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MPMI Vol. 16, No. 1, 2003, pp. 53–64. Publication no. M-2002-1107-01R.

Infection-Blocking Genes of a Symbiotic *Rhizobium leguminosarum* Strain That Are Involved in Temperature-Dependent Protein Secretion

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Submitted 20 May 2002. Accepted 19 September 2002.

Rhizobium leguminosarum **strain RBL5523 is able to form nodules on pea, but these nodules are ineffective for nitrogen fixation. The impairment in nitrogen fixation appears to be caused by a defective infection of the host plant and is host specific for pea. A Tn*5* mutant of this strain, RBL5787, is able to form effective nodules on pea. We have sequenced a 33-kb region around the phage-transductable Tn*5* insertion. The Tn*5* insertion was localized to the 10th gene of a putative operon of 14 genes that was called the *imp* (impaired in nitrogen fixation) locus. Several highly similar gene clusters of unknown function are present in *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Edwardsiella ictaluri,* and several other animal pathogens. Homology studies indicate that several genes of the *imp* locus are involved in protein phosphorylation, either as a kinase or dephosphorylase, or contain a phosphoprotein-binding module called a forkhead-associated domain. Other proteins show similarity to proteins involved in type III protein secretion. Two dimensional gel electrophoretic analysis of the secreted proteins in the supernatant fluid of cultures of RBL5523 and RBL5787 showed the absence in the mutant strain of at least four proteins with molecular masses of approximately 27 kDa and pIs between 5.5 and 6.5. The production of these proteins in the wild-type strain is temperature dependent. Sequencing of two of these proteins revealed that their first 20 amino acids are identical. This sequence showed homology to that of secreted ribose binding proteins (RbsB) from *Bacilus subtilis* and *V. cholerae*. Based on this protein sequence, the corresponding gene encoding a close homologue of RbsB was cloned that contains a N-terminal signal sequence that is recognized by type I secretion systems. Inoculation of RBL5787 on pea plants in the presence of supernatant of RBL5523 caused a reduced ability of RBL5787 to nodulate pea and fix nitrogen. Boiling of this supernatant before inoculation restored the formation of effective nodules to the original values, indicating that secreted proteins are indeed responsible for the impaired phenotype. These data suggest that the *imp* locus is involved in the secretion to the environment of proteins, including periplasmic RbsB protein, that cause blocking of infection specifically in pea plants.**

Additional keywords: imp locus, pathogenesis, protein phosphorylation, symbiosis.

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Bacteria of the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium,* and *Mesorhizobium*, collectively called rhizobia, can establish a host-specific symbiosis with leguminous plants, supplying the plant with fixed nitrogen in exchange for carbon sources. A clear example of host specificity is found in the *R. leguminosarum*–plant interaction. For instance, *R. leguminosarum* bv. *viciae* is limited in host range to plants of the pea and vetch groups, whereas *R. leguminosarum* bv. *trifolii* is limited to the clover group. The symbiosis occurs in specially developed organs, the root nodules that are induced by signal molecules produced by the rhizobia. These signal molecules have a common core structure of a chitin oligosaccharide backbone with a fatty acyl group at the nonreducing terminus, called lipochitin oligosaccharide (LCO). This core structure is modified with various kinds of side groups, depending on the bacterial species (Bladergroen and Spaink 1998). The large variation in the LCO structures is an important determinant for the specificity of the rhizobia-plant symbiosis. In *Rhizobium* spp., the genes that are involved in the synthesis and secretion of the LCOs are located on a plasmid, the symbiotic (Sym) plasmid (Long 1989). However, other factors have been shown to determine host specificity and these factors often are not plasmid encoded (Spaink 2000). For example, in three cases, loci have been identified which negatively influence nodulation. Using Tn*5* mutagenesis, mutants of *R. leguminosarum* bv. *trifolii* TA1 were isolated that are able to nodulate clover cv. Woogenellup, which normally cannot be nodulated by this strain (Lewis-Henderson and Djordjevic 1991; Roddam et al. 2002). These results indicated that cultivar specificity resulting from negative factors is mediated by at least two independent mechanisms or determinants. Similar cultivar-specific nodulation also was reported by the group of Pueppke (Chatterjee et al. 1990; Heron et al. 1989). Tn*5* induced mutants of *R. fredii* strain USDA257 are able to nodulate the soybean cv. McCall, which is not a natural host for this strain. However, a specific negative regulatory gene was not reported, so it cannot be excluded that specific *nod* genes were involved. The third example was found by Roest and associates (1997) in the interaction of *R. leguminosarum* bv. *viciae* with pea plants. These authors showed that an *R. leguminosarum* bv. *trifolii* strain in which the Sym plasmid is exchanged for that of an *R. leguminosarum* bv. *viciae* strain is able to nodulate pea (*Pisum sativum*) but is not able to fix nitrogen. A detailed analysis of this strain, called RBL5523, which was shown to behave as a normal wild-type strain in nodulation of *Vicia sativa* (Pollock et al. 1998; van Workum et al. 1997a, 1997b) showed an effect of the chromosomal background on pea nodulation. Roest and associates isolated a mutant of strain RBL5523 called RBL5787 or *imp* (impaired in

nodulation) mutant which nodulates pea more effectively and is restored in its ability to fix nitrogen. Lipopolysaccharide patterns as well as major cell envelope proteins, cell surface components, and the pattern of LCOs were found to be normal in this mutant strain. Finally, extracellular polysaccharides were assumed not to have any influence, because the colony morphology of wild-type and mutant strain were identical (Roest et al. 1997). A small part of the *imp* locus was identified by nucleotide sequencing, but homology studies did not indicate possible functions.

