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Bacteriocin *small* of *Rhizobium leguminosarum* Belongs to the Class of *N*-Acyl-L-Homoserine Lactone Molecules, Known as Autoinducers and as Quorum Sensing Co-Transcription Factors

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small **bacteriocin was isolated from the culture broth of the gram-negative bacterium *Rhizobium leguminosarum*, which forms symbiotic nitrogen-fixing root nodules on a number of leguminous plants. The structure of the molecule was elucidated by spectroscopic methods and identified as *N*-(3*R*-hydroxy-7-*cis*-tetradecanoyl)-L-homoserine lactone. The absolute configuration of both asymmetric carbon atoms in the molecule was determined by the use of the chiral solvating agents *S*-(+)- and *R*-(−)-2,2,2-trifluoro-1-(9-anthryl)-ethanol. *small* bacteriocin is structurally related to the quorum sensing co-transcription factors for genes from other bacteria such as *Vibrio fischeri*, *Pseudomonas aeruginosa*, *Erwinia carotovora*, and *Agrobacterium tumefaciens* which are involved in animal-microbe or plant-microbe interactions. The mechanism of regulation of such interactions by this kind of co-transcription factors is still unknown in *R. leguminosarum*.**

small bacteriocin (*small*) is produced by strains of all three biovars of *Rhizobium leguminosarum* and inhibits the growth of *R. leguminosarum* bv. viciae 248 and several other strains of this species which, like strain 248, contain a self-transmissible plasmid (12, 17). Two genes located close to the transfer (*tra*) genes (17) on the Sym plasmid pRL1JI of strain 248 are responsible for the fact that this strain does not produce *small* (*rps* [repression production *small*] gene) and that it is sensitive to *small* (*sbs* [*small* bacteriocin sensitivity] gene) (12, 17). When strain 248 is cured of its Sym plasmid, pRL1JI (as, for example, in strain RBL1390), it is insensitive to *small* and also produces *small* (12, 17). To our knowledge, *small* is produced only by *R. leguminosarum* strains, and in nonproducing strains of this species, a gene for *small* production like that in strain 248 is present (12, 17). Therefore, the presence of *small* is considered a characteristic of this species. Since another typical property of this species is symbiotic root nodule formation on certain leguminous plants, it is possible that both properties are related. However, a strain with a Tn*5* insertion in the *small* gene(s) could induce formation of normal root nodules, which shows that the *small* gene is not required for root nodule formation (16). However, this does not exclude an ecological link between *small* production and the interaction of *Rhizobium* spp. with plants, since other genes may complement the lost function or the function is not essential. In order to detect its biological significance, *small* was extracted from the bacterial culture medium with chloroform (16) and identified chemically. The *small* molecule appeared to contain an *N*-acyl homoserine lactone structure, as known from quorum sensing signal molecules, which function as co-transcription factors in bacteria that often interact with higher organisms (reviewed by Fuqua et al. [10]). The implications of this finding are discussed.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were the *small*-sensitive strain *R. leguminosarum* bv. viciae 248, which harbors Sym plasmid pRL1JI (16); and strain RBL1390, a derivative of 248 cured of the Sym plasmid pRL1JI, which produces *small*. Strain RBL1390 has reduced slime production, which facilitates extraction of *small*. The bacteria were grown at 28°C on B^- or B^+ medium (16). For plates, 1.8% agar was added.

For *small* extraction, the bacteria were cultured in aqueous B^- medium. Cultures up to 1 liter were aerated by shaking in a New Brunswick G25 incubator; 10-liter quantities underwent forced aeration.

small **bioassay.** *small*-containing fractions were pipetted on filter paper disks (diameter, 15 mm) and the solvent was evaporated in air under sterile conditions. The disks were transferred to fresh plates containing 25 ml of B^+ agar, in which 0.5 ml of B^+ medium culture of strain 248 (optical density at 660 nm of 0.1) was suspended. Inhibition zones around the disks could be observed after 2 days of incubation.

small **production and extraction.** Bacteria of strain RBL1390 were grown in B^- medium to an optical density at 660 nm of 0.3 to 0.5 and extracted with a 3× 50-ml/liter culture of chloroform-methanol (97.5:2.5 [vol/vol]). The combined fractions were evaporated, and the dry residue was dissolved in acetonitrile-H_2O (50:50 [vol/vol]).

Purification of *small*. *small*, dissolved in acetonitrile-H_2O, was applied to a C_{18} reversed-phase high-performance liquid chromatography (HPLC) column (Hypersil SpheriSorb) or a C_2-C_{18} reversed-phase HPLC column (SuperPacPepS) and eluted with a linear acetonitrile-H_2O gradient (50 to 100% acetonitrile). The fractions were monitored at A_{200} and checked for *small* activity with the bioassay. Active fractions were pooled and again checked for purity with an analytical Shandon C_{18} Hypersil column.

