Acyl Homoserine Lactone Production of Brewery Process Surface Bacteria

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Acyl Homoserine Lactone Production of Brewery Process Surface Bacteria

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ABSTRACT

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The aim of this study was to find out whether the first bacterial colonizers of brewery process surfaces after washing produce acyl homoserine lactone (AHL) quorum sensing signalling molecules. Microbial attachment and biofilm formation was studied by mounting sterile uncoated and coated stainless steel coupons onto critical places of the filling machines in three breweries. In the present study 26 previously deposited bacterial strains, as well as approximately 2,300 colonies from 76 process surface samples, were screened using reporter bacteria *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTLR4. AHL-producing bacteria were detected both from process samples and among the previous isolates. From the process samples, up to 15% of the screened colonies produced AHL molecules. Production of long chain AHLs was more common than short chain AHLs. The identified AHL-producing isolates belonged to the genera *Pseudomonas, Serratia, Hafnia*, *Rahnella*, *Enterobacter* and *Aeromonas,* which all belong to commonly found primary colonizers of brewery process surfaces. The same microbial genera producing AHL molecules were found from different breweries. Brewery filling equipment is susceptible to microbial attachment and accumulation on surfaces. In the future, inhibition of quorum sensing could be one additional way of controlling biofilm formation.

Key words: Acyl homoserine lactones, *Agrobacterium tumefaciens* NTLR4, brewery, *Chromobacterium violaceum* CV026, microbial attachment, quorum sensing.

INTRODUCTION

Brewery filling equipment is continuously exposed to moisture and the presence of nutrients between production runs, and is susceptible to microbial attachment and accumulation on surfaces. Secondary contamination, originating from bottling, canning or kegging, is responsible for at least half of the incidents of microbiological spoilage in breweries not using tunnel pasteurization[1,29]. Microbial attachment and biofilm formation in breweries has been studied by mounting sterile stainless steel coupons onto critical places of the filler machines in three breweries[30]. It was shown that the first, primary colonizers accumulated on new stainless steel surfaces within hours after the start of production. Regular daily cleaning reduced the number of microorganisms on the surfaces only momentarily, and biofilm formation occurred on certain surfaces despite daily cleaning and disinfection.

Bacteria are no longer regarded as undifferentiated cells focused on multiplication. Instead it has been shown that cell-to-cell signalling, known as quorum sensing (QS), is a widely spread phenomenon both within and between bacterial species. Autoinducer-2 (AI-2), revealed to be a furanosyl borate diester, is produced and detected by roughly half of all bacterial species, both Gram-positive and Gram-negative[25]. It is described as the universal signalling molecule for interspecies communication, but on the other hand it is not yet clear that it is always produced for the purposes of signalling[6]. *N*-acyl homoserine lactones (AHLs) are the major group of signalling molecules in Gram-negative bacteria. Some bacteria produce only a single detectable species of AHLs, while others, such as *Pseudomonas aeruginosa* and *Rhizobium etli,* can produce as many as seven compounds with detectable activity[7]. Quorum sensing also participates in biofilm formation[12]. Quorum-sensing-deficient mutants have been shown to form thinner and more unstructured biofilms compared to wild types[9,16,18,27], and to be more susceptible to chemicals[2,9]. Work with dental biofilms suggests that QS signalling is required for the formation of mixed-species biofilms[10]. A multitude of studies have found compounds that inhibit the quorum sensing signalling of bacteria, which may in the future provide means to control their biofilm formation capability. Examples of inhibiting substances include secondary metabolites of fungi, plant extracts, of which garlic has been shown to be especially efficient, and natural and synthetic furanones originating from algae[3,11,14,19,22].

Production of signalling molecules by bacterial strains may be screened with specific reporter bacteria, in which, for example, colour changes are induced by exogenous signalling molecules. Widely used reporter bacteria for AHL signalling molecules are *Chromobacterium violaceum* CV026, and *Agrobacterium tumefaciens* NTLR4[20,33]. Production of a violet pigment, violacein, of *C. violaceum* CV026 is inducible by unsubstituted short chain AHLs, whereas if an activating AHL is incorporated into the agar, long-chain AHLs can be detected by their ability to inhibit violacein production[20,28]. *A. tumefaciens* NTLR4 detects a broad range of substituted and long chain AHLs by reduction into a blue colour of an added substrate[7,28].

