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Short communication

Detection of acylated homoserine lactones in gram-negative proteolytic psychrotrophic bacteria isolated from cooled raw milk

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Abstract

Through a mechanism called quorum sensing, bacteria are able to express specific genes in response to population density. Cell-to-cell communication in bacteria is mediated by signal molecules such as acylated homoserine lactones (AHLs). This work aimed to detect AHL production in Gram-negative psychrotrophic bacteria isolated from raw milk. A total of 84.9% of the bacteria were identified as AHL producers eliciting a diversity of responses in the AHL-monitor systems. These results demonstrate that AHL-production is common among psychrotrophic bacteria isolated from milk, and indicate that quorum sensing may play an important role in the spoilage of this product.
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Keywords: Quorum sensing; Acylated homoserine lactones (AHL); Psychrotrophic bacteria; Raw milk

1. Introduction

Gram-negative proteolytic psychrotrophic bacteria are the predominant microorganisms responsible for spoilage of milk and milk products due to their ability to produce thermostable proteases that hydrolyze casein and decrease the yield and sensory quality of dairy products (Dogan & Boor, 2003; Sørhaug & Stepaniak, 1997). Some bacteria also secrete lecithinases and lipases that can play a significant role in the spoilage of these products (Dogan & Boor, 2003). It is known that activity of hydrolytic enzymes is detected at the end of logarithmic phase and at the beginning of the stationary growth phase of these bacteria (Matselis & Roussis, 1998; Rajmohan, Dodd, & Waites, 2002), conditions in which a high cell density is achieved.

Bacteria are able to regulate expression of phenotypic characteristics as a function of cell density in a mechanism termed quorum sensing (QS) (Fuqua, Winans, & Greenberg, 1994). In Gram-negative bacteria, this regulation is typically mediated by chemical signals such as *N*-acyl-L-homoserine lactones (AHL) (Fuqua et al., 1994; Whitehead, Barnard, Slater, Simpson, & Salmond, 2001). The acyl side chain of different AHL can vary from 4 to 18 carbons in length, degree of substitution, and saturation providing specificity to QS systems (Zhu, Chai, Zhong, LI, & Winans, 2003). The key regulatory components of these signaling systems are LuxI-type proteins which act as AHL synthases, LuxR-type proteins which serve as AHL receptors, and AHL-dependent transcription factors (Fuqua & Greenberg, 2002). Examples of phenotypes regulated by AHLs include production of antibiotics, biofilm development, competence for DNA uptake, cell differentiation, bioluminescence, growth, pigment production, conjugal plasmid transfer, virulence gene expression, and production of a range of degradative extracellular enzymes (Smith, Fratamico, & Novak, 2004).

Several works have been done to elucidate the role of QS in food spoilage. AHLs have been detected in some food products such as cold-smoked salmon (Gram, Christensen,

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Ravn, Molin, & Givskov, 1999), meat (Bruhn et al., 2004), and bean sprouts (Rasch et al., 2005). In this last work, it was shown that pectinase, protease, cellulase and siderophore-mediated iron chelation were regulated by QS, underlying the importance of this system in spoilage of bean sprouts. Christensen et al. (2003) demonstrated that several hydrolytic enzymes produced by *Serratia proteamaculans*, *a* typical member of a food spoilage flora, are regulated by QS. The involvement of QS in meat spoilage process, and biofilm formation was suggested by Jay, Vilai, and Hughes (2003). Furthermore, Liu and Griffths (2003) pointed out that spoilage of milk by *Pseudomonas fluorescens* is correlated with its ability to produce AHLs and the extracellular protease. However, currently, little is known about signal molecule production, such as AHLs, by bacteria isolated from food products.