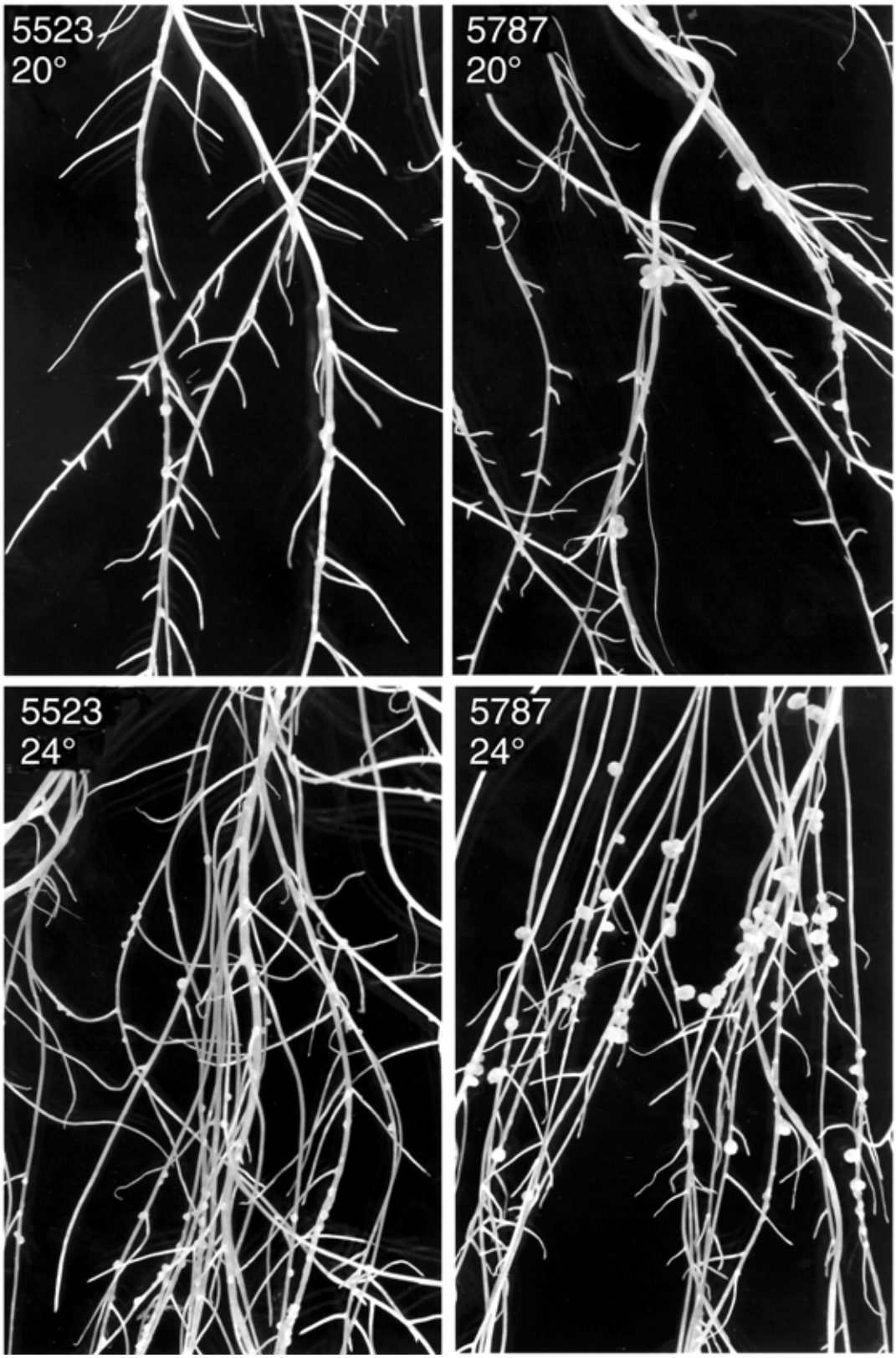

Fig. 1. Influence of growth temperature on pea nodulation and nitrogen fixation. The upper two panels show the roots of representative pea plants inoculated with RBL5523 (left) and RBL5787 (right) grown at 20°C. The lower two panels show the roots of pea plants inoculated with RBL5523 and RBL5787 grown at 24°C. It is clearly visible that RBL5787 forms much more nodules than RBL5523 at 24°C and that these nodules are pink.

In this article, we show that the gene mutated in RBL5787 is present in a putative operon of 14 genes, several of which show homology to avirulence genes of pathogens. These include genes that are members of the type III secretion system and genes that encode proteins that are normally secreted by this system, such as a protein phosphatase and a protein kinase. Furthermore, highly similar operons of unknown function are present in pathogens such as *Pseudomonas aeruginosa*, *Vibrio cholerae,* and various other animal pathogens. Nodulation experiments on pea and vetch indicated that the effect of the Tn*5* mutation is temperature sensitive and that the mutated gene influences symbiosis at the infection level. This suggests that the *imp* locus is a member of a new family of operons involved in modification of host responses. Our experiments indicate that such operons are involved in the secretion of proteins.

RESULTS

The role of the *imp* locus in symbiosis.

Strain RBL5787 was isolated as a derivative of strain RBL5523 restored in its ability to fix nitrogen on pea; this phenotype was shown to be due to a single, phage-transductable Tn*5* insertion (Roest et al. 1997). To investigate the influence of this Tn*5* insertion on pea infection and nodulation, strains RBL5523 and RBL5787, transconjugated with a plasmid-encoding green fluorescent protein (GFP), were inoculated on 3-day-old pea seedlings. Results of the nodulation experiment and the influence of the growth temperature on the formation of root nodules are shown in Figure 1. The effect of the mutation on root nodulation is most clearly visible at 24°C. At this temperature, the mutant forms approximately 10 times more nodules than the wild type (three independent experiments with nine plants). In addition, the nodules from the mutant are pink at 20 and 24°C, indicative of the occurrence of nitrogen fixation, in contrast to the nodules induced by strain RBL5523. Analysis of sections through 14-day-old nodules using fluorescence microscopy (Fig. 2) clearly showed that the nodules from plants infected with RBL5523 were empty, whereas the nodules of plants infected with RBL5787 were infected with green fluorescent bacteria. Therefore, we can conclude that the impairment in nitrogen fixation is due to a strong defect in the infection of the host plants.

Roest and associates also observed an improved nitrogen fixation phenotype of strain RBL5787 on vetch, although at a low level (Roest et al. 1997). To analyze the host-specific aspects of the infection-blocking factors of RBL5523, we also investigated the infection, nodulation, and nitrogen fixation phenotypes on *Vicia hirsuta* and *V. sativa* at different growth temperatures. The nodulation phenotype of RBL5787 did not differ from that of RBL5523 on these plants; in addition, infection thread counts with *lacZ*- or *gfp*-labeled bacteria did not yield any significant differences, although minor differences

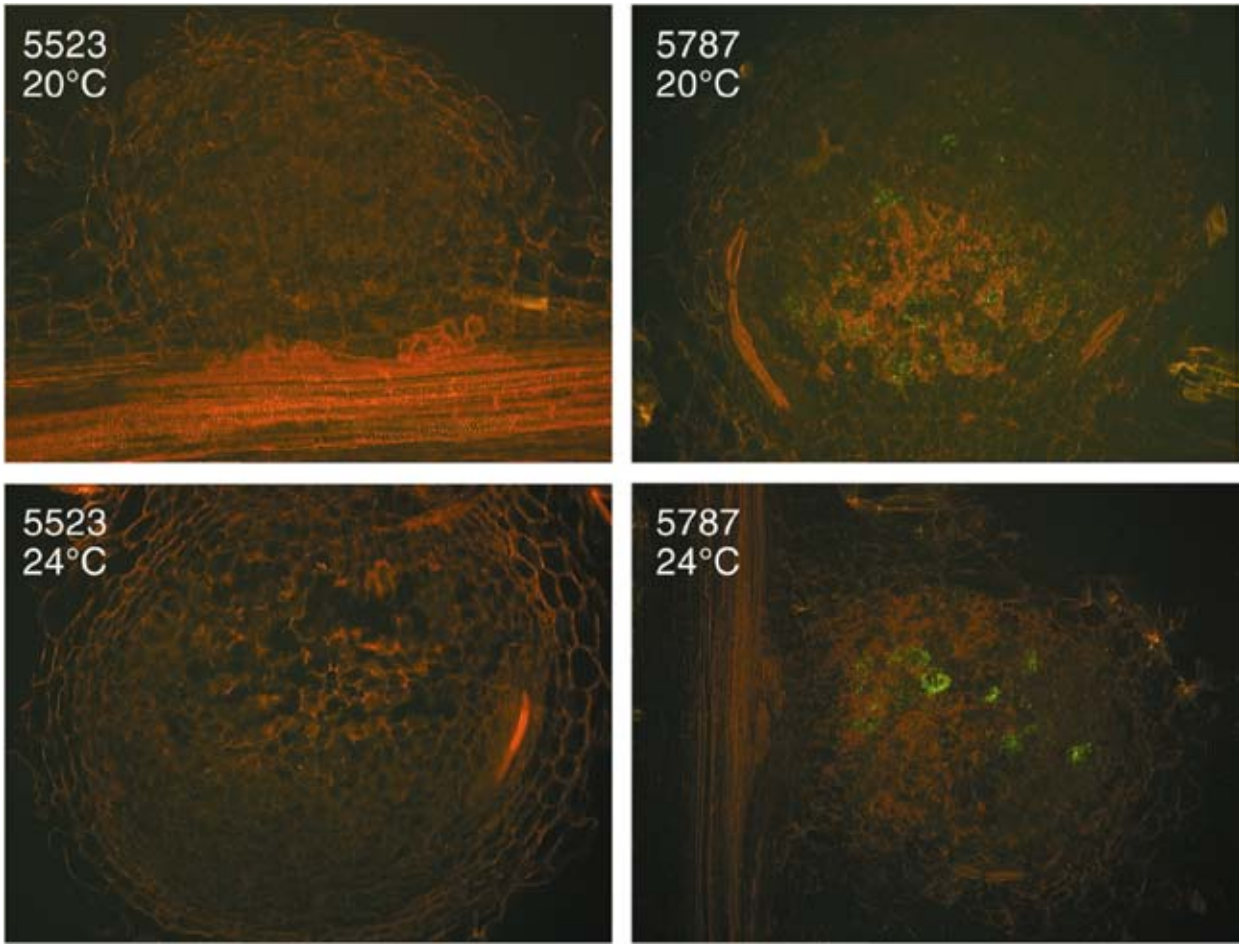

Fig. 2. Microscopic images from sections through 14-day-old nodules inoculated with green fluorescent protein–labeled bacteria. The bacteria were grown at two different temperatures, 20°C (upper panels) and 24°C (lower panels). The left panels show the situation for RBL5523 and the right panels for RBL5787. The images were recorded on a Leica IRBE fluorescence confocal microscope with 10-fold magnification, using an argon laser with an excitation wavelength of 488 nm and an emission wavelength of 505 to 540 nm.

cannot be excluded, considering the large overall number of infection threads (which can number over 100 per nodule in both the mutant and wild type) and the large variation of infection thread number in all nodules studied. The data suggest a tendency for higher acetylene reduction levels for RBL5787 compared with RBL5523 under several conditions both on *V. hirsuta* and *V. sativa* (Fig. 3). However, these differences seem too small to instigate further investigation at the current stage of research.