Spectra. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM-300 spectrometer equipped with an Aspect 2000 data system, on a Bruker MSL-400 spectrometer equipped with an Aspect 3000 data system, or on a Bruker AMX-600 spectrometer. Spectra were recorded in $CDCl_3$ at room temperature, except when the shift reagents were used (described below). Chemical shifts are presented in δ values relative to trimethylsilane. Standard procedures were used for the two-dimensional methods. Two-dimensional correlated spectroscopy (COSY) was measured according to the method of Aue et al. (1), two-dimensional CH correlation was performed according to the method of Bax (2), ^{1}H-detected heteronuclear single-quantum coherence (HSQC) was performed according to the method of Bodenhausen and Ruben (4), and heteronuclear multiple-bond connectivity (HMBC) was performed according to the method of Bax and Summers (3). The mass spectrum was obtained by gas chromatography-mass spectrometry by the system described by Dagnino et al. (7). Mass spectrum: *m/z* (relative intensity), 325 ($M^{+\cdot}$, 12%), 185 (52%), 143 (32%), 102 (100%).

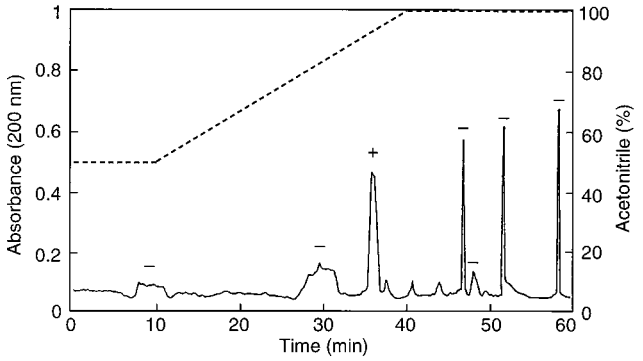

FIG. 1. Elution profile of reversed-phase HPLC purification of *small* bacteriocin. A crude *small* bacteriocin extract, dissolved in acetonitrile-H_2O (50:50 [vol/vol]) was applied to a C_{18} HPLC column and eluted with an acetonitrile-H_2O gradient. Only one peak fraction (indicated by +) was active in the bioassay for small bacteriocin. Inactive peak fractions are indicated by −.

Experiments with CSAs. To determine the stereochemistry of *small*, the amides of L-homoserine lactone with β-hydroxybutyric acid (both *R*- and *S*-) were prepared by a slightly modified version of the procedure reported by Cao and Meighen (5). Thus, 195 mg of D-(−)- or L-(+)-β-hydroxybutyrate (Sigma) and 467 mg of (−)-α-amino-τ-butyrolactone hydroiodide (Janssen) were treated for 4 h at room temperature with 370 mg of 1-ethyl-3-(dimethylaminopropyl)carbodiimide in 4 ml of water. Reaction mixtures were extracted four times with 25 ml of CH_2Cl_2. The organic phases were evaporated to near dryness and purified by short-column silica gel chromatography (2% CH_3OH in CH_2Cl_2). D-*N*-(3-hydroxybutyryl)homoserine lactone and L-*N*-(3-hydroxybutyryl)homoserine lactone (3′*R*-HBHL and 3′*S*-HBHL, respectively) were isolated in 26 and 22% yields, respectively. The shifts of the ^{1}H-NMR signals of both amides upon addition of CSA were compared with those of *small*. In this comparison, Δδ values were used, with Δδ defined as the difference in chemical shift of a certain proton as a result of the addition of 2,2,2-trifluoro-1-(9-anthryl)-ethanol (TFAE). A negative value denotes an upfield shift. The following chiral solvating agents (CSAs) were used: *S*-(+)-TFAE (Aldrich; stock no. 21,134-6) and *R*-(−)-TFAE (Aldrich; stock no. 21,135-4). The CSAs were added in a $CDCl_3$ solution (2.8 and 3.0 mg/ml, respectively). Spectra were measured at 253 K.

