The Role of Polygalacturonase in Root-Hair Invasion by Nodule Bacteria

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SUMMARY

The production of polygalacturonase in associations of nodule bacteria and seedlings of leguminous plants was investigated. Plants and bacteria were combined in the following ways: (a) different plant species were combined with infective strains (isolated from the same cross inoculation group) as well as non-infective ones (from foreign groups); (b) host plant species with different susceptibilities were combined with the same bacterial strain; (c) bacterial strains with different infectivities, as measured by the number of infection sites, were combined with the same host species; (d) clover strains which had lost their infectivity, as well as transformed and again infective subcultures of these strains, were tested on clover plants. The results indicate that infection of the seedlings was strongly correlated with the production of polygalacturonase. The conclusion is drawn that polygalacturonase plays an important part in the infection process. This function is thought to be a weakening of the cell wall of the root hair which would facilitate the bacterial invasion. The possible role also of indolyl-3-acetic acid in the infection process is discussed.

INTRODUCTION

In a study of the symbiotic relationships between leguminous plants and nodule bacteria one of the first questions which arises is how the bacteria enter the plant. This problem has been comparatively little studied, and no clear picture has so far been obtained of this important step in the development of the symbiosis. In previous work (Fåhraeus & Ljunggren, 1959) some evidence was found that pectic enzymes take part in the infection process. Significant amounts of polygalacturonase were found in associations of seedlings and their specific nodule bacteria, but not in those combinations where no infection occurred. An addition of nitrate, which stops the infection, also inhibited polygalacturonase production. Polygalacturonase was produced by the plant, not by the bacteria, which appears from the fact that an addition of cell-free preparations from the bacteria had the same effect as living bacteria in inducing formation of polygalacturonase (Ljunggren & Fåhraeus, 1959). Since in the above-mentioned work only rather few combinations of plants and bacteria had been examined, the investigation was extended by testing a larger number of plant species and bacteria. Bacterial strains with different degrees of infectivity and plant species with different susceptibilities were also studied. The present paper presents the results obtained, which seem to confirm the importance of the polygalacturonase in the infection process. On the basis of these findings a possible mechanism for rhizobial infection is discussed.

METHODS

Seed material. Commercially available seed of the following plants was used: *Trifolium repens* L. (varieties Morsö and Beta), *T. hybridum* L. (variety Svea), *Medicago sativa* L. (varieties Tuna and Alfa), *Pisum sativum* L. (variety Torsdags II). These were ordered from Svalöv, except *M. sativa*, variety Alfa, which was obtained from Weibullsholm, Sweden. In addition, several wild species were used for the experiments. The seed had been collected by Dr P. S. Nutman, who kindly put it at our disposal. These species were: *Trifolium ambiguum* M. Bieb., *T. glomeratum* L., *T. parviflorum* Ehrh., *T. patens* Schreb., *Melilotus alba* Desv., *Vicia hirsuta* (L.) S. F. Gray, *Lotus angustissima* L., *L. hispida* Desf., *Anthyllis vulneraria* L.

Bacterial strains. *Rhizobium trifolii*: strains 226, ClF, A 121111, SU 297, all infective and effective nitrogen fixers; strain Coryn, infective but forming ineffective nodules; strains Bart A and A 11, non-infective variants of originally infective strains. It has been possible to transfer infectivity to these strains by adding capsular material produced by the infective strain 226 (Ljunggren, 1961). The transformed strains are called Bart A2 and A 11.2, respectively. Other strains: *R. meliloti* AH₃, *R. leguminosarum* 311 and V 1, *Rhizobium* sp. (Lotus) L 1, *R. japonicum* 507.

The strains 226 and 311 were obtained by the courtesy of Dr G. Bjälfve (Leguminous Plant Laboratory, Uppsala); the others were generously supplied by Dr P. S. Nutman.

Substrates and chemicals. For cultivation of *Rhizobium* the medium A₅ given by Dorn (1956) was used throughout. For sterility control a standard Difco nutrient agar was used. The pectin used in the determinations of polygalacturonase was low-methoxyl citrus pectin Matheson, Coleman and Bell no. 7366 (Norwood, Cincinnati, Ohio, U.S.A.).

Experimental procedure

The experimental procedure was essentially the same as reported in a previous paper (Fähræus & Ljunggren, 1959). Some minor changes in the technique and details which we think are of special importance for the successful performance of the experiments are given in the following paragraphs.