The aim of this study was to investigate how common the production of acyl homoserine lactone (AHL) signalling molecules is among the colonizers of brewery filling

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machine surfaces. Both previously deposited isolates comprising primary colonizers, the first bacteria accumulating on the surfaces after washing, as well as new process surface samples were screened for AHL production by using the reporter bacteria *C. violaceum* CV026 and *A. tumefaciens* NTLR4.

MATERIALS AND METHODS

Microbial test strains

In an earlier study, primary colonisers after washing surfaces of filling machines were isolated, identified, and deposited into the VTT Culture Collection[30]. Twenty-six Gram negative bacteria from these isolates were screened for their AHL production using two reporter strains, *C. violaceum* CV026 and *A. tumefaciens* NTLR4 (current name *Rhizobium radiobacter*). *C. violaceum* CV026 produces a violet pigment, violacein, in the presence of short chain (C4–C8) AHL molecules[17,20]. The major *C. violaceum* AHL is *N*-hexanoyl-L-homoserine lactone (C6-HSL). When it is added to the medium, *C. violaceum* CV026 produces violacein, but the production is inhibited by long chain (C10-C14) AHL molecules[18]. *A. tumefaciens* NTLR4 reduces 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-Gal) into a blue indole colour when long chain AHLs or short-chain substituted AHLs are present[17,33].

Bioassays for AHL production

The test strains were incubated over-night in Luria-Bertani (LB) broth, Lennox (5 g L^{-1} NaCl, 10 g L^{-1} pancreatic digest of casein, 5 g L^{-1} yeast extract) (= LB) at 30°C. *C. violaceum* CV026 was incubated in LB + 20 µg mL^{-1} kanamycin, and *A. tumefaciens* NTLR4 was incubated in Agrobacterium broth (AB)[7] supplemented with 4 g L^{-1} mannitol (=ABM) and 20 µg mL^{-1} gentamicin. The test and reporter strains were streaked parallel (distance about 0.5 cm) onto agar plates to see the colour changes of the reporter bacteria, caused by production of signalling molecules by the test strains. Tests with *C. violaceum* were performed on LB plates (LB broth + 15 g L^{-1} agar) with or without 0.5 µM C6-HSL (Cayman Chemical). Tests with *A. tumefaciens* were performed on ABM plates with 40 µg mL^{-1} X-Gal (Promega). Negative and positive control strains were included in all tests. The reporter strains themselves were used as negative controls. The positive control for *C. violaceum* CV026 induction was *C. violaceum* ATCC 31532[20], and the positive control for *C. violaceum* inhibition and *A. tumefaciens* NTLR4 induction was *Pseudomonas aeruginosa* PAO1[26]. Positive colonies were identified by colour change in the surrounding agar.

Process samples

Process studies of microbial attachment on brewery filling machine surfaces have been described in detail elsewhere[21]. Briefly, sample coupons (18.75 cm^2) of stainless steel AISI 304 2B (Happoteräs, Helsinki, Finland) (Fig. 1) with different coatings (uncoated, TiO_2, TiO_2 + Ag, two different hydrophobic coatings) had been mounted onto the bottle filling machines of two breweries, A and B. In the selected filling machines, the most common product is beer. Strict hygiene of the filling area is ensured by frequent intermediate washing and rinsing during work shifts. Microbiological samplings of these coatings were performed at different time intervals by swabbing with a 5 × 10 cm sterile gauze, which was placed into 10 mL Ringer´s (Merck) solution, and homogenized with Stomacher lab blender for 10 min. The homogenates were plated onto Tryptic Soy Agar (TSA, Difco) and incubated for 3 days at 30°C. Plates with 1-50 colonies were selected for the AHL screening studies. From brewery A, altogether 96 TSA plates from 51 sample coupons were selected from sampling of 6 months. From brewery B, 59 TSA plates from 30 sample coupons were selected from sampling of 6 months, and 93 TSA plates from 50 sample coupons from sampling of 12 months.

