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Isolation of genes induced by autoinducer molecules from *Rhodobacter sphaeroides*

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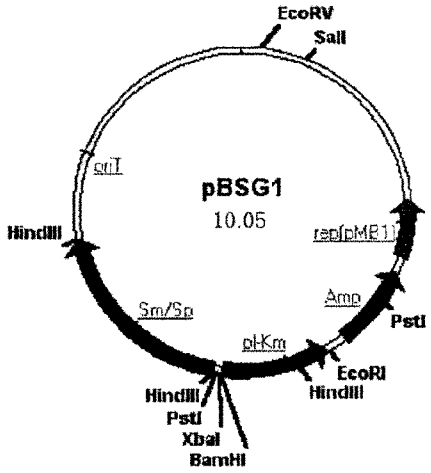
Since *N*-acyl homoserine lactone (HSL)-mediated gene regulation, called quorum-sensing regulation, was firstly found in the regulation of the *lux operon* expression in *Vibrio fischeri* (Eberhard, 1972; Nealson and Hasting, 1970), this type of regulation has been identified in the regulation of various genes in mostly gram-negative bacteria (for review, Dunlap and Greenberg, 1991; Ebel, 1999; Fuqua *et al.*, 1994; Jeon *et al.*, 1999). At least two major components are involved in common in the quorum-sensing regulation mechanisms; specific diffusable signal chemicals, called autoinducer, and a transcriptional activator molecule. Most of the autoinducer molecules belong to the *N*-acylhomoserine lactone (HSL) family, which the specificity is determined by the composition and the length of the acyl group. These molecules serve as signals to monitor population density and control expression of specific sets of genes in response to population size. Therefore, the presence of this type of molecule in a bacterial species is a good indication that a bacterium has some sets of genes that can be modulated by a quorum-sensing mechanism.

Rhodobacter is a free-living gram-negative purple nonsulfur bacterium capable of growth under diverse physiological conditions either as a chemoheterotroph or a photoheterotroph. The first report indicating that this bacterium may have quorum-sensing regulatory mechanism, was the finding of a diffusable molecule from the supernatant of culture identified by using an *Agrobacterium tumefaciens* HSL indicator strain-based TLC analysis method (Shaw *et al.*, 1997). Puska *et al.* (1977) determined the purified HSL molecule from the bacterium and determined the structure as 7,8-*cis*-*N*-(tetradecenoyl) homoserine lactone, and also identified genes corresponding to *luxR* and *luxI* (the genes encoding for transcriptional activator and for HSL synthase, respectively, from *V. fischeri*) from this bacterium. When the *cerI* gene is mutagenized, the resulting cells form large aggregates in liquid medium, and addition of the purified HSL resulted in dissolving of the clumping. Up to date, this is the only know phenotype associated with quorum sensing mechanism in this bacterium. Most of bacteria, that show quorum-sensing regulatory mechanism, are living in host-associated states, either as a pathogen or a mutualistic symbiont of an animal or plant. However, *R. sphaeroides* is known as free-living organism, and no such host-associated interaction has been described so far. Therefore, the exact physiological roles and target genes regulated by quorum-sensing mechanism in the organism remain to be elucidated.

