

Short Communication

Degradation of *N*-acylhomoserine Lactone Quorum-sensing Signals by *Azorhizobium caulinodans*, a Stem Nodule-forming Symbiont in *Sesbania rostrata*

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Azorhizobium caulinodans is a nitrogen-fixing bacterium that forms stem and root nodules on *Sesbania rostrata*. All tested *A. caulinodans* strains exhibited degradation activity against the quorum-sensing signaling compounds, *N*-acyl-L-homoserine lactones (AHL). The AHL-degrading gene homolog, *attM*, was identified in the genome sequences of *A. caulinodans* MAFF 210031^T and other *A. caulinodans* strains. Recombinant AttM functions as an AHL lactonase that hydrolyzes the lactone bond of AHL and retains its stable activity at environmental temperatures. The AHL-degrading activity of the *attM*-deletion mutant was completely diminished, which revealed that AHL degradation by MAFF 210031 was dependent on *attM*.

Key words: quorum sensing, acylhomoserine lactone, *Azorhizobium caulinodans*, lactonase, stem nodules

Quorum sensing is a regulatory mechanism for gene expression in response to an increase in cell density (Atkinson and Williams, 2009). *N*-acyl-L-homoserine lactones (AHLs) have been identified as quorum-sensing signaling compounds in many Gram-negative bacteria (Parsek and Greenberg, 2000). AHLs are synthesized by LuxI family proteins and diffuse outside and inside bacterial cells. When the AHL concentration increases and reaches a threshold, AHL receptor proteins belonging to the LuxR family bind to AHLs and regulate the expression of many genes (Parsek and Greenberg, 2000). Many Gram-negative plant pathogens produce AHLs and regulate their virulence factors by AHL-mediated quorum sensing (Von Bodman *et al.*, 2003). AHL-negative mutants generally show defects in pathogenicity; therefore, the disruption of quorum-sensing signals may inhibit the virulence and infection of host cells. To date, many AHL-degrading genes have been cloned and characterized (Uroz *et al.*, 2009). AHL lactonases are AHL-degrading enzymes that catalyze AHL ring opening by hydrolyzing lactones. AHL lactonases have also been used in the biocontrol of plant diseases. The expression of AHL lactonase genes in *Pectobacterium carotovorum* subsp. *carotovorum* was previously shown to significantly attenuate pathogenicity in some crops (Dong *et al.*, 2000).

Azorhizobium caulinodans is a nitrogen-fixing bacterium that forms nodules on the stem and roots of the tropical legume plant, *Sesbania rostrata* (Masson-Boivin *et al.*, 2009). *A. caulinodans* is not only a rhizobium of the leguminous plant *S. rostrata*, it is also an endophyte of non-

leguminous plants (Kurumisawa *et al.*, 2021). *S. rostrata* is widely used as a green manure in crop cultivation to supplement part of the inorganic fertilizer (Ladha *et al.*, 1989). Therefore, if *A. caulinodans* exhibits AHL-degrading activity, when *S. rostrata* with stem and root nodules formed by *A. caulinodans* is used as a green manure, *A. caulinodans* may spread throughout the soil and inhibit plant pathogens whose virulence factors are controlled by AHL-mediated quorum sensing. In the present study, we investigated the AHL-degrading activity of *A. caulinodans*, and identified and characterized an AHL-degrading lactonase encoded by an *attM* homolog.

