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Rapid and simple colorimetric method for the quantification of AI-2 produced from *Salmonella* Typhimurium

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ABSTRACT

The aim of this study was to evaluate the feasibility of Fe(III) ion reduction for the simple and rapid quantification of autoinducer-2 (AI-2) produced from bacteria using *Salmonella* Typhimurium as a model. Since the molecular structure of AI-2 is somewhat similar to ascorbic acid it was expected that AI-2 would also act as a reducing agent and reduce Fe(III) ions in the presence of 1,10-phenanthroline to form the colored $[(o\text{-phen})_3\ Fe(II)]SO_4$ ferroin complex that could be quantified colorimetrically. In support of this, colony rinses and cell free supernatants from cultures of all tested AI-2 producing strains, but not the AI-2 negative *Sinorhizobium meliloti*, formed a colored complex with a λ_{max} of 510 nm. The OD_{510} values of these culture supernatants or colony rinses were in broad agreement with the % activity observed in the same samples using the standard *Vibrio harveyi* bioluminescence assay for AI-2 detection, and with previously reported results. This methodology could potentially be developed as an alternative method for the simple and rapid quantification of AI-2 levels produced in bacterial cultures.

1. Introduction

Among the responses of free living bacteria to their environment is the ability to communicate with other bacteria of the same or even different species. This communication, known as quorum sensing, allows the bacteria to respond in an environmental- and population density-dependent manner and influences behaviors as diverse as bioluminescence, sporulation, sex (transfer of DNA) and biofilm formation, as well as the production of antibiotics, pathogenic factors and other metabolites (Barnard et al., 2007; Miller and Bassler, 2001). Although some quorum signaling molecules are seemingly species specific, others may be used by different species and so form intra-species communication. In general, quorum sensing Gram-negative bacteria typically use N-acyl homoserine lactones (AHLs), while Gram-positive bacteria use autoinducing peptides for intra-species communication. However, autoinducer-2 (AI-2), a group of interconverting molecules derived from 4,5-dihydroxy-2,3-pentane-dione (DPD), is unique in that it is used for inter-species communication as well as intra-species communication in both Gram-negative and Gram-positive bacteria (Federle and Bassler, 2003; Miller and Bassler, 2001; Walters and Sperandio, 2006a,b).

Indeed, AI-2 is likely to play an important role in bacteria as it has been reported to influence the expression of a wide variety of genes responsible for the regulation of important microbial processes, such as growth, sporulation, toxin production, virulence, antibiotic synthesis, motility and colonization, in a variety of bacteria. These effects are mediated through alterations in gene expression patterns in response to the cell density and conditions in the environment (Federle and Bassler, 2003; Surette and Bassler, 1999; Taga and Bassler, 2003; Xavier and Bassler, 2003). The biosynthesis of DPD (the precursor of AI-2) in *Vibrio harveyi* is dependent upon the *lux*S gene, with the genomes of more than 50 species of bacteria being reported to contain homologs of this gene and a number of these bacteria being shown to produce AI-2, including the food pathogens *Escherichia coli*, *Bacillus cereus*, *Salmonella* spp. and *Vibrio* spp. (Federle and Bassler, 2003; Miller et al., 2004; Winzer et al., 2003). In addition, AI-2 is also suspected to be involved in microbial attachment and biofilm formation that can lead to food spoilage. To better understand the role and mechanism of microbial cell to cell signaling in foods, especially since it is related to pathogen persistence, biofilm formation and food spoilage, the development of methods to quantify AI-2 molecules are required.

