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Bacteriocin *small* of Fast-Growing Rhizobia Is Chloroform Soluble and Is Not Required for Effective Nodulation

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***small* bacteriocin is a low-molecular-weight bacteriocin which is common in fast-growing rhizobia. As its activity could not be detected in chloroform-sterilized culture supernatants (P. R. Hirsch, J. Gen. Microbiol. 113:219–228, 1979), the bacteriocin could not be purified in order to study its mechanism of action. We report here that *small* is soluble in chloroform, an observation which led to effective and simple (partial) purification. Other properties of *small* are its low molecular weight, which is estimated to be between 700 and 1,500, its resistance to proteolytic enzymes, pectinase, and lysozyme, and its heat stability at pH 5.5 but not at pH 7.0. Its bactericidal action on exponentially growing sensitive cells was not detected until 11 h after its addition. The bactericidal action was preceded by inhibition of cell division. To determine whether *small* activity is required for nodulation or nitrogen fixation, a transposon Tn*5*-induced *small*-negative mutant was isolated. The observation that this strain formed normal, acetylene-reducing root nodules showed that *small* production is not a prerequisite for the formation of effective nodules.**

Many, if not all, species of *Rhizobium* produce bacteriocins, designated rhizobiocins (11). Recently part of our research interest was focused on a class of low-molecular-weight bacteriocins which are present in most fast-growing rhizobia (5, 15). These rhizobiocins were designated *small* by Hirsch (5) because the large inhibition zones (>20 mm) caused by a *small*-producing strain in agar plates with a top layer of a *small*-sensitive strain are presumably due to fast diffusion of the low-molecular-weight "small" bacteriocin molecule. These small rhizobiocins of various strains are closely related, if not identical, since they all inhibit the growth of several sensitive strains and since strains producing *small* are cross-resistant (5, 15). Another remarkable feature is that those non-*small*-producing fast-growing *Rhizobium* strains which were investigated for the presence of genes involved in the production of *small* did in fact harbor them. The appearance of *small* in the culture medium of nonproducing strains turned out to be repressed by the presence of a highly self-transmissible plasmid with a function (Rps) that represses the production of *small* (2, 15). Thus, genes involved in the production of *small* are present in all fast-growing rhizobia. When Sym plasmids from fast-growing rhizobia are transferred to other species of fast-growing rhizobia, nitrogen-fixing root nodules can be formed on plants by the cross-inoculation group of the donor bacterium (7, 13, 14).

Purification of *small* has been hampered by the fact that it could not be detected in chloroform-sterilized culture supernatants. In this paper we report the partial purification and some of the properties of *small*. Moreover, the question whether functional *small* genes are required for the formation of effective nitrogen-fixing root nodules was answered by using a *Rhizobium leguminosarum* mutant impaired in the synthesis or excretion of *small*.

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MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains listed in Table 1 were maintained on solidified medium A, containing yeast extract, mannitol, and glucose (13). The compositions of B^- minimal salt medium, B^+ medium (B^- medium supplemented with yeast extract), and RMM medium have been described earlier (6, 13).

Transposon mutagenesis and transduction. Mutagenesis with transposon Tn*5* was achieved as previously described (1). Tn*5* mutants were selected on RMM plates containing 0.2 mg of kanamycin per ml. Transduction with phage RL38 was performed as described previously (3).

Bacteriocin sensitivity and production. Sensitivity to *small* was tested on B^- plates containing inhibitory concentrations of *small*. The production of *small* was tested as described elsewhere (15). Sterilization of *small* solutions was achieved by using cellulose nitrate filters, pore size 0.45 μm (Sartorius, Gottingen, West Germany).

Isolation of *small*-negative mutants. The nonmucoid strain *R. leguminosarum* RBL1086 rather than its parental strain RBL1 was used for mutagenesis because it formed smaller, nonmucoid colonies which therefore were better suited for replica plating. Selection of *small*-negative mutants was carried out on B^- plates with a 20-ml bottom layer containing approximately 5×10^7 CFU of the *small*-sensitive strain 248 and a 3.5-ml top layer without bacteria. Independent kanamycin-resistant colonies from selection plates obtained after Tn*5* mutagenesis were replica-plated on these double-layer B^- plates. After 48 h of incubation at 28°C, colonies producing *small* could be differentiated from non-*small*-producing ones by the presence of a halo in the bottom layer. Colonies not surrounded by such an inhibition zone were purified and tested again for the production of *small*.