Sequence analysis of the *imp* gene locus.

The locus that is responsible for the impaired phenotype of RBL5523 was isolated on several overlapping cosmids by Roest and associates (1997). One of these cosmids, pMP3717, was used for further analysis. Sequencing of subclones from cosmid pMP3717 resulted in a DNA sequence of 33.1 kb in which the Tn*5* insertion of RBL5787 could be localized at position 11,899 based on prior data (Roest et al. 1997) (Fig. 4). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AF361470. BLASTX searches in the databases at the National Center for Biotechnology Information (NCBI) yielded 28 open reading frames (ORFs) with a significant homology to known sequences. Comparison of the gene organization of our sequence with that of the homologous genes indicates that we have identified genes of at least seven different operons (Fig. 4). The genes located in the operon in which the Tn*5* insertion of RBL5787 was located were designated *impA* through *impN*. The other genes were designated ORF1 through ORF14, because we have no evidence that these ORFs are involved in the *imp* phenotype. The BLAST search results in the NCBI databases with the obtained sequences are shown in Table 1, listing the most similar homologs identified and the hypothetical function, if any could be proposed, of any other member of the found gene families. As we can observe from this table and from Figure 4, many ORFs show homology to ORFs from *P. aeruginosa* and *Vibrio cholerae*. Remarkably, they not only show homology at the amino acid level but, in addition, the operon structure of the first two operons is very similar in all three bacterial species, both directionally and locationally. Also, in two other completely sequenced *Rhizobiaceae* family members, *Agrobacterium tumefaciens* strain C58 (accession AE009362) and *Mesorhizobium loti* (accession AP002999), homologous gene clusters are found. However, no homologous genes were found in the entire genomic sequence of *Sinorhizobium meliloti*.

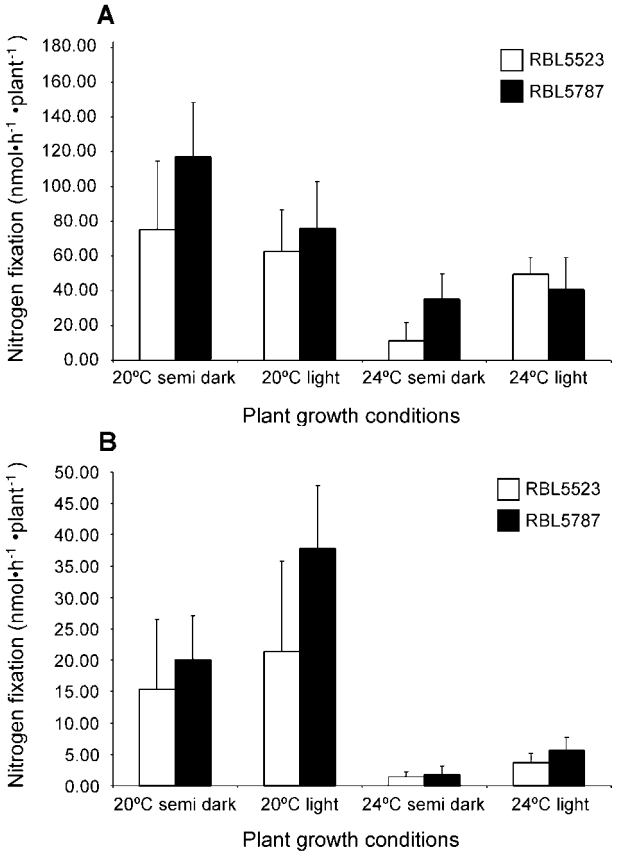

Fig. 3. Acetylene reduction levels of **A,** *Vicia sativa* and **B,** *V. hirsuta* inoculated with RBL5523 and RBL5787 and grown at various growth conditions. Open columns represent RBL5523 and black columns represent RBL5787. Standard deviations are shown for each experiment. Approximately 10 plants per experiment were analyzed.

In addition to the listed overall sequence homologies that were identified by BLAST searches, the predicted ImpI protein contains a clearly recognizable forkhead-associated (FHA) domain of at least 55 amino acids (aa). The FHA domain is a phosphoprotein-binding domain that is found in a wide variety of prokaryotic and eukaryotic proteins (Li et al. 2000; Pallen et al. in press).

Analysis of culture supernatant proteins of RBL5523 and RBL5787.

The Tn*5* insertion in RBL5787 is located in an operon that contains homologues of avirulence genes of pathogens suggesting a function in protein translocation. To determine if this insertion has an effect on protein secretion, culture supernatant proteins from the mutant and wild-type strains grown at 24°C were isolated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Sypro-Ruby staining, which revealed a clear difference between the proteins from RBL5523 and RBL5787 (data not shown). Notably, one protein band of approximately 27 kDa was not detectable in the *imp* mutant. Comparison of supernatant proteins of strains grown at different growth temperatures showed that the band of 27 kDa was not detectable in spent growth medium of RBL5523 grown at 20°C (two independent experiments). To further investigate protein composition of the samples, 2D-protein analysis was performed. The 2D-gels from culture supernatant proteins from RBL5523 and RBL5787 are shown in Figure 5. A number of protein spots are reduced in intensity in the samples of strain RBL5787. Most clearly, at a molecular weight of approximately 27 kDa, four proteins with a pI between 5.5 and 6.5 can be observed in RBL5523 that are absent from RBL5787 (Fig. 5, arrowheads). By use of Western blotting and Coomassie blue staining, we were able to isolate a significant amount of protein from the two biggest spots (Fig. 5, spot 1 and 2) for protein sequencing using Edman degradation. The sequence of the first 50 aa from spot 1 are shown in Fig. 6. The first 20 aa of spot 2 appeared to be identical to those of spot 1; therefore, further sequence analysis of spot 2 was discontinued. These results suggest that the difference between spots 1 and 2, and probably also between spots 3 and 4, is caused by a posttranslational modification, such as phosphorylation, resulting in a different charge. However, it cannot be excluded that product diversification resulted from trichloroacetic acid (TCA) precipitation.

