, P.E., and Phucas, C.S. 1984. Effect of saliva on coaggregation of oral
572 *Actinomyces* and *Streptococcus* species. Infect. Immun. **44**(2): 228-233.
573 Langsrud, S., Seifert, L., and Møretrø, T. 2006. Characterization of the microbial flora in
574 disinfecting footbaths with hypochlorite. J. Food. Protec. **69**(9): 2193-2198.
575 Lewis, S.J., Gilmour, A., and Johnston, D.E. 1989. Factors influencing the detachment of a
576 polymer-associated *Acinetobacter* sp from stainless-steel. Int. J. Food Microbiol. **8**(2): 155-
577 164. doi: 10.1016/0168-1605(89)90070-6.
578 Mah, T.F.C., and O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial
579 agents. Trends Microbiol. **9**(1): 34-39. doi: 10.1016/s0966-842x(00)01913-2.
580 Malik, A., Sakamoto, M., Ono, T., and Kakii, K. 2003. Coaggregation between *Acinetobacter*
581 *johnsonii* S35 and *Microbacterium esteraromaticum* strains isolated from sewage activated
582 sludge. J. Biosci. Bioeng. **96**(1): 10-15. doi: 10.1016/s1389-1723(03)90090-9.
583 McIntire, F.C., Vatter, A.E., Baros, J., and Arnold, J. 1978. Mechanism of co-aggregation
584 between *Actinomyces viscosus* t14v and *Streptococcus sanguis* 34. Infect. Immun. **21**(3): 978-
585 988.
586 Min, K.R., and Rickard, A.H. 2009. Coaggregation by the freshwater bacterium *Sphingomonas*
587 *natatoria* alters dual-species biofilm formation. Appl. Environ. Microbiol. **75**(12): 3987-3997.
588 doi: 10.1128/aem.02843-08.
589 Min, K.R., Zimmer, M.N., and Rickard, A.H. 2010. Physicochemical parameters influencing
590 coaggregation between the freshwater bacteria *Sphingomonas natatoria* 2.1 and
591 *Micrococcus luteus* 2.13. Biofouling **26**(8): 931-940. doi: 10.1080/08927014.2010.531128.
592 Møretrø, T., Langsrud, S., and Heir, E. 2013. Bacteria on meat abattoir meat production
593 process surfaces after sanitation: Characterisation of survival properties of *Listeria*
594 *monocytogenes* and the commensal bacterial flora. Adv. Microbiol. **3**: 255-264.
595 Reid, G., McGroarty, J.A., Angotti, R., and Cook, R.L. 1988. *Lactobacillus* inhibitor production
596 against *Escherichia coli* and coaggregation ability with uropathogens. Can. J. Microbiol. **34**(3):
597 344-351.
598 Rickard, A.H., Gilbert, P., and Handley, P.S. 2004. Influence of growth environment on
599 coaggregation between freshwater biofilm bacteria. J. Appl. Microbiol. **96**(6): 1367-1373. doi:
600 10.1111/j.1365-2672.2004.02297.x.
601 Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E., and Handley, P.S. 2003. Bacterial
602 coaggregation: an integral process in the development of multi-species biofilms. Trends
603 Microbiol. **11**(2): 94-100. doi: 10.1016/s0966-842x(02)00034-3.
604 Rickard, A.H., Leach, S.A., Hall, L.S., Buswell, C.M., High, N.J., and Handley, P.S. 2002.
605 Phylogenetic relationships and coaggregation ability of freshwater biofilm bacteria. Appl.
606 Environ. Microbiol. **68**(7): 3644-3650. doi: 10.1128/aem.68.7.3644-3650.2002.
607 Schachtsiek, M., Hammes, W.P., and Hertel, C. 2004. Characterization of *Lactobacillus*
608 *coryniformis* DSM 20001(T) surface protein Cpf mediating coaggregation with and

24

aggregation among pathogens. Appl. Environ. Microbiol. **70**(12): 7078-7085. doi: 10.1128/aem.70.12.7078-7085.2004.

Schirmer, B.C.T., Heir, E., Møretrø, T., Skaar, I., and Langsrud, S. 2013. Microbial background flora in small-scale cheese production facilities does not inhibit growth and surface attachment of *Listeria monocytogenes*. J. Dairy Sci. **96**(10): 6161-6171. doi: 10.3168/jds.2012-6395.

Simões, L.C., Simões, M., and Vieira, M.J. 2008. Intergeneric coaggregation among drinking water bacteria: Evidence of a role for *Acinetobacter calcoaceticus* as a bridging bacterium. Appl. Environ. Microbiol. **74**(4): 1259-1263. doi: 10.1128/aem.01747-07.

