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Role of Plant Root Exudate and Sym Plasmid-Localized Nodulation Genes in the Synthesis by *Rhizobium leguminosarum* of Tsr Factor, Which Causes Thick and Short Roots on Common Vetch

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In a previous paper it was shown that cocultivation of *Rhizobium leguminosarum* with the plant *Vicia sativa* subsp. *nigra* on solid medium causes a changed mode of growth of the plant roots, resulting in thick and short roots (Tsr). The Sym plasmid present in the bacterium appeared to be essential for causing Tsr (A. A. N. van Brussel, T. Tak, A. Wetselaar, E. Pees, and C. A. Wijffelman, Plant Sci. Lett. 27:317–325, 1982). In the present paper, we show that a role in causing Tsr is general for Sym plasmids of *R. leguminosarum* and *Rhizobium trifolii*. Moreover, mutants with transposon insertions in the Sym plasmid-localized nodulation genes *nodA*, *B*, *C*, and *D* are unable to cause Tsr, in contrast to nodulation mutants localized in other parts of the Sym plasmid. The observation that Tsr could also be brought about in liquid medium enabled us to show that Tsr is caused by a soluble factor. Experiments in which plants and bacteria were grown separately in the sterile supernatant fluids of each other resulted in establishing the following sequence of events. (i) The plant produces a factor, designated as factor A. (ii) Factor A causes the Sym plasmid-harboring bacteria to produce Tsr factor. (iii) Growth of young plants in the presence of Tsr factor results in the Tsr phenotype. Models explaining this example of molecular signalling between bacteria and plants are discussed.

Root nodule formation by fast-growing *Rhizobium* bacteria on leguminous plants requires the presence of a symbiosis plasmid (Sym plasmid) in the bacterium (6, 10). These plasmids harbor genes involved in host-specific nodulation (*hsn*), specific root hair curling (*hac*), and root hair deformation (*had*) as has been shown after Tn*5* mutagenesis (5, 14, 17, 21a). Mutations in the Sym plasmid which cause delayed nodulation have also been found (14, 17, 21a), and it has been suggested that these genes influence the efficiency of nodulation (21a).

Vicia sativa subsp. *nigra* (common vetch), inoculated with a *Rhizobium leguminosarum* strain harboring Sym plasmid pJB5JI, forms thick and short roots, designated as the Tsr phenotype (19). The roots have a reduced length, and they are locally at least 50% thicker than uninfected roots. One or more of the bacterial genes required for expresing the Tsr phenotype are located on this Sym plasmid (19). This pronounced, macroscopically visible, Tsr phenotype is uncommon among plants which nodulate upon infection with *R. leguminosarum* strains. It is, for example, not obvious on pea, *Vicia hirsuta*, *Vicia tetrasperma*, and several other plants of the pea cross-inoculation group (19). Apparently *V. sativa* subsp. *nigra* is extremely sensitive to the Tsr factor(s), and this plant therefore can be used to detect the presence of Tsr factor(s) (19). It is not known whether the gene(s) involved in the expression of the Tsr phenotype also plays a role in root nodule formation. However, it is evident that expression of the Tsr phenotype is not a prerequisite for nodulation since in most plants nodulation apparently is not accompanied by this phenomenon.

Obviously the Tsr phenotype is an example of an interaction between bacteria and plants. As our current research interest includes molecular aspects of signals involved in interactions between bacteria and plants (e.g., see reference 12), we studied the molecular mechanism causing expression of the Tsr phenotype in more detail. Using a genetic approach, we show that expression of certain *nod* genes of Sym plasmid pRL1JI is required to cause the Tsr phenotype. In addition, we attempted to collect information on the mechanism leading to the Tsr phenotype by growing plants and bacteria separately on the excretion products of each other. The results show the following sequence of events leading to the Tsr phenotype: a factor present in plant root exudate stimulates the bacterium to produce the Tsr factor, which in turn induces expression of the Tsr phenotype on *V. sativa* subsp. *nigra*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The relevant characteristics of *Rhizobium* strains and Sym plasmids are listed in Table 1. *Agrobacterium tumefaciens* 202, a derivative of C58 obtained by curing for its Ti plasmid, was obtained from P. J. J. Hooykaas (8). The bacterial strains were maintained on agar slants containing medium A (18). The compositions of the bacterial growth media A (yeast extract, mannitol, and glucose), B^- (mannitol and nitrate) (18), and TY (tryptone and yeast extract) (21) have been described previously. CFU were determined after plating on solid TY medium.

