A target-specific bioassay for screening of bioactive AHL-analogues from natural products

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Abstract

Acylated homoserine lactones (AHLs) are membrane-permeant signal molecules responsible for biofilm formation of gram-negative bacteria via a unique mechanism known as quorum-sensing. A target specific bioassay employing the AHL-responsive *Agrobacterium tumefaciens* reporter strain has been developed to identify new AHL-like compounds from natural products, which could be developed into antifouling compounds. By varying the X-gal concentration, incubation time, solvent for sample preparation and the sample loading procedure, it was possible to detect low level AHLs up to 10^1nM. The length of the acyl chain of the AHLs was found to affect the sensitivity of this bioassay.

Introduction

Biofilms are found in many places in nature including the human body and are of great concern because they can result in virulence of infected microorganisms or environmental hazards. They are known to be formed through a unique mechanism called "quorum sensing" which involves membrane-permeant signaling molecules, AHLs (acyl-homoserine lactones).[1-3] The cells in biofilm, are more resistant to antibiotics than their free-living counterpart. de Nys et al.[4] have recently isolated halogenated furanone analogues having antifouling activity from the red algae, *Delisea pulchra,* that were chemically similar to the AHLs. Considering the abundant marine resources in Korea, novel bioactive AHL analogues are also expected. However, the absence of a sensitive bioassay has hindered the screening of active compounds, since the bioactive secondary metabolites are usually present at very low concentrations. Herein, we report the results of a parameter study for micro-detection of AHL-analogues based on the cross-feeding assay developed by Fuqua *et al* with an AHL-responsive *A. tumefaciens* strain.[2]

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains *A. tumefaciens* NTL4(pCF218)(pCF372) and *A. tumefaciens* KYC6(pCF218) were kindly provided by Dr. Fuqua at Indiana University, USA. NTL4 harbors Ti plasmidless, *tra*R plasmid(218)(Tc^R, Sp^R) and *tra*I-*lacZ*(372), provides extremely sensitive detection of AHLs. KYC6 harbors *tra*M null mutant with *tra*R overexpression plasmid(pCF218)(Tc^R, Km^R) and overproduces high levels of the *Agrobacterium* autoinducer. Prior to use, both strains were incubated on AT medium agar plates supplemented with Tc(4.5㎍/ml) and Sp(50 ㎍/ml)(NTL4) or with Km(100㎍/ml) and Tc(4.5㎍/ml)(KYC6) at 30℃.

Preparation of AHLs and X-gal solution

The AHLs used in this study are : N-hexanoyl-DL-homoserine lactone (HHL; mw, 199.25), N-octanoyl-DL-homoserine lactone (OHL; mw, 227.30), N-decanoyl -DL-homoserine lactone (DHL; mw, 255.35) and N-tetradecanoyl-DL-homoserine lactone (TDHL; mw, 311.46). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galatop yranoside) and AHLs were dissolved in DMF. The concentration of AHLs tested were 10^5, 10^3, 10^1, 10^{-1} and 10^{-3}nM. X-gal solutions were prepared in 100, 80, 60, 40, 20, 10, 5 and 2mg/ml. All solutions of X-gal and AHL were filtered by a 0.2㎛ membrane after preparation.

Bioassay with *A. tumefaciens* NTL4(pCF218)(pCF372)

A modified method of McLean *et al.*[2] was used. LB agar plates were prepared and covered with different concentrations of X-gal in DMF. Then, AHLs and the reporter strain NTL4 were streaked on the plates in various concentrations and methods. The effective AHL binding and activity was indicated by the activation of the traI-lacZ fusion by TraR[2] and a blue coloration by the expression of the β-galactosidase activity. (Fig. 1)

Results and Discussion

The assay of AHLs is based on the blue coloration produced from the metabolism of X-gal by β-galactosidase which is produced when AHLs bind to the traI-lacZ of the reporter strain NTL4(pCF218)(pCF372). This bioassay is effective only when living microorganisms are continuously producing the AHLs. To improve the sensitivity of this bioassay, various parameters affecting the coloration of NTL4 were examined.

Table 1 shows the effect of X-gal concentration on color formation. The low

concentration of 5mg/ml could give blue coloration, but the optimum concentration to give a clear coloration was about 20mg/ml. Further increase in the X-gal concentration gave a more intense color, but was not related with the sensitivity. The distance of streaks between the AHLs and the reporter strain also affected the sensitivity. When AHLs were streaked with, or overlaid on the reporter strain, better sensitivity was observed than when AHLs and the reporter strain were streaked apart from each other. The closer the distance was, the less AHLs were lost during the migration through solid agar medium. Incubation time also affected the sensitivity and the optimum time was determined to be 24hours.

The study conducted with the different AHLs suggested that some correlation between the molecular weight of the AHLs and the color response existed. (Table 2) The activity was observed to increase when the carbon number in AHLs increase up to 8 or 10, but decreased at 12. This implies that the binding affinity of AHLs are affected by their lipopholicity and there may exist an optimum lipophilicity. The parabola-shaped correlation has been observed in other similar studies.[5] Under optimized conditions, the minimum detectable concentration was 10^{1}nM for OHL and DHL, and 10^{3}nM for HHL and TDHL. (Table 2)

This study showed that the sensitivity of the cross-feeding assay can be improved significantly by controlling various factors affecting the coloration of the NTL4 strain. Bioactivity-guided fractionation of natural product is under progress, with the purpose of isolating novel AHL-like analogues.

Fig. 1. Bioassay with A. T NTL4(pCF218) (pCF372) and A. T KYC6(pCF218)

Conc. of X-gal (mg/ml)	Color change of reporter strain
100	+++++++
80	+++++++
60	+++++++
40	++++++
20	+++++
10	+++
5	++
2	-

Table 1. The effect of X-gal concentration on the color change of the reporter strain

Table 2. Color response observed in the reporter strain at different concentration and molecular size of the AHLs

	HHL	OHL	DHL	TDHL
10^{0}nM	+++++	+++++	+++++	+++++
	+++++	+++++	+++++	+++++
	+++++	+++++	+++++	+++++
10^{-1}nM	+	+++++	++++	+
	+	+++++	+++	+
	-	++++	+++	-
10^{-2}nM	+	++	++	-
		+	+	-
		+	+	-
10^{-3}nM	+	-	+	-
		-	-	-
		-	-	-
10^{-4}nM	+	-	-	-
			-	-
		-	-	-

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