For seed disinfection a HgCl₂ + formaldehyde mixture (Fähræus, 1957) was ordinarily used. Extreme care was exercised to remove traces of mercury and possibly harmful seed exudates (Dadd & Jacobs, 1958; Jacobs & Dadd, 1959). To this end, the seed was allowed to swell overnight in the last wash water and was then rinsed again several times. For hard-coated seeds the procedure with sulphuric acid (Nutman, 1959) was more satisfactory and it was used for *Trifolium pratense*, *T. ambiguum*, *T. subterraneum*, *Lotus hispida*, *Anthyllis vulneraria*, *Melilotus alba* and *Vicia hirsuta*.

Certain batches of seed were very difficult to disinfect by any of these methods. Better results were sometimes obtained when the seed after disinfection was allowed to germinate in 0.01 % (w/v) hydroxylamine (personal recommendation by Dr G. Bjälfve). Nevertheless, in many cases we obtained vigorous growth of a Gram-negative, non-sporulating rod which gave yellow colonies on nutrient agar, it was probably a *Flavobacterium*.

Following disinfection the seed was transferred to sterile 10 cm. diam. Petri dishes, and the water volume adjusted to 2–3 ml. in each dish. Usually on the

second day the seedlings were inoculated with *Rhizobium* suspensions made up in distilled water. The suspensions were prepared from agar slopes with 3-day bacterial growth; 0.5 ml. containing about 500 million organisms/ml. was added to each dish, the total volume being adjusted to about 5 ml. with sterile distilled water. The use of distilled water seems to be an important point in this type of work, because even traces of Ca disturb the viscometric analysis. For that reason it is also advisable to rinse all glassware carefully in distilled water.

The dishes after inoculation were kept in an incubator at $20^{\circ} \pm 1^{\circ}$, and were illuminated with fluorescent light.

The experiments were terminated 3–4 days after inoculation. The solution was separated from the roots and solid particles by filtration through a layer of cotton wool.

For the determination of polygalacturonase the viscometric technique described previously (Fåhræus & Ljunggren, 1959) was used throughout. The pectin was dissolved in 0.1 M-acetate buffer (pH 5.0) containing 0.5 % (w/v) NaCl. The solution was filtered on a Buchner funnel through double papers to free it completely from suspended particles. Together with the enzyme solutions to be tested the pectin solution was kept in a constant temperature bath at 30° for temperature equilibration. At zero time the solutions were mixed in Ostwald viscometers (3 ml. test solution + 8 ml. substrate in buffer) and the flow time recorded immediately. Measurements were again made after 1 hr. and 24 hr. The viscometers used had a flow time for water (t_w) between 31 and 35 sec. To get the t_w -value for a 'standard viscometer' with a flow time of 40 sec. we multiplied the values obtained with the different viscometers by the factor $k = 40/t$, where t represents the flow time of water in the individual viscometers.

The decrease in viscosity given in percentage of initial values was calculated from the expression

$$P = \frac{t_0 - t_t}{t_0 - t_w} \times 100,$$

where P = percentage decrease in viscosity; t_0 = initial flow time; t_t = flow time after t min.; t_w = flow time of water.

Sterility control. Since pectin-decomposing micro-organisms are common and the presence of contaminants especially of this kind in our experiments would largely invalidate the results, it was of the utmost importance to keep infection hazards as low as possible. The following precautions were therefore strictly followed.

(a) When the seed had been transferred to Petri dishes for germination, about 50 seeds from the disinfection flask were distributed over two plates of nutrient agar. When microbial growth occurred on these plates, the whole experiment was discarded.

(b) Immediately before inoculation, nutrient agar plates were streaked with liquid from each dish. All dishes which showed microbial growth were discarded.

(c) Before taking samples for analysis, the liquid and a number of roots were examined microscopically. Uninoculated dishes which showed microbial growth and inoculated dishes with growth morphologically distinct from *Rhizobium* were discarded. Fungal mycelium, when present, usually grew along the roots and was easily observed microscopically.

RESULTS

Cross-infection group specificity

In a series of experiments leguminous seedlings were combined with different species and strains of *Rhizobium*. As some seeds were available in small quantities only, experiments could not always be performed to determine the most suitable period for incubation. As a rule, however, satisfactory results were obtained by inoculating the dishes when the seedlings were 8–10 mm. long and incubating for another 3–4 days. The experiments are summarized in Table 1, from which it is evident that polygalacturonase was always present, when the seedlings were inoculated with the appropriate bacteria and that there was no, or very little, activity in other combinations. In this connexion it is interesting to notice Nutman's (1959) report that the *Lotus* and *Anthyllis* species showed deformed root hairs and produced nodules but that no infection threads were observed. In our test, however, they did not significantly differ from the other species tested.