Plasmid transfer and transductions. Plasmids were transferred by the method of Beringer et al. (1). The transfer of the non-self-transmissible plasmids pPRE and pSym1 was accomplished with the aid of the mobilizing plasmid pRL180 (8). Transductions were performed as described by Buchanan-Wollaston (2). From each transduction, 20 kanamycin-resistant colonies were tested for nodulation and for formation of thick and short roots on *V. sativa* subsp. *nigra*. When appropriate, antibiotics were used at final concentra-

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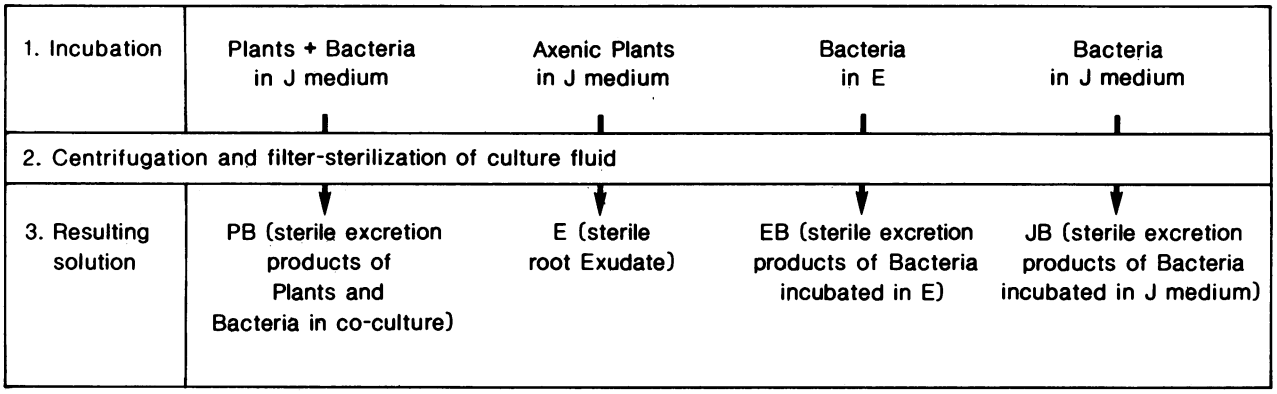

FIG. 1. Flow scheme showing the preparation of various solutions containing excretion products of bacteria or plants or both.

tions of (milligrams per milliliter): kanamycin, 200; rifampicin, 20; and spectinomycin, 100.

Plant cultivation. The methods used for surface disinfection of *Vicia sativa* L. subsp. *nigra* (L.) seeds and subsequent germination were the same as those published previously (19) except that the cold period at 4°C after surface disinfection was extended from 4 to 7 days since this improved the germination of some seed batches. The 25 ml of water used to swell the seeds was checked for bacterial contamination after mixing 10 ml with 10 ml of liquid TY medium and subsequent incubation at 28°C for 4 days. In case of growth, the seeds were discarded. Germinated seeds with roots approximately 1 cm in length were used to cultivate plants under the conditions described (19) in J (Jensen) medium, a mineral medium without fixed nitrogen (20). When appropriate, the medium was solidified with 1% agar.

Investigations of the Tsr phenotype in agar cultures. The germinated seeds were inoculated by dipping them in bacterial colonies grown on medium A. These seeds were then transferred to slants containing solidified J medium. After incubation for 14 days at 20°C, the expression of the Tsr phenotype was judged. A positive Tsr phenotype was defined as roots shorter than 6 cm and at least 0.6 mm wide at the thickest part of the root.

TABLE 1. *Rhizobium* spp. strains and Sym plasmids

Strain or plasmid[a]	Relevant characteristics	Reference or source
Strain		
LPR5039	*R. trifolii* RCR5 cured of Sym plasmid pRtr5a	P. J. J. Hooykaas (9)
RBL1387	*R. leguminosarum* 248 cured of Sym plasmid pRL1JI	13
RBL5505	LPR5039 *rif spc*	13
RBL5601	LPR5039 *rif spc* pRL1JI *mep2*::Tn*5*	13
RBL607	RBL5505 pRL1JI *kan7*::Tn*5*, *nodC7*	C. A. Wijffelman
RBL611	RBL5505 pRL1JI *nodB*11::Tn*5*	C. A. Wijffelman
Plasmid from *R. leguminosarum*		
pRL1JI	248	7
pSym1	RCC1001	P. J. J. Hooykaas (8)
pAB4	RBL4	21
pPRE	PRE	R. C. van den Bos (15)
Plasmid from *R. trifolii*		
pRtr5a	RCR5	P. J. J. Hooykaas (9)

[a] All plasmids used were derivatives labeled with transposon Tn*5* inserted in genes not essential for nodulation unless otherwise indicated (e.g., the *nod*::Tn*5* mutants).