Thirteen strains of *A. caulinodans* were obtained from the NARO Genebank (Tsukuba, Japan) and are listed in Table 1. *A. caulinodans* strains were routinely grown on TY medium (5 g L⁻¹ peptone, 3 g L⁻¹ yeast extract, and 0.9 g L⁻¹ CaCl₂·2H₂O) containing 1.5 wt% agar. *A. caulinodans* strains were inoculated into 4 mL of TY liquid medium containing 20 μM *N*-hexanoyl-L-homoserine lactone (C6-HSL) or *N*-decanoyl-L-homoserine lactone (C10-HSL). After an 18-h incubation, the remaining AHLs in the culture supernatant were visualized using the AHL biosensors *Chromobacterium violaceum* CV026 (for C6-HSL) and VIR07 (for C10-HSL), which produce the purple pigment violacein in response to AHLs (McClellan *et al.*, 1997; Morohoshi *et al.*, 2008). AHLs were detected on agar plates containing biosensors using a previously described method (Morohoshi *et al.*, 2024). All *A. caulinodans* strains completely degraded AHLs within the 18-h incubation (Fig. 1).

Table 1. *Azorhizobium* strains used in the present study

Strains	Source	Site	Location	Culture collection
<i>Azorhizobium caulinodans</i>				
MAFF 210031 ^T (=OSR 571 ^T)	<i>Sesbania rostrata</i>	stem nodule	Senegal	MAFF
MAFF 210032	<i>Sesbania rostrata</i>	root nodule	Philippines	MAFF
MAFF 210033	<i>Sesbania rostrata</i>	stem nodule	Philippines	MAFF
MAFF 210034	<i>Sesbania rostrata</i>	stem nodule	Philippines	MAFF
MAFF 210035	<i>Sesbania rostrata</i>	stem nodule	Philippines	MAFF
MAFF 210036	<i>Sesbania rostrata</i>	stem nodule	Philippines	MAFF
MAFF 210164	<i>Sesbania rostrata</i>	stem nodule	Philippines	MAFF
MAFF 210165	<i>Sesbania rostrata</i>	root nodule	Philippines	MAFF
MAFF 210166	<i>Sesbania rostrata</i>	stem nodule	Philippines	MAFF
MAFF 210287	<i>Sesbania rostrata</i>	stem nodule	Thailand	MAFF
MAFF 210309	<i>Sesbania rostrata</i>	stem nodule	Thailand	MAFF
MAFF 210459	<i>Sesbania rostrata</i>	stem nodule	Japan	MAFF
MAFF 210460	<i>Sesbania rostrata</i>	stem nodule	Japan	MAFF
<i>Azorhizobium doebereineriae</i>				
NBRC 107856	<i>Sesbania virgata</i>	root nodule	Brazil	NBRC