TABLE 1. ^{1}H-NMR signals from *small* bacteriocin at room temperature and at −20°C and shifts obtained with *R*-(−)-TFAE and *S*-(+)-TFAE at −20°C

Proton (designation)[a]	^{1}H-NMR signal δ (ppm)		Splitting pattern [coupling constant(s) (Hz)]	Δδ (ppm) at −20°C	
	Room temp	−20°C		+*R*-(−)-TFAE	+*S*-(+)-TFAE
NH (A)	6.40	6.53	dbr (5.1)	−0.040	−0.038
H-8′ (B)	5.38	5.38	dt (11.0; 7.0)	0.000	−0.002
H-7′ (C)	5.33	5.31	dt (11.0; 7.0)	−0.004	−0.007
H-2 (D)	4.55	4.58	ddd (11.7; 8.6; 6.0)	−0.051	−0.028
H-4a (E)	4.49	4.52	t (9.1)	−0.020	−0.022
H-4b (F)	4.29	4.32	ddd (11.1; 9.3; 5.9)	−0.027	−0.020
H-3′ (G)	4.03	4.02	m	−0.030	−0.024
OH	3.53	3.53	sbr		
H-3a (H)	2.84	2.84	ddd (12.4; 8.0; 6.2)	−0.039	−0.033
H-2′a (I)	2.46	2.46	dd (15.4; 3.6)	−0.039	−0.041
H-2′b (J)	2.34	2.34	dd (15.4; 9.1)	−0.046	−0.047
H-3b (K)	2.18	2.21	dddd (11.7; 11.7; 11.7; 8.9)	−0.036	−0.040
H-6′ (L)	2.06	2.05	ddd	−0.011	−0.017
H-9′ (M)	2.01	1.99	ddd	0.000	−0.002
H-4′/5′a (N)	1.5	1.5	m		
H-5′b (O)	1.4	1.4	m		
H-10′-13′ (P)	1.25	1.25	m		
H-14′ (Q)	0.88	0.86	t (6.8)	+0.002	+0.002

[a] Proton designations as used in Fig. 2, 3, and 4.

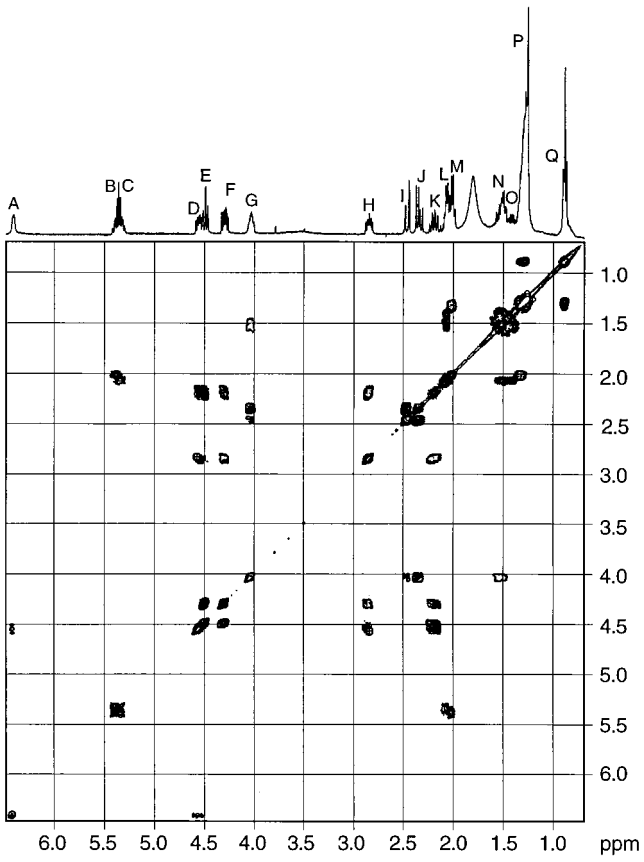

FIG. 2. COSY spectrum of *small* bacteriocin ($CDCl_3$; 400 MHz).

RESULTS

Extraction and assay of *small*. Chloroform extracts from supernatants of *R. leguminosarum* bv. viciae RBL1390 cultures inhibited strain 248 in the bioassay described in Materials and Methods. The chloroform was evaporated and the residue, dissolved in an acetonitrile-water mixture, was applied to the HPLC columns. Only one column fraction, monitored at a wavelength of 200 nm, was active in the bioassay (Fig. 1). The active fractions from several HPLC runs were pooled and checked for purity as described in Materials and Methods. After drying, a very small quantity (about 0.5 mg from 10 liters of culture medium) of a yellow oil was obtained.

Identification of *small*. The ^{1}H-NMR spectrum of *small* displayed 18 different signals (Fig. 2 and Table 1). The presence of a fatty acid chain was indicated by the superimposed signals of aliphatic CH_2 signals around 1.3 ppm and the terminal CH_3, which gave a signal at 0.88 ppm. Furthermore, the two overlapping signals from olefinic protons at 5.3 ppm indicated the presence of a double bond in the fatty acid chain. These signals coupled to each other with a coupling constant of 11.0 Hz, thus establishing the *cis* configuration of the double bond. A two-dimensional COSY spectrum (Fig. 2) indicated the presence of two spin systems. One consisted of six protons and included the most downfield signal, which coupled to a single other proton at 4.55 ppm. This signal coupled to two other protons at 2.84 and 2.18 ppm, which coupled again to two other protons at 4.49 and 4.29 ppm. The high chemical shift value of the most downfield signal at 6.40 ppm indicated that this proton was connected to a nitrogen and not to a carbon. It should then be