Table 1. *Formation of polygalacturonase (PG) in associations of leguminous plants and nodule bacteria*

PG activity expressed as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

	Plant									
	<i>Trifolium repens</i>	<i>Trifolium ambiguum</i>	<i>Trifolium hybridum</i>	<i>Medicago sativa</i>	<i>Melilotus alba</i>	<i>Pisum sativum</i>	<i>Vicia hirsuta</i>	<i>Lotus hispida</i>	<i>Lotus angustissima</i>	<i>Anthyllis vulneraria</i>
Rhizobium strains	PG activity									
Clover { CIF	12.0	—	—	—	2.0	—	—	—	—	—
226	16.1	—	11.0	—0.8	—	0.0	3.5	3.1	—	1.9
A121111	25.4	10.6	—	—	5.6	—	—	—	0.8	—
Lucerne AH ₂	1.0	0.5	—	22.2	20.6	—	—	—	3.7	0.5
Lotus L1	2.8	2.3	—	1.9	—	—	3.5	14.3	46.6	12.5
Vetch V1	2.0	—	—	2.4	—	—	16.6	0.9	—	0.5
Pea 311	1.2	—	—	—	—	16.4	—	—	—	—
Soybean 507	1.1	—	—	—	—	—	—	—	—	—

Host/bacteria effects within the clover group

Host differences. Nutman in a series of papers (reviewed in Nutman, 1956) studied the genetic factors affecting host/bacterial compatibility. An examination of infection density was also performed by Nutman (1959), but in this case without genetical analysis. An examination of his data, however, shows that there was a difference in infection density between different host species inoculated with the same bacterial strain. For instance, the mean number of infected hairs/plant after 11 days was 91.5 in *Trifolium parviflorum* and 7.0 in *T. patens*, when inoculated with *Rhizobium* strain CIF. The amounts of polygalacturonase produced by the above-mentioned hosts inoculated with one bacterial strain is shown in Table 2. The results indicate that there was a higher polygalacturonase activity in the more susceptible plant species.

Bacterial strain differences. In his work on root hair infection by nodule bacteria Nutman (1959) found great differences in infection density in the same host plant when inoculated with various strains of *Rhizobium trifolii*, and stated that these differences were independent of the host. Table 3 shows the results of two experiments with *Trifolium repens* and *T. glomeratum* inoculated with different strains of clover bacteria. The number of infection threads on the same level on both sides of the root were counted by using the slide technique earlier described (Fåhræus, 1957) and were compared with polygalacturonase activities found in simultaneous experiments. From Table 3 it is evident that there was a certain correlation between the number of infection threads and the polygalacturonase activity found.

Table 2. *Formation of polygalacturonase (PG) by two species of Trifolium inoculated with the same bacterial strain.*

PG activity as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Host plant	Rhizobium strain	PG activity
<i>Trifolium parviflorum</i>	—	1.4
	CIF	27.6
<i>T. patens</i>	—	0.9
	CIF	14.9

Table 3. *The relation between polygalacturonase (PG) activity and number of root-hair infections in two species of Trifolium in association with different strains of Rhizobium trifolii*

PG activity as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Host plant	Rhizobium strain	PG activity		Mean number of infected root hairs
<i>Trifolium repens</i>	—	—0.7	1.4	0
	CIF	11.2	12.2	3.5
	Coryn	17.6	22.7	18.5
	226	17.0	23.0	21.5
<i>T. glomeratum</i>	—	2.0	—	—
	CIF	16.0	—	48.6*
	S.U. 297	11.6	—	9.8*

* Figures from Nutman (1959).

Experiments with non-infective clover strains. The origin of the *Rhizobium* strains Bart A, A11 and A121111 was recorded by Nutman (1946). Of these three strains Bart A and A11 have lost their ability to invade the root hairs of their original host plants and hence they are no longer able to induce nodule production. Table 4 shows that the non-infective bacteria were unable to induce polygalacturonase production. In this respect there was no difference between them and quite unrelated bacteria. We have, however, been able to transfer infectiveness to these bacteria from the infective strain 226 by adding capsular material from the latter to seedlings planted on an agar slope and inoculated with *Rhizobium* strains Bart A and A11. These seedlings produced nodules, and the bacteria isolated from these nodules (Bart A2, A11.2) are infective (Ljunggren, 1961). As shown in Table 4, the transformed

strains are also active polygalacturonase producers. These experiments have in fact given the only unequivocal proof, except serological tests, that Bart A and A11 really are *Rhizobium* strains.