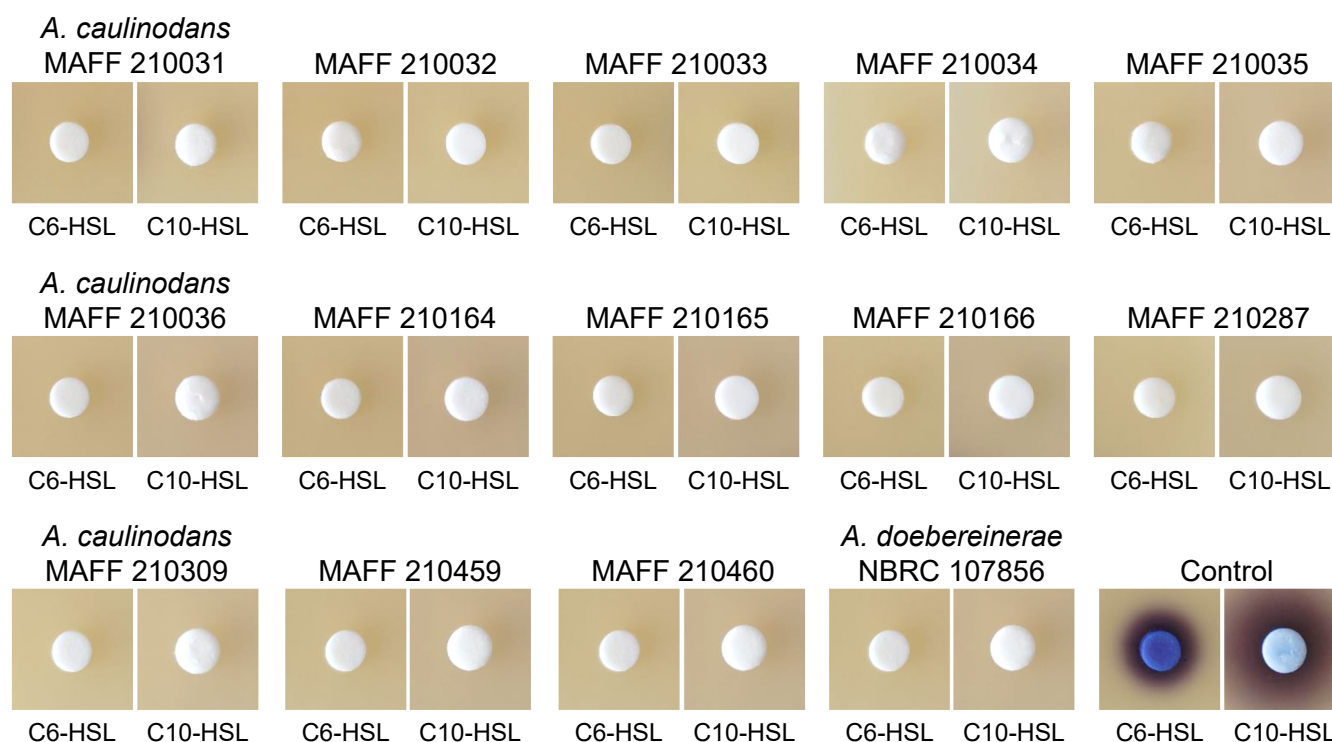


Fig. 1. AHL-degrading activities of 13 strains of *Azorhizobium caulinodans* and *Azorhizobium doebereineriae* NBRC 107856. The strains were inoculated into TY medium containing 20 μ M C6-HSL or C10-HSL and then incubated at 30°C for 18 h with shaking. C6-HSL and C10-HSL remaining in the supernatant were detected on LB agar plates containing *Chromobacterium violaceum* CV026 and VIR07, respectively. The plates were incubated at 30°C overnight, and the appearance of a purple pigment was assessed. The disappearance of the purple pigment indicated the degradation of the AHLs being tested.

Azorhizobium doebereineriae has been isolated from the root nodules of another leguminous plant, *Sesbania virgata*, but is phylogenetically different from *A. caulinodans* (Maria de Souza Moreira *et al.*, 2006). When the AHL-degrading activity of *A. doebereineriae* type strain NBRC 107856 was examined using the same method as that for *A. caulinodans*, NBRC 107856 exhibited similar AHL-degrading activity to *A. caulinodans* (Fig. 1). These results suggest that AHL-degrading activity is widespread in the genus *Azorhizobium*.

The complete genome sequence of *A. caulinodans* type strain MAFF 210031 (=ORS 571) has been deposited in the DDBJ/ENA/GenBank databases (RefSeq accession no.

GCF_000010525.1). When the presence of an AHL lactonase gene homolog in the genome sequence of MAFF 210031 was investigated, a putative Zn-dependent hydrolase (locus tag AZC_RS03915) showed high identity (86.3%) with the AHL lactonase AttM from *Agrobacterium tumefaciens* A6 (accession no. AY052389) (Khan and Farrand, 2009). The genome sequences of eight strains belonging to the genus *Azorhizobium* have been deposited in the RefSeq database at NCBI (Table 2) as of August 25, 2025. The *attM* gene homolog was present in the genomes of most strains, except for *Azorhizobium oxalatophilum* CCM 7897. Although a specific species of *Azorhizobium* sp.