Preparation of sterile, soluble, excretion products of bacteria and plants cultivated separately, together, or one after the other. To investigate the sequence of events leading to the production of the factor that causes expression of the Tsr phenotype, experiments were designed to grow bacteria and plants in liquid medium separately, together, or on the sterile excretion products of the other. Subsequently, the sterile supernatant fluids were prepared and tested for the ability to induce expresssion of the Tsr phenotype on *V. sativa* subsp. *nigra* plants. The procedure for the preparation of the supernatant fluids is outlined in Fig. 1 and will be described in detail here.

Bacteria to be used as inocula were pregrown at 25 to 27°C in B^- medium to the logarithmic-growth phase, centrifuged for 15 min at 6,000 × *g*, suspended in deposit-free J medium, and starved by incubation for 24 h on a rotatory shaker. The bacteria were then centrifuged again and resuspended in deposit-free J medium to an A_{660} of 0.1, which corresponds to 5×10^8 CFU ml^{-1}. J medium-bacteria fluid, plant-bacteria fluid, and exudate-bacteria fluid (JB, PB, and EB, respectively; see below) were then prepared with an initial bacteria concentration of 5×10^5 CFU ml^{-1}.

To obtain root exudate, five germinated seeds were directly transferred to a support of stainless steel wire netting located 5 mm above 25 ml of J medium in culture tubes (28 by 280 mm) plugged with cotton. Sterile root exudate (E) was prepared after growth of uninoculated plants for 7 days either on 25 ml of liquid J medium as described above or, for larger quantities from similar cultures, after growth of 100 to 150 seeds in 750 ml of J medium in a 2-liter beaker covered with a large petri dish lid. After removal of the plants, the medium was centrifuged for 10 min at 10,000 × *g*, prefiltered with prewashed cellulose nitrate filters (pore diameter, 0.8 μm; type SM 11104; Sartorius, Göttingen, Federal Republic of Germany), and filter sterilized with prewashed Sartorius cellulose nitrate filters (pore diameter, 0.2 μm; type SM 11102) or Duran no. 5 glass filters (Schott, Mainz, Federal Republic of Germany). Before centrifugation and after the final filtration step, 0.1-ml volumes of culture fluid were inoculated on TY plates to check for contamination. Only sterile exudates were used further. The root exudate so

obtained could be stored at 4°C for at least 1 week without significant loss of biological activity. Coculture fluids from plants inoculated with bacteria were prepared and sterilized in the same way as E and designated as PB.

In a number of cases bacterial suspensions, prepared as described above, were added to sterile E or to J medium, incubated for 1 to 4 days, and sterilized as described. The resulting fluids were designated EB and JB.

Investigation of the Tsr phenotype in liquid medium. Experiments designed to test the ability of bacterial suspensions or filter-sterilized supernatant fluids to cause expression of the Tsr phenotype in liquid medium were carried out in duplicate with five *V. sativa* subsp. *nigra* plants per tube. When the activity of the fluids in which bacteria or plants or both had been grown (E, EB, PB, or JB) was to be tested, this solution (based on J medium) was used undiluted and supplemented with 0.05 volume of J medium, which contains sufficient mineral salts to ensure normal plant development. Tubes containing plants (with 5×10^5 bacteria ml^{-1} or without bacteria) were incubated at 20°C under the conditions described previously (19), with day 1 in the dark and the following days in the light. The Tsr phenotype was quantified by measuring the length of the main root of plants after 7 days. All inoculated plant cultures were checked for bacterial contamination at the end of the experiment as described for E.

Root hair phenotypes. To check for possible root hair changes, root systems were inspected by bright-field microscopy. Hac (marked root hair curling) was defined as a curling of root hair, which was frequently more than 360°. Had (root hair deformation) was defined as those zigzags, bends, local swellings, and top swellings that occur neither on the uninfected plants nor on plants infected with bacteria cured of their Sym plasmid. Increases in number of root hairs and root hair deformation were judged by visual inspection with incubations that should cause positive and negative effects as controls.

