

1 **Degradation of *N*-acyl- L-homoserine-lactones by *Bacillus cereus* in culture media and**  
2 **pork extract**

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12 Running title: Degradation of AHL by *Bacillus cereus*

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25 **Degradation of *N*-acyl-L-homoserine-lactones by *Bacillus cereus* in culture media and**  
26 **pork extract**

27

28 **Abstract**

29 Degradation of the quorum sensing signal molecule *N*-acyl-L-homoserine lactones (AHL) in  
30 co-cultures was verified with *Bacillus cereus* and *Yersinia enterocolitica* in culture media  
31 and in pork extract. Results showed evidence of microbial interaction when the AHL  
32 degrading bacterium and AHL producing bacterium were co-cultured in a food simulating  
33 condition.

34

35 **Keywords:** quorum sensing, *Yersinia enterocolitica*, *Bacillus cereus*, *Paenibacillus*  
36 *polymyxa*, *N*-acyl-L-homoserine lactone, lactonase

37 The term “quorum sensing” has been proposed to describe the ability of bacteria to monitor  
38 their own population density and modulate gene expression accordingly (17). This  
39 communication system uses chemical signal molecules called autoinducers, which are  
40 produced and released by the bacterial cell. Intracellular response occurs when concentration  
41 of autoinducers rises above minimum concentration threshold. Some Gram-negative food  
42 associated bacteria can produce *N*-acyl-L-homoserine lactones (AHLs) as signal molecules.  
43 Examples include *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Yersinia*  
44 *enterocolitica* (11, 12, 20, 24, 26). In these bacteria, a relationship between AHL production  
45 and expression of some mechanisms such as biofilm formation, motility and exoprotease  
46 production have been described (2,15,17, 25).

47 The signal molecule AHL is the key factor in this bacterial communication process.  
48 Degradation of the molecule prevents its sufficient accumulation in the immediate vicinity of  
49 bacterial cell, and will consequently lead to a disruption of the communication system. In  
50 several fields, blocking of quorum sensing by degradation of AHLs molecules has been  
51 proposed as a promising alternative for diminishing bacterial virulence (4, 5, 6, 9, 18). In  
52 food systems quorum sensing may have a role in food spoilage, in the growth and/or toxin  
53 production of pathogens present in food, in biofilm formation, bacteriocin production,  
54 virulence responses etc. Possible role of some bacteria in biological degradation of AHL has  
55 been suggested (5, 6, 7, 10, 13, 14, 19, 22). Most of the reports on quorum sensing  
56 degradation have been focused on plant pathogens (5, 6, 18). Although it is well known that  
57 the lactonase gene is widely spread among certain strains of foodborne *Bacillus* spp. no  
58 actual data are available on the interaction of mixed populations of AHL producing and AHL  
59 degrading bacteria in food simulating conditions.

60 The aim of the present study was to screen for the AHL degrading capacity of *B. cereus* and  
61 some other (former) *Bacillus spp.* and establish whether degradation capacity is noticed in  
62 the co-culture environment. The AHL degrading capacity of *Bacillus spp.* strains was  
63 evaluated with both synthetic AHL molecules and AHL produced by *Y. enterocolitica*. The  
64 AHL degradation ability was further assessed in one *B. cereus* strain inoculated in co-culture  
65 with a *Y. enterocolitica* strain in culture media and pork extract.

66 **Screening of synthetic-AHL degradation at 30 °C.** Twenty *B. cereus* and 29 (former)  
67 *Bacillus spp.* isolates were screened for their AHL degradation capacity. *N*-hexanoyl-L-  
68 homoserine lactone (C6-HSL) (Biochemika, Sigma-Aldrich) and 3-oxo-hexanoyl-L-  
69 homoserine lactone (3-oxo-C6-HSL) (Sigma-Aldrich) were used as target molecules in the  
70 degradation assay. *Bacillus* strains were inoculated with a loopful culture in 50 ml LB  
71 medium and incubated at 30 °C for 14 h. After incubation, a volume of 900 µl of each  
72 culture was mixed with 100 µl of each of synthetic standards obtaining the final  
73 concentrations of C6-HSL and 3-oxo-C6-HSL of 50 µmoles l<sup>-1</sup> and 46 µmoles l<sup>-1</sup>,  
74 respectively. The mixtures were incubated for 24 h at 30 °C, and the sterile supernatant was  
75 prepared by centrifuging cultures at 6000 x g for 5 min in a microcentrifuge (Biofuge, Pico,  
76 Osterode, Germany) and filtration (0.45 µm, HPLC filters, Alltech IL, USA). The sterile  
77 supernatants were screened for presence of AHL using an indirect fluorescence based  
78 method (18). Three replicate fluorescence measurements per supernatant were performed.  
79 *Escherichia coli* JB523, containing the plasmid pJBA130 responsible for the production of a  
80 green fluorescent protein (gfp) was used as AHL biosensor (1).