TABLE 2. ^{13}C-NMR signals from *small* bacteriocin

Carbon (designation)[a]	^{13}C-NMR signal δ (ppm)
C-1 (a)	175.22
C-1′ (b)	172.86
C-8′ (c)	130.63
C-7′ (d)	129.00
C-3′ (e)	68.48
C-4 (f)	66.07
C-2 (g)	49.25
C-2′ (h)	42.45
C-4′ (i)	36.40
C-12′ (j)	31.76
C-3 (k)	30.32
C-11′ (l)	29.67
C-10′ (m)	28.98
C-9′ (n)	27.27
C-6′ (o)	26.92
C-5′ (p)	25.54
C-13′ (q)	22.65
C-14′ (r)	14.10

[a] Carbon atom designations as used in Fig. 3 and 4.

exchangeable with deuterium atoms from D_2O, and this was indeed found, thus establishing the spin system as derived from a -NH-CH-CH_2-CH_2- substructure.

The other spin system was more difficult to derive because of heavy overlapping of signals. Two signals at 2.34 and 2.46 ppm showed a common coupling constant of 15.4 Hz, thus indicating that they were derived from a single CH_2. They both coupled to one other proton showing a signal at 4.03 ppm. The latter proton coupled to one or two protons at 1.5 ppm. The subsequent coupling pattern was difficult to follow, but clearly no direct coupling between the large signal of aliphatic protons at 1.3 ppm and the signal at 1.5 ppm was observed. A cross signal of the signal at 1.5 ppm with a quartet at 2.06 ppm was observed. This in turn showed two further cross peaks: one to a signal at 1.4 ppm and another to the aforementioned olefinic protons, and in this case, to the lower of the two. The other olefinic proton coupled to a signal at 2.01 ppm, also a quartet, which in turn coupled to the complex signal at 1.3 ppm. This signal showed subsequent coupling only to the terminal methyl of the fatty acid chain. By integration, the number of protons responsible for this signal was estimated to be about 10.

A ^{13}C-NMR spectrum yielded 18 signals (Table 2). To assign the signals, a ^{1}H-^{13}C-correlated spectrum was recorded on a 400-MHz apparatus. However, because of the limited quantity of compound, several connectivities could not be observed. An HSQC spectrum measured with a 600-MHz apparatus did, however, reveal all of the direct connectivities between the protons and carbons. With the 600-MHz apparatus, an HMBC spectrum could also be measured from the small amount of compound available (Fig. 3). The long-range correlations thus obtained enabled the complete assignment of all ^{1}H and ^{13}C signals. The observed connectivities are displayed in Fig. 4. The structure deduced from the NMR spectra was in accordance with the molecular weight of 325 obtained from the mass spectrum.

Elucidation of the stereochemistry of the asymmetric centra. The configuration of the two asymmetric centra of the molecule was determined by comparing the displacements of the ^{1}H-NMR signals of *small* induced by the CSAs *S*-(+)- and *R*-(−)-TFAE with those of the reference compounds 3′*R*- and 3′*S*-HBHL induced by *S*-(+)-TFAE. An initial observation was that, upon application of the CSA, *small* did not show any doubling of signals (Table 2), thus excluding the possibility that a mixture of stereoisomers could be present. In Table 3, the ^{1}H-NMR signals of both 3′*R*- and 3′*S*-HBHL molecules are tabulated, together with the displacements induced by the CSA. Correlation of the displacements of the *small* signals with those of the reference compounds yielded a series of graphs (Fig. 5, lines a to d). Comparison of the graphs shows that in only one case is a linear relationship observed: the graph in which the displacements of the 3′R-HBHL signals induced by the *S*-(+) reagent are correlated with those of *small* induced by the same reagent. This is a strong indication that *small* has the same stereochemistry of both asymmetric centra as 3′*R*-HBHL (thus, 3′*R*,2*S*).