FIG. 2. *V. sativa* subsp. *nigra* inoculated with Sym plasmid pRL1JI-harboring strain RBL5601 (A) or Sym plasmid-devoid strain RBL5505 (B). The Tsr phenotype is only observed in Fig. 2A. The phenotype of uninoculated plants (not shown) is similar to that shown in Fig. 2B. The plants were cultured on solid medium as described in Materials and Methods and photographed on day 14.

TABLE 2. Influence of the presence of a Sym plasmid on the Tsr phenotype of *V. sativa* sv *nigra*

Source and designation of Sym plasmid	Sym plasmid-less recipient strain[a]		
	R. trifolii LPR5039	*R. leguminosarum* RBL1387	*A. tumefaciens* LBA202
None	–	–	–
R. leguminosarum			
pRL1JI	+	+	+
pAB4	+	ND	ND
pPRE	+	ND	+
pSym1	+	ND	ND
R. trifolii			
pSym5	+	+	+

[a] + and –, Presence and absence of Tsr phenotype, respectively; ND, not determined.

Statistical analysis. The significance of root length differences between two sets of plant roots was calculated by the Mann-Whitney one-tailed U test (16).

RESULTS

A Sym plasmid is required for the induction of the Tsr phenotype on solid medium. The influence of the presence of Sym plasmid pRL1JI on expression of the Tsr phenotype of *V. sativa* subsp. *nigra* on solid medium is shown in Fig. 2. To see whether other Sym plasmids also can cause induction of the Tsr phenotype, various *R. leguminosarum* and *Rhizobium trifolii* Sym plasmids were transferred to the cured *R. trifolii* LPR5039 and the cured *R. leguminosarum* RBL1387. It appeared that all seven resulting strains gained the ability to induce the Tsr phenotype (Table 2), indicating that the presence of genes involved in the induction of the Tsr phenotype is a property of *R. leguminosarum* and *R. trifolii* Sym plasmids in general. For all three cases tested, even the chromosomal background of *Agrobacterium tumefaciens* LBA202 was sufficient to cause expression of the Tsr phenotype provided that a Sym plasmid was present (Table 2).

***nod* genes are required for expression of Tsr.** As the Sym plasmid is required for induction of the Tsr phenotype (Table 2) and as a large collection of nodulation-deficient Tn*5* insertion mutants in the Sym plasmid pRL1JI have been isolated in our laboratory (Wijffelman et al., in press), the latter mutants were tested for the ability to induce the Tsr phenotype. The genes of the Sym plasmid pRL1JI involved in nodulation are located on a DNA fragment of 10 kilobases (kb) (4). A 6.6-kb *Eco*RI fragment of this DNA is involved in root hair deformation (14). Further analysis of this 6.6-kb *Eco*RI fragment revealed that Tn*5*-generated mutations in the *nodD*, *A*, *B*, and *C* genes, which are located in a 3.5-kb piece of DNA on the right-hand side of this fragment, result in a nodulation-negative phenotype, whereas delayed nodulation occurs when the mutation is located in the left-hand part of the 6.6-kb *Eco*RI fragment (14; Wijffelman et al., in press). None of the eight mutants in the genes *nodA*

TABLE 3. Induction of Tsr phenotype on *V. sativa* subsp. *nigra* by sterile supernatant fluids from cocultures of *Rhizobium* spp. and *V. sativa* subsp. *nigra* in liquid medium[a]

Test solution	Length of main root (mm)
E	81.7 ± 11.8
PB of RBL5505 (Sym^-)	79.8 ± 5.7
PB of RBL5601 (Sym^+)	47.4 ± 4.9 [b]
JB of RBL5601	77.7 ± 6.8

[a] Tubes with five plants each were incubated in duplicate with PBs produced as described in Materials and Methods and Fig. 1 with 5×10^5 bacteria ml^{-1} as the starting concentration. Incubations in which PB was replaced by E and JB served as controls. The root length was measured after 7 days.

[b] Value is significantly different ($P < 0.005$) from the other values.