Extraction and properties of *small*. A 500-ml volume of cell-free culture supernatant fluid of strain RBL1082 was extracted three times with 10 ml of chloroform. Aqueous solutions of the bacteriocin were obtained by evaporating

TABLE 1. *R. leguminosarum* strains

Strain	Relevant characteristics	Source or reference
248	*small* sensitive	5
RBL1	Wild type	14
RBL1(pJB5JI)	Contains Sym plasmid pRL1JI::Tn*5*, introduced by conjugation	8
RBL1082	Derivative of RBL1 impaired in slime production	This paper
RBL1086	Rough-colony derivative of RBL1	This paper
RBL1309	RBL1, spectinomycin resistant	15

the chloroform in the presence of water by bubbling air through the liquid. Dialysis and Sephadex gel filtration were used to estimate the molecular weight. Spectrapor membranes type 1 (molecular weight cutoff, 6,000 to 8,000) and type 3 (molecular weight cutoff, 3,500) (Spectrum Medical Industries, Inc., Los Angeles, Calif.) and Visking dialysis tubing (molecular weight cutoff, 12,000 to 14,000) were used for dialysis, which was performed by hanging a dialysis bag containing 1.0 ml of *small* solution in tubes containing 15 ml of water. The *small* present in the dialysate was isolated by three successive extractions with 1.0 ml of chloroform and redissolved in water by chloroform evaporation as described above. The activity of *small* was then determined by an agar diffusion assay (15) (see below). Sephadex G10 and G15 columns (Pharmacia, Woerden, The Netherlands) with a void volume of 3 ml were used for molecular sieve chromatography of *small*. The heat sensitivity of *small* was investigated by incubating 1.0 ml of a *small* solution in 10 mM potassium phosphate buffer at the pH values indicated below in a water bath at various temperatures. The remaining *small* activity was estimated by using an agar diffusion assay (see below). Sensitivity to enzymes (purchased from Sigma Chemical Co., St. Louis, Mo.) was determined by incubating 1.0-ml volumes of *small* dissolved in water with 1.0 ml of buffer and 0.5 ml of enzyme solution (1.0 mg/ml). Pepsin and pectinase were incubated with 0.1 M acetate buffer at pH 2.0 and 4.0, respectively. Lysozyme, peptidase, and protease types 6, 7, and 8 were incubated with 0.1 M potassium phosphate buffer at pH 6.2, 7.0, 7.5, 7.5, and 7.5, respectively. The incubation temperature was 37°C except for pectinase and lysozyme, which were incubated at 28°C. *small* was recovered from the incubation mixtures by chloroform extraction and estimated as described above.

Quantitative determination of *small*. Double-layer plates of B^+ agar with a top layer containing 10^8 bacteria of *R. leguminosarum* 248 and three 12-mm holes in the agar made with a cork borer were used. The bottom layer and top layer contained approximately 25 and 3 ml of agar, respectively. *small*-containing solutions (0.1 ml) were pipetted into the holes, and the plates were incubated for 2 days at 28°C. An average culture supernatant caused an inhibition zone 35 mm in diameter. One unit of *small* was defined as 10^{-2} of the activity giving an inhibition zone of 35 mm in this agar diffusion assay.

Experiments with plants. Nodulation tests with *Vicia sativa* subsp. *nigra*, determination of nitrogenase activity, and isolation of bacteria from root nodules were performed as described in reference 14.

RESULTS

Molecular weight of *small*. Our experiments with crude, filter-sterilized preparations of *small* indicated that *small* diffused through cellophane, which is consistent with the observations of Hirsch (5). In addition, we observed that *small* can diffuse through Spectrapor type 1 and type 3 membranes, indicating a molecular weight lower than 3,500. By using gel filtration with Sephadex G10 and G15 columns, *small* was found in the void volume of the G10 column (exclusion limit, 700) and in the bed volume of the G15 column (exclusion limit, 1,500). Therefore *small* most likely has a molecular weight between 700 and 1,500.

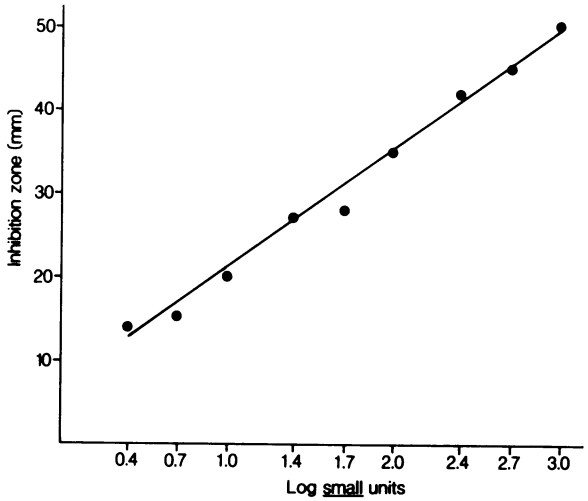

FIG. 1. Quantitative estimation of *small* by the agar diffusion assay. Various concentrations of *small* were applied on B^+ agar plates containing *small*-sensitive *R. leguminosarum* 248 cells. Conditions were those described in the text. A straight line was obtained when the diameter of the zone of growth inhibition was plotted against the logarithm of the *small* concentration. One unit is 10^{-2} times the amount of *small* giving an inhibition zone of 35 mm.