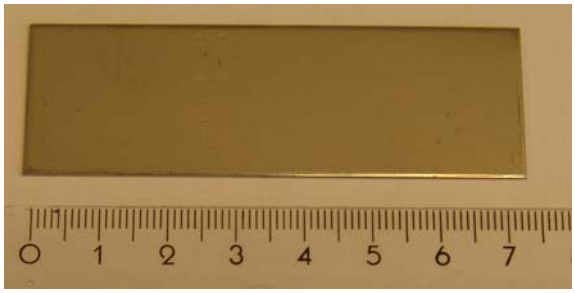

Fig. 1. Example of a stainless steel test coupon.

Colonies from selected TSA plates were replicated with velvet cloths to plates with or without reporter bacteria (100 mL overnight culture + 400 mL LB). The plates included 1) LB + *C. violaceum* CV026, 2) LB + 0.5 µM C6-HSL, 3) ABM + 40 µg mL^{-1} X-Gal + *A. tumefaciens* NTLR4, and 4) ABM + X-Gal (control plate). Plates were incubated overnight at 30°C and colour changes of the reporter bacteria were recorded. To LB + 0.5 µM C6-HSL plates, 5 mL molten semi-solid (0.3%, w/v) agar with a 50 µL over-night culture of *C. violaceum* CV026 was poured the next day, and incubation was continued for another day. Antibiotics were omitted from the overlay assays. Positive colonies were identified by a colour change in the surrounding agar.

Identification of isolates

Twelve AHL-producing different looking colony types from process samples were purified and identified by partial sequencing of their 16S rRNA gene. An approximately 500-bp fragment from the 5′ end of the 16S rRNA gene was amplified with universal primers BSF8/20 (5′-AGAGTTTGATCCTGGCTCAG-3′) and BSR534/18 (5′-ATTACCGCGGCTGCTGGC-3′)[36]. DNA extraction, PCR amplification and sequencing reactions have been described in detail by Katina et al.[13] The obtained sequences were compared against GenBank sequences with BLASTN search[5] sequences of RDP (Ribosomal Database Project) type strains[8].

RESULTS

AHL-producing strains were detected both from process samples and among the previously isolated bacterial

Table I. AHL-production of brewery process surface isolates from the VTT culture collection[30].

VTT no	Species	*C. violaceum* CV026		*A. tumefaciens* NTLR4
		Induction	Inhibition	Induction
E-022167	*Achromobacter piechaudii*	-	+[a]	-
E-022166	*Acinetobacter baumannii*	-	+	+
E-052820	*Pantoea agglomerans*	-	++[b]	-
E-022169	*Pantoea agglomerans*	-	++	-
E-042562	*Pseudomonas fluorescens*	-	++	-
E-042565	*Pseudomonas fluorescens*	-	++	-
E-031889	*Pseudomonas fluorescens*	-	++	-
E-052840	*Pseudomonas libaniensis*	-	++	+++[c]
E-042561	*Pseudomonas* sp.	-	++	+
E-042563	*Pseudomonas* sp.	-	+++	-
E-042564	*Pseudomonas* sp.	-	++	+
E-052801	*Pseudomonas* sp.	-	++	-
E-052817	*Pseudomonas* sp.	-	++	-
E-052833	*Pseudomonas* sp.	-	++	-
E-052843	*Pseudomonas* sp.	-	++	-
E-052800	*Rahnella aquatilis*	-	++	-
E-052816	*Rahnella aquatilis*	-	++	-
E-052841	*Rahnella* sp.	+	+	+++
E-022170	*Serratia marcescens*	-	++	-
E-022171	*Serratia marcescens*	-	++	-
E-022172	*Serratia marcescens*	-	++	-
E-031888	*Serratia marcescens*	-	++	-
E-042566	*Serratia sp.*	+	++	+++
E-052839	*Sphingomonas* sp.	-	-	-
E-052831	*Stenotrophomonas maltophilia*	-	++	-
E-052834	*Stenotrophomonas* sp.	-	+	-

[a] Weak colour production.
[b] Medium colour production.
[c] Strong colour production.

Table II. Numbers of AHL-producing strains from process samples.