For the detection or determination of AI-2(s), the AI-2 bioassay is still commonly used, where the reporter *V. harveyi* BB170 strain at lower cell densities (10^5–10^7 colony forming units (CFU)/mL) is able to specifically bioluminate in response to AI-2 (Bassler et al., 1994, 1997). The bioluminescence produced as a result of the presence of AI-2 in the different samples is measured using a luminometer plate reader. This method has been utilized in a number of studies in an attempt to characterize the mode of AI-2 production in various microorganisms (Antunes et al., 2005; Bassler et al., 1997; Cloak et al., 2002; Sivakumar et al., 2008), and many bacterial species have been shown to produce AI-2 through the use of this bioassay (Antunes et al., 2005; Bassler et al., 1997). However, the cultivation conditions of the inoculums for the bioassay (i.e. media components and especially the glucose concentrations and pH) and the assay conditions, including even the

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blank reference medium, have a profound impact on the ability to detect AI-2 with this bioassay and can result in false negative or false positive results. In addition, components in some matrices can mimic AI-2 like molecules giving false positives, making it difficult to standardize this method and to reliably detect traces of AI-2 in complex media (Blair and Doucette, 2013; DeKeersmaecker and Vanderleyden, 2003; Lu et al., 2004; Tavender et al., 2008; Thiel et al., 2009; Turovskiy and Chikindas, 2006). Combined with its high variability, and that the standard *V. harveyi* strains used as indicators can produce their own AI-2 (although this is avoidable by using the MM32 mutant isolate), the standard bioluminescent method is at best qualitative only.

For other alternatives, the analytical chemical detection of AI-2 by LuxP-FRET reporters, high performance liquid chromatography or gas chromatography are difficult at present due to the complexity of the respective extraction and purification methods that are required for these procedures (Farine et al., 1993; Rajamani et al., 2007; Thiel et al., 2009). In addition, the operation cost is high. The same rational applies against the recent development of liquid chromatography coupled to tandem mass spectrometry (Campagna et al., 2009), where in addition the low efficiency of the derivatization of AI-2 in a complex matrix may result in inaccurate measurements.

The molecular structure of AI-2 is somewhat similar to ascorbic acid (Fig. 1), in that both molecules are furanones and contain a dihydroxyl group that is a site of one dimensional substitution with metal ions to yield a stable complex. Therefore, it was expected that AI-2 would also act as a reducing agent, including for the reduction of Fe(III) ions that forms the basis for the quantification of ascorbic acid (Besada, 1987). In this reaction, ascorbic acid reduces Fe(III) ions (as ferric ammonium sulphate) and in the presence of 1,10-phenanthroline the Fe(II) ions form the orange–red colored $[(o\text{-phen})_3Fe]SO_4$ ferroin complex, as shown in Eq. (1).

$$C_6H_8O_6 + FeNH_4(SO_4)_2 + 3phen \rightarrow C_6H_6O_6 + [(o-phen)_3Fe]SO_4 + 2H + SO_4 + NH_4. \quad (1)$$

The resulting $[(o\text{-phen})_3Fe]SO_4$ ferroin complex is then quantified spectrophotometrically by monitoring the absorbance at 510 nm. Accordingly, the proposed quantification of AI-2 was based on this simple reaction and spectrophotometric detection, where this study aimed to evaluate the feasibility of such, using A1-2 producing *Salmonella* Typhimurium isolates as a model system in direct comparison to the standard bioluminescence detection assay for AI-2. The application of the developed method for the investigation of the factors reported to influence AI-2 production by *S.* Typhimurium was also evaluated to allow a further indirect comparison with that already reported.

2. Materials and methods

2.1. Bacterial strains, cultivation media and inoculum preparation

Four strains of the AI-2 producing *S.* Typhimurium (ATCC 13311, ATCC 14028, DMST 28913 and DMST 28914), plus the AI-2 producing *E. coli* ATCC 4212, *Staphylococcus aureus* ATCC 65388 and *Vibrio parahaemolyticus* DMST 22093, were obtained from the Department of Medical Sciences, National Institute of Health of Thailand. The non-AI-2 producing *Sinorhizobium meliloti* SM 1021 isolate was obtained from the Chulabhorn Research Institute, Thailand. *V. harveyi* BAA-1117 (=*Vibrio campbellii* BB-170), used as the bioluminescent marker for the detection of AI-2, was obtained from the American Type Culture Collection, USA. All strains of bacteria, except for *V. harveyi*, were cultured in Nutrient Broth (NB) and maintained on Nutrient Agar (NA) (Himedia, India). *Vibrio* strains were maintained on Marine Agar and grown in Marine Broth (Difco, France). For the induction of AI-2 production in the liquid media, the bacteria were grown in peptone water (PW; 1.0% (w/v) peptone from casein (Himedia, India), 0.5% (w/v) glucose and 0.5% (w/v) NaCl), or (where stated) Luria broth (LB) or NB. For the solid media, the bacteria were cultured on peptone agar (PA; PW plus 1.5% (w/v) agar).

