In Search of Homoserine Lactone or Homoserine Lactone-like Autoinducer Production by Microbes from Local Environments

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Abstract

The ability of certain gram-negative bacteria to modulate the expression of genes in response to cell density is termed "quorum sensing" (qs). The list of bacteria capable of this type of communication is mostly comprised of symbionts and pathogens of animals and plants. However, recent research suggests that microbes in natural assemblages, such as biofilms, are also capable of qs. Thus, there is reason to believe that microbes from diverse local habitats of Woods Hole (the Great Sippewissett Salt Marsh mat layers, berries, etc.) may also regulate gene expression by qs. A modified version of the *Agrobacterium* homoserine lactone autoinducer (HSL AI) assay described by S.K. Farrand was used to assay a diverse array of microbes and environmental samples for such activity. Of the 46 samples that were tested, a methylotroph, isolated on Nitrate Minimal Salts/MeOH, by C. Francis, and whole berries, were potential HSL AI producers. These findings suggest that HSL AI production may be more common than is presently known and that further testing should be performed on a more extensive list of microbes and environmental samples. In addition, the specific source of HSL AI production in the berries should be elucidated.

Introduction

N-acylated homoserine lactone autoinducer (HSL AI) is a small, diffusible molecule produced by certain bacteria that passes freely through the cell envelope. As the population of HSL AI-producing bacteria increases, so does the concentration of this signaling molecule. Once the HSL AI concentration within a bacterial cell has reached a threshold, enough of these signaling molecules will be bound to specific intra-cellular proteins (such as LuxR in *Vibrio fischeri*) to effect transcription. These HSL-AI complex transcriptional activators can then bind to specific stretches of DNA within a promoter

Introduction

to turn on the adjacent genes. There is now a growing list of phenotypic traits (including luminescence, virulence, antibiotic production, enzyme synthesis, secondary metabolite synthesis, etc.) which are under cell density-dependent control in various bacteria. For example, in *Agrobacterium tumefaciens*, a plant pathogen, an HSL AI regulates expression of genes required to transmit the Ti (or tumor-inducing) plasmid into a susceptible plant cell. In *A. tumefaciens*, *traR* (coding for the HSL AI binding protein), and *traI* (coding for the HSL AI synthase) in the Ti plasmid, combine to form the quorum sensing system. Recently, these genes were modified or deleted from an *A. tumefaciens* strain to create strain NTL4 (pZLR4) that could be used in an assay to detect HSL AI from other bacteria. This "indicator strain" contains a *traG*::*lacZ* reporter fusion and *traR*, but does not contain *traI*. When this bacterium is exposed to exogenous HSL AI that is recognized by TraR, the resulting complex can bind to the promoter region of *traG*, and thus, can induce expression of β-galactosidase (bg). The expression of bg can be recognized by the addition of x-gal to an agar plate containing NTL4 and the sample being tested for HSL AI production.

Materials and Methods

Strains

Agrobacterium tumefaciens strain NT1 (pTiC58Δ*accR*) is a constitutive producer of Homoserine Lactone (HSL) autoinducer (AI).

A. tumefaciens NT1 lacks the Ti plasmid and does not produce detectable levels of AI.

Materials and Methods

Strains

A. tumefaciens NTL4(pZLR4) is the AI indicator strain and was maintained on nutrie t agar containing 30 μg/ml gentamicin (NA/g; to maintain the plasmid containing the *traG::lacZ* fusion under control of the HSL AI transcriptional activation binding site).

Preparation of HSL AI Extracts

Solid samples (mat layers, sediments, and berries) and bacteria grown on solid media were either directly assayed or were placed into either nutrient broth (NB) or nutrient broth with 2% NaCl (final concentration) and incubated overnight at 24°C. Liquid cultures were centrifuged at high speed in a microfuge for 5 min. The resulting cell free supernatants were removed and stored at 4°C until assayed.

The Assay

A 0.1 ml aliquot of an overnight culture of strain NTL4 grown in NB/g was added to 10 ml of NB/g and shaken for 6-8 h at 24°C. A 0.3 ml aliquot of the shaken culture and 40 μg/ml (final concentration) x-gal were then added to 3 ml of melted soft agar (0.7% agar in water; held at 42°C), mixed, and spread onto NA. The soft agar was allowed to dry for 5 min before 30 μl spots of cell-free supernatants or solid samples were added to the agar surface. Plates were incubated at 24°C overnight and checked for diffuse formation of blue x-gal cleavage product indicating that HSL AI was present.