	C. violaceum CV026		*A. tumefaciens* NTLR4
	Induction	Inhibition	Induction
Brewery A 6 months (n ~ 690[a])	26 (4%)	8 (1%)	90 (13%)
Brewery B 6 months (n ~ 970)	5 (0.5%)	82 (8%)	50 (5%)
Brewery B 12 months (n ~ 560)	2 (0.4%)	87 (15%)	22 (4%)

[a] Approximate number of screened colonies.

strains (Tables I and II). From 26 primary colonisers deposited into the VTT Culture Collection, all except VTT-E-052839 *Sphingomonas* sp., produced long chain AHLs detected by inhibition of *C. violaceum* CV026 violacein production. Six strains produced AHLs detected by *A. tumefaciens* NTLR4. Short chain AHLs detected by induction of *C. violaceum* CV026 violacein synthesis were produced by two strains, *Serratia* sp. and *Rahnella* sp.

With the in process samples from brewery A, 4% of the screened colonies produced short chain AHLs detected by induction of *C. violaceum* CV026, 1% produced AHLs detected by inhibition of *C. violaceum*, and 13% produced AHLs detected by *A. tumefaciens* NTLR4. In two samplings from brewery B, the corresponding percentages were 0.5%, 8% and 5% for sampling of 6 months, and 0.4%, 15% and 4% for sampling of 12 months (Table II). In all screened bacterial strains, production of long chain AHLs seemed to be more common than production of short chain AHLs. The highest percentages of AHL positive strains were found from the previously isolated primary colonisers.

The identified AHL-producing isolates belonged to the genera *Pseudomonas, Serratia, Hafnia*, *Rahnella*, *Enterobacter* and *Aeromonas* (Table III). Isolates producing short chain AHLs belonged to genera *Serratia, Hafnia, Rahnella* and *Aeromonas*.

DISCUSSION

This work demonstrated that AHL-producing bacteria are common on brewery process surfaces. The production of signalling molecules by brewery surface bacteria has had little study to date. Timke[32] studied 106 isolates from seven different sample locations in German breweries with *A. tumefaciens* NTL4. Nine of the isolates (8%) were AHL positive. These all belonged to the Proteobacteria and most of them were members of the genus *Pseudomonas*. The AHL-secreting isolates were often cultivated from the places with a thin biofilm, whereas isolates from places with no visible biofilm did not appear to produce AHLs. Timke concluded that this might indicate the role of AHL-producing isolates as surface colonizing organisms. It was noteworthy that almost all primary colonizers from the VTT Culture Collection produced long chain AHLs, detected by inhibition of *C. violaceum* CV026 in the presence of C6-HSL, which could indicate that one or

Table III. Identification and AHL-production of selected process isolates.

Isolate	Closest type strain in RDP	Similarity score	*C. violaceum* CV026		*A. tumefaciens* NTLR4
			Induction	Inhibition	Induction
A1	*Hafnia alvei*	0.953	+	-	+
A2	*Hafnia alvei / Obesumbacterium proteus*	0.956	+	-	+
B1	*Obesumbacterium proteus*	0.974	+	-	-
A13	*Serratia quinivorans / plymuthica*	0.934	+	-	-
A15	*Serratia quinivorans / plymuthica*	0.937	+	-	-
A23	*Serratia plymuthica*	0.902	+	-	-
B3	*Serratia plymuthica*	0.942	+	-	-
A5	*Enterobacter kobei*	0.964	-	-	+
A22	*Aeromonas punctata*	0.974	+	-	-
A9	*Pseudomonas marginalis*	0.982	-	+	-
B20	*Pseudomonas koreensis*	0.991	-	-	+
B36	*Pseudomonas koreensis*	1.000	-	-	+

several of these AHL molecules are important in primary attachment. In our study, samples were taken from different locations and different coatings from two breweries but due to the very variable numbers of screened colonies from different locations, it was not possible to draw conclusions on the effect of the sampling location or coating type on the presence of AHL-producing bacteria.