Table 2. Genomic information on *Azorhizobium* species deposited in the RefSeq database at NCBI

Strains	RefSeq accession no.	Locus tag of <i>attM</i>
<i>Azorhizobium caulinodans</i>		
ORS 571 ^T (=MAFF 210031 ^T)	GCF_000010525.1	AZC_RS03915
CNM20220104	GCF_036600915.1	V5728_RS01715
CNM20190194	GCF_036600855.1	V5726_RS04185
CNM20190462	GCF_036600875.1	V5730_RS00645
CNM20190156	GCF_036600895.1	V5727_RS01090
<i>Azorhizobium doebereineriae</i>		
UFLA1-100 ^T	GCF_000473085.1	YU1_RS0114775
<i>Azorhizobium oxalatophilum</i>		
CCM 7897 ^T	GCF_014635325.1	—
<i>Azorhizobium</i> sp.		
AG788	GCF_004364705.1	DFO45_RS24290

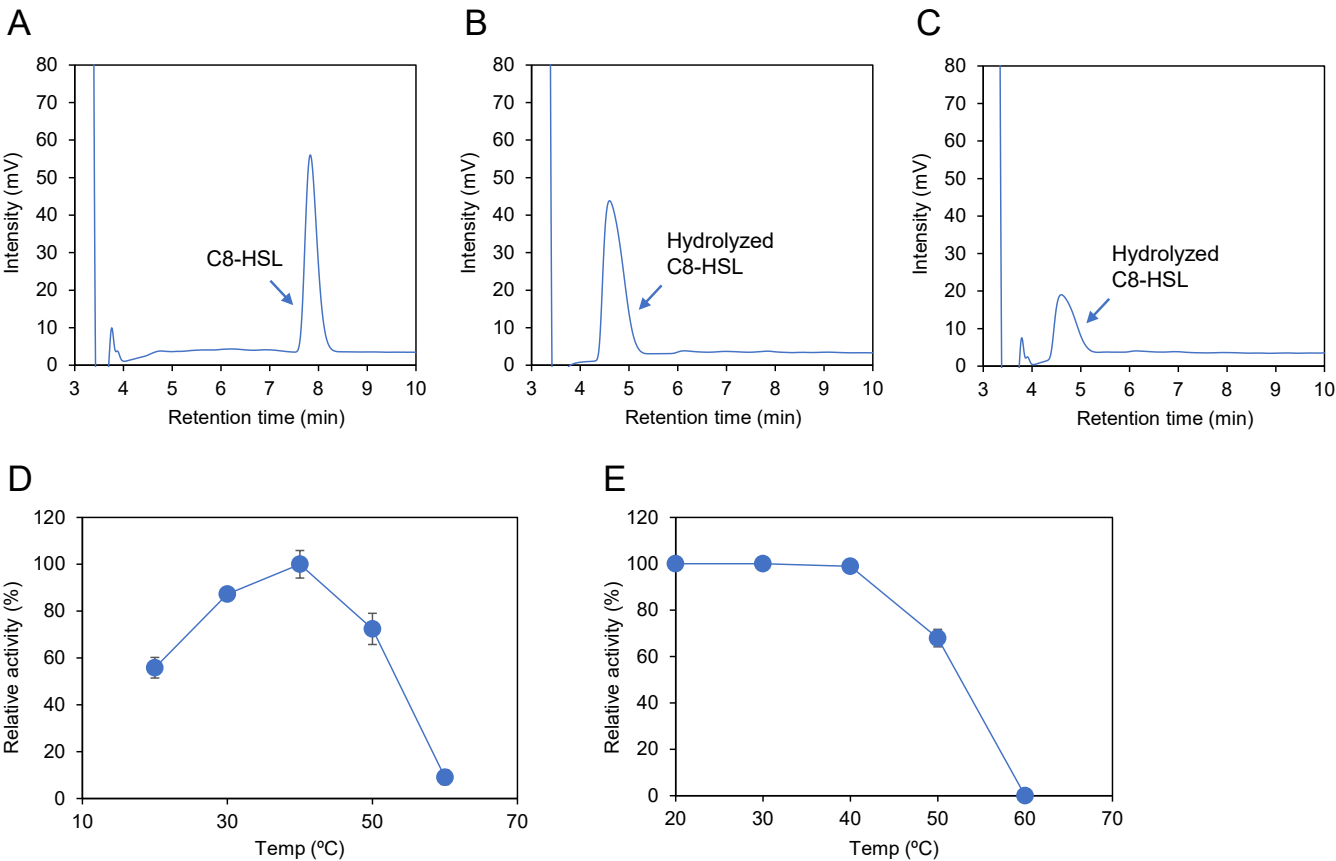


Fig. 2. HPLC profiles of C8-HSL (A), C8-HSL hydrolyzed with 10 mM NaOH (B), and C8-HSL treated with binding buffer (D). The peaks corresponding to C8-HSL (retention time of approximately 7.8 min) and hydrolyzed C8-HSL (4.6 min) are indicated by arrows. To select the optimal temperature, His-tagged AttM was mixed with C8-HSL and incubated at temperatures ranging from 20°C to 60°C (D). To assay thermostability, AttM was pre-incubated at temperatures ranging from 10°C to 60°C for 10 min. Pre-incubated AttM was mixed with C8-HSL and incubated at an optimal temperature of 40°C. After an incubation for 1 h, the residual substrate was quantified using HPLC. The maximum activity of each AHL lactonase was defined as 100%. Data were reproduced at least three times, and error bars indicate standard deviations.

AG788 has not been identified, based on the average nucleotide identity (ANI) value between AG788 and ORS 571, AG788 may be identified as *A. caulinodans* (ANI>97%). Therefore, it was assumed that the *attM* gene homolog was widely conserved in the genomes of *A. caulinodans*.

To characterize the AHL-degrading activity of AttM from MAFF 210031, a plasmid expressing His-tagged AttM at the C terminus was generated. The *attM*-coding region was amplified using KOD FX Neo DNA polymerase (Toyobo) with forward (5'-CATATGACCGACATCCGCTCTATATG

CTTCAGTC-3') and reverse (5'-GTCGACGTCGTAATAG CCGGGGCCTTCTGAAGG-3') primers. The PCR product was digested with *NdeI* and *SalI* and inserted into the same restriction site in a pET21b vector (Novagen). The expression and purification of His-tagged AttM was performed using a previously described method (Morohoshi *et al.*, 2024). Purified His-tagged AttM was mixed with *N*-octanoyl-L-homoserine lactone (C8-HSL) and analyzed by HPLC. The HPLC conditions used were described in a previous study (Morohoshi *et al.*, 2024) and the data obtained were reproduced at least three times. Fractionation of the