A temperature effect was observed in one dimensional gel analysis; therefore, we also analyzed the secreted proteins at the growth temperature of 20°C in 2-D analysis. At this temperature, the proteins from spots 1 and 2 are found in the growth medium of strain RBL5523, but in much lower amounts than at 24°C. Spots 3 and 4 are no longer visible using silver staining (Fig. 5C). It also could be noted that spot 1 appeared to consist of two spots at 20°C (Fig. 5C, spot 1A and B). This also could be the case for the samples obtained at 24°C growth tem-

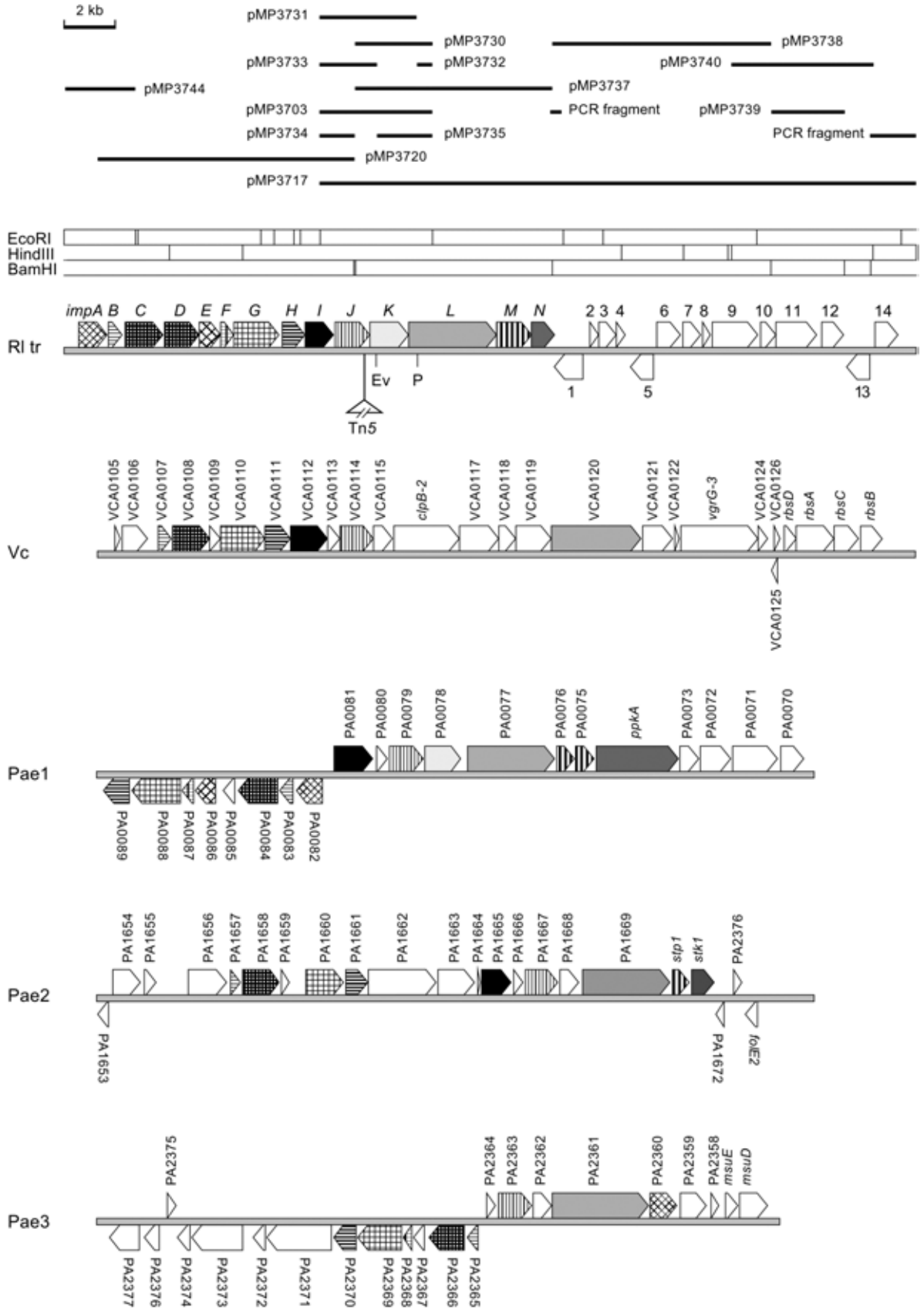

Fig. 4. Schematic representation of the *imp* (impaired in nodulation) gene locus of *Rhizobium leguminosarum* strain RBL5523 and of the cloned fragments from this locus in comparison with the homologous regions in *Pseudomonas* and *Vibrio* spp. pMP3703 and pMP3720 have been published before (Roest 1995; Roest et al. 1997). The positions and lengths of the open reading frames and the direction of transcription were determined by DNA sequence analysis. The gene organization of the *imp* gene locus is compared with that of *Pseudomonas aeruginosa* and *Vibrio cholerae* in the corresponding regions. For *P. aeruginosa,* three regions with substantial similarity were identified (Pae 1, 2, and 3). The corresponding genes are indicated by corresponding shading. B = *Bam*HI, E = *Eco*RI, Ev = *Eco*RV, and P = *Pst*I. The Tn*5* insertion is represented by the triangle; // indicates regions which are not drawn to scale.

perature, but this probably is not notable because of the larger quantity of protein.

Cloning of the gene encoding the secreted protein.

Backtranslation of the protein sequence of spot 1 into DNA provided a template for the synthesis of polymerase chain reaction (PCR) primers (Fig. 6). We designed degenerate primers on amino acids 1 to 12 and amino acids 33 to 44 to perform a low-stringency PCR on chromosomal DNA. This PCR yielded a fragment of the expected size (132 bp) from both RBL5523 and RBL5787, as well as the phage-transducted *imp*-mutant strain RBL5961, whereas it was not found following PCR of total DNA of *S. meliloti* strain GMI766 (Renalier et al. 1987). Sequencing of this fragment showed that all three strains contain identical DNA fragments encoding the sequenced part of the secreted protein. This fragment was used as a probe to screen a cosmid library of LPR5020 by colony blotting. From one positive clone, the nucleotide sequence of a 1,642-bp *Eco*RI fragment was determined showing the presence of the probe sequence (Fig. 6). The sequence of this region has been submitted to the DDBJ/EMBL/GenBank databases under accession number AF361471. The sequence predicted the probe to be part of an ORF of 963 bp, which was analyzed against the protein database and showed highest similarity to the secreted ribose binding protein (RbsB) of *Bacillus subtilis* (Fig. 6). This sequence was followed by a gene homologous to the *rbsA* gene and preceded by a gene homologous to the *rbsC* gene of *B. subtilis*. Interestingly, the homologous *imp* region of *V. cholerae* also harbors the *rbsA, rbsB,* and *rbsC* genes.

The influence of secreted proteins of RBL5523 on the rhizobia–pea symbiosis.

To investigate whether secreted proteins were involved in the fix$^-$ phenotype of RBL5523, we inoculated the mutant bacteria in the spent growth medium from a culture of RBL5523 on pea plants. This culture supernatant caused a reduction in nodules and nitrogen fixation. Denaturation of the proteins by boiling the supernatant fluid before inoculation reversed the nodulation and nitrogen fixation levels to the values of RBL5787 in its own culture supernatant (Fig. 7A, columns III and IV; B, columns III to V). Consistent with these results, co-inoculation of the two strains reduced the number of nodules. However, nitrogen fixation was even slightly increased compared with the mutant inoculated in its own growth medium. Confocal laser scanning microscopic analysis of nodules from pea plants co-inoculated with RBL5523-pMP4656 (CFP) and RBL5787-pMP4639 (YFP) showed the presence of only RBL5787-pMP4639 (YFP) in those nodules, confirming the defectiveness for infection of RBL5523. However, the infection frequency of RBL5787 was not decreased, which seems contrary to the negative effect of the protein samples from strain RBL5523. Perhaps this is caused by the fact that produc-