Partial purification and quantification. The properties of *small* can best be studied in purified preparations. However, Hirsch reported that *small* could not be detected in the chloroform-sterilized culture supernatant, and she had to use agar agar as a source of *small* (5). As we found high *small* activity in filter-sterilized culture supernatants from *small*-producing strains, the influence of chloroform on the solubility of *small* was investigated. It turned out that *small* dissolved more easily in chloroform than in water, which explains the differences between our results and those of Hirsch. As outlined above, we used these data to extract, concentrate, and partially purify *small*. After subsequent solubilization in water, *small* activities up to 500 times that in a culture supernatant, as estimated with the agar diffusion assay, could easily be obtained. Straight lines were found when the diameter of the inhibition zone was plotted against the logarithm of the *small* concentration (Fig. 1).

TABLE 2. Temperature sensitivity of *small*

pH	% *small* activity remaining[a]	
	85°C	99°C
5.5	73 ± 11	45 ± 7
6.0	45 ± 7	24 ± 4
6.5	23 ± 4	0
7.0	0[b]	0[b]

[a] *Small* was incubated for 45 min in 10 mM phosphate buffer of the indicated pH and temperature. Remaining *small* activity was estimated by the agar diffusion method.

[b] Complete inactivation occurred after incubation for less than 10 min.

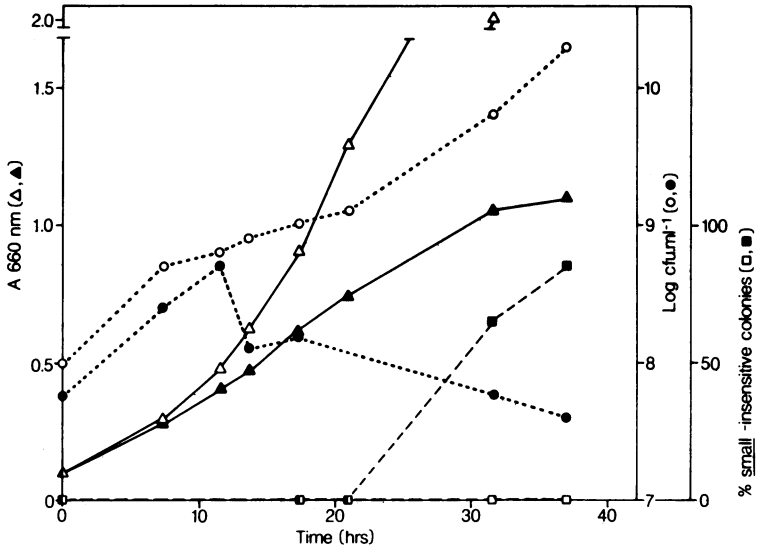

FIG. 2. Inhibition of RBL1(pJB5JI) by *small* in B^- medium. A 0.25-ml volume of a 500× concentrated solution of *small* in water was added to 5 ml of an exponentially growing culture and incubated under aeration at 28°C. The optical density at 660 nm, the logarithm of the number of CFU per milliliter, and the percentage of *small*-insensitive colonies were plotted against time. Open and solid symbols represent cultures without and with *small*, respectively.

Properties of *small*. The thermostability of *small* appeared to be dependent on the pH (Table 2) in that lower pH values increased the stability of the bacteriocin. Incubation with various enzymes showed that *small* was not inactivated by pepsin, peptidase, protease types 6, 7, and 8, pectinase, or lysozyme. To test the influence of *small* on the growth behavior of *R. leguminosarum*, cells of the *small*-sensitive strain RBL1(pJB5JI) were grown in liquid B^- medium with and without *small*. *small* did not influence the increase in the optical density during the first 7 h. Thereafter, the increase in optical density in the culture supplemented with *small* changed from exponential to linear. Microscopic examination of the cells after 7 h of growth in the presence of *small* revealed high numbers of elongated forms. The number of viable RBL1(pJB5JI) cells, as determined by plating on B^- agar (Fig. 2), increased during the first 11 h in the presence of *small*, decreased rapidly during the next 2 h, and then continued to decrease at a lower rate. To detect the appearance of *small*-insensitive clones in the culture, 20 colonies from the platings on B^- agar were streaked on B^- agar with and without *small*. In the first 21 h, only *small*-sensitive colonies were found. Later, at 32 and 37 h, 65 and 85%, respectively, of the cells in the culture with *small* were *small* insensitive (Fig. 2). All *small*-sensitive and *small*-insensitive colonies were kanamycin resistant, indicating stable maintainance of Tn*5* and therefore of pJB5JI. *Small*-insensitive colonies were not found in the control culture without *small*, even after incubation for 37 h (Fig. 2).