Results /Discussion

Results are summarized in Table 1. From a set of over 40 environmental samples or strains, two, a methylotroph isolated by C. Francis, and whole berries, produced evidence for HSL AI production. Due to a lack of time, the methylotroph was not studied further. A second assay should be performed in which HSL AI is extracted and purified from this bacterium using ethyl acetate and is added to cultures of the luminous bacterium *Vibrio fischeri* in order to detect induction of luminescence. Also, its identity and phylogenetic classification should be determined in order to begin to understand its physiology and ecology. This information will be useful in assessing the potential role of an HSL AI in the cellular processes of the bacterium. Similarly, HSL AI activity was also detected in berries from the Great Sippewissett Salt Marsh. However, because this is a complex microbial consortium, the identity of the specific producer(s) of the HSL AI activity or its purpose in their environment will be difficult to assess. Eight pure cultures of bacteria from berries were also tested for HSL AI activity but were non-reactive. The potential HSL AI activity detected in the berries should also be ethyl acetate extracted and exposed to various other HSL AI indicating assays. Also, more bacteria isolated from the berries should be assayed. *In situ* hybridization using probes specific for known HSL AI-producing bacteria should be used to determine if this activity is produced by known HSL AI-producer(s) or is novel. Although the various microbial mat layers that were sampled were non-reactive in this assay, this is not conclusive proof that such activity is not present. In the future, freshly collected mat samples should be used (rather than those collected and stored at 4°C for several days), mat layers should be soaked in a variety of broth media and assayed at regular intervals for HSL AI activity rather than solely assaying cell-free extracts from overnight inocula. Recent work by Bachofen and Schenk (Microbiological Research 153(1). 1998. 61-63) revealed that microbial mats and cyanobacterial blooms in their studies produced HSL AI activity. Perhaps the assay described in this paper (using a mutant of *Chromobacterium violaceum* as the reporter) is more sensitive to an HSL AI among such a consortium within the berry. For example,

the HSL AI of *V. harveyi* does not induce luminescence in the related bacterium *V. fischeri* due to unique R proteins. In conclusion, the discovery of potential HSL AI activity in a methylotrophic salt pond isolate and within the berries of the salt marsh suggests that HSL AI production may be widespread and that further studies are required to better assess "who" are the producers and what is the purpose of such HSL AIs.

Table 1 Results

Sample	1°Enrichment Medium	2°	HSL-like activity
Mat Layers			
pink	NB		ND
green	"		"
all[a]	"		"
pink	NB +2% NaCl		"
green	"		"
all	"		"
pink	EtOH + SO_4		"
all	"		"
pink	EtOH		"
green	"		"
pink	MeOH		"
pink	SW75 + N		"
green	SW50 + N		"
black	SW75 + N		"
green	phototroph medium (no H_2S)		"
green (pink org)	"		"
green	(direct addition)		"
pink	"		"
black	"		"
grey	"		"
Berries			
colony A	phototroph medium		"
colony B	SWC	NB +2%NaCl	"
colony D	"	"	"
colony E	"	"	"
colony G	"	"	"
agar digestor	"	"	"
swarmer	"	"	"
berry consortium *	NB +2% NaCl		
berry	(direct additon)		+
berry (concentrated xtract)*	"		
HDH*	NB+2% NaCl		

Table 1 Results

Sample	1°Enrichment Medium	2°	HSL-like activity
Luminous Bacteria			
Eel Pond isolate	SWC	NB +2%NaCl	ND
MJ1	"	"	"
Beach isolate	"	"	"
Salt Pond			
SF A	PFW	NB	"
SF B	"	"	"
SF C	"	"	"
methylotroph sediment *	NMS/MeOH (direct addition)	"	+
***Loligo* ANG**			
JS isolate A	SFM	NB +2%NaCl	"
JS isolate B	"	"	"
JS isolate C	"	"	"
Extract	(direct addition)		"
Sepia* Pathogen	SWC	NB +2%NaCl	"
"Shewanella"	TYE	NB	"
Slim Jim	FWnr		"

ND= none detected

EtOH= ethanol
FWnr= freshwater non-reduced
MeOH= methanol
PFW= peptone freshwater
NB= nutrient broth
Nitrite Mineral Salts
SFM= sporeformer medium
SWC= seawater complete
SW#+N= seawater%+nitrogen
TYE= tryptone yeast extract

* Experiment was not completed due to a lack of growth of the indicator strain, perhaps due the unexpected decrease in incubator temperature from 24°C to 14°C overnight