Table 1. Identified open reading frames and their homologous proteins

Name[a]	Most homologous protein	Percent identity/ similarity[b]	Possible function or other homologous proteins[c]
impA	PA2360 *Pseudomonas aeruginosa*	40/53 (65)	Hypothetical, PA0082
impB	Eip20 *Edwardsiella ictaluri*	51/67 (131)	Hypothetical, PA2365, PA0083, PA1657, VCA0107
impC	Eip55 *E. ictaluri*	56/72 (479)	Hypothetical, PA2366, PA0084, PA1658, VCA0108
impD	PA2366 *P. aeruginosa*	40/57 (430)	Hypothetical, Eip55, PA0084, PA1658, VCA0108
impE	PA0086 *P. aeruginosa*	27/43 (274)	Hypothetical
impF	Eip19 *E. ictaluri*	28/47 (153)	Hypothetical, PA2368, PA0087
impG	PA0088 *P. aeruginosa*	34/48 (628)	Hypothetical, PA2369, PA1660, VCA0110
impH	PA2370 *P. aeruginosa*	28/45 (239)	Hypothetical, PA0089, PA1661, VCA0111
impI	PA0081 *P. aeruginosa*	28/41 (143)	Proliferation-related Ki-67 antigen (*Homo sapiens*), PA1665, FHA domain
impJ	PA0079 *P. Aeruginosa*	44/65 (104)	Contains Tn*5* mutation in RBL5787
impK	PA0078 *P. aeruginosa*	35/52 (140)	Flagellar torque generating protein, MotB
impL	PA0077 *P. aeruginosa*	31/47 (1098)	IcmF *Legionella pneumophila*, putative macrophage toxin *Escherichia coli*, PA2361, PA1669, VCA0120
*imp*M[d]	PA0076 *P. aeruginosa*	40/56 (136)	Hypothetical
	PpkB *P. aeruginosa*	31/55 (145)	Protein phosphorylation/ dephosphorylation, PA0075, PA1670 (=Stp1)
impN	Putative serine/threonine kinase, *Myxococcus xanthus*	36/50 (199)	Serine/threonine kinase, PA0074 (=PpkA), PA1671, PA1782
ORF1	CpxD *Actinobacillus pleuropneumoniae*	34/51 (375)	Capsular polysaccharide exporter outer membrane protein, PssN *Rhizobium leguminosarum* bv. *trifolii,* PA2234
ORF2	Hypothetical protein, *Streptomyces coelicor*	50/68 (109)	Hypothetical, PA1065, very short stretch of this putative protein is homologous to Heat Shock proteins
ORF3	PA2134 *P. aeruginosa*	31/45 (190)	Hypothetical
ORF4	Amicyanin precursor *Thiobacillus versutus*	24/50 (82)	Methylamine dehydrogenase *Paracoccus denitrificans*, Amycianin precursor *Paracoccus denitrificans*
ORF5	GstR *Bradyrhizobium japonicum*	31/51 (293)	Positive transcriptional regulator, PA0056, PA2316, PA1738, PA2334, PA2383, PA2447, PA2681, PA1201, PA1067,
ORF6	YdfO *Bacillus subtilis*	44/58 (304)	aromatic metabolite ABC transporter
ORF7	Hydrolase	82/89 (229)	Hydrolase, PA2698, PA1202, PA2419
ORF8	PA2697 *P. aeruginosa*	71/94 (52)	Hypothetical
ORF9	PA2699 *P. aeruginosa*	62/75 (624)	Exoenzymes regulatory protein, YtcJ *Bacillus*, PA2448
ORF10	PA4205 *P. aeruginosa*	34/47 (110)	No homolog, just identified as a putative open reading frame
ORF11	PA2701 *P. aeruginosa*	34/52 (520)	Transporter of the Major Facilitator Superfamily
ORF12	PrxC *P. pyrrocinia*	80/88 (278)	Haloperoxidase, PA2717, PA1622
ORF13	PA2696 *P. aeruginosa*	26/45 (267)	AraC-like transcriptional regulator *Azorhizobium caulinodans,* PA1619
ORF14	PA2696 *P. aeruginosa*	29/52 (278)	AraC-like transcriptional regulator *A. caulinodans,* AgpT *Sinorhizobium meliloti,* PA1619, PA2332, PA2337

[a] Similarity between *impC* and *impD* at the DNA level is 45% and at the protein level 29% (identity) or 44% (similarity). Eip55 and PA2366 are homologues of each other. ORF = open reading frame.
[b] Values in parentheses are the number of amino acids on which the identity score is based.
[c] PA numbers are from *Pseudomonas aeruginosa*, VCA numbers are from *Vibrio cholerae*, annotation from sequence project.
[d] Two separate regions of homology are present in this protein.

tion of infection-blocking proteins by RBL5523 in vivo under co-inoculation conditions is strongly diminished or delayed compared with the situation in culture medium.

DISCUSSION

In this article, we describe the identification of a locus, called *imp*, that is involved in the repression of formation of effective root nodules by *R. leguminosarum* strain RBL5523 on pea roots. With a combination of genetics and reverse genetics we have shown that the phenotype is caused by genes that are most likely involved in the temperature-dependent secretion of proteins. In the original description of the locus by Roest and associates (1997), it was hypothesized that the gene mutated in strain RBL5787 is involved in the synthesis of a compound that evokes a hypersensitive response on plants that are not natural hosts for these bacteria. Such genes are common in avirulence loci of phytopathogenic bacteria with homologs in several mammalian pathogens. As described in this article, the following findings support the defense response hypothesis. First, some of the genes in the identified locus are homologous to those located in avirulence loci of pathogenic bacteria. As further discussed below, ImpK is homologous to type III secretion genes and ImpN and ImpM are homologous to protein phosphatases and kinases that are substrates of avirulence secretion systems of animal pathogens (DeVinney et al. 2000; Young et al. 1999). Second, avirulence factors are often highly species specific. They interact with plant-specific complementary resistance genes, which leads to a hypersensitive response. The same avirulence factors often act as virulence factors on susceptible host plants, leading to disease symptoms. We demonstrated that the effect of the *imp* genes was most pronounced on pea and not clearly detectable on vetch. Third, the phenotype exhibited by classical avirulence genes is often temperature dependent (van Dijk et al. 1999), which also was suggested by our nodulation experiments (Fig. 1), because the largest difference between mutant and wild-type nodulation was noticed at 24°C, whereas hardly any effect was observed at 20°C. Finally, microscopic examination of the few nodules formed by RBL5523 revealed that this strain was unable to infect the plant tissue consistent with a defense response of the host. The same phenotype was observed with the mutants of *S. fredii* strain USDA257 on soybean cv. McCall. The mutated genes were proposed to be involved in preventing infection thread initiation or development in McCall (Chatterjee et al. 1990; Heron et al. 1989). However, we have not tested whether the blocking of infection caused by the *imp* operon is due to the elicitation of plant defense re-