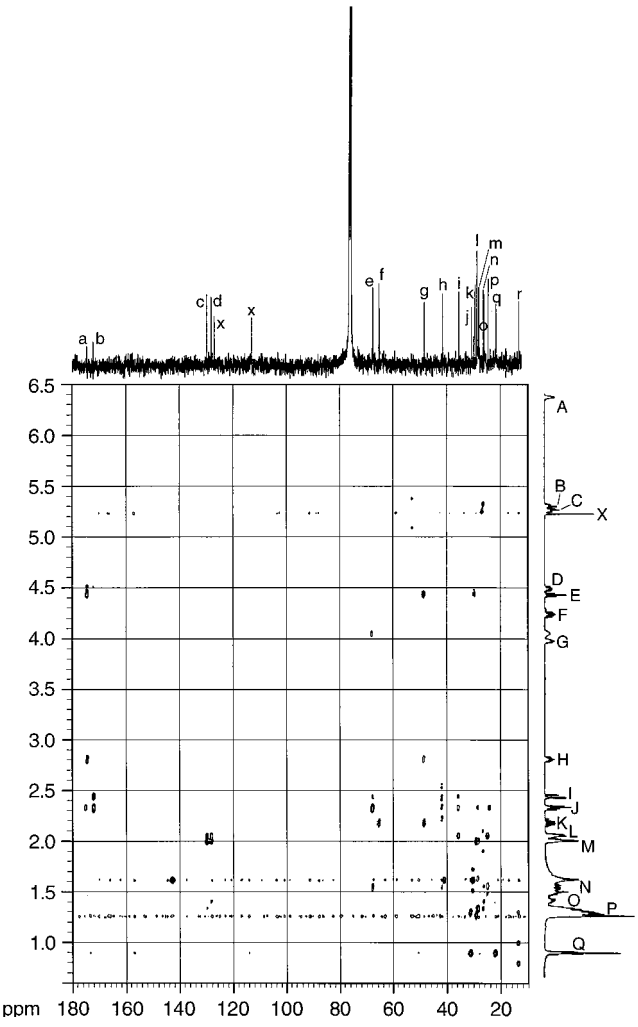

FIG. 3. HMBC spectrum of *small* bacteriocin ($CDCl_3$; 600 MHz).

DISCUSSION

Bacteriocin *small* of *R. leguminosarum* was isolated by bioassay-guided fractionation. The inhibition of growth of strain 248 of *R. leguminosarum* bv. viciae was used as a bioassay. By spectroscopic means, *small* was identified as an *N*-acyl-L-homoserine lactone (aHSL). These molecules are known as autoinducers and as quorum sensing co-transcription factors (8). In the accompanying paper by Gray et al. (11), partial identification of the autoinducer of the *rhi* genes of *R. leguminosa-*

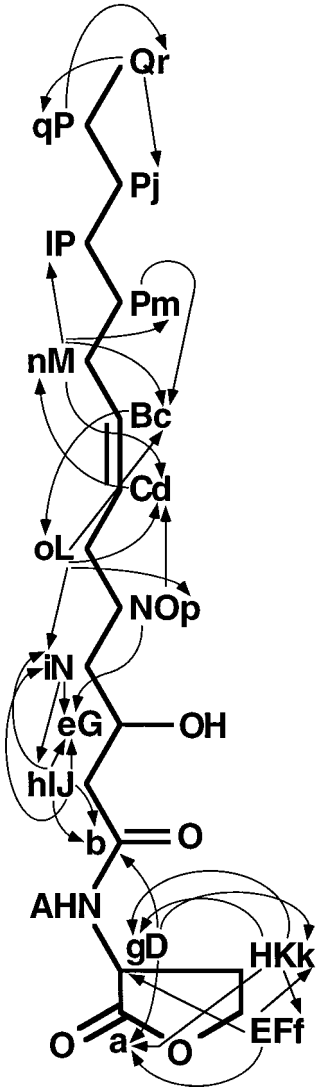

FIG. 4. Chemical structure of *small* bacteriocin. Arrows indicate long-range ^{1}H-^{13}C correlations as observed in the HMBC spectrum (Fig. 3). Protons are indicated by capital letters corresponding to those in Table 1. The carbon atoms are indicated by lowercase letters corresponding to those in Table 2.

rum has been described. Gray et al. used a bioassay in which the activation of the *rhi* promoter was assessed by measuring the β-galactosidase activity of a *rhiA-lacZ* construct. The compound they isolated and called RLAI (*R. leguminosarum* autoinducer) was shown by spectroscopic data to be identical to our bacteriocin *small*. Gray and coworkers expected to identify an aHSL molecule as the autoinducer of the *rhi* genes because of the homology of the *rhiR* gene with the aHSL-regulated *luxR* gene of *V. fischeri*. In contrast, we were surprised to identify the bacteriocin *small* as an aHSL molecule. On the other hand, the growth-inhibiting properties of RLAI were a surprise to Gray et al. (11). Our approach to chemically identify a molecule in order to get more knowledge about its possible biological function proved to be very useful, because we now know that we have to study autoinduction and quorum sensing to unravel the mechanism of growth inhibition by *small* and to determine its symbiotic function(s).