(two strains), *nodB* (one strain), *nodC* (four strains), or *nodD* (one strain) was able to induce the Tsr phenotype (data not shown). The mutations which cause the Nod^- Tsr^- phenotype were found to be coupled to the Tn*5* insertion in seven of the eight cases tested since transduction showed 100% coupling of the kanamycin resistance of Tn*5* with Nod^- and Tsr^-. With the other mutant, strain RBL607, this coupling was 10% showing that this Tn*5* was not inserted in the *nod* region of the plasmid. However, also in strain RBL607 Nod and Tsr were found to be 100% coupled. All eight *nod* mutants with transposon insertions in the left-hand part of the 6.6-kb *Eco*RI were able to cause the Tsr phenotype. The most likely explanation of these results is that the four genes *nodA*, *B*, *C*, and *D*, which already have been implied in root hair curling (14; Wijffelman et al., in press), are also required for the induction of the Tsr phenotype.

Induction of Tsr phenotype in liquid medium. For the purification of excreted signal molecules involved in this bacterium-plant interaction, the use of a liquid medium would be superior to the use of agar cultures. Moreover, if the Tsr phenomenon could be brought about in liquid medium we could incubate bacteria and plants in chosen sequences in the filter-sterilized excretion products of each other, an approach which might allow us to elucidate the sequence of events in which those signal molecules mediate the communication between bacterium and plant. *V. sativa* subsp. *nigra* plants appeared to grow very well in liquid J medium, forming long, thin roots (76.2 ± 12 mm long) with few undeformed root hairs on the main root. These roots had a habit comparable to that observed on uninfected plants grown on solid medium. Inoculation of such plants with the *Rhizobium* strains RBL5505 (cured from its Sym plasmid) and RBL5601 (harboring Sym plasmid pRL1JI) resulted in no visible effect on root length (75.7 ± 8.0 mm long) and root hairs and in a Tsr habitus with deformed root hairs and specific root hair curling (root length; 32.6 ± 7.8 mm), respectively. Plants incubated with the Sym plasmid had significantly shorter roots ($P < 0.005$) than both untreated plants and those treated with bacteria cured of the plasmid. The root hair curling mentioned for strain RBL5601 was not very abundant, the majority of the root hairs being straight not deformed. Unlike strain RBL5505, strain RBL5601 formed infection threads and effective root nodules in liquid medium.

Tsr phenotype is caused by soluble factor(s). To determine whether the physical presence of bacterial cells is required for expression of the Tsr phenotype or whether the role of bacterial cells is only to mediate the production of a molecule which in itself is sufficient to cause expresssion of the Tsr phenomenon, PB was prepared from the plant-bacteria cultures mentioned above (Fig. 1) and used as the substrate for fresh axenic *V. sativa* subsp. *nigra* plants. It appeared that PB of strain RBL5601 was indeed able to cause a Tsr phenotype, similar to that observed by cocultivation (Table 3). Therefore, it was concluded that physical contact between *Rhizobium* bacteria and plant roots is not required and that a soluble factor is sufficient to cause expression of the Tsr phenomenon. Although we cannot exclude the possibility that the Tsr phenomenon is caused by a combination of factors, we tentatively designate the factor(s) as Tsr factor. The observation that PB of Sym plasmid-less strain RBL5505 was unable to cause expression of Tsr (Table 3) showed that in liquid medium also the presence of the Sym plasmid is a prerequisite for production of Tsr factor. Control experiments, which showed that root exudate (E) alone or bacterial excretion products (JB) alone were unable to cause expression of Tsr, indicate that products of both organisms are required for the production of the Tsr factor, although the order in which these products act still was to be established. The fact that JB does not cause expression of Tsr also indicates that the Tsr factor is not simply produced by conversion of a plant factor by bacterial excretion products.

Sequence of events leading to the Tsr phenotype. To investigate the individual roles of bacterium and plant in the production of Tsr factor, plants and bacteria were grown separately. Bacteria were also incubated in filter-sterilized root exudate. The resulting culture fluids, EB, were examined for Tsr phenotype-inducing properties on *V. sativa* subsp. *nigra* plants after filter sterilization.

The results (Table 4) show that only EB of the Sym plasmid-harboring strain induces the Tsr phenotype. Apparently root exudate contains a factor which somehow induces the formation of Tsr factor by the Sym plasmid-harboring bacterium. Control incubations in JB, J medium, and E are consistent with this conclusion.