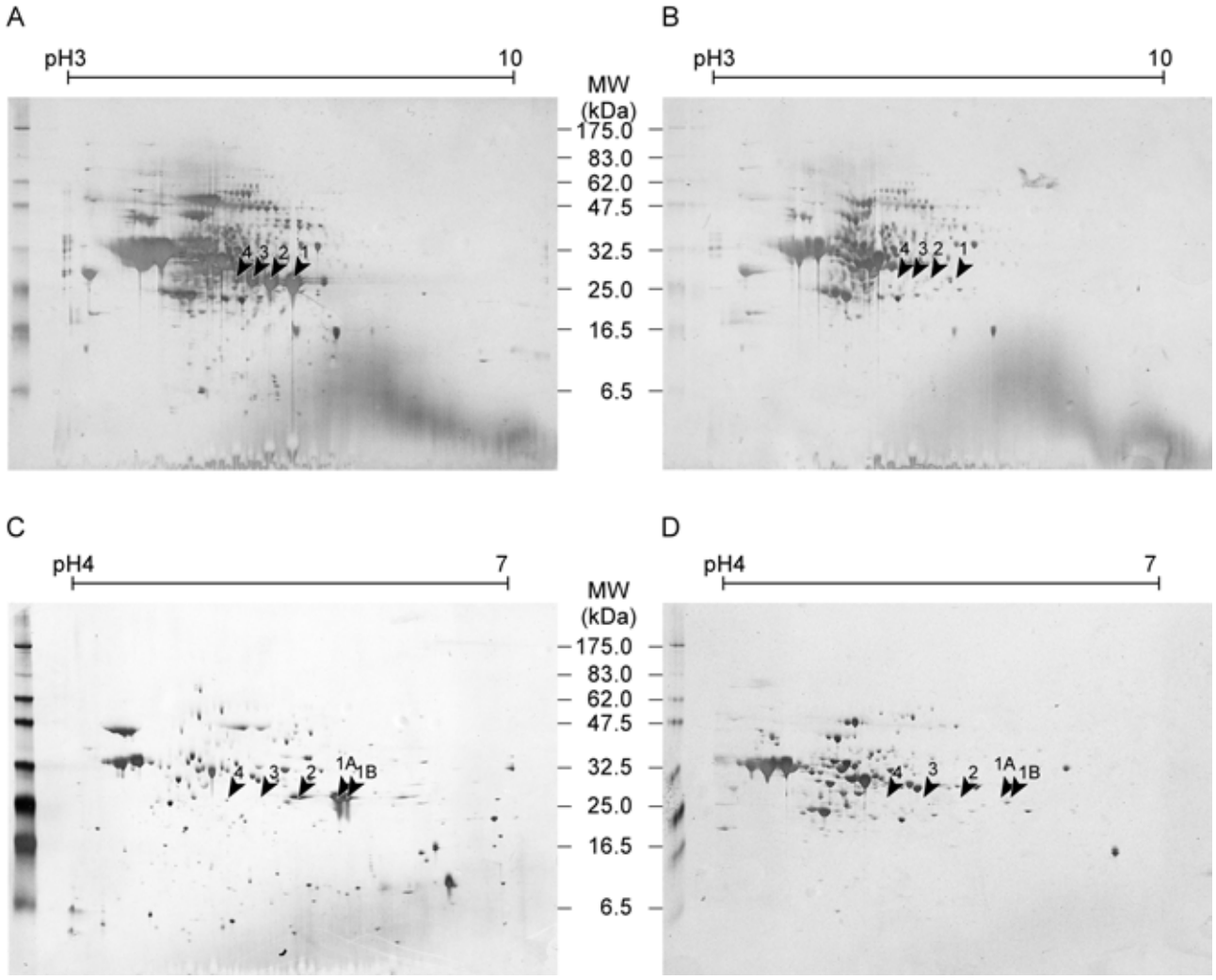

Fig. 5. 2D-gel electrophoretic analysis from the spent growth medium of RBL5523 compared with that of the *imp* (impaired in nodulation) mutant. The culture supernatant fluids of **A** and **C,** RBL5523 and **B** and **D,** RBL5787 were analyzed at **A** and **B,** 24°C and **C** and **D,** 20°C. The pH scale in the first dimension is indicated horizontally. The molecular weight of standard proteins is indicated vertically. Arrowheads indicate protein spots that are different between the two strains; for clarity, they are also shown in **B** and **D**, where the protein spots are absent. As can be seen in panel **C,** spot 1 appears to consist of two spots at 20°C (1A and 1B).

sponses; therefore, the indicated similarities with avirulence loci of pathogens still could be co-incidental.

The following observations indicate that the *imp* phenotype is caused by one or more secreted proteins. First, 2D PAGE analysis of proteins present in the spent growth medium showed a different protein profile between the wild-type and the mutant strains. Most notably, the intensity of four protein spots, two of which appeared to share N-terminal sequence identity, was greatly affected in the mutant strain. Second, external application of sterile growth supernatant of RBL5523 inhibited the infection of pea nodules by RBL5787, which was reversed by boiling this supernatant before application. Of course, at this moment we cannot rule out the possibility that the phenotype is caused by secreted proteins other than the one identified in this study. This is suggested by the fact that the presence of these proteins in the spent growth medium was temperature dependent but was not directly correlated with the temperature dependence of the symbiotic phenotype. For instance, the *impJ* mutant shows a higher number of nodules at 24 than at 20°C, whereas the secreted protein is not detectable at either growth temperature. The identified protein showed homology to the secreted ribose binding protein from *B. subtilis*. This protein contains a signal peptide sequence for secretion by a type I secretion system. This signal sequence is also present in the rhizobial counterpart, and is not present in the purified secreted protein, indicating a periplasmic localization. With the protocol we have utilized, it is very unlikely that we would have purified periplasmic proteins in the spent growth medium at such high quantities, although we cannot completely exclude the possibility that the protein was released from the cells as a result of cell damage. However, considering the fact that, in the *imp* mutant, the RbsB protein was no longer detectable in the spent growth medium, it is most likely that the *imp* operon is involved in secretion of the RbsB protein to the extracellular environment, either directly or indirectly. Interestingly, a gene encoding a homologue to this ribose-binding protein also was located close to the *imp* homologues from *V. cholerae* (Fig. 4).

Homology studies indicate that some genes in the *imp* locus share limited homology with some elements of type III secretion genes. For instance ImpK is homologous to MotB which is part of a flagellar motor apparatus. However, we could not detect any motility changes upon bacterial growth on medium with substrates that influence the flagellar motion (data not shown). The flagellar motor assembly complex is highly homologous to type III secretion systems and recently it has been shown that the flagellar export apparatus can serve as a protein secretion system (Young et al. 1999). Interestingly, we also found homology of the ImpL protein to IcmF that is a gene product from a type IV secretion system. IcmF of *Legionella pneumophila* is directly involved in the killing of macrophages (Purcell and Shuman 1998). The *icm/dot* gene cluster of *L. pneumophila* is homologous to the conjugative plasmid transfer system or type IV secretion system (Christie and Vogel 2000; Komano et al. 2000). In the *icmF* gene cluster, another protein is located, *lphA* (Segal et al. 1998) that shows homology to *motB* and therefore also to *impK*. However, this protein was not shown to be involved in Icm/Dot mediated secretion (Komano et al. 2000). To summarize, our data suggest that the *imp* locus may utilize an unconventional secretion system with characteristics of both types III and IV. The effect of the *impJ* mutation on RbsB secretion indicates an involvement of this gene or other downstream-localized genes in the secretion of a periplasmatically localized protein to the extracellular environment. Such a function does not exclude the involvement of a type III secretion mechanism, because it has been shown that substrates for the Hrp type III secretion system from *P. syringae* are secreted into the growth medium (van Dijk et al. 1999) even though their biological function is presumably based on translocation into the host cell.

It remains interesting to note that the action of several avirulence proteins of phytopathogens is dependent on a functional type III secretion system. The functions of the proteins secreted by a type III secretion system of animal pathogens are diverse, but many belong to the protein phosphatase and protein kinase families (DeVinney et al. 2000), two functions that are very important in the regulation of stress responses in the host (Rodriguez 1998). Such proteins frequently have been identified in pathogenicity islands, such as YopH and YpkA of *Yersinia* spp., EspB/D of enteropathogenic *Escherichia coli* and SptP of *Salmonella enteritica* (Hueck 1998). After transfer of these proteins to the host cells, their phosphatase or kinase activity leads to events like actin cytoskeletal rearrangements and apoptosis (Frankel et al. 1998; Juris et al. 2000; Würtele et al. 2001). However, a molecular function has been shown for none of the phytopathogenic bacteria-derived effector proteins. Interestingly, in the *imp* operon, we also identified a putative protein kinase (ImpN) a protein phosphatase (ImpM), and a phosphoprotein-binding protein (ImpI), indicating similarities with type III secretion substrates from animal pathogens and suggesting a role of these proteins in regulation host cell responses. However, we have not been able to identify these proteins in the culture supernatant.

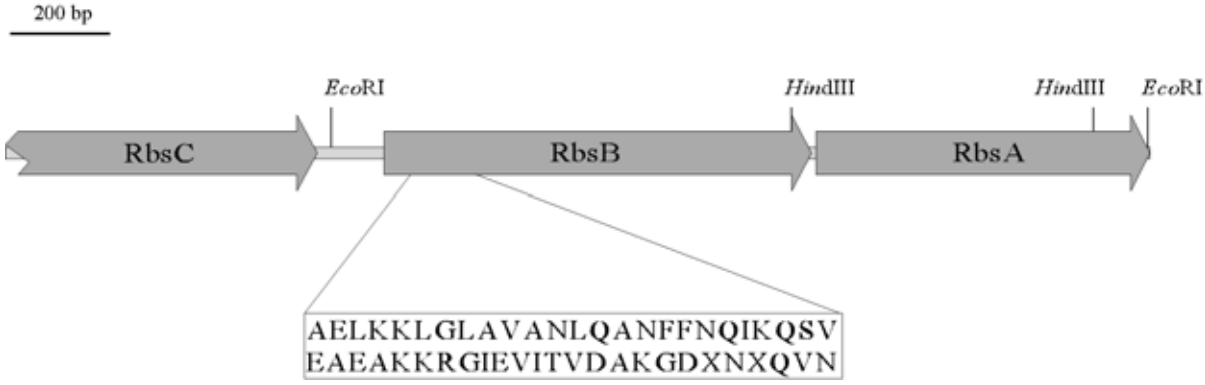

Fig. 6. The identified DNA region encoding the ribose binding proteins (Rbs) C, RbsB, and RbsA homologues. Amino acid sequence of spot 1 from Figure 5A is shown below. Primers used to amplify the fragment used for screening the cosmid library were (i) GCN GAR CTS AAR AAR CTS GGY CTS GCN GTN GCNAAC and (ii) YAG NGG RAA NCG YAG NTG NCA HTA NTG RAG HTA NGG. Sequencing of polymerase chain reaction–amplified fragments using these primers revealed that the DNA sequence was identical in all three rhizobial strains tested: RBL5523, RBL5787 (*impJ*::Tn*5*), and RBL5961 (phage tranducted *impJ*::Tn*5*).