81 Controls prepared with each synthetic standard in LB medium were running in parallel. The  
82 recombinant *Pseudomonas fluorescens* P3/pME 6863 strain, and the derivate *P. fluorescens*  
83 P3/PME6000 were used as degrader and non-degrader reference strains (18). The screening

84 results showed evidence of AHL degrading factors occurring in *B. cereus* and *Paenibacillus*  
85 *polymyxa* (formerly *Bacillus polymyxa*). At 30 °C, 15 of out of 20 (75 %) tested *B. cereus*  
86 isolates tested were able to degrade AHLs (Table 1). These results are in agreement with  
87 other reports presenting AHL degradation by several *Bacillus* species (5, 10). The two *P.*  
88 *polymyxa* strains degraded both AHLs synthetic standards. Antibacterial properties of *P.*  
89 *polymyxa* have been reported (21, 23), however, to the best of our knowledge no reports on  
90 AHL degradation ability of this microorganism have been presented so far.

91 **Screening of synthetic-AHL degradation at 7 °C.** The ability of some psychrotrophic *B.*  
92 *cereus* isolates to degrade synthetic AHL molecules at 7 °C was investigated. A selection of  
93 psychrotrophic strains, previously confirmed as AHL degraders at 30 °C, showed AHL  
94 degrading capacity at 7 °C, too.

95 **Degradation of naturally produced AHL.** *P. polymyxa* 625 and *B. cereus* 720, previously  
96 determined as C6-HSL and 3-oxo-C6-HSL degrading strains were selected as AHL  
97 degraders. *Y. enterocolitica* 057 was selected as AHL producer (16). The sterile supernatant  
98 of a 24 h culture of *Y. enterocolitica* was mixed respectively with an overnight culture of two  
99 AHL degrading strains and AHL degradation was evaluated as described above. Results  
100 obtained indicated a reduction of relative fluorescence units (RFU) values, and thus  
101 degradation of AHLs present in the sterile supernatants of co-cultures of *Y. enterocolitica*  
102 057 with *B. cereus* 720 or *P. polymyxa* 625, in comparison to the sterile supernatant of mono-  
103 culture of *Y. enterocolitica* 057.

104 **AHL degradation with sterile *Bacillus spp.* supernatants.** In order to determine if the  
105 degrading factor is excreted out of the cell or it is cell-bound, the filter sterilized supernatants  
106 of the overnight AHL degrading cultures of *B. cereus* 720 and *P. polymyxa* 625 were tested  
107 in the degradation assay, as described above. Current findings suggest that the degrading

108 factor is not excreted to the cell exterior, which agrees with reports of Molina et al. (18)  
109 hypothesizing that AHL molecules diffuse into the AHL degrader bacterial cells, where  
110 molecule inactivation takes place.

111 **AHL degradation in co-cultures.** Different bacterial relationships and behaviours can occur  
112 when mixed bacterial populations are present, which is often the case is in food. Therefore,  
113 the performance of these AHL degrading mechanisms was evaluated in food simulating  
114 conditions using AHL producer-AHL degrader model. The assay comprised co-cultures  
115 *B. cereus* 720 – *Y. enterocolitica* 057 on solid and in liquid culture media. Pork extract (solid  
116 and liquid) was used as model food matrix. For co-culture on solid media *Y. enterocolitica*  
117 057 and *Bacillus cereus* 720 were streaked perpendicularly to each other onto the surface of  
118 LB agar previously inoculated with *E. coli* JB523. LB agar inoculated with *E. coli* JB523  
119 was prepared as described for top layer agar used in Thin Layer Chromatography (TLC)  
120 identification of AHL (16). The pork extract, prepared as described elsewhere (3), was  
121 mixed with agar (Agar Bacteriological N° 1, OXOID LTD, Basingstoke, Hampshire,  
122 England) (1.6 %) and sterilized. Pork extract agar was inoculated with the *E. coli* JB523  
123 culture, as described above for LB agar. The non-AHL degrader *B. cereus* 258 was used as a  
124 negative control. After 24 h incubation at 30 °C, a fluorescent signal was determined using a  
125 Transilluminator (Clare Chemical Research DR – 45 M) under the conditions of 230 V, 50  
126 Hz, and 9 W. Absence of the fluorescence in the proximity of the point of contact of *B.*  
127 *cereus* 720 and *Y. enterocolitica* 057 compared to the obvious fluorescence with non-  
128 degrading *B. cereus* 258 indicates that *B. cereus* 720 can degrade AHL molecule produced by  
129 *Y. enterocolitica* if present in the close proximity (Figure 1). Appropriate controls showed  
130 that pure cultures of *B. cereus* 720 and *B. cereus* 258 did not produce detectable AHL by the  
131 fluorescence assay. Similar results of AHL degradation were observed in pork extract agar.