We employed some special analytical techniques to allow for determination of the complete structure of *small.*

The inverse-detected carbon proton correlation spectrum (HMBC [Fig. 3]) proved to be an extremely powerful tool for elucidation of the *small* structure, the more so since the sample contained only about 0.5 mg of the compound. The spectrum displayed nearly all of the expected signals due to two- or three-bond couplings (Fig. 4). The structure deduced contains two asymmetric carbon atoms, one in the homoserine lactone part, which could be D or L configurated (*R* or *S*, respectively). The L configuration would be most probable, but experimental proof was needed for the confirmation. The other asymmetric carbon bears the β-hydroxyl moiety in the fatty acid chain. Any of the four possible stereoisomers or even a mixture of stereoisomers could thus be present.

The small amount of compound available limited the number of methods for the determination of the stereochemistry of the asymmetric centra of the molecule. The conventional methods such as optical rotatory dispersion and circular dichroism were not feasible. NMR spectroscopy in combination with an asymmetric shift reagent seemed to offer the best possibilities for tackling this problem.

The large number of asymmetric shift reagents can be divided into two groups (14): chiral lanthanide shift reagents and CSAs. They are paramagnetic and diamagnetic, respectively, and furthermore there are differences in the respective additive-solute association mechanisms and differences in the origin and appearance of the spectral perturbations. Chiral lanthanide shift reagents are more suitable for the determination of enantiomeric purity, mainly because they are able to induce much larger shifts and thus nonequivalence of enantiomeric solutes (15). However, for the assignment and comparison of absolute configuration, the chiral lanthanide shift reagents are less suitable because of the complexity of the lanthanide-solute systems. An additional advantage of CSAs for solving our present problem would be the availability of both enantiomers of the shift reagent. If the exact conformation of the CSA-solute complex is predictable, the stereochemistry of the solute might be deduced from the displacements of the NMR signals. However, in the case of *small*, the exact conformation of the CSA-solute complex would be very difficult to predict; therefore, suitable reference compounds were essential. The amides of L-homoserine lactone with both *R*- and *S*-β-hydroxybutyric acid were synthesized (3′*R*-HBHL and 3′*S*-HBHL, respectively).

As a shift reagent, TFAE was chosen because it is well-known that TFAE induces relatively large chemical shift nonequivalence. Moreover, this shift reagent was available in both the *R* and *S* configurations, which would enable solution of the stereochemistry of both asymmetric centra at the same time.

In Table 3, the ^{1}H-NMR signals of both HBHL molecules are tabulated. A comparison of these spectra revealed that the chemical shifts of the H-2′ proton signals were slightly different and that the ones of 3′*R*-HBHL most resembled those of *small*. To both reference compounds, the CSA *S*-(+)-TFAE was applied. For *small*, both the *S*- and *R*-configurated CSAs were used. In all cases, application of the CSAs gave small but significant perturbations of the chemical shifts. In the case of *small*, no doubling of signals was observed, which excludes the possibility that a mixture of stereoisomers could be present.

Unfortunately, the magnitudes of the Δδ values of *small* and of the reference compounds cannot be compared directly, because it is extremely difficult to prepare NMR samples with exactly the same concentrations of CSA and solute. Moreover, small amounts of impurities in either of the samples can affect the magnitude of the Δδ values. For these reasons, we used a graphical approach. In Fig. 4, the Δδ value of each proton from a reference compound was plotted against the Δδ value of the

TABLE 3. ^{1}H-NMR signals from 3′*R*-HBHL and 3′*S*-HBHL at room temperature and at −20°C and shifts obtained with *S*-(+)-TFAE at −20°C

Proton	δ (ppm) in ^{1}H-NMR signal of 3′*R*-HBHL		Δδ (ppm) at −20°C with *S*-(+)-TFAE	δ (ppm) in ^{1}H-NMR signal of 3′*S*-HBHL		Δδ (ppm) at −20°C with *S*-(+)-TFAE	3′*R*-HBHL and 3′*S*-HBHL splitting pattern [coupling constant(s) (Hz)]
	Room temp	−20°C		Room temp	−20°C		
NH	6.50	6.72	−0.087	6.53	6.75	−0.068	dbr (6.4)
H-2	4.57	4.59	−0.072	4.58	4.64	−0.094	ddd (11.8; 8.7; 6.4)
H-4a	4.49	4.52	−0.061	4.49	4.51	−0.100	ddd (9.1; 9.1; 1.0)
H-4b	4.30	4.32	−0.045	4.30	4.32	−0.084	ddd (11.4; 9.3; 5.9)
H-3′	4.23	4.23	−0.060	4.23	4.23	−0.084	dddq (9.0; 3.4; 2.9; 6.3)
OH	3.18	3.59	−0.043	3.19	3.61	+0.025	d (3.4)
H-3a	2.83	2.80	−0.081	2.83	2.81	−0.123	dddd (12.4; 8.7; 5.8; 1.0)
H-2′a	2.46	2.46	−0.091	2.44	2.44	−0.113	dd (15.6; 3.6)
H-2′b	2.35	2.35	−0.095	2.37	2.37	−0.114	dd (15.6; 9.0)
H-3b	2.20	2.24	−0.102	2.19	2.22	−0.164	dddd (12.4; 11.9; 11.4; 9.0)
H-4′	1.26	1.25	−0.058	1.26	1.25	−0.068	d (6.3)