Secretion of proteins by a type III secretion system in animal pathogens is usually contact dependent, although it is possible to induce secretion in culture by the application of the right growth conditions (such as low calcium and 37°C for *E. coli*). However, external application of these proteins to host cells usually does not have the effects that are observed during infection. Type IV secretion also is thought to be contact dependent and to be able to deliver effector molecules directly into the host cytosol (Christie and Vogel 2000). In contrast to the results reported with the external application of type III and type IV secreted proteins, application of the culture supernatant of RBL5523 to pea plants inoculated with RBL5787 did inhibit nodulation and nitrogen fixation by this strain.

The presence of a type III secretion system in several rhizobial species has been reported before (Viprey et al. 1998), as well as a type IV secretion system in *R. etli* (Christie and Vogel 2000). A mutation in the regulatory gene or in a gene that codes for a structural compound of the type III secretion system in strain NGR248 also increased the ability to nodulate *Pachyrhizus tuberosus*. This system was dependent on flavonoid induction and had the same genetic organization as the classical systems. Therefore, these systems are very different from the *imp* locus. However, several highly similar loci of unknown function have been described in *P. aeruginosa*, *V. cholerae,* and other animal pathogens such as *E .coli* strain O157 (Pallen et al. in press). Interestingly, homologues from *impB*, *impC*, and *impF*, were identified in the catfish pathogen *Edwardsiella ictaluri* on basis of antiserum from convalescent fish (Moore et al. in press), suggesting an extracellular localization. Furthermore, it has been shown that mortality caused by *E. ictaluri* in the catfish host occurs most frequently (97.8%) at 25°C (Baxa Antonio et al. 1992; Francis-Floyd et al. 1987), whereas low mortality rate (46.6%) is found at 20°C, implying another possible resemblance to the rhizobial system. On basis of these results, we can hypothesize that both these loci are also involved in protein secretion and perhaps pathogenesis. Considering the widespread occurrence of these gene families, it can be expected that many more homologous loci will be discovered in the future.

MATERIALS AND METHODS

Bacterial strains and plasmids.

A list of bacterial strains and plasmids with relevant characteristics is shown in Table 2. *Rhizobium* spp. were grown on tryptone yeast (TY) (Beringer 1974) or yeast mannitol broth (Hooykaas et al. 1977) medium at 28°C with the proper antibiotics, except when used for nodulation tests. *Escherichia coli* strains were grown on Luria complete medium (Sambrook et al. 1989) at 37°C.

pMP3717 (Roest et al. 1997) is a cosmid containing a partially digested *Eco*RI fragment of approximately 25 kb of chromosomal DNA from *Rhizobium* strain LPR5020. This cosmid contains the gene that was mutated by a Tn*5* insertion in strain RBL5787. To sequence the complete 25-kb insert, pMP3717 was digested with *Bam*HI, *Eco*RI, or *Hin*dIII and subcloned in pBluescript or pIC20H, resulting in plasmids pMP3703, pMP3720, pMP3737, pMP3738, pMP3739, pMP3740, and pMP3741. pMP3703 was subcloned further, resulting in plasmids pMP3730, pMP3731, pMP3732, pMP3733, pMP3734, and pMP3735 (Table 2, Fig. 1).

Plant material.

Pea (*Pisum sativum* cv. *rondo*) and vetch (*Vicia sativa* subsp. *nigra* and *V. hirsuta*) seeds were surface sterilized and germinated as described previously (van Brussel et al. 1982). Pea was grown in brown pots filled with Jensen medium (van Brussel et al. 1982). Vetch was grown in tubes on stainless steel grids, with the roots in liquid or solidified (0.75% agar) Jensen medium. The plants were cultured with the roots in the light or shielded from the light as indicated in the experiment. Roots growing in the light produce an increased amount of ethylene leading to thick, short roots and reduced nodulation, which can be overcome by the addition of aminoethoxyvinylglycine (AVG) (Zaat et al. 1989). If indicated, AVG was added to a final concentration of 0.1 mg/liter.

The plants were grown in growth chambers (20°C, 70% relative humidity or 24°C, 80% relative humidity) at a light intensity of approximately 15,000 to 20,000 lux (Philips TLF 60W/33 fluorescent tubes) and a day and night cycle of 16 and 8 h, respectively.

DNA manipulation and sequence analysis.

Standard DNA manipulations were carried out according to Sambrook and associates (Sambrook et al. 1989). Sequencing was done at Eurogentec (Seraing, Belgium) or BaseClear (Leiden, The Netherlands). All sequencing was done on one strand unless otherwise indicated. Sequence analysis was carried out with Vector NTI (Informax Inc, North Bethesda, MD,

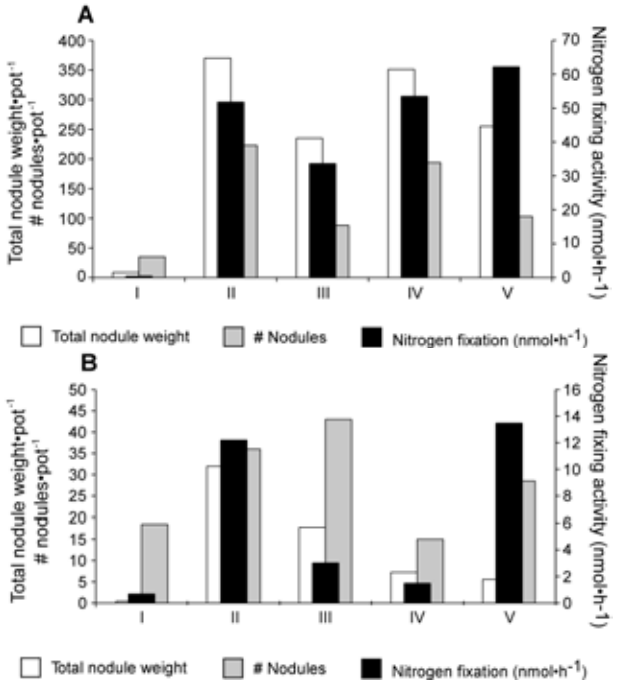

Fig. 7. Phenotypic analysis of the effect of bacterial secreted proteins on pea plants inoculated with RBL5787 in the presence or absence of spent growth medium of RBL5523. The features tested were number of nodules (gray bars), total nodule weight (white bars), and acetylene reduction levels (black bars). The figure displays two independent experiments that show considerable variation in overall total nodulation and nitrogen fixation levels. **A,** I, RBL5523 supplemented with its own growth medium; II, RBL5787 supplemented with its own growth medium; III, RBL5787 supplemented with 2 ml of growth medium from RBL5523; IV, RBL5787 supplemented with 2 ml of boiled growth medium from RBL5523; V, RBL5523 inoculated together with RBL5787. **B,** I, RBL5523 supplemented with its own growth medium; II, RBL5787 supplemented with its own growth medium; III, RBL5787 supplemented with 2 ml of growth medium from RBL5523; IV, RBL5787 supplemented with 6 ml of growth medium from RBL5523; V, RBL5787 supplemented with 6 ml of boiled growth medium from RBL5523. All values are per pot, which contained three plants each. The values for the number of nodules and the total nodule weight (mg) are shown on the left axis. The values for nitrogen fixation levels are shown on the right axis. Spent growth medium was derived from strains grown at 24°C.