Table 4. *Formation of polygalacturonase (PG) by Trifolium repens in association with infective and non-infective strains of Rhizobium trifolii*

PG activity as % decrease in viscosity of 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Rhizobium strain	PG activity	
	Expt. 1	Expt. 2
—	—1.0	0.5
Bart A	1.5	2.1
A11	2.5	1.1
A121111	15.0	15.2
226	16.2	15.4
Bart A2	—	10.8
A11.2	—	11.3

DISCUSSION

The correlation found between the infection of leguminous plants by nodule bacteria and the increased production of polygalacturonase in the bacteria + plant associations can be interpreted in two ways: (i) polygalacturonase formation may be the primary effect of bacterial action, (ii) the infection might come first and lead to an increased production of polygalacturonase. We have drawn the conclusion that the formation of polygalacturonase is the primary effect resulting in a partial depolymerization of the cell-wall pectin which, in its turn, facilitates the bacterial invasion. For this succession of events speaks the fact that infection rarely can be observed until after 3 days, whereas an increased production of polygalacturonase may occur as soon as 1 to 2 days following inoculation.

The infection mechanism may be visualized in the following manner. The bacteria secrete water-soluble substances which are highly specific (Humphrey & Vincent, 1959; Ljunggren & Fåhræus, 1959). Our active preparations are mainly of polysaccharide nature, but transformation experiments carried out by one of us (Ljunggren, 1961) suggest that they might also contain deoxyribonucleic acid. The active principle will pass through the cell wall, reach the protoplasm, and react with some specific cell component. The nucleus, which at this stage is situated in the root hair near the tip, may possibly be involved in the interaction with the bacterial compound. Previous observations (Fåhræus, 1957; Nutman, 1959) suggest that the nucleus takes an active part in the infection process, but the mechanism is still obscure.

The reaction between the bacterial substance and the specific cell compound results in the formation of an 'organizer' which governs the production of polygalacturonase. This enzyme is always present in the growing root hair in small amounts. The apical hair wall consists mainly of pectic substances, and the function of polygalacturonase may be a continuous softening of this wall, which leads to cell elongation (Ekdahl, 1953). However, when appropriate nodule bacteria are present, they induce, in some hairs at least, a much stronger production of polygalacturonase. This might result in a more pronounced depolymerization of the pectic layer, which

would allow the bacteria to penetrate the cell wall. But it appears also possible that the increased polygalacturonase activity can result in an intensified but very localized growth process giving an infection thread by invagination, in accordance with Nutman's hypothesis (1956). It seems to us that an important role in the infection process can be ascribed to polygalacturonase regardless of whether Nutman's hypothesis be accepted or not.

The mechanism outlined in this way could explain the high specificity which is characteristic for the cross-inoculation groups of leguminous plants, since the

Fig. 1. Induced formation of polygalacturonase (PG) in root hair tip of clover plant.

specificity of polygalacturonase formation, as shown by our experiments, is quite as high. This is further illustrated by the tentative scheme given in Fig. 1. A pre-requisite for infection as well as for polygalacturonase production is a certain nitrogen deficiency (Fåhræus & Ljunggren, 1959). The reason why polygalacturonase formation is stopped at higher nitrate concentrations might perhaps be that nitrogen stimulates synthetic mechanisms in the plant which decrease the formation of depolymerizing enzymes. Even at suboptimal nitrogen concentrations, the increased polygalacturonase activity is probably not a sufficient condition for infection to take place. The well known fact that only a very restricted number among thousands of root hairs are invaded indicates that the infection is a rather complex process. Possibly the cell wall must in some way be 'sensitized' before the polygalacturonase becomes active. It lies near at hand in this connexion to think of indolyl-3-acetic acid (IAA). IAA increases the plasticity of cell walls (Galston & Purves, 1960), and is produced by nodule bacteria (Link, 1937; Kefford, Brockwell & Zwar, 1960). According to Thornton (1936) the deformation of root hairs, which is believed to be caused by bacterial IAA, is a necessary prelude to infection. However, our present knowledge of the action of IAA on cell walls—which might involve an immobilization of pectin methylesterase (Glasziou, 1957; Fåhræus & Ljunggren, 1959)—are too vague to permit detailed conclusions about the role of

IAA in infection. It is certainly not possible to explain the whole infection process only as an IAA effect (Kefford, Brockwell & Zwar, 1960). Such an explanation does not account for the marked specificity of rhizobial infection, since IAA is produced by almost all types of nodule bacteria, and also by *Agrobacterium radiobacter* which is unable to infect any leguminous plant (Georgi & Beguin, 1939).

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