corresponding proton in *small*. If the stereochemistries of both asymmetric centra are identical in both compounds, a linear relationship between the points in the graph would be expected. Comparison of the graphs shows that in only one case is a linear relationship observed: the graph in which the shifts of 3′*R*-HBHL with the *S*-(+) reagent are correlated with those of *small* with the same reagent. This is a strong indication that *small* has the same stereochemistry of both asymmetric centra as 3′*R*-HBHL (3′*R*,2*S*). The proposed structure of *small* is therefore *N*-(3*R*-hydroxy-7-*cis*-tetradecanoyl)-L-homoserine lactone. It should be noted here that this approach relies on the assumption that the two HBHL compounds are representative model compounds and that they interact with TFAE in a way similar to that in which *small* does. However, the very small Δδ values of the protons further down the fatty acid chain support this assumption.

The structure deduced for *small* is new, but several other aHSLs with interesting biological activities have already been described (for a review, see reference 10). These aHSL molecules vary in the length of the fatty acid chain, the presence of an unsaturated bond, and in having a β-oxy group instead of the β-hydroxy group as in *small*. Very typically, the molecules (i) have mostly been found in bacteria having interactions with higher organisms and (ii) are co-transcription factors for functions that are useful at higher cell densities. These functions are expressed only after a certain aHSL concentration threshold has been reached, which is generally at the end of the logarithmic growth phase and during the stationary growth phase.

small bacteriocin is the first chemically identified aHSL molecule reported for *Rhizobium* spp. Up to now, little has been known about the function of aHSL molecules in *Rhizobium* spp. In the soil, the bacteriocinic properties of the aHSL molecule *small* may be important in competition for nodule occupancy between *Rhizobium* strains in the host plant rhizosphere. In relation to *small*, there are two types of *R. leguminosarum* strains: (i) non-*small*-producing strains and (ii) *small*-producing strains (see the introduction). Examples of both types are found in all three biovars of *R. leguminosarum* (17), bv. viciae, bv. trifolii, and bv. phaseoli, each one nodulating a different set of host plants.

In the non-*small*-producing strain of *R. leguminosarum* bv. viciae, strain 248, two gene clusters are candidates for regulation by aHSL as a co-transcription factor. Both gene clusters are located on the Sym plasmid pRL1JI. One of these is the *rhi* gene cluster (6, 8, 9), which contains the *rhiA*, *-B*, *-C*, and *-R* genes. The RhiR protein has homology with the LuxR regulatory protein, which requires an aHSL molecule as a co-transcription factor. The *rhiA* gene is transcribed at the end of the logarithmic growth phase, as is expected for aHSL-regulated proteins. It has now been confirmed by Gray et al. (11) that *small* (the RLAI molecule) functions as a co-transcription factor for the *rhiA* gene. As to its function, the cytoplasmic RhiA protein is not expressed in the nitrogen-fixing bacteroid forms of *R. leguminosarum* bv. viciae in root nodules. However, RhiA protein has been found in rhizobia present in the rhizosphere and may function in this niche in interactions of *R. leguminosarum* bv. viciae with its host plant (6, 8). In normal wild-type *R. leguminosarum* bv. viciae strains, mutations in the *rhi* genes do not affect nodulation, whereas in the absence of functional *nodFEL* genes, mutations in the *rhiA* and *rhiR* genes almost completely abolish nodulation of *Vicia hirsuta* (6).

The fact that *rhi* gene transcription requires RLAI or *small* indicates that *small* has a function in nodulation which is not essential in the wild-type rhizobia but which is required for normal nodulation of *nodFEL* mutants.

The other gene cluster which possibly is regulated by aHSL molecules is the *tra* gene cluster of the Sym plasmid pRL1JI, which contains the *rps* and *sbs* genes involved in *small* function (see the introduction). Analogous to a *tra* gene cluster of *Agrobacterium tumefaciens* (18), this *tra* gene cluster might be regulated by an aHSL molecule as a co-transcription factor.