The AHL detection is based on different bacterial bioassays. The use of reporter bacteria, in which the *luxI* homologue gene responsible for AHL production had been inactivated, has shown to be a valuable system for AHL detection (Ravn, Christensen, Molin, & Givskov, 2001). Then, the expression of a reporter gene is possible only in the presence of exogenous AHLs. Plasmid reporter vectors that respond to activation of LuxR homologues have also been used in *Escherichia coli* strains (Winson et al., 1998). Because of the specificity of each LuxR homologue, reporter strains display specificity towards different AHL molecules, allowing detection of a wide range of AHLs and differentiation between AHL production patterns (Van Houdt, Aerstsen, Jansen, Quintana, & Michiels, 2004). For instance, LuxR of *Vibrio fisheri*, used in several reporter plasmids such as pSB403, is activated by AHLs with carbon chains of C6 or C8 with or without 3-oxo substitutions (Winson et al., 1998). On the other hand, CviR of *Chromobacterium violaceum* is sensitive to unsubstituted chains varying in size from C4 to C8, and also is able to detect long-chain AHLs by their ability to inhibit violacein production if an activating AHL is incorporated to the medium (McClean et al., 1997). The TraR of *Agrobacterium tumefaciens* is sensitive to most 3-oxo AHLs (Shaw et al., 1997).

In order to improve the understanding of the process of cell-to-cell communication among bacteria found in milk, this work aimed to evaluate the production of AHL in proteolytic psychrotrophic bacteria isolated from cooled raw milk.

2. Material and methods

2.1. Bacterial strains and culture conditions

Gram-negative psychrotrophic bacteria isolated from cooled raw milk (Pinto, 2004) and strains from American type culture collection – ATCC (Table 1) were investigated for AHL-production. The bacteria were stored in brain heart infusion (BHI) with the addition of glycerol to 20% v/v and maintained at −80 °C. The strains were activated in Luria Berthani broth (LB) and incubated at 25 °C for 24 h with agitation (150 r.p.m.). Ultracompetent *E. coli* DH5α was transformed with pSB403 (Winson et al., 1998) using standard methods described by Sambrook, Fritsch, and Maniats (1989). *Chromobacterium violaceum* CV026 (McClean et al., 1997) and *Agrobacterium tumefaciens* a136 (Fuqua & Winans, 1996) were also used to detect AHL-production. These monitor strains were grown in LB supplemented with appropriate antibiotics (Ravn et al., 2001), and incubated at 28 °C during 24 h, with agitation (150 r.p.m.), prior the assay. *C. violaceum* ATCC 6357 and *P. aeruginosa* 15442 were used as positive controls in the experiments and the monitor strains as negative control themselves.

2.2. Screening for AHL-production

The screening for AHL-production was performed according to Ravn et al. (2001). The tested strains were streaked in parallel to the monitor strains on LB agar plates. *E. coli* (pSB403) is unable to produce luminescence without exogenous AHL. The luminescence was observed after approximately 3 min for adaptation of the eyes to a darkened room, under weak red light, as a positive result in response to AHL produced by the tested strain. *C. violaceum* CV026 produces the pigment violacein only in the presence of exogenous AHL. Thus, the pigment production indicated a positive result. Testing for AHL production against *A. tumefaciens* A136 assay, was done in a similar assay supplementing the LB agar with 50 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). This monitor strain has the *lac*Z gene fused to the promoter of *tra*I gene, which is regulated by autoinduction. The strain produces a blue pigment in response to AHL when the medium is supplemented with X-gal. All plates were incubated for 24 h at 25 °C, except the ones streaked with *A. tumefaciens* A136 that were incubated up to three days.

Long chains AHLs were detected observing the inhibition of the pigment production by the induced monitor strain *C. violaceum* CV026. LB agar plates were supplemented with 75 nM of *N*-hexanoyl-L-homoserine lactone (HHL) obtained commercially (Fluka, Switzerland). The psychrotrophic isolates were streaked in the medium and incubated for 24 h at 25 °C. Then, the monitor strain was streaked in parallel to the previous strains and the plates were re-incubated at the same conditions. Experiments were repeated at least twice.

2.3. Thin-layer chromatography (TLC)

Extracts for TLC were prepared from 100 ml cultures after growth in LB medium for 20 h at 25 °C with agitation of 150 r.p.m. Bacteria were removed by centrifugation, the supernatants were extracted twice with equal volumes of ethyl acetate acidified with 0.5% of formic acid, and the combined extracts were dried, filtered, and evaporated to

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