U.S.A.) and the Gene Finder program (Baylor College of Medicine, Houston, TX, U.S.A.). Homologous proteins in the data library at the NCBI were identified with the BLASTX program.

Nitrogen fixation.

Nitrogen fixation was measured with acetylene reduction assays as described previously (van Brussel et al. 1982). In short, detached roots (pea) or whole plants (vetch) were put in tubes capped with Suba seal rubber stoppers and 10% acetylene was added. After 2 h, ethylene formation was measured with a Varian 3350 gas chromatograph on an alumina column (30 m, 0.53-mm internal diameter) with a column temperature of 70°C and nitrogen as the carrier gas, and detection was done with a flame ionization detector (200°C).

Isolation and detection of secreted proteins.

Rhizobium spp. were grown in TY medium until stationary phase. The culture was centrifuged at 24,000 × *g* for 30 min followed by filtering of the spent growth medium through a 0.22-µm filter (Millipore Millex-GV). Subsequently, proteins were precipitated with 10% TCA, centrifuged at 24,000 × *g* for 60 min, and the pellet was washed twice with acetone. The pellet was dissolved in 0.1% SDS so that a 3-µl sample contained the proteins from 1 ml of culture. The proteins were separated on a 12% polyacrylamide gel, stained with Sypro-Ruby (Molecular Probes), and visualized under a Fluor-S (BioRad). 2D-gel electrophoresis was done on the multiphor II horizontal electrophoresis apparatus (Pharmacia-Biotech, Uppsala, Sweden) according to the manufacturer's protocol, which was in principle according to Görg and associates (1988). The first dimension was isoelectric focusing (IEF) with ready-made IPG-strips in a pH-range of 3 to 9 or 4 to 7 (Pharmacia-Biotech) followed by SDS-PAGE in the second dimension on ready-made ExcelGel Gradient 12-14 gels (Pharmacia-Biotech). For IEF, the sample was dissolved in IEF sample buffer (8.5 M urea, 2% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate [CHAPS], 0.8% Pharmalytes, 15 mM dithiothreitol [DTT]) instead of 0.1% SDS, so that 400 µl of sample contained the amount of proteins from 40 ml of culture.

Coinoculation.

Rhizobia were grown in Jensen medium supplied with 0.1% glucose and 3 mM potassium nitrate until stationary phase (optical density at 660 nm [OD_{660}] = 0.3). The culture was centrifuged at 24,000 × *g* for 30 min, and the medium was filtered through a 0.22-µm filter and stored for later use to resuspend the bacteria. RBL5523 or RBL5787 was resuspended from a fresh plate in the desired medium to an OD_{660} of 0.1. Pea plants were inoculated with 2 ml of this culture in a total volume of 200 ml and grown at 24°C. To denature the proteins, the medium was boiled for 5 min before resuspending the bacteria.

Microscopy.

Plasmid pMP2464, containing eGFP, was mobilized from *E. coli* to the *Rhizobium* strains used in this study, using the helper plasmid pRK2013, following the procedure described by Ditta and associates (1980). The antibiotics kanamycin and rifampicin were used for selection at concentrations of 50 and 20 µg ml^{-1}, respectively. The GFP expressing *Rhizobium* was dispersed in Jensen medium to an OD of 0.1 at a wavelength of 660 nm. Suspended bacteria (100 µl) was applied to the roots of *V. sativa* and *V. hirsuta* seedlings as indicated above. For *P. sativum,* 10 µl of suspended bacteria was used.

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strain		
RCR5	Wild-type *Rhizobium leguminosarum* bv. *trifolii*	Rothamsted Culture Collection, Harpenden, U.K.
LPR5020	RCR5; Str	Hooykaas et al. 1981
RBL5502	LPR5020 cured of pRtr5a, supplied with pRL1JI[b], Str	Priem and Wijffelman, 1984
RBL5515	LPR5020 cured of pRtr5a, Rif, Str	Priem and Wijffelman, 1984
RBL5523	RBL5515 pRL1JI::Tn1831[c]	Priem and Wijffelman, 1984
RBL5787	RBL5523 *impJ*::Tn5[c]	Roest et al. 1997
RBL5961	RBL5502 *impJ*::Tn5[c]	This study
Plasmid		
pBluescript	IncColE1, multicopy cloning vector, Cb	Stratagene
pIC20H	IncColE1, multicopy cloning vector, Cb	Marsh et al. 1984
PMP2464	PBBR1MCS5 derivative, eGFP, Gm	Stuurman et al. 2000
pMP3703	pIC20H clone from a LPR5020 genomic library	Roest 1995
pMP3717	pLAFR1 clone from an LPR5020 genomic library	Roest et al. 1997
pMP3720	pIC20H clone from an LPR5020 genomic library	This study
pMP3730	pMP3703Δ*Bam*HI fragment	This study
pMP3731	pMP3703Δ*Pst*I fragment	This study
pMP3732	*Pst*I fragment from pMP3703 cloned in pIC20H	This study
pMP3733	pMP3703Δ*Eco*RV fragment	This study
pMP3734	*Bam*HI fragment from pMP3703 cloned in pIC20H	This study
pMP3735	*Eco*RV fragment from pMP3703 cloned in pIC20H	This study
pMP3737	9 kb *Bam*HI fragment from pMP3717 cloned in pBluescript	This study
pMP3738	8 kb *Bam*HI fragment from pMP3717 cloned in pBluescript	This study
pMP3739	1.2 kb *Bam*HI fragment from pMP3717 cloned in pBluescript	This study
pMP3740	6 kb *Hin*dIII fragment from pMP3717 cloned in pBluescript	This study
pMP3741	132 bp PCR fragment from RBL5523 in pGemT vector	This study
pMP3742	132 bp PCR fragment from RBL5787 in pGemT vector	This study
pMP3743	132 bp PCR fragment from RBL5961 in pGemT vector	This study
pMP3744	5 kb EcoRI fragment from pMP3717 cloned in pBluescript	This study
pMP4639	pME6010 derivative, eYFP, pLac, Tc	Stuurman et al. 2000
pMP4656	pME6010 derivative, eCFP, pLac, Tc	Stuurman et al. 2000

[a] Antibiotic resistance: Str = streptomycin, Rif = rifampicin, Cb = carbenicillin, Gm = Gentamycin, and Tc = Tetracycline; PCR = polymerase chain reaction.

[b] pRL1JI is the Sym-plasmid of *Rhizobium leguminosarum* bv. *viciae* strain 248.

[c] Tn*5* encodes kanamycin resistance and Tn*1831* encodes spectinomycin resistance.

GFP expression in planta was analyzed at 7, 14, 21, and 28 days after inoculation. Sections of root bearing nodules were excised from the plants. The nodules were then longitudinally serially sectioned with a freeze microtome at 50 µm of section thickness, carefully lifted onto glass slides, and examined with a Leitz Laborlux D microscope, equipped with a fluorescent light source. The fluorescence was observed using a filter set for FITC (Ex. 450 to 490 nm, LP 520 nm, FT 510 nm). Images of the plant tissues were taken using a color video camera (Sony CCD-iris with integration unit, Sony DKR700; Sony Corp., Tokyo).

ACKNOWLEDGMENTS

We thank N. Sedee of the Center for Phytopathology (TNO) for help with the 2D protein gels; T. Tak for technical assistance; I. H. M. Mulders for the construction of *Rhizobium* strain RBL5961; O. Stronk and A. Wijfjes for assistance in the initial stage of this work; and R. Thune (Louisiana State University, Baton Rouge, LA, U.S.A.) and M. Pallen (University of Birmingham, Birmingham, U.K.) for communicating results prior to publication. M. R. Bladergroen was supported by the Dutch organization for Scientific Research (NWO) and K. Badelt was supported by an EU-TMR grant (ERB FMR XCT 960039).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Forkhead-associated domain page, Swiss Institute of Bioinformatics Expert Protein Analysis System (ExPASy) website: www.expasy.ch/-cgi-bin/nicedoc.pl?PDOC50006

Protein families (Pfam) database of alignments and HMMs, Wellcome Trust Sanger Institute website: www.sanger.ac.uk/cgi-bin/Pfam/-getacc?PF00498