Bacterial conditioned culture medium, in the form of a cell free supernatant, was prepared for evaluation of the extracellular AI-2 activity by both the standard bioluminescence assay and the colorimetric method developed here. The specified bacterial isolate was cultivated in the indicated liquid media and then clarified by centrifugation at 14,000 ×*g* for 5 min. The supernatant was harvested and filtered through a 0.2 μm filter. The resulting cell free supernatant was immediately analyzed and/or stored at −20 °C until analyzed.

2.2. Bioluminescence assay

V. harveyi BAA-1117 was cultured overnight at 30 °C with aeration, and then diluted 1/5000 prior to use to assay the cell free supernatant (5 log CFU/mL). The test cell free supernatant or colony wash (150 μL) was mixed with 1.35 mL of the above diluted *V. harveyi* and incubated at 30 °C for 6 h with aeration, while fresh culture media were used as the negative control (Surette and Bassler, 1998). The luminescence of each sample was analyzed using a Microplate Luminometer (SpectraMax M5). The AI-2 level in the sample was evaluated as the bioluminescent activity (%) from 100x/y, where x and y are the emitted light from the test sample and control sample, respectively. Thus, the AI-2 level is represented by the % activity over 100%.

2.3. Characterization of substances in the colony rinses of AI-2 producing and non-producing bacteria by one-dimension (1D)-^{1}H NMR spectroscopy

Salmonella Typhimurium ATCC 13311, *V. parahaemolyticus* and *S. meliloti* were cultured in NB at 37 °C with shaking at 150 rpm overnight (~16 h) to stationary phase. The overnight-grown cultures were then swabbed on the surface of PA plates and incubated at 37 °C for 24–48 h. Colonies were removed and resuspended in deuterium oxide, for determination of AI-2 with one dimension proton nuclear magnetic resonance (1D-^{1}H NMR) spectroscopy using a Varian Mercury plus 400 MHz instrument operated with VNMR 6.1C software under Solaris, and in sterile normal saline solution (SNSS; 0.85% (w/v) NaCl) for the bioluminescence assay. For the NMR analysis, each colony rinse was clarified of cells by centrifugation at 15,000 ×*g*, 4 °C for 5 min, harvesting the supernatant and filtering through a sterilized 0.2 μm membrane filter (BD, Singapore) and then analyzed immediately or stored at −20 °C until analyzed. For the bioluminescence assay, the colony rinses (150 μL) were mixed to the diluted *V. harveyi* culture and assayed as per the protocol described in Section 2.2.

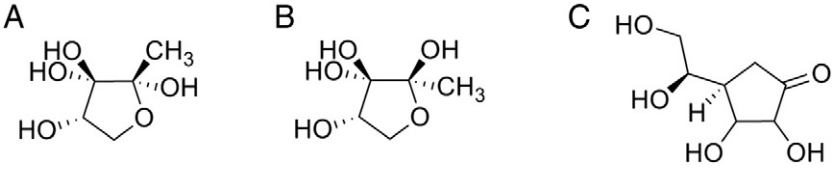

Fig. 1. The AI-2 isomers (A) (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) and (B) (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*S*-THMF), and (C) ascorbic acid (5*R*,1*S*)-1,2-dihydroxyethyl-3,4-dihydroxyfuran-2(5*H*)-one.