Isolation and symbiotic properties of mutants not producing *small*. With the double-layer technique described previously, 2 mutants with a *small*-negative character, designated RBL10861 and RBL10863, were selected from 13,500 independent Tn*5* insertion mutants. As the parental strain RBL1086 is unable to form root nodules on *V. sativa*, we transduced the *small*$^-$ mutations of these two mutants to the effectively nodulating strain RBL1309 by using phage RL38 and selecting for kanamycin resistance. In all 12 cases tested, the *small* mutation of strain RBL10861 was coupled to the kanamycin resistance marker of Tn*5*. However, in none of six cases tested was the kanamycin resistance of strain RBL10863 coupled to the *small* mutation. Therefore, in the latter strain Tn*5* is either not inserted in a gene involved in *small* production or, less probable, is also inserted in another gene. To test the requirement of *small* production for nodulation, *V. sativa* subsp. *nigra* plants were inoculated with the *small*-producing strain RBL1309 and the non-*small*-producing, Tn*5*-carrying RBL1309 transductants. Both strain RBL1309 and the 12 transductants gave rise to acetylene-reducing root nodules (50 to 100 nmol $plant^{-1}$ h^{-1}). Bacteria isolated from the nodules of plants inoculated with the transductants turned out to be kanamycin resistant and did not produce *small*, showing that these root nodules had been formed by *small* mutants and not by their *small*$^+$ revertants. Therefore it was concluded that the presence of functional *small* production genes is not required for the formation of effective root nodules.

DISCUSSION

The most important finding reported in this paper is that *small* readily dissolves in chloroform. This explains why Hirsch (5) and others (9) who sterilized culture supernatants with chloroform could not detect *small* in such supernatants. *small* can be extracted and purified with chloroform and estimated with an agar diffusion test. With this knowledge it will now be possible to obtain sufficient amounts for chemical analysis.

Our finding that *small* is a molecule with a low molecular weight is consistent with the data of Hirsch (5), who found that it diffused through cellophane. We report now a most likely molecular weight between 700 and 1,500. Hirsch (5) reported that *small* is resistant to proteolytic enzymes, which was confirmed by our experiments. *small* is heat labile at pH 7.0 but more stable at pH 5.5, since 45% of the *small* activity was still present after incubation for 45 min at 99°C. Hirsch (5) reported total disappearance of *small* activity after incubation for 45 min on agar plates at 85°C. This result may be explained by the effect of the pH on the heat stability of *small* (Table 2).

It is clear from our results that *small* does not belong to the conventional class of high-molecular-weight proteinace-

ous bacteriocins. Rather, it probably is not a protein, and its molecular weight is similar to that of agrocin 84, a bacteriocin of *Agrobacterium radiobacter* K84 that inhibits the growth of *Agrobacterium tumefaciens*. Agrocin 84 is an adenine nucleotide analog with a molecular weight of 1,350 (12). However, *small* differs from agrocin 84 in at least two respects. First, chloroform-sterilized supernatants of an agrocin 84-producing strain still contain active agrocin 84 (10). Second, agrocin 84 kills more than 99% of the sensitive cells within 1 h (10), whereas *small* does not kill within the first 11 h (Fig. 2).

The *small*-insensitive clones found by plating after 21 h of culture with added *small* did not arise from cells which had lost pJB5JI because the Tn*5* marker of the plasmid was still present. Probably a mutation in the *small* bacteriocin sensitivity gene (*sbs*) present on pJB5JI (15) was responsible for this phenomenon.

We conclude that functional *small* production genes are not needed for the formation of effective nitrogen-fixing root nodules. The question then is whether there is another function for *small* in symbiosis. An ecological function has been ascribed to agrocin 84 in crown gall disease (4). This bacteriocin kills the crown gall-inducing strain *A. tumefaciens* by entering it via a transport system for agrocinopine A, an opine produced by the plant tumor. *A. radiobacter* K84 can catabolize nopaline, another opine, produced by this tumor and thus grows at the expense of the strain which induced the tumor. Presently an ecological function for *small* in rhizobia is not known. However, the idea of a function similar to that of agrocin 84 remains intriguing, also because the *R. leguminosarum* genes for *sbs*, *rps*, (repression production *small*) and *tra* (plasmid transfer) on pJB5JI (15) and the genes on the nopaline Ti plasmid for agrocin 84 sensitivity, repression of agrocin 84 production, and *tra* (4) are organized in a similar way.

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