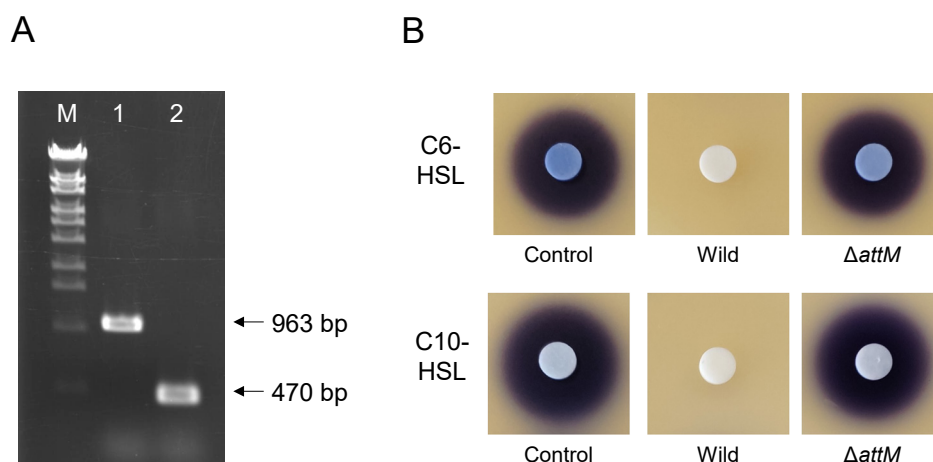


Fig. 3. (A) PCR amplification of the *attM*-coding region in MAFF 210031 and $\Delta attM$. PCR products were electrophoresed on a 1.5% agarose gel. Lane M: One Step Marker 6 (Nippon Gene) as the DNA size marker, Lane 1: MAFF 210031 wild-type, and Lane 2: $\Delta attM$. (B) AHL-degrading activities of MAFF 210031 and $\Delta attM$. The strains were inoculated into TY medium containing 20 μ M C6-HSL or C10-HSL and then incubated at 30°C for 18 h with shaking. C6-HSL and C10-HSL remaining in the supernatant were detected on LB agar plates containing *Chromobacterium violaceum* CV026 and VIR07, respectively. The plates were incubated at 30°C overnight, and the appearance of a purple pigment was assessed. The disappearance of the purple pigment indicated the degradation of the AHLs being tested.

C8-HSL standard and lactone ring-opened C8-HSL revealed one major HPLC peak with retention times of approximately 7.8 and 4.6 min, respectively (Fig. 2A and B). When AttM was mixed with C8-HSL and incubated at 30°C for 1 h, the fractionation of AttM-treated C8-HSL revealed one HPLC peak corresponding to lactone ring-opened C8-HSL (Fig. 2C). These results demonstrate that AttM from MAFF 210031 is an AHL lactonase that catalyzes lactone ring opening by hydrolysis. Although general AHL lactonases degrade a wide range of AHL structures, including 3-oxo-substituted AHLs, an analysis of the degrading activity of AttM for various structures of AHL will be the subject of future work. The optimal temperature and thermostability of AttM were then examined using a previously described method (Morohoshi *et al.*, 2024). The optimal temperature for AttM was approximately 40°C (Fig. 2D), and AttM retained its maximum activity after a pre-incubation at temperatures below 40°C (Fig. 2E). These results demonstrate that AttM from *A. caulinodans* exhibited high and stable degradation activity in the temperature range found in the natural environment.

The *attM* deletion mutant was constructed to investigate the role of AttM in the AHL-degrading activity of MAFF 210031. The internal region of *attM* in the chromosome of MAFF 210031 was deleted using the homologous recombination method described in a previous study (Morohoshi *et al.*, 2017). When the deletion of the internal region of *attM* was confirmed by PCR, the size of *attM* was approximately 500 bp smaller than the wild type (Fig. 3A). MAFF 210031 and the *attM* mutant ($\Delta attM$) were inoculated into 4 mL of TY liquid medium containing 20 μ M C6-HSL or C10-HSL, and the remaining AHLs in the culture supernatant were detected after the 18-h incubation. C6-HSL and C10-HSL were completely degraded in the culture supernatant of the wild type; however, the majority of AHL remained in the supernatant of $\Delta attM$ (Fig. 3B). Although *attM* complementation in $\Delta attM$ was not performed, these results suggest that AHL degradation within MAFF 210031 was dependent on *attM*.

In summary, we herein demonstrated that *A. caulinodans*, which forms nodules on the roots and stem of *S. rostrata*, exhibited AHL-degrading activity and also that the AHL-lactonase gene *attM* was widespread in *A. caulinodans*. When *S. rostrata* with stem or root nodules formed by *A. caulinodans* is used as a green manure, the AHL-degrading activity of *A. caulinodans* spread throughout the field may exert inhibitory effects on plant pathogenic bacteria to control their pathogenicity through AHL-mediated quorum sensing. Furthermore, since AttM stably degraded AHLs, even at natural environmental temperatures, it is conceivable that the AHL-degrading activity of *A. caulinodans* may be maintained in the long term.

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