AHL molecules are produced and are active at very low concentrations, which is why they are most often detected by bioassays. The problem with bioassays however is that a single reporter bacterium is not capable of detecting the whole spectrum of AHLs. Use of several reporters with complementary specificities helps to partly solve this problem. Induction of violacein production of *C. violaceum* CV026 is known to detect very specifically unsubstituted C4-C8-AHLs, but this strain cannot detect any of the 3-hydroxyderivatives and lacks sensitivity to most 3-oxo derivatives[7,20]. *A. tumefaciens* NTLR4 is sensitive to most 3-oxo AHLs, but the 3-unsubstituted AHLs and the long-chain AHLs (over C12) are less active, and C4-HSL does not give any signal[26,37]. These two bacteria therefore complement each other in the detection of AHLs. *C. violaceum* CV026 induction has a narrow range and was not common among the samples. The same was previously noted in vacuum-packed meat samples[4]. It has to be borne in mind, however, that from the absence of a signal it cannot be concluded that a strain does not produce AHLs, since they may produce signals which are not detectable by the reporter strain, or the level of molecules may be below the detection level of the bioassay.

The difficulty in using *A. tumefaciens* NTLR4 was that some test strains caused a blue colour in the ABM + X-Gal medium, probably having β-galactosidase activity, a phenomenon also noted by Cha et al.[6] We therefore had to compare all the isolates from the replica plates with ABM + X-Gal without *A. tumefaciens* NTLR4, and re-test the ones giving a blue colour without the reporter strain. In replica plating, the number of colonies on the plate to be replicated has to be low enough in order to prevent spreading and overgrowing of the colonies, which in turn may prevent accurate quantification of positive colonies. Bruhn et al.[4] used replica plating in screening for AHL-producing bacteria from meat. They concluded that the technique may not be applicable to samples where the level of AHL-producing bacteria constitutes less than 1% of the microbial community, since the replica plates will be overgrown with non-AHL-producing organisms. Another factor to be considered in replica plating is the limited number of replicas that can be copied from one plate without losing colonies. We performed four replicates from each plate, which was the absolute maximum in our case.

The identified AHL-producing isolates belonged to the genera *Pseudomonas, Serratia, Hafnia*, *Rahnella*, *Enterobacter, Obesumbacterium* and *Aeromonas* (Table I, Table III), which are commonly found as primary colonizers of brewery process surfaces. These genera have also been shown to be common AHL-producers in other environments. *Hafnia alvei* was the most frequently identified AHL-producing bacterium from vacuum-packed meat samples[4], and *Acinetobacter* spp., *Pseudomonas aeruginosa* and *Serratia marcescens* from human chronic wound isolates[25].

It had been thought that QS genes were activated at a certain population density. However, it has been shown that there is not a single population density at which all QS genes are activated, but different genes are activated at different population densities[23]. It is also possible that signalling between bacterial cells is not only for sensing population density, but also for decentralizing the decision-making process, enabling all individuals in a community to participate in reaching a consensus[37]. Davies et al.[9] concluded that QS does not participate in the initial stages of biofilm formation, but only in the maturation phase. However, more recent studies have shown that attachment of *Pantoea stewartii* may be repressed by QS[15], and that QS was the main regulatory pathway in adhesion of *Serratia marcescens* to the biotic surface[16]. They suggested that it is possible that as a bacterium approaches a surface, the surface acts as a diffusion barrier, resulting in an increased concentration of AHLs and the activation of genes coding for adhesins, a theory also proposed by Redfield[24]. QS may thus have a previously undiscovered role in the attachment of microbes to surfaces.

REACH (registration, evaluation, authorisation and restriction of chemicals) and the 3rd Emission Trading Scheme 2013–2020 of the European Union, also covering breweries[34], aim at improving energy and water saving and reducing chemicals loads. These aims encourage the finding of new alternative or synergistic ways to control brewery hygiene, of which QS inhibition is one alternative. Because QS is not involved in bacterial growth, inhi-

bition of QS should not yield a strong selective pressure for development of resistance. QS inhibitors are much studied in clinical applications, and the success of macrolide antibiotics against *Pseudomonas aeruginosa* pulmonary infections is suggested to be due to QS inhibition[31]. QS inhibitors could also have synergistic effects with existing biocides. Vestby et al.[35] showed that the effect of disinfectants on *Salmonella* in a biofilm was significantly enhanced when the biofilm was grown in the presence of a QS inhibitor. One way to utilize QS inhibitors might be their inclusion in functional coatings. Our future work on QS will include inhibition studies and investigation of the possibility to utilize QS inhibition in the management of brewery hygiene.

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