132 The possible explanation may lay in the diffusion of *Y. enterocolitica* short chain AHLs  
133 through the media into *B. cereus* cells where they are then degraded.

134 For co-culture in liquid media, *Y. enterocolitica* 057 and test strain *B. cereus* 720 were  
135 cultured in 7 ml LB medium and incubated overnight at 30 °C. The AHL non-degrader  
136 *B. cereus* 258 was used as a negative control for AHL degradation. Following, a mix of  
137 *B. cereus* - *Y. enterocolitica* (10:1) was prepared and 30 µl of this mix was used to inoculate  
138 30 ml of LB broth and/or pork extract (initial inoculum level of approx. 3 log CFU ml<sup>-1</sup> and  
139 approx. 4 log CFU ml<sup>-1</sup> for *Y. enterocolitica* and *B. cereus*, respectively). The co-cultures  
140 were incubated for 24 h at 30 °C and then ten-fold serially diluted in Physiological Peptone  
141 Solution (PPS). The spread plate enumeration (detection limit 100 CFU ml<sup>-1</sup>) was performed  
142 on the surface of Egg Yolk Polymyxin agar (LAB M, Lancashire, UK) and Plate Count Agar  
143 (Oxoid, Hampshire, England) containing crystal violet (1.5 % w/v) to enumerate *B. cereus*  
144 and *Y. enterocolitica*, respectively. Centrifugation, filter-sterilization and screening for the  
145 presence of AHL in the supernatant with fluorescence assay were performed as described for  
146 synthetic-AHL degradation. Results obtained indicated no inhibition of the bacterial growth.  
147 Both microorganisms reached similar stationary phase levels when grown in co-culture in  
148 comparison to respective mono-culture. A reduction in the fluorescence signal (RFU) values  
149 was noted for the co-culture *B. cereus* 720 - *Y. enterocolitica* 057, indicating AHL  
150 degradation (Figure 2). In contrast, in the co-culture of *B. cereus* 258 and *Y. enterocolitica*  
151 057 the obtained fluorescence signal (RFU values) was similar to the one produced by *Y.*  
152 *enterocolitica* in mono-culture, indicating lack of AHL degradation (Figure 2). However, no  
153 difference in the counts of *B. cereus* 720 and *B. cereus* 258 in the respective co-cultures were  
154 observed. A reduction of RFU values with co-cultures *B. cereus* 720 - *Y. enterocolitica* 057  
155 was also observed in pork extract (Figure 3). However, in pork extract, a limited AHL

156 degradation was observed also with *B. cereus* 258. As AHL molecules are chemically  
157 unstable under alkaline conditions (8) the pH value of pork extract (pH 5.7) could not have  
158 caused observed AHL degradation. Further investigation is suggested to elucidate observed  
159 results.

160 The present study provides evidence of AHL degrading capacity of *B. cereus* and *P.*  
161 *polymyxa*, also under food simulating conditions. These bacteria may use this AHL  
162 degrading mechanism as competitive advantage toward bacterial competitors and may help  
163 them to dominate the ecological niche (6, 19).

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- 258

259 Table 1. AHL degradation assay for *Bacillus cereus* and (former) *Bacillus* spp. at 30°C,  
 260 as determined with fluorescence assay (18).