Srey, S., Jahid, I.K., and Ha, S.-D. 2013. Biofilm formation in food industries: A food safety concern. Food Control **31**(2): 572-585. doi: 10.1016/j.foodcont.2012.12.001.

Stoodley, P., Sauer, K., Davies, D.G., and Costerton, J.W. 2002. Biofilms as complex differentiated communities. Annu. Rev. Microbiol. **56**: 187-209. doi: 10.1146/annurev.micro.56.012302.160705.

Tareb, R., Bernardeau, M., Gueguen, M., and Vernoux, J.-P. 2013. In vitro characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic *Lactobacillus* strains and interaction with foodborne zoonotic bacteria, especially *Campylobacter jejuni*. J. Med. Microbiol. **62**: 637-649. doi: 10.1099/jmm.0.049965-0.

Towner, K.J. 2009. Acinetobacter: an old friend, but a new enemy. J. Hosp. Infect. **73**(4): 355-363. doi: 10.1016/j.jhin.2009.03.032.

Vornhagen, J., Stevens, M., McCormick, D.W., Dowd, S.E., Eisenberg, J.N.S., Boles, B.R., and Rickard, A.H. 2013. Coaggregation occurs amongst bacteria within and between biofilms in domestic showerheads. Biofouling **29**(1): 53-68. doi: 10.1080/08927014.2012.744395.

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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.

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

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

641   sequences of 800 to 1450 bp. Where more than one species are reported, identical 16S rDNA matches to the reported species were obtained.

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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)

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

Line	Label	Value
668	Heat	
669		4
670		0
671		NT§
672		3
673		0
674		NT
675		2
676		0
677		NT
678	ProtK	
679		4
680		NT
681		1
682		3
683		NT
684		1
685		2
686		NT
687		0

688 *Heat treated (85° C, 30 min)

689 [†]Proteinase K treated

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

691 [§]Not tested

692
693
694
695

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 (◼), 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
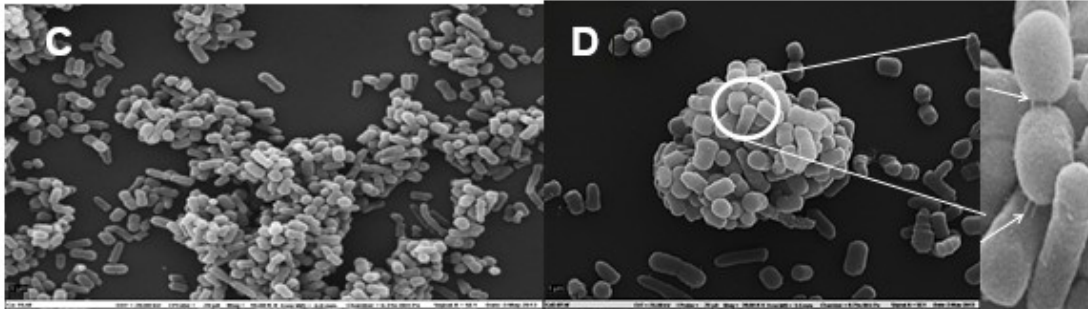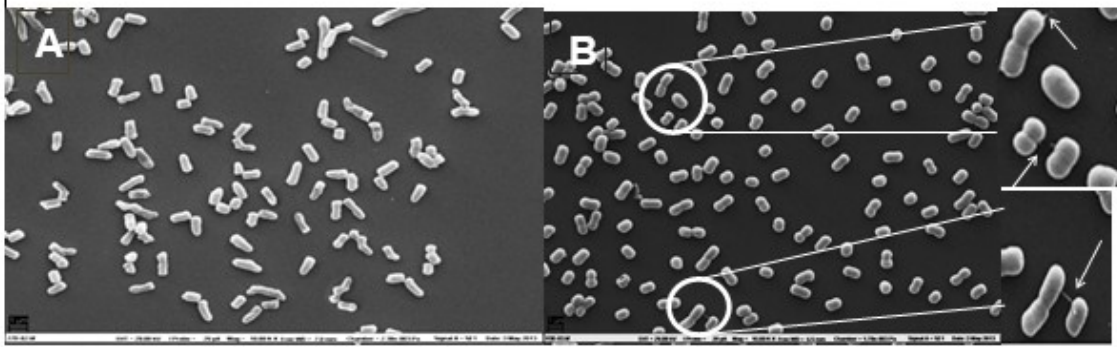
730   and approximate.

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Fig.1.

Fig. 2.

Fig. 3.

Fig 4a

Fig 4b

Fig. 5

Fig. 6