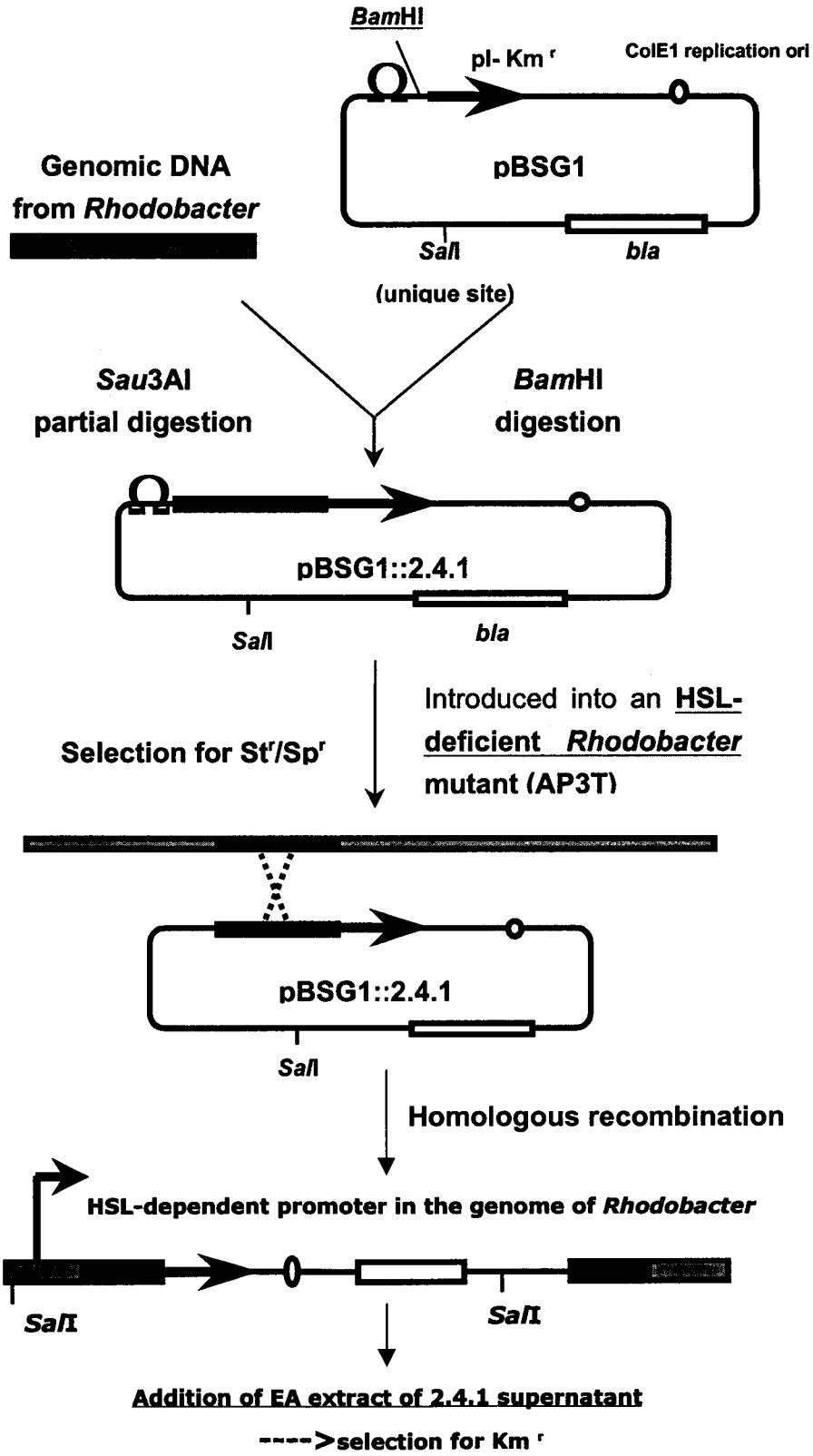
In order to identify the quorum-sensing related genes, we have developed a genetic system to isolate genes, the expression of which is modulated by HSL molecules from the supernatant of *Rhodobacter sphaeroides* 2.4.1 culture at the stationary phase. In the system, a reporter vector, named pBSG1was employed. The vector is a derivative of

ColE1, in which a promoterless *aph* gene (pl-Km^r gene) is positioned downstream to MCS and streptomycin^r/spectinomycin^r (St^r/Sp^r) cassette upstream to MCS (Figure 1).