261

<i>B. cereus</i>			(former) <i>Bacillus</i> spp		
Isolate	C6-HSL	3-oxo-C6-HSL	Isolate	C6-HSL	3-oxo-C6-HSL
<i>B. cereus</i> 527	+++	+	<i>B. alvei</i> 110	-	-
<i>B. cereus</i> 720	+++	+	<i>B. brevis</i> 107	-	-
<i>B. cereus</i> 396	+++	+	<i>B. brevis</i> 111	-	-
<i>B. cereus</i> 729	+++	+	<i>B. circulans</i> 196	-	-
<i>B. cereus</i> 254	+++	-	<i>B. circulans</i> 681	-	-
<i>B. cereus</i> 710	+++	-	<i>B. circulans</i> 682	-	-
<i>B. cereus</i> 711	+++	-	<i>B. circulans</i> 683	-	-
<i>B. cereus</i> 712	+++	-	<i>B. circulans</i> 684	-	-
<i>B. cereus</i> 718	+++	-	<i>B. circulans</i> 685	-	-
<i>B. cereus</i> 727	+++	-	<i>B. firmus</i> 118	+	-
<i>B. cereus</i> 005	++	+	<i>B. lentus</i> 631	-	-
<i>B. cereus</i> 004	++	+	<i>B. licheniformis</i> 628	-	-
<i>B. cereus</i> 724	+	+	<i>B. licheniformis</i> 629	-	-
<i>B. cereus</i> 431	++	-	<i>B. megaterium</i> 104	-	-
<i>B. cereus</i> 435	-	+	<i>B. megaterium</i> 630	-	-
<i>B. cereus</i> 258	-	-	<i>P. polymyxa</i> 625	+++	+++
<i>B. cereus</i> 426	-	-	<i>P. polymyxa</i> 115	+++	+++
<i>B. cereus</i> 428	-	-	<i>P. polymyxa</i> 072	-	-
<i>B. cereus</i> 434	-	-	<i>B. pumilus</i> 108	-	-
<i>B. cereus</i> 552	-	-	<i>B. pumilus</i> 626	-	-
			<i>B. pumilus</i> 627	-	-
			<i>B. sphaericus</i> 109	-	-
			<i>G. stearothermophilus</i> 007 <sup>a</sup>	-	-
			<i>G. stearothermophilus</i> 113 <sup>a</sup>	-	-
			<i>G. stearothermophilus</i> 008 <sup>a</sup>	-	-
			<i>B. subtilis</i> 102	-	-
			<i>B. subtilis</i> 623	-	-
			<i>B. subtilis</i> 624	-	-
			<i>B. thuringiensis</i> 092	-	-

262 -No degrading activity observed; + Weak degrading activity; ++ Medium degrading

263 activity; +++ High degrading activity; <sup>a</sup> *Geobacillus stearothermophilus*

264 **FIGURE CAPTIONS**

265

266 Figure 1: 1. Degradation assay on LB agar: 1. *Y. enterocolitica* 057-*B. cereus* 258 (AHL  
267 non-degrading strain); 2. *Yersinia enterocolitica* - *B. cereus* 720 (AHL degrading strain).  
268 Area around *B. cereus* 258 (fluorescence) and around *B. cereus* 720 (lack of  
269 fluorescence) indicates inability of the former one and ability of the later one to degrade  
270 AHL molecule.

271

272 Figure 2: AHL detection by fluorescence assay in supernatant derived from co-culture *Y.*  
273 *enterocolitica* - *B. cereus* in LB medium: a) (●) *Y. enterocolitica* 057 (positive control),  
274 (□) *Y. enterocolitica* 057 - *B. cereus* 720 (AHL degrader), (■) LB medium (blank). b) (●)  
275 *Y. enterocolitica* 057 (positive control), (□) *Y. enterocolitica* 057 - *B. cereus* 258 (AHL  
276 non-degrader), (■) LB medium (blank).

277

278 Figure 3 AHL detection by fluorescence assay in supernatant derived from co-culture *B.*  
279 *cereus* - *Y. enterocolitica* in pork extract: (□) *Y. enterocolitica* 057 - *B. cereus* 720 (AHL  
280 degrader), (▲) *Y. enterocolitica* 057 - *B. cereus* 258 (AHL non-degrader), (■) *Y.*  
281 *enterocolitica* 057 (positive control), (●) pork extract (blank).

282

*Y. enterocolitica* 057

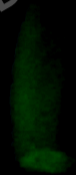


*B. cereus* 258

1

ACCEPTED

*Y. enterocolitica* 057



*B. cereus* 720

2