TABLE 4. Test of excretion fluids of bacteria, plants, or both for the presence of Tsr factor to elucidate the sequence of events required for synthesis of Tsr factor

Test solution[a]	Bacterial strain	Length of main root (mm)[b]
JB	RBL5601	82.3 ± 8.4
JB	RBL5505	78.8 ± 5.6
EB	RBL5601	42.7 ± 4.8[c]
EB	RBL5505	74.4 ± 11.6
J		95.2 ± 8.8
E		91.6 ± 11.6
JB obtained after incubation[d] In J medium plus 0.01 mM potassium glutamate	RBL5601	74.0 ± 7.9
In J medium plus 0.1 mM potassium glutamate	RBL5601	78.6 ± 13.6

[a] E, JB, and EB were produced as described in Materials and Methods and Fig. 1. The Tsr phenotype-inducing properties of these liquids were quantified by incubating duplicate tubes with five plants each and measuring the root length after 7 days.

[b] These values were obtained with JB, EB, and J medium plus glutamate prepared after incubation for 3 days. Fluids prepared after 1,2, and 4 days gave similar results.

[c] This value is significantly different from the other values ($P < 0.005$).

[d] Potassium glutamate was added to J medium as growth substrate for strain RBL5601. In this specific experiment, the media were inoculated with bacteria (5×10^5 CFU ml^{-1}) and incubated for 3 days at 25 to 27°C. Growth was stimulated to the same extent by the addition of 0.01 M glutamate as by incubation in exudate. A 10-fold higher glutamate concentration resulted in a fivefold higher bacterial concentration.

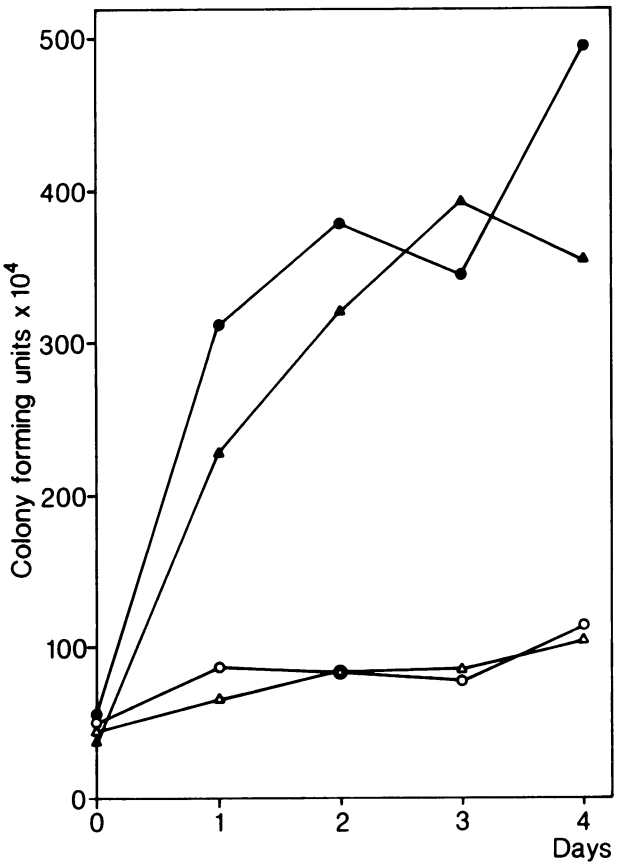

FIG. 3. Viable count of strains RBL5505 (Sym⁻) (●, ○) and RBL5601 (Sym⁺) (▲, △) after incubation in J medium (○, △) and root exudate (●, ▲).

Comparison of the viable counts of bacteria in J medium and in exudate suggested the possibility of an indirect role of exudate in inducing the synthesis of Tsr factor. As the presence of exudate stimulates growth of the bacteria considerably (Fig. 3), the effect of exudate might be nonspecific as it could be caused only by allowing bacterial growth, which in itself could result in production or excretion of Tsr factor. To study the possible effect of bacterial growth on Tsr factor production, the growth-stimulating effect of exudate was mimicked by the addition of 0.01 mM glutamate to J medium. However, although the same number of bacteria was reached as after incubation in E, the filter-sterilized supernatant fluid of this culture was unable to induce the Tsr phenotype (Table 4). Also, a 10-fold higher glutamate concentration, which resulted in a fivefold higher bacterial concentration, did not result in a Tsr phenotype (Table 4). Replacement of glutamate by 0.001 and 0.01 volumes of B⁻ medium also resulted in considerable bacterial growth. The filter-sterilized supernatant fluids of these cultures were also unable to induce the Tsr phenotype. These results indicate that factor A of exudate plays a specific role in stimulating Tsr factor formation.