<Figure 1. Structure of the reporter vector pBSG1>

Rhodobacter genomic DNA fragments in sizes of 3- to 5-kb prepared by *Sau3*AI-partial digestion followed by the sucrose gradient centrifugation was inserted into the *Bam*HI site at the MCS of pBSG1. The resulting library clones were transformed into *E. coli* strain S17-1. Over 10,000 transformants were subsequently mated with the strain AP3T, which is a *cerI* (AI-producing gene)-null mutant of *R. sphaeroides* 2.4.1. Since pBSG1 cannot replicate in *R. sphaeroides*, transconjugants with St^r/Spr are assumed to have the plasmid cointegrated into the chromosome of AP3T by an odd-number single crossover event between the insert and homologous region of the chromosomal DNA (Figure 2). The Km resistance of each of the resulting transconjugants was examined on the solid Sistrom's minimal media in the absence or in the presence of ethyl acetate (EA)-extracted supernatant presumably containing AI. We have isolated clones, which grew faster on the medium containing Km upon the addition of EA-extract. However, no clone was isolated that grew slower in the presence of the EA-soluble fraction than in the absence of that. The genomic DNA of each of those transconjugants, that are Km^r upon the addition of the extract, was digested with *Sal*I that cuts a single site at pBSG1 and ligated to retrieve pBSG1 containing a genomic DNA fragment which allows the pl-Km^r gene to express. The ligation reaction was transformed into *E. coli* strain DH5α, and Ap^r transconjugants were isolated. From the Ampicillin-resistant DH5α transconjugants, the pBSG1-derivative plasmids were isolated. The DNA nucleotide sequences of the inserted DNA fragments from the retrieved plasmids were determined and compared in the GenBank database. Table 1. shows the list of genes in the GenBank database showing homologies with the DNA nucleotide sequences of retrieved DNA fragments.



< Figure 2. Strategy for the isolation of clones modulated by ethyl acetate-extracted supernatant of 2.4.1 >

Possible functions	Putative protein product	Clone #
Fatty acid β-oxidation	Long-chain enoyl-CoA hydratase (*fadB*)	22
	Acyl-CoA dehydrogenase (*fadE*)	23
	Acyl-CoA dehydrogenase (*fadE*)	60
	Long-chain fatty acid-CoA ligase (*fadD*)	66
Helicase	ATP-dependent helicase (*uvrD*), HelicaseII	52
	ATP-dependent helicase (*uvrD*)	63
Carboxylase	Phosphoribosylaminoimidazole carboxylase (*purK*)	28
ATPase	pp-loop superfamily ATPase	43
Unknown	No homology with any known genes	Ten clones

<Table 1. Genes in the GenBank database showing homologies with the retrieved DNA>

We could identify several putative genes, several of which appear to encode genes associated with fatty acid β-oxidation such as acyl-CoA dehydrogenase, acyl-CoA ligase, acetyl-CoA synthetase, enoyl-CoA hydratase. Putative genes showing homologies with phosphoribosyl-aminoimidazole carboxylase and ATP-dependent DNA helicase were also included. We are currently focusing our study on those clones showing homologies with genes encoding for fatty acid β-oxidation. At the moment, we do not understand their physiological role in the bacterium. We are generating mutations on those genes in *R. spharoides*. Genetic, physiological, and biochemical studies using the mutants will allow us to understand the roles of those genes.

It appears that the strategy employed in this study can be successfully applicable for the systematic isolation of genes from gram-negative bacteria, which are modulated by quorum-sensing regulation. Recently, Whiteley *et al.* (1999) identified quorum-sensing inducible genes from *Pseudomonas aeruginosa* by generating transposable element-based random *lacZ* fusion onto the genome of the bacterium. In the study, they could isolate more than thirty genes, the expression of which was enhanced upon the addition of HSL molecules from the bacterium. To our surprise, none of the postulated functions of those identified genes appears to be matched with those of the putative genes isolated in our study. This may be due to the differences either in organism or in screen methods. In our study, we used promoterless-kanamycin resistant gene as a reporter gene. It is thought that even a basal level of expression of the reporter gene confers the resistance to the antibiotic on bacterium. Therefore, in our strategy, only those genes, that are very tightly regulated, may be isolated. We are currently construct *lacZ* fusion onto those genes isolated in our study. Quantitative analysis of the expression of the isolated genes will allow us to assess the nature of the expression level.

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