Because strain 248 does not produce *small*, it probably produces a different aHSL molecule (aHSL248) as a co-transcription factor which, in the absence of *small*, functions in *rhi* gene transcription and eventually in *tra* gene transcription. Our current hypothesis is that aHSL248 differs from *small* and that *small* probably functions as a growth-inhibiting analog of aHSL248.

In *R. leguminosarum* strains, there is a another difference between biovars in that all strains of *R. leguminosarum* bv. viciae investigated show RhiA protein production, whereas in the other two biovars this is only very rarely the case (8, 9). Because in *small*-producing strains, the production of *small* likely has a function, we expect that transcription of genes different from the *rhi* genes is regulated by *small* in strains of these two biovars and possibly in strains of *R. leguminosarum* bv. viciae as well. These genes may also have a function in root

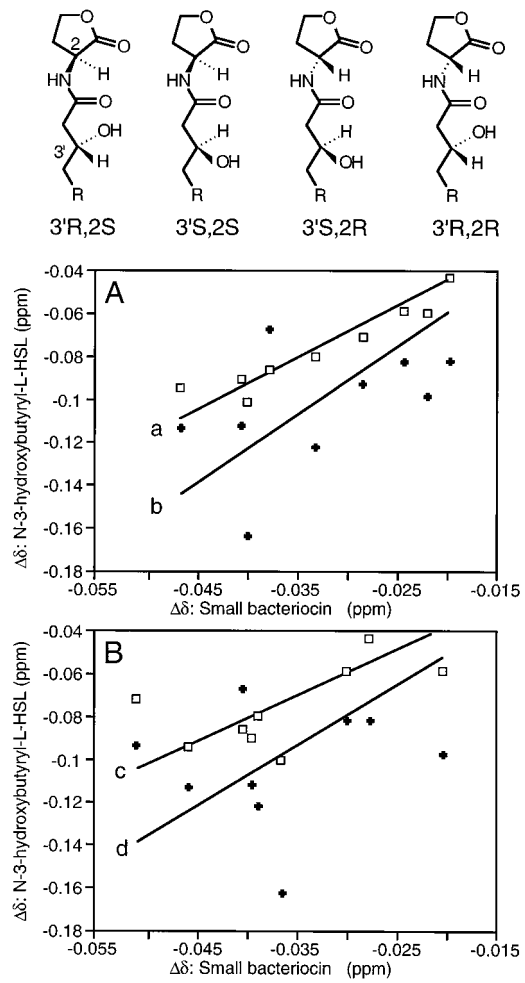

FIG. 5. Correlation of the Δδ values of the *small* bacteriocin signals (Table 1) with those of the corresponding signals of *N*-(3*R*-hydroxybutyryl)-L-homoserine lactone (□) or those of *N*-(3*S*-hydroxybutyryl)-L-homoserine lactone (+) (Table 3). Δδ values were obtained with *S*-(+)-TFAE except in panel B, in which the shifts of *small* bacteriocin were obtained with *R*-(−)-TFAE. The lines were obtained by linear regression through zero. A good correlation would indicate an identical stereochemistry at both asymmetric centra in panel A (because the same shift reagent was used) or a mirror image stereochemistry at both centra in panel B (because the mirror image shift reagent was used for *small* bacteriocin). In the case of a good correlation, line a would thus indicate a 3′*R*,2*S* stereochemistry, line b would indicate 3′*S*,2*S*, line c would indicate 3′*S*,2*R*, and line d would indicate 3′*R*,2*R*. The structures are given above the graphs [*small* bacteriocin, $R=(CH_2)_2CH=CH(CH_2)_5CH_3$; *N*-(3-hydroxybutyryl)-L-homoserine lactone, *R*=H]. Only line a yielded a good correlation, clearly indicating the 3′*R*,2*S* stereochemistry for *small* bacteriocin.

nodule symbiosis, since in other bacteria, aHSL molecules very often play a role in interactions with higher organisms (see reference 10).

In bacteria, nutritional stress and other kinds of stresses, such as heat shock and UV irradiation, lead to the induction of a great number of stress-related genes. In *Escherichia coli*, a specific stress-related sigma factor, σ^s, encoded by the *rpoS* gene is involved in the transcription of at least 30 stress-related genes (see reference 13). In this connection, it is of interest that the inducer of the *rpoS* gene is homoserine lactone, which is reported to be a starvation signal in *E. coli* by Huisman and Kolter (13). Huisman and Kolter suggest a connection between homoserine lactone as the internal stress signal and an *N*-acylated homoserine lactone molecule as an external, membrane-diffusible stress signal by which cells of a bacterial population communicate with one another under stress circumstances. Therefore, as root nodules are formed under conditions of nitrogen starvation and other growth limitations, the study of aHSL-regulated transcription in *Rhizobium* strains might lead to the identification of some interesting features of root nodule morphogenesis.

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