DISCUSSION

In a previous paper from our laboratory it has been described that the presence of *R. leguminosarum* strains harboring Sym plasmid pJB5JI on the roots of *V. sativa* subsp. *nigra* causes an altered type of root growth resulting in thick and short roots, designated as the Tsr phenotype. The importance of Sym plasmid pJB5JI, a derivative of pRL1JI, in causing Tsr was demonstrated by showing that the Sym plasmid-less strain was unable to cause Tsr (15). The Tsr phenotype is observed before the nodules can be seen. So, presumably this phenotype is connected with an early step in the nodulation process. As Tsr is an example of bacterium-plant interaction, a field of increasing importance, and as the demonstrated involvement of the Sym plasmid provided an opportunity to approach this problem genetically, we decided to study Tsr in more detail.

In the present paper our results confirm the role of the Sym plasmid pRL1JI and extend it in that each of the five tested *R. leguminosarum* and *R. trifolii* Sym plasmids in a Sym plasmid-less *R. leguminosarum* background caused Tsr. In addition to the chromosomal background of the latter bacterium, that of *R. trifolii* and even that of *A. tumefaciens* also allowed the Tsr effect (Table 2).

Transposon mutants of Sym plasmid pRL1JI with transposon insertions in the *nod* genes *nodA*, *B*, *C*, and *D* appeared to be unable to cause Tsr and Hac. The finding that these genes are common *nod* genes, i.e., that they are functionally conserved among rhizobia of various cross-inoculation groups (3, 11, 14, 21a), is consistent with the observation that they are active in various chromosomal backgrounds (Table 2). Using the observation that the Tsr phenomenon can also be observed in liquid medium, we found that a physical interaction between bacterium and plant, which is a prerequisite for specific root hair curling (Hac), is not required for Tsr. Instead, a soluble factor, synthesized by cooperation between bacterium and plant, is sufficient to cause Tsr (Table 3). A series of experiments designed to elucidate the order in which the factors of plant and bacterial origin act (Table 4) show that a combination of a root exudate factor(s), simply designated as factor A, and the Sym plasmid-harboring bacterium is required to synthetize Tsr factor.

The role of factor A in stimulating the formation of Tsr factor is not that of allowing growth of the Sym plasmid-containing strain, since growth of this strain on other substrates did not stimulate Tsr factor formation. Moreover, the Sym plasmid-less strain showed the same growth behavior (Table 4). Questions like which other specific plants are able to mediate the production of Tsr factor and what the molecular nature of the responsible exudate substance is remain to be answered.

Based on the established facts that factor A is a plant product and that the *nodA*, *B*, *C*, and *D* genes are required for Tsr factor production by the bacterium, two types of models can be proposed to explain the results. In the first type, factor A directly or indirectly causes induction or derepression of the *tsr* (*nod*) genes leading to the synthesis of Tsr factor. These genes could either have a structural or a regulatory function. In the case of indirect induction it is conceivable that factor A is a plant enzyme, acting on a bacterial excretion product or on the bacterial cell surface, whose product induces the synthesis of Tsr factor. In the second type of model, plant factor A does not induce the *nodA*, *B*, *C*, and *D* genes but is involved in another way in Tsr factor synthesis. These genes must be expressed, however, either constitutively or by factors other than factor A present in the root exudate. According to this model, factor A can be a precursor molecule which is converted into Tsr factor by a bacterial enzyme that requires the *nodA*, *B*, *C*, and *D* genes for activity. Alternatively, factor A can be a plant enzyme which converts a bacterial substrate, whose synthesis or modification depends on the *nodA*, *B*, *C*, and *D*

genes, into Tsr factor. It should be noted that the models mentioned above do not exclude the possibility that other factors are involved in Tsr factor production, e.g., they leave open the possibility that genes other than the *nod* genes of the Sym plasmid are involved.

Our future research will be directed toward the role of the *nodA*, *B*, *C*, and *D* genes in nodulation. These genes are involved in Hac. Unfortunately, Hac cannot be brought about by soluble factors, as it has been reported (22) and confirmed by us that physical contact between plant and bacterium is required to cause Hac. Using expression of Tsr as a bioassay, we will try to purify Tsr factor. Its subsequent chemical characterization might be helpful in elucidating the nature of the factor causing Hac. In addition, we will test the models mentioned above. Also, for these experiments the characterization of Tsr factor and factor A will be crucial.

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